ENZYMES REGULATING THE HOMEOSTASIS OF AGONISTS AND ANTAGONISTS OF THE N-METHYL D-ASPARTATE RECEPTORS

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ENZYMES REGULATING THE HOMEOSTASIS OF AGONISTS AND ANTAGONISTS OF THE N-METHYL D-ASPARTATE RECEPTORS

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EDITORIAL
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Editorial: Enzymes Regulating the Homeostasis of Agonists and Antagonists of the N-Methyl D-Aspartate Receptors

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Keywords: NMDAR = N-methyl-D-aspartate receptor, enzymes - metabolism, enzyme drug target, D-serine, L-serine

Editorial on the Research Topic

Enzymes Regulating the Homeostasis of Agonists and Antagonists of the N-Methyl D-Aspartate Receptors

N-Methyl-D-aspartate receptors (NMDAR) are ligand-gated ion channels, involved in numerous neurological functions, including memory, learning, and synapsis plasticity. The main agonist is L-glutamate, and D-serine and glycine are co-agonists, D-serine being produced by serine racemase and glycine produced by L-serine degradation or direct synthesis. A metabolite of the kynurenine pathway, kynurenic acid, produced by kynurenine aminotransferase-II, acts as an antagonist whereas another metabolite of the same pathway, quinolinate, is a potent agonist. Other NMDAR ligands, such as magnesium and zinc ions, play regulatory roles.

High activity of NMDAR is associated with several neuropathologies, including Parkinson's disease, Alzheimer's disease, lateral amyotrophic sclerosis, and ischemia, whereas low activity is associated with schizophrenia. Presently, the pharmacological treatment is based on ligands targeting NMDAR, that are endowed with severe side effects.

This Research Topics is focused on the enzymes that are involved in the synthesis and degradation of the main agonists and antagonists of NMDAR, thus controlling their homeostasis. The understanding of the structure, dynamics, function, and regulation of these enzymes is the prerequisite for the development of drugs that allow a fine tuning of NMDAR activity.

Billard's review sets the stage describing structural and functional properties of NMDARs. NMDAR activity is modulated by the level of D-serine that, in turn, primarily depends on serine racemase catalysis. It has been found that the levels of D-serine decrease with aging whereas the opposite occurs in the progress of the Alzheimer's disease. In spite of a different origin, the net outcome is a memory loss due in aging to sublevels of NMDAR stimulation and in the Alzheimer's disease to an excessive stimulation causing neurotoxicity.

The pathway leading to L-serine is composed of three enzymes. The first enzyme, D-3-phosphoglycerate dehydrogenase, catalyzes the oxidation of the glycolytic intermediate 3-phosphoglycerate to 3-phosphohydroxylpyruvate, that by the action of the pyridoxal 5'-phosphate-dependent phosphoserine aminotransaminase in the presence of glutamate is converted into phosphoserine. The final step is catalyzed by phosphoserine phosphatase producing L-serine. Grant reports on the structure-function relationships of 3-phosphoglycerate dehydrogenase, present in different types. The physiological and pathophysiological relevance of the distinct types is still under investigation. However, a deficiency of the enzyme or inactive forms results in metabolic defects of the nervous system.

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Both Graham et al. and Raboni et al. reports on serine racemase with the former describing the role played by key amino acid residues in controlling the reversible conversion of L-serine to D-serine as well as L- and D-serine β-elimination, a pathway potentially of physiological relevance because it leads to a decrease in D-serine concentration. The role of the so-called "triple serine loop" and its modification might alter the rate along the two alternative pathways. Raboni et al. more deeply discuss the multiple regulation of serine racemase activity, including the dependence on divalent cations, ATP, nitrosylation, and several proteins. Evidence of crosstalk among the active site and allosteric sites strongly supports the notion of a significant plasticity of serine racemase conformation.

D-serine is degraded by D-amino acid oxidase, a FAD-dependent enzyme. Pollegioni et al. review the available structural, functional, and regulatory information on this enzyme, pointing to the low efficiency in D-serine degradation. Interaction with proteins, such as pLG72, as well as post-translational modifications and point mutations, strongly modulate enzyme activity. Given its proposed relevance in controlling D-serine homeostasis, several inhibitors have been designed and tested.

Two metabolites, both originating from the kynurenine pathway, bind to NMDAR: kynurenic acid and quinolinic acid. Kynurenic acid acts as an antagonist, playing a protective role toward excitotoxicity. However, high levels are associated with schizophrenia. Kynurenic acid synthesis is catalyzed by kynurenine aminotransferase, a pyridoxal 5'-phosphate-dependent enzyme. This enzyme is present in at least four types, with type II being the prevalent form in the brain. Rossi et al. reviews the enzyme structural features that have served the basis for the development of potent inhibitors of potential interest for treating diseases associated with low NMDAR activity.

Opposite to kynurenic acid, quinolinic acid is a strong agonist of NMDAR, causing excitoxicity. The key enzyme for quinolinic acid synthesis is kynurenine monooxygenase, a FAD-dependent enzyme. Phillips et al. reviews the biochemical and structural properties, and, in light of its therapeutic relevance, the strategies that have been pursued for the development of potent inhibitors.

Overall, we hope that this Topic issue triggers further interest on the enzymes here presented as well as on other enzymes which products bind to NMDAR. It is well-established that only via the modulation of the enzymes that synthesize agonists, co-agonists or antagonists of the NMDAR is it possible to "gently" tune NMDAR activity, thus curing both hypo- and hyper-excitatory signals associated with several neuropathologies.

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AM and RP equally contributed to the topics and in the writing of the editorial.

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REVIEW
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Human Serine Racemase: Key Residues/Active Site Motifs and Their Relation to Enzyme Function

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Serine racemase (SR) is the first racemase enzyme to be identified in human biology and converts L-serine to D-serine, an important neuronal signaling molecule that serves as a co-agonist of the NMDA (N-methyl-D-aspartate) receptor. This overview describes key molecular features of the enzyme, focusing on the side chains and binding motifs that control PLP (pyridoxal phosphate) cofactor binding as well as activity modulation through the binding of both divalent cations and ATP, the latter showing allosteric modulation. Discussed are catalytically important residues in the active site including K56 and S84—the si- and re-face bases, respectively,—and R135, a residue that appears to play a critical role in the binding of both negatively charged alternative substrates and inhibitors. The interesting bifurcated mechanism followed by this enzyme whereby substrate L-serine can be channeled either into D-serine (racemization pathway) or into pyruvate (β-elimination pathway) is discussed extensively, as are studies that focus on a key loop region (the so-called "triple serine loop"), the modification of which can be used to invert the normal *in vitro* preference of this enzyme for the latter pathway over the former. The possible cross-talk between the PLP enzymes hSR and hCBS (human cystathionine β-synthase) is discussed, as the former produces D-serine and the latter produces H_2S, both of which stimulate the NMDAR and both of which have been implicated in neuronal infarction pursuant to ischemic stroke. Efforts to gain a more complete mechanistic understanding of these PLP enzymes are expected to provide valuable insights for the development of specific small molecule modulators of these enzymes as tools to study their roles in neuronal signaling and in modulation of NMDAR function.

Keywords: D-serine, pyridoxal phosphate (PLP), serine racemase, racemization, elimination, mechanism, ATP, allosteric activation/regulation

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INTRODUCTION

Pyridoxal phosphate (PLP) enzymes have been extensively studied owing to interest in both the molecular details of their chemistry and the physiologic importance of the reactions that they catalyze, particularly in the area of neuroactive amine homeostasis (Walsh, 1979; Toney, 2005). This review will focus on key residues, binding sites and catalytically important motifs of human serine racemase, and its mammalian counterparts. In the literature to date, there are several reviews on topics such as the importance of D-serine and serine racemase in glial neurotransmission (Mothet, 2008; Wolosker, 2011, 2018; Wolosker and Mori, 2012; Wolosker et al., 2016) and in neurodegeneration (Campanini et al., 2013; Coyle and Balu, 2018). In addition, recent reviews

by Mozzarelli (Raboni et al., 2019) and by Mori (Mori, 2014) discuss the SR energy landscape and mechanism in the context of structure. The current review also builds upon previous reviews that discuss inhibition of hSR (Jirásková-Vanícková et al., 2011), in focusing upon key residues and structural motifs to consider in generating future inhibitors.

Traditionally thought to be restricted almost exclusively to the domain of bacterial cell wall biosynthesis, D-amino acids are now clearly seen as playing important and as yet incompletely understood roles in human biology, particularly in neuronal signaling (Wolosker et al., 2008; Li et al., 2017; Weatherly et al., 2017; Du et al., 2018). Although the receptor is named after its ability to bind N-methyl-D-aspartate (NMDA), L-glutamate is the primary agonist of the NMDA receptor with D-serine (D-Ser), serving as co-agonist, as illustrated in **Figure 1**. The NMDAR operates primarily as a ligand-gated channel that dislodges a Mg^{2+} or Zn^{2+} ion, allowing for depolarization and Ca^{2+} influx. Ca^{2+} ions are critical for synaptic plasticity and appropriate levels of each agonist are required for neuronal homeostasis and long-term potentiation (LTP) associated with learning and memory.

Whereas D-serine binds to the so-called "glycine site" of the NMDAR, it displays >2 orders of magnitude more potent activation of the NMDAR than glycine (Gly) itself (Berger et al., 1998; Wolosker, 2007). Perhaps the best experiments demonstrating this are elegant *in vitro* measurements of miniature excitatory postsynaptic currents (mEPSCs). In response to coagonist stimulation, 0.3 μM D-serine produces a higher level of NMDA charge transfer than 30 μM glycine (Berger et al., 1998). Consistent with these observations, the crystal structures of the NR1 subunit of the NMDAR with bound D-Ser (PDB code: 1PB8) and with bound Gly (PDB code: 1PB7) demonstrate that the former ligand engages in several additional hydrogen bonds as compared with the latter (Furukawa and Gouaux, 2003). This topic has been more extensively reviewed elsewhere (Schell, 2004). Recent reports also show that D-Ser, and not Gly, is responsible for LTP in the visual cortex (Meunier et al., 2016), and demonstrate that D-Ser concentrations in compartments of the cerebellum are much more tightly controlled than those of Gly, with the former being concentrated in the neocortex where complex thinking is taking place (Suzuki et al., 2017).

At the turn of the millennium, it was established that biosynthesis of D-Ser is mediated by a PLP-dependent serine racemase enzyme. This constituted the first known example of a mammalian racemase enzyme (Wolosker et al., 1999; De Miranda et al., 2000). Interestingly, human serine racemase (hSR) has an apparent dual role as it funnels neuronal L-serine into bifurcating pathways toward either D-Ser (racemization) or pyruvate (β-elimination).

MECHANISM

The generally accepted mechanism by which human SR catalyzes both the racemization of L-Ser to D-Ser and the elimination of L-Ser to pyruvate is illustrated schematically in **Figure 2**. Substrate L-Ser displaces K56 via an initial transaldimination reaction to form the external aldimine. The displaced K56 residue serves as the *si*-face base, α-deprotonating to yield a common carbanionic or quinonoid intermediate (see Discussion below). This is the point at which the path bifurcates with *re*-face reprotonation by S84 giving the racemization product, D-Ser, or with expulsion of the β-OH-leaving group, presumably following protonation, giving rise to an enamine that eventually is released as pyruvate, the β-elimination product. The wt-hSR enzyme displays an ∼4-fold preference for the β-elimination pathway over racemization under *in vitro* steady-state enzyme kinetic conditions (Nelson et al., 2017). However, Toney and co-workers showed that this ratio can be significantly altered by selected mutations (Foltyn et al., 2005) as will be discussed. Moreover, given the number of important protein-protein interactions (PPI) that have been implicated for hSR *in vivo* (Fujii et al., 2006; Baumgart et al., 2007; Hikida et al., 2008; Ma et al., 2013, 2014), one must consider that these may influence hSR activity and the racemization to β-elimination ratio seen *in vivo* as well.

SEQUENCE OVERVIEW

A global overview of SR primary structure with an eye toward highlighting key functional domains is presented in **Figure 3**. This review will discuss conserved motifs displayed there, including all the elements of the PLP binding pocket—the essential lysine residue, the tetraglycine loop for phosphate binding (Smith et al., 2010), the H-bond donor for the PLP ring nitrogen and the edge-to-face π-π interaction that serves to anchor the pyridine ring (Wang and Barger, 2012). The enzyme is stimulated by both divalent metal cation binding and ATP binding, each with established contact residues, with good evidence for allostery in the case of the nucleotide binding site. Finally, an interesting "triple Ser loop" is present that appears to have significant influence on the bifurcation, i.e., L-serine racemization vs. β-elimination activity.

DIVALENT METAL BINDING SITE

The importance of divalent metal cation binding to hSR was first reported, in parallel, by the laboratories of Wolosker (De Miranda et al., 2002) and of Cook (Cook et al., 2002). The latter group performed the most extensive study of divalent cations, finding that Mn^{2+} leads to the highest increase in activity of the enzyme (153% @10 μM), followed by Ca^{2+} (134% @1 mM) and Mg^{2+} (112% @10 μM) relative to the purified hSR without divalent cation supplementation. The effect of divalent metal identity upon activity does not appear to be due to major structural changes, as evidenced by circular dichroism studies. Smith et al. have deposited the coordinates of several mammalian SR crystal structures including PDB code: 3L6B displayed in **Figure 4A**, a structure that highlights the formally octahedral divalent metal (Mn^{2+})-coordination sphere involving residues E210 and D216 (∼2.1 Å metal-ligand bond lengths), an amide carbonyl and three water molecules. A similar divalent metal coordination environment is seen in the *S. pombe* SR enzyme (PDB code: 1WTC).

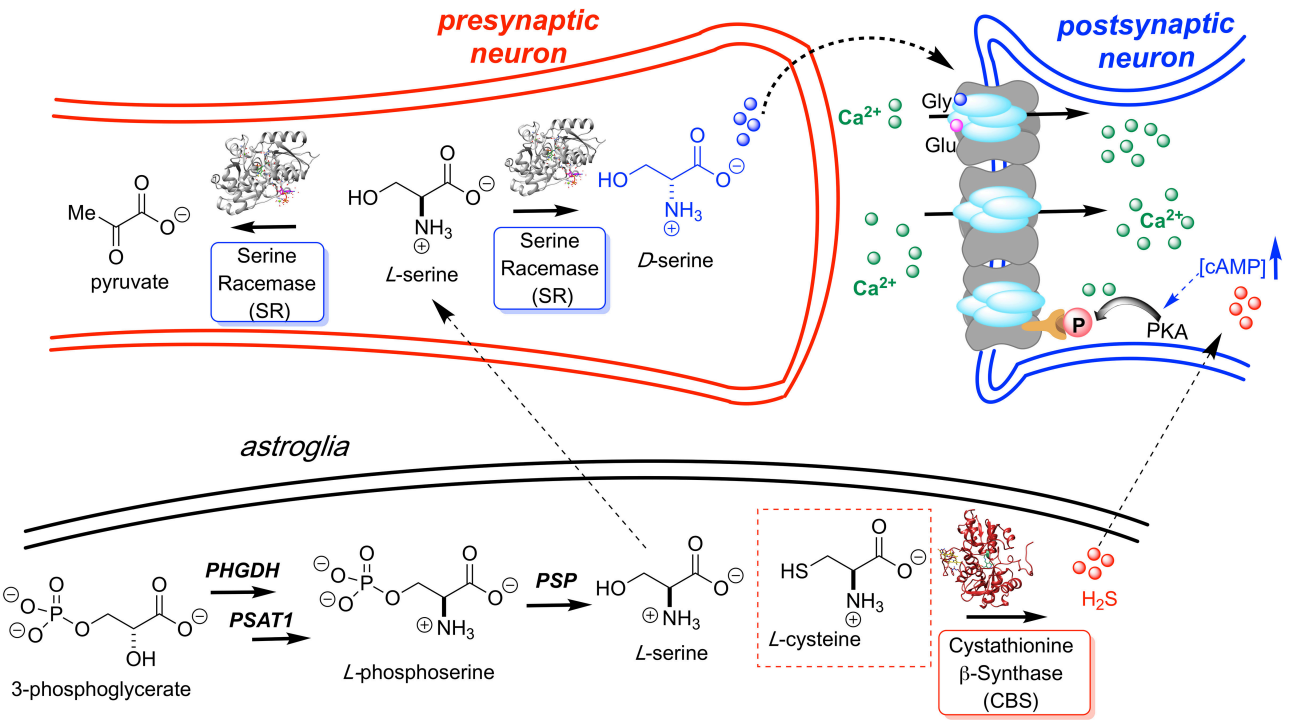

FIGURE 1 | L-Serine is first produced in the astroglia from 3-phosphoglycerate by way of a three enzyme cascade involving phosphoglycerate dehydrogenase (PHGDH), phosphoserine aminotransferase 1 (PSAT1), and phosphoserine phosphatase (PSP). L-Serine is then transported to the presynaptic neuron where it is converted to D-Ser by hSR. D-Serine acts as a potent NMDAR co-agonist at the glycine site. H_2S, generated by CBS, is also thought to activate the NMDA receptor. Models for H_2S-NMDAR activation include a cAMP-dependent PKA-mediated phosphorylation and H_2S- or sulfane sulfur-mediated NMDAR disulfide bond reduction (Kimura, 2000; Kimura et al., 2013).

ATP BINDING SITE

Of the crystal structures currently available for SR, the ATP-binding site is best seen in the *Schizosaccharomyces pombe* serine racemase (SpSR) structure (1WTC) that features a bound AMP-PCP ligand, a hydrolytically stable β,γ-CH_2-phosphonate analog of ATP (**Figure 4B**). In this structure, residues K52 and N25 coordinate to the terminal phosphonate group and a Mg^{2+} ion bridges across the β,γ-phosphono groups. Tyrosine-119 appears to be engaged in hydrogen bonding interactions with the proximal phosphate, and Asn311 appears to be engaged in a similar H-bond with the 3'-hydroxyl group of the ribose.

It is well-known that ATP-binding leads to enhanced catalytic activity for SR; an overlay of the ATP-free structure (e.g., 3L6B) with the ATP bound structure (1WTC) provides evidence that this amounts to allosteric activation (**Figure 4C**). Specifically, it has been argued that allostery arises through an extensive hydrogen binding network (T52, N86, Q89, E283, N316) connecting the ATP ribose 3'-hydroxyl group to the active site (T52 corresponds to M53 in SpSR). Similarly, this hydrogen binding network is predominantly conserved within close evolutionary homologs serine dehydratase (SDH) and threonine deaminase. By aligning 186 sequences, Mozzarelli and co-workers found that the T52 position showed the highest variability while Q89 is conserved in enzymes that are allosterically regulated by nucleotides (i.e., hSR, spSR, threonine deaminases) (Canosa et al., 2018). On the other hand, *Hordeum vulgare* SR and SDH have either an alanine or methionine at this position and are not regulated by ATP.

Upon mutation of the Q89 residue to either a methionine or an alanine, ATP activation is reduced from a 7-fold increase in the wt-enzyme to only 4-fold and 2-fold for the Q89M and Q89A mutants, respectively (Canosa et al., 2018). Moreover, this effect is not due to the decrease in ATP affinity, as addition of ATP at higher concentrations still fails to show activation in the mutants. Interestingly, the Q89 mutants maintain the same activity as the wt-enzyme in the absence of ATP. Studies show that these mutants exhibit non-cooperative binding with respect to ATP. This can also be observed in the crystal structures solved to date in which two different conformations of Q89 have been observed (**Figure 4**). It is postulated that this residue acts as a key gating residue, playing a central role in the conformational change associated with allosteric activation of the enzyme (Canosa et al., 2018).

PYRIDOXAL PHOSPHATE SITE

Phosphate Binding Pocket

Human serine racemase displays a classical PLP binding site, including all the hallmark attributes as follows: (i) the tetraglycine loop for binding of the 5′-phosphate (Smith et al., 2010), (ii) π-stacking interaction to engage the pyridine ring (Smith et al.,

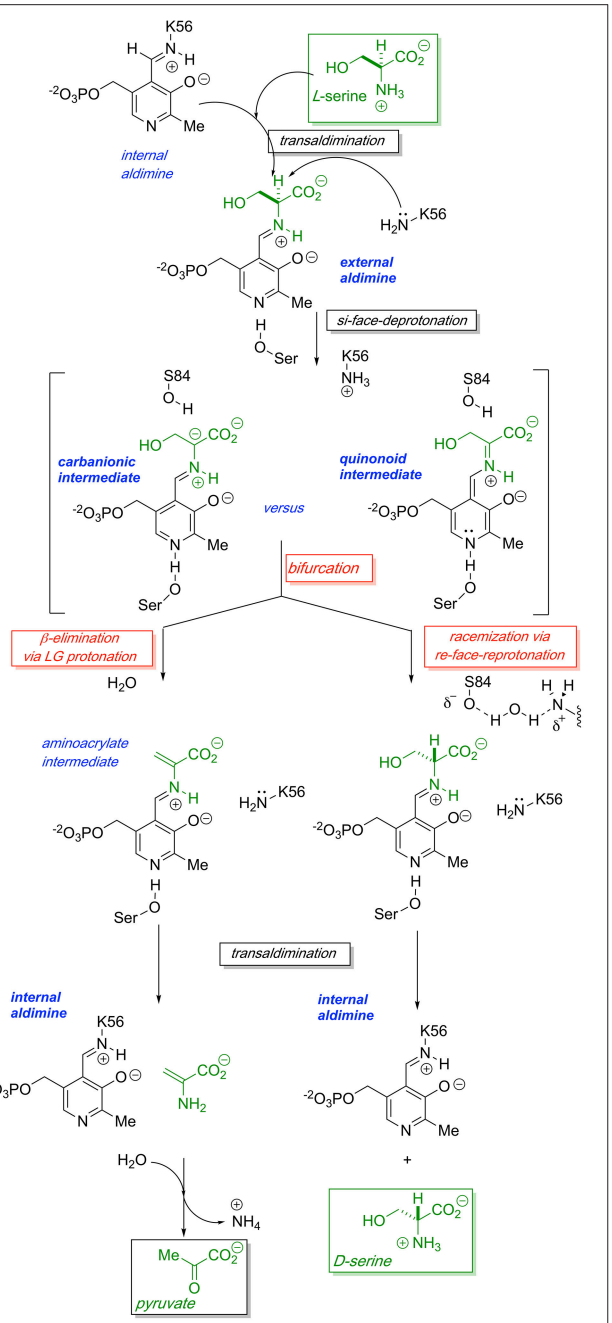

FIGURE 2 | Proposed bifurcating mechanism of hSR showing the racemization reaction vs. the competing β-elimination reaction via a common carbanionic or quinonoid intermediate.

2010), and (iii) hydrogen-bonding to the pyridine-nitrogen. The tetraglycine loop for hSR consists of a string of glycines from position 185 to 188, each utilizing an amide N-H to donate a hydrogen bond for phosphate binding (Smith et al., 2010). This canonical PLP enzyme feature is present in most cofactor binding sites and is quite evident in the hSR structure (**Figure 5**). While the PLP binding site is highly conserved across most PLP enzymes (see **Figure 6**) it is important to note that PLP-dependent enzymes fall into a wide range of fold types, which has been discussed nicely elsewhere (Schneider et al., 2000). For our purposes here, it is notable that PLP-dependent racemases themselves fall into more than 1-fold type, with serine racemase (Yoshimura and Ito, 2014) and aspartate racemase (Takahashi, 2009) being members of the fold type II family and alanine racemase being a fold type III enzyme (Azam and Jayaram, 2016).

π-Stacking

The second key stabilizing feature often found in PLP cofactor binding sites is an aromatic amino acid side chain engaged in a favorable π-π interaction with the pyridine ring. In enzymes such as serine racemase (**Figure 6A**) (Smith et al., 2010), aspartate racemase (Mizobuchi et al., 2017) (**Figure 6C**) and serine dehydratase (**Figure 6D**) (Wang et al., 2012), the aromatic ring for π-stacking is provided by a Phe residue immediately preceding the essential lysine in the primary sequence. The aromatic ring of this Phe side chain is engaged in an edge-to-face π-π-interaction at the *si*-face of the PLP-ring. Other racemases, such as α-amino ε-caprolactam racemase (**Figure 6E**) (Frese et al., 2017) and isoleucine 2-epimerase (**Figure 6F**) (Hayashi et al., 2017), utilize a tyrosine side chain for a similar edge-to-face interaction.

Pyridine Nitrogen

The third feature of most PLP-binding pockets is the presence of a hydrogen bond-donating side chain that partially protonates the pyridine nitrogen in the ground state. For transaminase enzymes, this residue is generally an aspartate residue (Chan-Huot et al., 2013; Fesko et al., 2018), presumably promoting formation of a quinonoid intermediate with broad charge delocalization. It has been argued that the intermediacy of such a charge-delocalized species facilitates the required azallylic isomerization (i.e., C4'-protonation) for such enzymes. For racemase enzymes, however, it is clear that such an acidic proton donor is not required. For example, perhaps the most well-studied PLP-dependent racemase, alanine racemase, utilizes an arginine residue in this position, a weak hydrogen bond donor (**Figure 6B**) (Shaw et al., 1997).

A survey of current PLP-dependent racemase structures in the pdb shows that, in fact, these enzymes feature a broad range of proton donors for the pyridine ring. In aspartate racemase (Mizobuchi et al., 2017), Cys321 serves as H-bond donor (**Figure 6C**-PDB code: 5YBW), whereas α-amino ε-caprolactam racemase (**Figure 6E**-PDB code: 5M46), (Frese et al., 2017) and isoleucine 2-epimerase (**Figure 6F**-PDB code: 5WYA) (Hayashi et al., 2017) utilize aspartatic acid residues Asp238 and Asp250, respectively, as PLP-nitrogen protonating residues. Serine racemase (**Figure 6A**–PDB code: 3L6B) (Smith et al., 2010) employs a serine residue, Ser313, reminiscent of β-eliminase enzymes such as tryptophan synthase or O-acetylserine sulfhydrylase (OASS). This is interesting because SR, like tryptophan synthase, catalyzes the β-elimination of water from L-serine. That said, not all β-eliminase enzymes employ a serine residue, as serine dehydratase utilizes a cysteine (**Figure 6D**–PDB code: 4H27) (Wang et al., 2012).

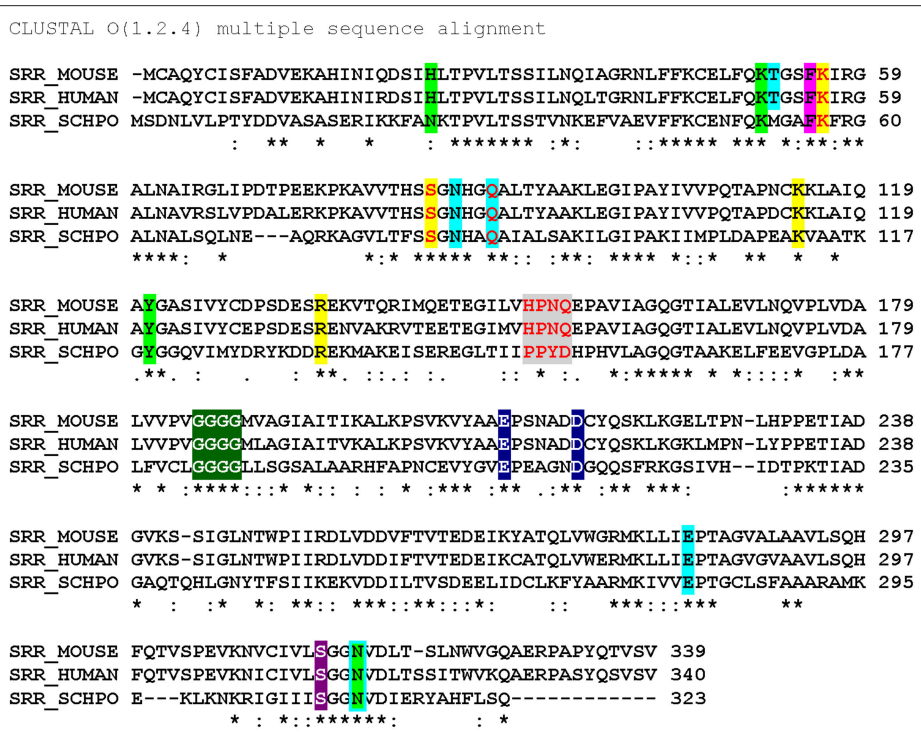

FIGURE 3 | Sequence alignment (Clustal O) of SR from mouse, human and *S. pombe*. Color code: ATP binding site (lime); ATP hydrogen bonding network (cyan highlight or outline); π-stacking residue (magenta) and catalytic residues (yellow); "triple serine loop" region by analogy with aspartate racemase (gray); tetraglycine loop (green); divalent cation binding site (navy blue); pyridine-N-protonating residue (violet). Red letters indicate positions that have been mutated. *conserved residue: strongly similar residues. Weakly similar residues.

The greater need for charge delocalization (and hence pyridine N-protonation) for PLP-enzyme-mediated transamination, as opposed to β-elimination or racemization chemistry, is supported by the results of a seminal study employing deaza-PLP (Griswold and Toney, 2011). These workers compared all three of these classes of PLP enzymes with both the native cofactor and its synthetic deaza-analog. Upon removing the ring nitrogen, by far the biggest penalty paid in k_{cat} is for transamination, with aspartate aminotransferase suffering an $\sim 10^9$-fold decrease in activity. On the other hand, the β-eliminase OASS experiences only a 260-fold decrease and alanine racemase sees an ∼700-fold decrease in k_{cat}. Griswald and Toney conclude that upon breaking the Cα-H bond, transaminases delocalize charge fully into the PLP ring, while β-eliminases and racemase enzymes operate via a "carbanionic intermediate" with a more localized azallylic charge distribution across Cα and C4'.

Nature of the Electron Sink: Quinonoid vs. Carbanionic Intermediate

The PLP-imine π-system is often described as a four-electron sink. The pyridine nitrogen is thought of as the primary locus for the first two electrons stored in the extended π-system, with the imine nitrogen then in position to accept a second pair of electrons as, for example, would be required for a γ-replacement enzyme. Prior to such elegant physical organic chemical tools that are now available to interrogate reaction mechanism, it was long assumed, following the pioneering stereoelectronic arguments of Dunathan (Dunathan, 1966), that PLP enzymes catalyzing Cα-H, Cα-C, or Cα-COOH bond cleavage would fully delocalize the resultant electron density into the π-system of the PLP-imine, the most stable resonance form of which would place those electrons on the pyridine nitrogen (Walsh, 1979). The ability to form a charge-balanced, net-neutral quinonoid intermediate would then require the pyridine nitrogen to be protonated. While this is clearly possible when an Asp (or potentially Glu) residue is so positioned in the active site, full protonation of the PLP ring nitrogen with a Ser, Cys, or Arg residue, for example, would require that these residues have abnormally low pK_a values in the given PLP enzyme active site.

Consistent with this reasoning, to our knowledge, quinonoid intermediates have only been observed for PLP enzyme active sites that do feature an acidic residue donating a proton to the PLP ring nitrogen. Because such quinonoid intermediates feature an extended quinone-like π-system, these species absorb well into the visible, typically with $\lambda_{max} \sim$ 480–550 nm. Quinonoid intermediates have been observed by stopped flow spectrophotometry in enzymes that natively feature aspartate residues protonating the pyridine nitrogen (Metzler et al., 1988; Phillips et al., 1998; Karsten et al., 2005). In enzymes in which this residue is natively a serine, such as tryptophan synthase (Jhee et al., 1998) or an arginine such as alanine racemase (Sun and Toney, 1999), mutation of these residues to Asp or Glu, respectively, allows for the observation of quinonoid intermediates that had otherwise been unobservable.

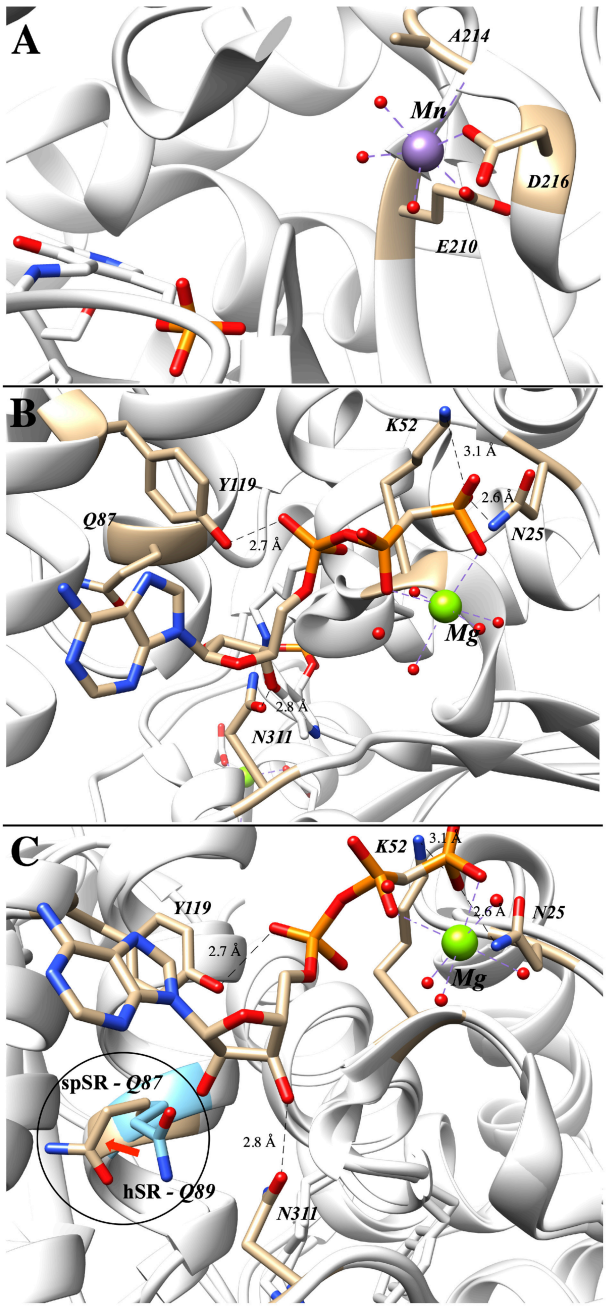

FIGURE 4 | (A) Mn^{2+} occupying the metal binding site in hSR; both E210 and D216 are within 2.1 Å of the divalent metal (3L6B). **(B)** Binding of the second ATP–associated metal from the AMP-PCP-bound structure of *S. pombe* SR (1WTC). **(C)** Overlay of human (blue) and *S. pombe* (tan) SR highlighting the movement of Q89 upon ATP-binding (Q89 in hSR and Q87 in spSR).

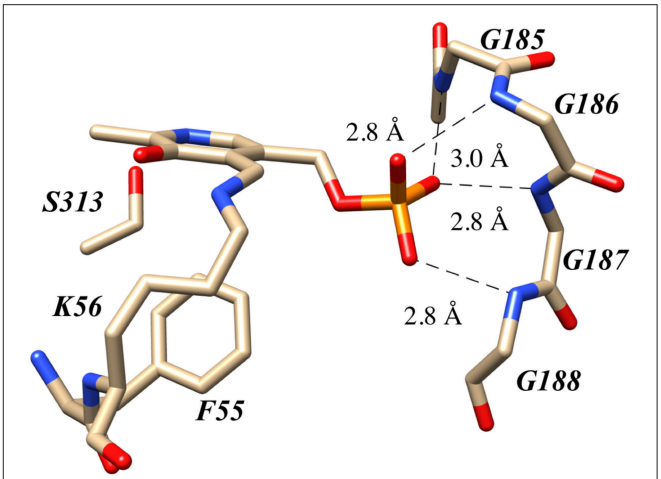

FIGURE 5 | The canonical tetraglycine loop that serves as a PLP phosphate binding pocket as seen in the hSR structure PDB code:3L6B.

These experiments suggest several alternative possibilities for catalysis with an enzyme such as tryptophan synthase. Catalysis might proceed (i) in a concerted fashion without buildup of negative charge in an intermediate or transition state, (ii) via a more localized carbanionic intermediate in which the charge is not delocalized significantly into the aromatic π-system of the PLP ring, or (iii) via a fully delocalized quinonoid intermediate with a lifetime that is too short to observe with typical stopped flow instruments. In fact, collaborative work by Dunn and Mueller, utilizing a combination of NMR, X-ray crystallography and computational modeling (Caulkins et al., 2016; Huang et al., 2016), provides evidence for the intermediate case just described; namely, for the formation of such a localized "carbanionic intermediate." As is shown in **Figure 7A,** this non-planar intermediate is thought to distribute electron density across the Cα-N-C4'-azallylic system rather than into the pyridine π-system. The active site lysine ε-ammonium ion is seen in close enough proximity to electrostatically stabilize this "carbanionic intermediate."

A related observation was made for the enzyme drosophila cystathionine β-synthase (CBS), by Banerjee, Smith and co-workers via x-ray crystallography (**Figure 7B**) (Koutmos et al., 2010). Here, too, a non-planar structure is seen for the putative "carbanionic intermediate" with L-serine substrate at pH 7. The azallylic C4'-N-Cα-anion is puckered upward toward the *re*-face, out of the plane of the pyridine ring system. Upon lowering the pH to 6.5, β-elimination apparently occurs, and one sees the resultant aminoacrylate intermediate in the crystal. Consistent with these crystallographic results, stopped flow spectrophotometric analysis provides evidence for an aminoacrylate intermediate at 460 nm. Another intermediate is also seen at 315 nm, potentially the azallylic carbanionic species, as such a lower λ_{max} value would be expected for such a system with limited charge delocalization.

It should be noted that both cystathionine β-synthase and tryptophan synthase have similar active sites to that of serine racemase, as all three contain a serine hydroxyl in position to protonate the pyridine ring nitrogen. These studies thus suggest that the bifurcating racemase/β-eliminase activity of SR may proceed by way of such an incompletely delocalized "carbanionic intermediate."

ACTIVE SITE

Essential Lysine

The essential lysine residue is found in all PLP-dependent enzymes, serving as a handle for the covalent attachment of

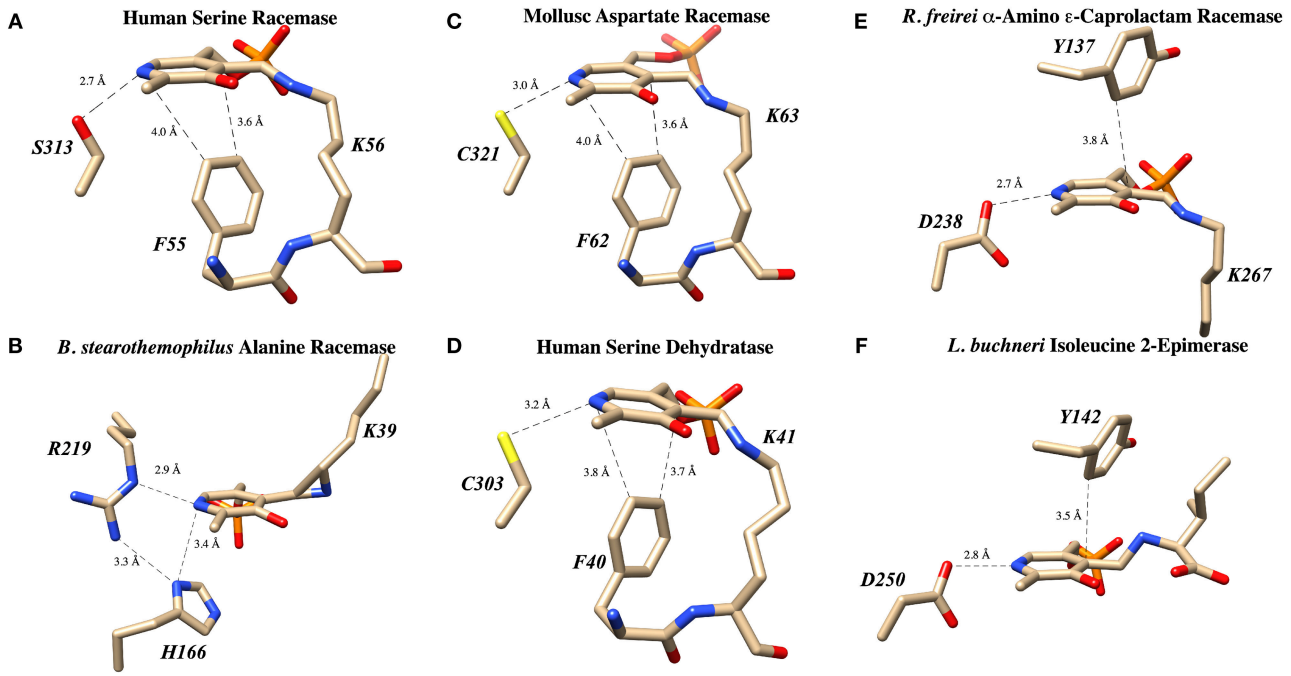

FIGURE 6 | PLP binding sites in known racemase structures illustrating hydrogen bonding and π-facial interactions of the pyridine ring. **(A)** hSR: pyridine H-bonding with S313 and edge-to-face π-π interaction with F55 (PDB code: 3L6B). **(B)** Alanine racemase: presumably weak pyridine H-bonding with R219 (PDB code: 1SFT). **(C)** Aspartate racemase: pyridine H-bonding with C321 and edge-to-face π-π interaction with F62 (PDB code: 5YBW). **(D)** Human serine dehydratase: pyridine H-bonding with C303 and edge-to-face π-π interaction with F40 (PDB code: 4H27). **(E)** α-Amino ε-caprolactam racemase: pyridine H-bonding with D238 and edge-to-face π-π interaction with Y137 (PDB code: 5M46). **(F)** Isoleucine 2-epimerase: pyridine H-bonding with D250 and edge-to-face π-π interaction with Y142 PDB code: 5WYA.

the PLP cofactor in the internal aldimine. There are clear kinetic advantages of such an aldimine linkage, as this allows the amino acid substrate to rapidly form the external aldimine via a facile transaldimination reaction that passes through a *gem*-diamine intermediate. Essential lysine mutants generally lose orders of magnitude in catalytic efficiency but are still useful for the study of enzyme structure and mechanism. These mutants often retain the ability to non-covalently bind the PLP cofactor and support formation of the external aldimine by a much less efficient amine-aldehyde condensation rather than by the usual transaldimination mechanism. Perhaps, more importantly, such a mutation also means that the *si*-face base has been lost. For example, in possibly the most well-studied PLP enzyme, aspartate aminotransferase, the K258A mutant exhibits a 10^8-fold decrease in activity (Toney and Kirsch, 1993; Griswold and Toney, 2011) relative to the wild type. In CBS, the K119A mutant displays a 10^3-fold decrease in activity. However, addition of the exogenous base ethylamine allows for a 2-fold gain in activity, perhaps reflecting external compensation for this lost *si*-face base activity (Evande et al., 2004).

Putative *re*-face Base

In hSR, the essential lysine, Lys56, is thought to be the *si*-face base responsible for α-deprotonation of L-serine or related substrates in human serine racemase. Ser84 has been proposed to be the complementary *re*-face base, responsible for reprotonation at the α-carbon to form D-serine. Crystallography demonstrates that this serine is highly conserved for hSR and its homologs in various other organisms (Goto et al., 2009; Koutmos et al., 2010). However, at physiological pH, the serine hydroxyl pK_a is estimated to be too high to serve as a catalytically efficient general acid. Recently, based upon crystallographic considerations, Berkowitz and co-workers suggested a possible mechanism through which the effective pK_a of Ser84 may be lowered in the hSR active site. Namely, these workers noted that the hSR structure features a potential Ser84-Wat372-Lys114 hydrogen bonding network (Nelson et al., 2017) resembling the Ser-*cis*-Ser-Lys catalytic triad of the amidase signature enzyme family (Ekici et al., 2008; Mileni et al., 2009; Pratt and McLeish, 2010; Lee et al., 2015; Cerqueira et al., 2017).

Several groups have expressed the *re*-face base Ser to Ala mutant in serine racemase enzymes from *Dictyostelium discoideum* (slime mold), *S. pombe,* and humans (Goto et al., 2009; Bodhinathan et al., 2010; Nelson et al., 2017). In all cases, as expected, racemization activity is lost. For the mammalian enzyme, β-elimination of L-serine to pyruvate persists but undergoes a 6-fold reduction in catalytic efficiency (k_{cat}/K_m), as can be seen in **Table 1**. The normal hSR preference for the negatively charged β-elimination substrates L-threo-β-hydroxy-aspartate (L-THA) and L-serine-*O*-sulfate (L-SOS), also persists in the S84A mutant (Strísovský et al., 2005).

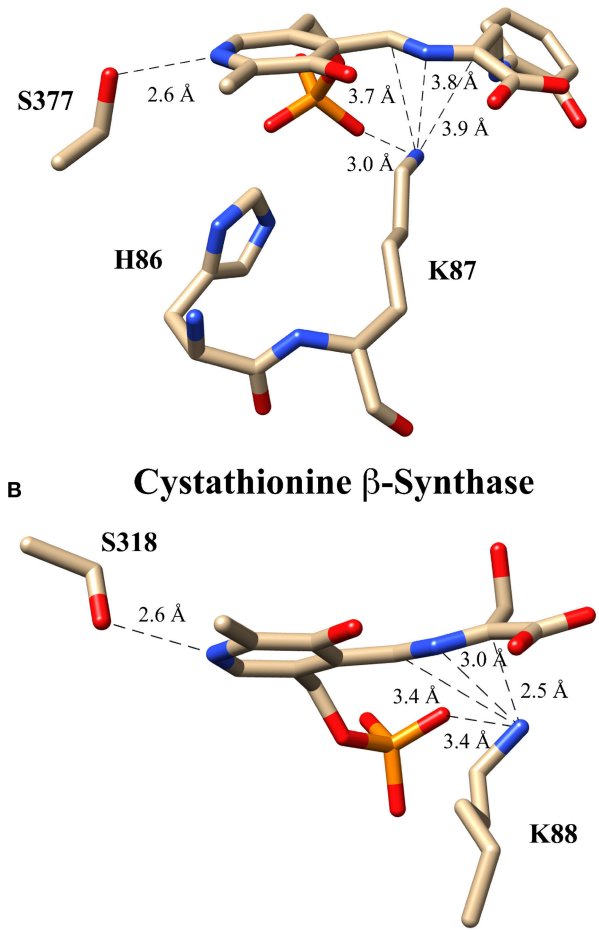

FIGURE 7 | Putative carbanionic intermediates as observed via x-ray crystallography. **(A)** Tryptophan synthase structure evidencing a "carbanonic intermediate" formed by addition of 2-aminophenol to the aminoacrylate intermediate (PDB code: 4HJP). This model is supported by Gaussian 09-based computations. **(B)** Cystathionine β-synthase structure featuring a similar "carbanionic intermediate" observed at pH 7 and hypothesized to be electrostatically stabilized by K88 (PDB code: 3PC4).

S84D Mutant Reveals Importance of R135 in Controlling β-Elimination Substrate Preferences

When Ser84 is mutated to an acidic aspartate residue, the S84D mutant again loses the ability to catalyze the racemization reaction, as expected. The β-elimination chemistry of this mutant, however, demonstrates a surprising reversal of substrate preference. The native enzyme prefers the elimination substrates L-SOS and L-THA, each of which displays a negatively charged side chain over L-Ser ∼100:1. This ratio changes to 50:1 in favor of L-Ser in the S84D mutant. This corresponds to a ∼5,000-fold swing in L-Ser to L-THA preference and a ∼1,200-fold change in L-Ser to L-SOS processing efficiency. The S84D hSR mutant thus displays a an inverted β-elimination substrate bias toward L-Ser of 50:1 vs. L-THA and of 12:1 vs. L-SOS (Nelson et al., 2017).

TABLE 1 | Kinetics of hSR mutants highlighting the S84D mutants switch in preference to elimination of serine over charged substrates and the interesting preference of S84T for L-SOS over L-THA [table adapted with permission of the American Society for Biochemistry and Molecular Biology (ASBMB) (Nelson et al., 2017)].

	k_{cat}/K_m (Sub.): k_{cat}/K_m (L-Ser)		
Variant	**L-SOS**	**L-THA**	**L-Ser vs. L-SOS vs. L-THA**
WT	100:1	93:1	1:100:93
S84A	71:1	34:1	1:71:34
S84D	1:12	1:50	50:4:1
S84N	2.5:1	7:1	1:2.5:7
S84T	370:1	50:1	1:370:50

Utilizing molecular dynamics simulation and docking, the Berkowitz group put forth a model based upon the Dunathan hypothesis (Dunathan, 1966) that is consistent with this finding (Nelson et al., 2017). The model is based upon stereoelectronics and the notion that the Cα-H bond to be broken must be aligned with the extended π-system of the PLP-imine (Dunathan, 1966). For the wild-type enzyme, negatively charged substrates are predicted to be oriented via a salt bridge with R135 resulting in the proper alignment for deprotonation (**Figure 8A**). This model is also consistent with the crystal structure of hSR bound to malonate (3L6B), in which the β-carboxylate of the inhibitor forms a salt bridge with R135 (Koutmos et al., 2010). Molecular dynamics simulations of the S84D mutant suggest that D84 moves to form a new salt bridge with R135, thereby preventing the positively charged arginine guanidinium group from interacting with the negatively charged side chains of L-THA and L-SOS. This results in a less-than-optimal positioning of these substrates in their respective enzyme-bound external aldimines, with the Dunathan angle (dihedral angle = H-Cα-N-C4′) distorted from the ideal 90–46° and 33° for L-THA and L-SOS, respectively (**Figure 8B**) (Nelson et al., 2017).

In light of the S84D results, the S84N hSR mutant was also studied. Intermediate results were observed, with only a modest preference for L-SOS (2.5:1) and L-THA (7:1) over L-Ser being observed. L-Serine racemization was not detected but the β-elimination was nearly at wild-type catalytic efficiency. Molecular modeling suggests that the intermediate activity seen with L-THA may be due to two different conformations of the hSR-bound external aldimine; in one, the R135 guanidinium group is engaged with the β-carboxylate of the substrate (better Dunathan alignment ∼ 82°), and in the other, R135 forms a salt bridge with the α-carboxylate (Nelson et al., 2017).

Finally, the S84T mutant was also studied. This is the only hSR mutant studied that retains L-Ser racemization activity, albeit with a 10-fold loss in efficiency. The β-elimination reaction of L-Ser is also less efficient by ∼1 order of magnitude. As for the β-elimination reaction of the negatively charged substrates, this mutant shows a marked preference for L-SOS elimination (370:1 vs. L-Ser) over L-THA (50:1 vs. L-Ser) elimination. This L-SOS preference may be due to the fact that this substrate features a favorable β-sulfate leaving group that need not be protonated to leave, as compared with L-THA, for which the

β-OH leaving group would require protonation to leave. It may simply be that in the S84T mutant, the general acid required for β-OH protonation in L-THA is not optimally positioned to do so (Nelson et al., 2017).

Lys-N$^{\varepsilon}$-Ala57 Extender Arm Variant

An interesting apparent self-catalyzed active site modification reaction has been reported for the serine racemase from *Schizosaccharomyces pombe* (SpSR) by Esaki and Hirotsu and co-workers (Goto et al., 2009; Yamauchi et al., 2009). In one of the first three crystal structures published by this group for the *S. pombe* SR, it was observed that the essential lysine had been modified to a L-lysino-D-alanyl-residue upon extended incubation with L-serine. This amounts to a three-atom extension of the active site Lys-57 residue. The authors provide both mass spectrometric (+87) and x-ray crystallographic evidence in support of this structure. They claim that a 97% level of modification is seen.

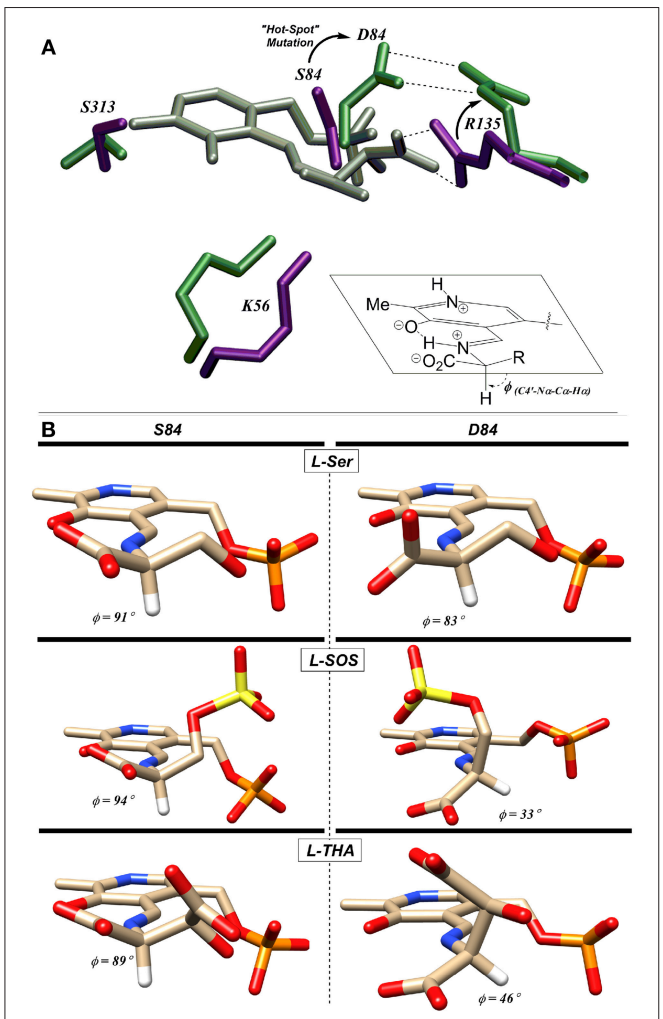

FIGURE 8 | (A) L-THA-derived external aldimine (gray) docked with native-hSR (violet) and S84D hSR (green). The putative L-THA-R135 salt bridge is replaced with a D84-R135 salt bridge in the mutant. **(B)** Docked external aldimines of L-Ser, L-SOS, and L-THA exhibiting much better Dunathan alignment in wt-hSR (left) than in S84D-hSR (right) [Figure adapted with permission of the American Society for Biochemistry and Molecular Biology (ASBMB) (Schell, 2004; Nelson et al., 2017)].

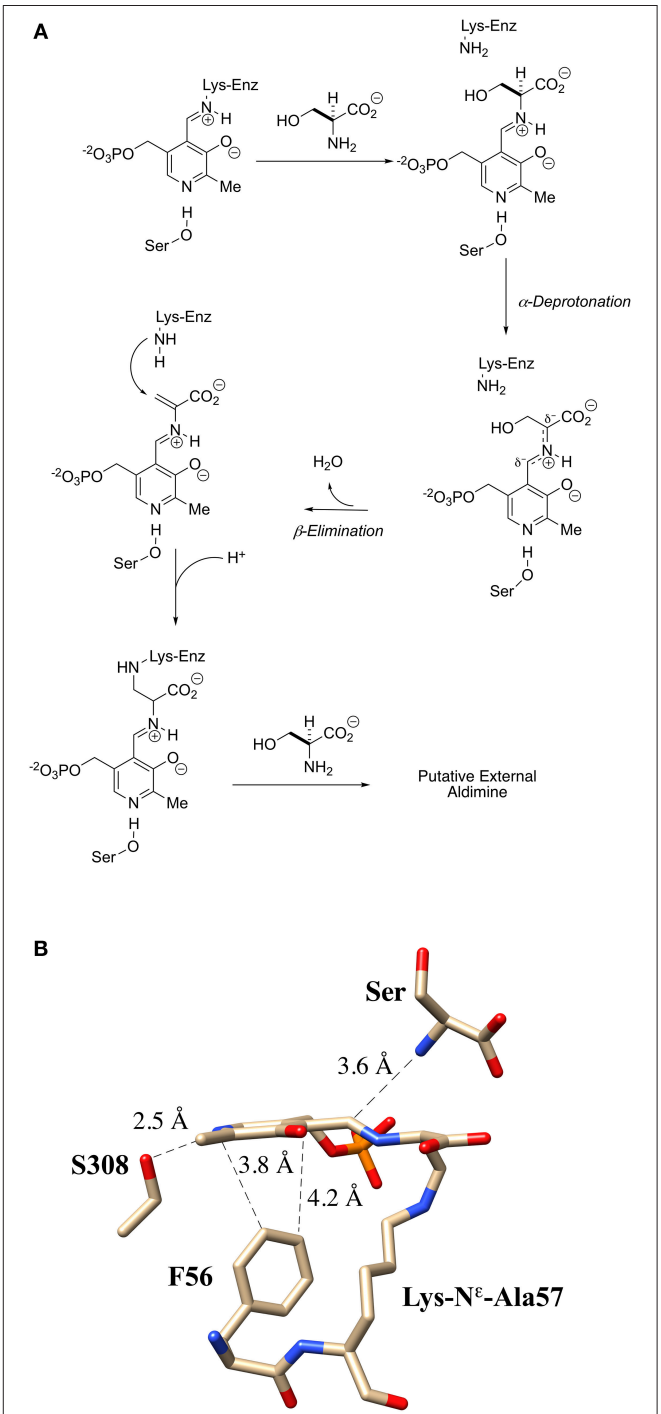

FIGURE 9 | Extender arm of the essential lysine observed by crystallography of *S. pombe* serine racemase. **(A)** Potential mechanism for formation of the extender arm. **(B)** Crystal structure of the internal aldimine with the "extended"-lysine residue (2ZR8).

Even though this modification places a carboxylate group alpha- to this active site "extended" lysine residue, the modified SpSR maintains 54% of the racemization activity and 68% of the β-elimination activity of the wt-enzyme. This self-catalyzed modification of the essential lysine of SpSR is proposed to occur by elimination of water from serine and the conjugate addition of the essential lysine into the resultant PLP-bound aminoacrylate intermediate (**Figure 9A**). In addition to revealing the three-dimensional structure of this modified enzyme, the crystal structure also appears to show a near attack conformation of an L-serine molecule approaching the lysino-D-alanyl-internal aldimine as would be expected for a transaldimination reaction of this modified enzyme (**Figure 9B**) (Goto et al., 2009). It remains to be seen if similar behavior will be observable in mammalian SR enzymes.

Residues Influencing Racemization vs. β-Elimination—"Triple Serine Loop"

As is illustrated schematically in **Figure 2**, one observes a bifurcating L-Ser racemization (giving D-Ser product) vs. L-Ser β-elimination (giving pyruvate product) mechanism for the enzyme serine racemase. This raises several important questions. (1) Is this observed bifurcating activity biologically relevant, incidental, or even an artefact of the assay conditions *in vitro*? (2) If the bifurcation is biologically relevant, what are the key biological determinants of whether hSR drives L-Ser substrate more toward D-Ser or pyruvate? (3) What are the key structural/mechanistic features along the reaction coordinate that control the "decision" to racemize or β-eliminate substrate?

As to the relevance of the *in vitro* assay, it is necessary to note here that hSR is known to be engaged in protein-protein interactions *in vivo* that may influence catalytic activity and/or bifurcation ratio. These include reported interactions with PICK 1 (protein interacting with C-kinase) (Hikida et al., 2008), GRIP (glutamate receptor interacting protein) (Baumgart et al., 2007), stargazin and PSD95 (postsynaptic D protein 95) (Ma et al., 2014) and DISC 1 (disrupted in schizophrenia) (Ma et al., 2013; Xia et al., 2016). That said, for now, in the absence of compelling evidence that the overall kinetic profile is significantly altered by such PPIs, we will proceed to analyze hSR performance by steady-state kinetic analysis in isolated enzyme assays, with both divalent cation and ATP present.

As we and others have noted (Strísovský et al., 2003; Nelson et al., 2017), under such conditions, wt-hSR favors the L-Ser

TABLE 2 | Kinetic indication that mutations of SR residues in the "triple serine loop" to the corresponding aspartate racemase (AR) residues tends to bias enzyme activity toward racemization over β-elimination.

Variant	Position				Racemization Efficiency‡	k_{cat}(rac): k_{cat} (β-elim)	K_{cat}/K_m(rac): k_{cat}/K_m (β-elim)	References
	152	153	154	155				
"Triple Ser Loop"- Relation to Racemase Activity								
hSR+	H	P	N	Q	100	1:4	1:3.7	Hoffman et al., 2009; Nelson et al., 2017; Canosa et al., 2018
mSR+	H	P	N	Q	133	1:2.3	1:1.3	Uda et al., 2016
mSR	S	P	N	Q	–	–	1:1.4	Foltyn et al., 2005
mSR	S	P	N	Q	121	***rac only****	***rac only****	Uda et al., 2017
mSR	H	S	N	Q	–	–	**4:1**	Foltyn et al., 2005
mSR	H	S	N	Q	1140	**23:1**	**2:1**	Uda et al., 2017
mSR	H	P	S	Q	174	**6.8:1**	**2.3:1**	Uda et al., 2017
mSR	H	P	N	D	46 (700)‡	**7:1**	**7.3:1**	Foltyn et al., 2005
mSR	S	S	N	Q	100	***rac only****	***rac only****	Uda et al., 2017
mSR	H	S	S	Q	376	**3.5:1**	1:12.8	Uda et al., 2017
mSR	S	P	S	Q	160	***rac only****	***rac only****	Uda et al., 2017
mSR	S	S	S	Q	55	***rac only****	***rac only****	Uda et al., 2017
spSR+	P	P	Y	D	**	1:29¶	1:26§	Yamauchi et al., 2009
amAR+	H	S	S	D	24	**8.6:1**	**2.2:1**	Uda et al., 2017
cgAR+	S	S	S	D	18	***rac only****	***rac only****	Uda et al., 2017

*Residue numbering corresponds to the hSR sequence. SR wild type residues are in **blue**; AR from Acropora millepora (amAR) and Crassostrea gigas (CgAR) are being compared here with the CgAR wild type residues appearing in **green**. Emboldened entries indicate enzymes for which racemization is preferred over elimination.*

+designates the wild type enzyme.

‡These values are normalized to the average value of k_{cat} for racemization for wt hSR (30 ± 15 min^{-1}) which is arbitrarily set at 100.

***no β-elimination activity observed.**

the value in parentheses here represents the value of kcat(rac) for this mutant normalized to the k_{cat}(rac) of the wt-mSR reported by these authors which is more than an order of magnitude lower than that determined by other groups.

***these authors report "V_{max} = 30 U/mg," but this is actually a specific activity reported for a standard assay @ [L-Ser] = 10 mM. Since this concentration is very close to K_m for L-Ser, the velocities reported are well-below V_{max}.*

¶this value represents relative velocity for racemization vs. elimination at 10 mM concentration rather than a ratio of kcat values.

§whereas the K_m values for these activities are reported, $k_{cat}(V_{max})$ values are not. For the latter, reported relative velocity for racemization vs. elimination at 10 mM concentration is given.

β-elimination reaction over the racemization reaction by a 4-5:1 ratio. It may be that the β-elimination reaction serves as a sort of "bleed valve," potentially allowing local stores of L-Ser to be diverted to pyruvate and away from D-Ser as a mechanism for muting D-Ser signaling. This could be a sort of secondary checkpoint, providing a mechanism for managing steady-state L-Ser levels in the neuron, beyond the control that is exercised by the rate at which L-Ser is produced from 3-phosphoglycerate in the astroglia and shuttled to the neuron (**Figure 1**) (Ishiwata et al., 2015; Wolosker et al., 2016). There has been a related discussion on hSR-mediated D-Ser β-elimination being a mechanism for controlling D-Ser concentrations in the neuron (Foltyn et al., 2005; Wolosker, 2011).

As is illustrated in **Figure 2**, probably the most streamlined mechanism for this bifurcation would involve initial external aldimine formation of L-Ser, followed by *si*-face deprotonation by K56 to a common "carbanionic" or quinonoid intermediate. As has been discussed earlier, elegant model studies by the Toney group (Griswold and Toney, 2011) suggest that neither racemase nor eliminase activity requires a completely delocalized quinonoid intermediate. These observations are consistent with the observation of "carbanionic intermediates" for the β-eliminase/replacement enzymes, CBS (Koutmos et al., 2010) and tryptophan synthase (Caulkins et al., 2016), as noted in **Figure 7**. As is shown in **Figure 2**, a likely decision point for bifurcation would then occur at the protonation step, with *re*-face protonation, presumably by Ser84, leading to racemization and with OH-leaving group (LG) protonation leading to β-elimination.

Both the groups of Toney (Foltyn et al., 2005) and of Uda et al. (2016, 2017) have reported studies demonstrating that mutation of targeted residues can profoundly influence the racemization to β-elimination ratio in hSR and homologs. These results are summarized in detail in **Table 2**. Uda et al. performed a detailed phylogenetic analysis of the serine/aspartate racemase family and deduced that a so-called "triple serine loop" (**Figure 10**; named after the wt-AR sequence) may be critical for racemization function as residues here appear to correlate with a likely evolution from SR to AR activity.

Specifically, L-THA dehydratases and SRs from lower order organisms possess a loop region from amino acids P150, P151, and Y152. Tracing this loop region up the phylogenetic tree demonstrates that these residues change to H152, P153, and N154 in human and mouse SR. On the other hand, in aspartate racemases, these residues morph into a "triple serine loop" of sequence S150, S151, S152 as for example in the AR from *Crassostrea gigas* (CgAR) (Uda et al., 2016). For CgAR, this SSS motif appears to dictate the substrate preference for L-Asp over L-Ser. Wild-type *S. pombe* SR with the PPY sequence exhibits no AR activity, and mouse SR with the MPN sequence shows limited AR activity with $k_{cat}/K_m \sim 65\ mM^{-1}min^{-1}$ (Bodhinathan et al., 2010; Uda et al., 2016).

A secondary finding from these studies is that, in general, introducing residues representing the AR sequence into this loop in hSR tends to favor L-Ser racemization, by a combination of suppressing L-Ser β-elimination (or dehydrase activity) and promoting L-Ser racemization. Uda and co-workers cloned nearly a dozen SR and aspartate racemase enzymes and studied both the wt- and mutant versions of these enzymes kinetically. Earlier, the Toney group had also looked at SR mutants in this loop. From **Table 2,** it can be seen that installation of S residues at positions 153 and 154 in hSR in particular tends to increase the k_{cat} for racemization. Introduction of S into position 152 in mSR significantly decreases L-Ser β-elimination activity; a similar effect appears to result from introduction of a D residue into position 155.

Most importantly, these studies demonstrate that for hSR, the bifurcation ratio is controlled significantly at the level of the primary sequence, with particular sensitivity to modifications in this "triple serine loop." **Figure 10** illustrates that this loop is located just above the PLP-imine functionality in the external aldimine crystal structures for both the SR and AR enzymes. To understand the molecular basis for how specific mutants morph hSR activity from the native predilection for β-elimination to a preference for racemization, the tools of structural biology could be of great value.

DISCUSSION AND CONCLUDING REMARKS

From an evolutionary standpoint, it appears that serine racemase activity may have evolved from L-*threo*-hydroxyaspartate (L-THA) eliminase activity and may also have served as the

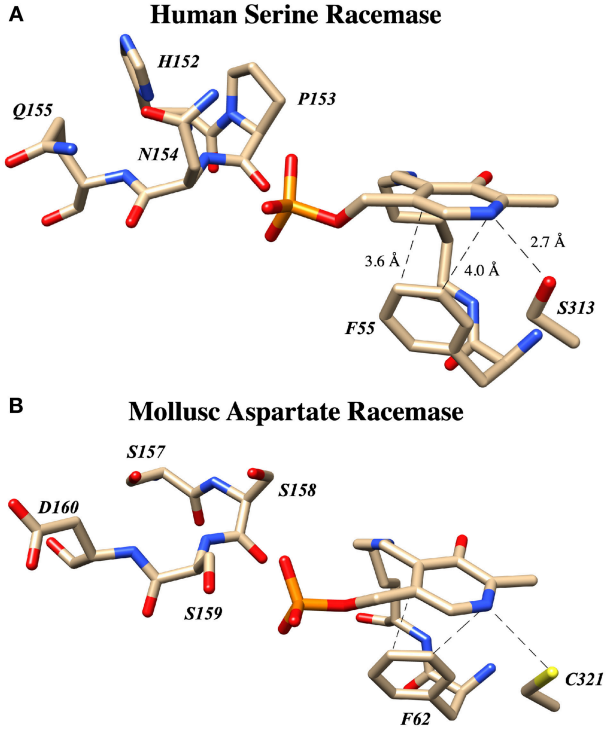

FIGURE 10 | The "Triple serine loop" in hSR and *Anadara broughtonii* (AbAR) is situated near the presumed locus of the external aldimine and the carbanionic intermediate derived therefrom. **(A)** Crystal structure of human serine racemase (3L6B) loop region containing H152, P153, N154, and Q155. **(B)** Corresponding region of aspartate racemase (5YBW) with residues S157, S158, S159, D160.

evolutionary precursor to aspartate racemase (AR). Consistent with this view, both the *re*-face base S84 and R135 in the human isoform are highly conserved across the SR family, and these residues are also conserved among enzymes demonstrating L-THA dehydratase activity (Nelson et al., 2017). Additionally, modifications in the loop region corresponding to positions 152–155 in hSR, from HPNQ to SSSD, appear to have been primary determinants in the evolution of AR function from SR function. Interestingly, a smaller subset of modifications here, specifically the P153S and N154S mutations, seem to confer a much greater L-Ser racemization bias into hSR, over competing L-Ser β-elimination activity, than is seen in the native enzyme. This observation suggests that there may be an advantage to maintaining β-eliminase activity in the native enzyme, perhaps as an additional control point for L-Ser homeostasis.

As has been discussed herein, the core of the hSR enzyme features an archetypical PLP binding site; this includes the active site lysine (K56) covalently engaging the cofactor, the tetraglycine loop binding the phosphate moiety (**Figure 5**), and both an H-bond donor (S313) in the ring plane engaging the pyridine nitrogen and an edge-to-face π-π interacting partner residue (F55) orthogonal to the ring plane (**Figure 6**). In the case of the SpSR enzyme, at least, that core can apparently be modified via an unusual β-elimination-K56/conjugate addition sequence, leading to an apparent lysino-D-alanyl extender arm version of the active site (**Figure 9**). Surrounding this PLP binding pocket are key catalytic residues, including K56, which doubles as the *si*-face base; S84, which serves as the *re*-face base for the racemization; and R135, which appears to help position negatively charged substrates, such L-SOS and L-THA, for elimination (**Figure 8**), and which can be exploited for inhibitor binding, as is seen in the binding motif for malonate. The acidity of S84 may be modulated by K114 via an H-bonding network through a bound water molecule (Nelson et al., 2017).

Activity of the hSR enzyme is stimulated by both divalent cations and ATP, with the latter likely operating via an allosteric mechanism associated with a conformational change upon nucleotide binding that depends upon interactions with Q89 (**Figure 4**). Catalytic activity of the enzyme follows a bifurcated pathway from L-Ser to either D-Ser (racemization) or pyruvate (β-elimination), likely through a common "carbanionic intermediate," the molecular nature and charge distribution of which is yet to be established (**Figure 2**). Whereas, wt-hSR favors the β-elimination reaction over the racemization reaction, this preference can be inverted through specific mutations in the hSR152-155 loop region ("triple serine loop" in AR;

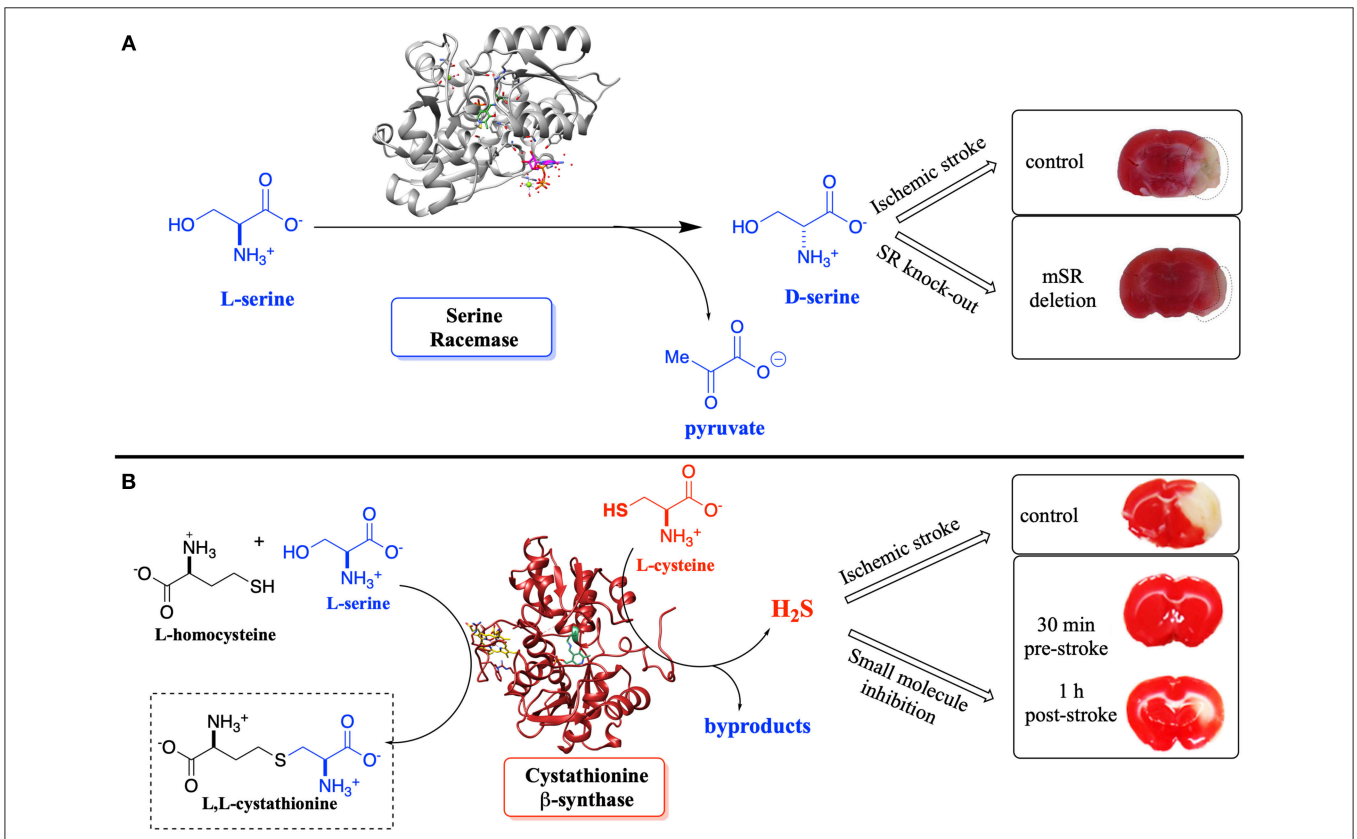

FIGURE 11 | (A) Effect of D-Ser upon infarction volume post-ischemic stroke induced by transient middle cerebral arterial occlusion (tMCAO)–SR-knockout mice vs. control. **(B)** Effect of H_2S upon infarction volume–inhibition of CBS vs. control; pre- and post-tMCAO. Adapted from the original articles by permission of the Society for Neuroscience (Mustafa et al., 2010) (image in **A**) and the American Chemical Society (Toney, 2005; McCune et al., 2016) (image in **B**; https://pubs.acs.org/doi/abs/10.1021/acscentsci.6b00019), respectively.

Table 2 and **Figure 10**). Elucidation of the molecular basis of this reaction pathway tuning will likely require more precise structural biological studies of appropriate (mutant) enzyme-substrate combinations in the future.

Such detailed studies of hSR structure/function relationships are critical given the importance of the enzyme in neuronal signaling via the NMDAR, in neuronal infarction pathways, and potentially in the etiology of neurodegenerative disease. Note that D-Ser and H_2S, a product of CBS, another PLP-enzyme that controls neuronal signaling, are thought to stimulate the NMDAR (**Figure 1**) (Kimura, 2000, 2015; Kimura et al., 2013). Both D-Ser and H_2S are thought to be elevated pursuant to ischemic stroke, and model studies in a tMCAO (transient middle cerebral arterial occlusion) rat stroke model suggest that both hSR (Mustafa et al., 2010) and hCBS (McCune et al., 2016) may be potential targets for inhibition to mitigate against neuronal infarction in ischemic stroke (**Figure 11**).

In a recent cell biology-based study on apoptosis, HEK 293T cells expressing the Q155D-hSR mutant (favoring L-Ser-to-D-Ser racemization over β-elimination) demonstrated a reduced rate of cell death when apoptotic agent staurosporine was introduced, indicating that the racemization reaction (i.e., D-Ser) may have a protective role against apoptosis (Talukdar et al., 2017). While these results are compelling, they also call out as a challenge to chemists the need to develop selective small molecule modulators of hSR that either inhibit or stimulate the enzyme or that modulate the inherent β-eliminase to racemase preference of the enzyme. This serves as motivation in our own laboratory to develop reaction-specific PLP enzyme inhibitors based upon mechanistic understanding (Berkowitz et al., 1994, 1996, 2004, 2008; Berkowitz and Smith, 1996; Karukurichi et al., 2007; McCune et al., 2016, 2017; Tu et al., 2018). If such hSR inhibitors/modulators can be developed, they will serve as tools for chemical biology, and potentially as leads for medicinal chemistry in the effort to understand hSR function in the context of neuronal signaling and D-serine neurobiology.

DEDICATION

We wish to dedicate this article to Christopher T. Walsh on the 40th anniversary of his seminal treatise on enzymatic reaction mechanisms.

DATA AVAILABILITY

Publicly available datasets were analyzed in this study. This data can be found here: https://www.rcsb.org/pdb/home/sitemap.do.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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The Synthesis of Kynurenic Acid in Mammals: An Updated Kynurenine Aminotransferase Structural KATalogue

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Kynurenic acid (KYNA) is a bioactive compound that is produced along the kynurenine pathway (KP) during tryptophan degradation. In a few decades, KYNA shifted from being regarded a poorly characterized by-product of the KP to being considered a main player in many aspects of mammalian physiology, including the control of glutamatergic and cholinergic synaptic transmission, and the coordination of immunomodulation. The renewed attention being paid to the study of KYNA homeostasis is justified by the discovery of selective and potent inhibitors of kynurenine aminotransferase II, which is considered the main enzyme responsible for KYNA synthesis in the mammalian brain. Since abnormally high KYNA levels in the central nervous system have been associated with schizophrenia and cognitive impairment, these inhibitors promise the development of novel anti-psychotic and pro-cognitive drugs. Here, we summarize the currently available structural information on human and rodent kynurenine aminotransferases (KATs) as the result of global efforts aimed at describing the full complement of mammalian isozymes. These studies highlight peculiar features of KATs that can be exploited for the development of isozyme-specific inhibitors. Together with the optimization of biochemical assays to measure individual KAT activities in complex samples, this wealth of knowledge will continue to foster the identification and rational design of brain penetrant small molecules to attenuate KYNA synthesis, i.e., molecules capable of lowering KYNA levels without exposing the brain to the harmful withdrawal of KYNA-dependent neuroprotective actions.

Keywords: kynurenine pathway, kynurenic acid, kynurenine aminotransferase, PLP enzyme, crystal structure

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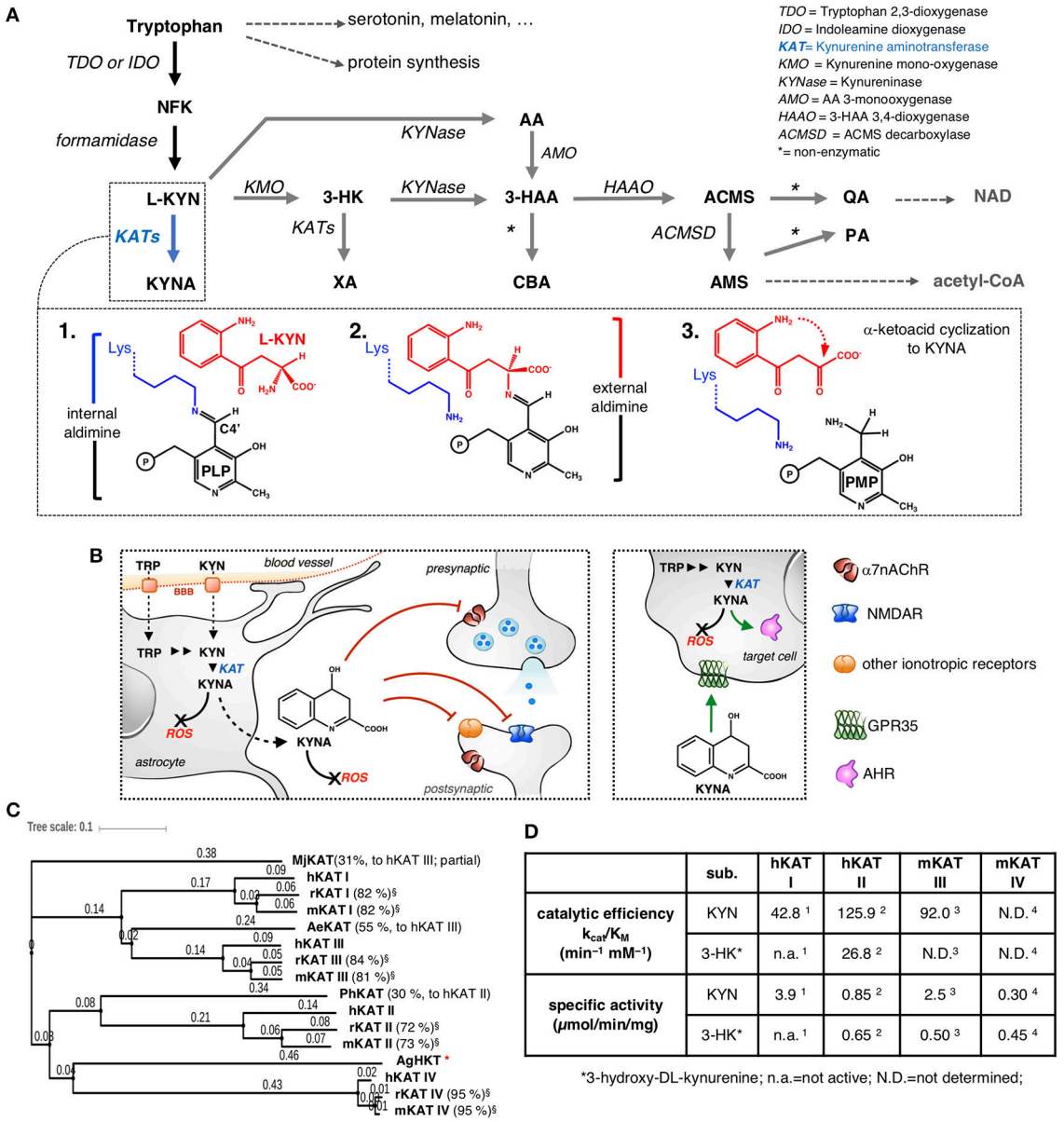
INTRODUCTION

In mammals, approximately 95% of the essential amino acid L-tryptophan that is not used for protein synthesis is metabolized through the kynurenine pathway (KP). Research interests in the KP find a unifying rationale in the ever-increasing demonstrations that the majority of the compounds formed along the pathway play a role in modulating fundamental aspects of biology (Schwarcz et al., 2012; Stone et al., 2013; Schwarcz and Stone, 2017). The KP is a complex catabolic cascade consisting of a multi-step main branch and several lateral arms (**Figure 1A**). The relative abundance of the molecules generated through the KP in peripheral tissues and inside the central nervous system (CNS) is thought to be governed at different interdependent levels (Badawy, 2017).



	sub.	hKAT I	hKAT II	mKAT III	mKAT IV
catalytic efficiency k_{cat}/K_M (min^{-1} mM^{-1})	KYN	42.8 [1]	125.9 [2]	92.0 [3]	N.D. [4]
	3-HK*	n.a. [1]	26.8 [2]	N.D.[3]	N.D. [4]
specific activity (μmol/min/mg)	KYN	3.9 [1]	0.85 [2]	2.5 [3]	0.30 [4]
	3-HK*	n.a. [1]	0.65 [2]	0.50 [3]	0.45 [4]

*3-hydroxy-DL-kynurenine; n.a.=not active; N.D.=not determined;

FIGURE 1 | The kynurenine pathway with a focus on KYNA synthesis. **(A)** NFK, N-formylkynurenine; L-KYN, L-kynurenine; KYNA, kynurenic acid; 3-HK, 3-hydroxykynurenine; XA, xanthurenic acid; AA, anthranilic acid; 3-HAA, 3-hydroxyanthranilic acid; ACMS, 2-amino-3-carboxymuconate-6-semialdehyde; AMS, 2-aminomuconate-6-semialdehyde; PA, picolinic acid; CBA, cinnabarinic acid; QA, quinolinic acid; NAD, nicotinamide adenine dinucleotide. *Inset:* **1.** At the beginning of the transamination reaction, the catalytic lysine of KAT is bound to the PLP molecule. **2.** The α-amino group of L-KYN substitutes for Lys and binds to the cofactor, forming an external aldimine intermediate. **3.** At the end of the first half-reaction, the resulting α-keto acid undergoes an intramolecular condensation reaction, releasing the final product, KYNA, and leaving the cofactor in the pyridoxamine phosphate (PMP) form, then, an α-keto acid is required to bring the enzyme back to its initial state through a series of equivalent reversed reactions (not shown). **(B)** The multiplicity of KYNA actions inside and outside the CNS. KYNA acts as an antagonist of specific receptors both at the synapses and at extra-synaptic sites (only selected targets are shown); KYNA possesses immunomodulatory properties mainly by behaving as an agonist/ligand of GPR35 and AHR. KYNA participates in the direct scavenging of ROS; BBB, blood-brain barrier. **(C)** Analysis of protein sequence similarity among different KATs, including representatives of non-mammalian KATs (Mj, *Methanocaldococcus jannaschii*; Ph, *Pyrococcus horikoshii*; Ae, *Aedes aegypti*; Ag, *Anopheles gambiae*; HKT, 3-hydroxykynurenine aminotransferase). The values in brackets refer to the percentage sequence identity to the corresponding human isoenzyme; *no significant sequence identity. The phylogenetic tree was generated by iTOL (Letunic and Bork, 2016). **(D)** Comparison of selected kinetic constants for the transformation of KYN to KYNA and 3-HK to XA catalyzed by the indicated KATs; (Han et al., 2004, 2008a, 2009a, 2010a).

The whole complement of enzymes operating in the KP is present in the CNS, where the bioactive kynurenines produce multiple and often antithetical effects, including control of synaptic transmission, hyper-stimulation or hypo-functioning of receptor-mediated signaling, and direct excitotoxicity or neuroprotection (Ruddick et al., 2006). In particular, kynurenic

acid (KYNA) is considered a "Janus-faced" compound in brain physiology (Rózsa et al., 2008; Wirthgen et al., 2018). In fact, KYNA limits the neurotoxicity associated with the action of excitatory amino acids (Birch et al., 1988) by acting as an endogenous antagonist at the glycine co-agonist site of the *N*-methyl-D-aspartate receptor (NMDAR). In addition, KYNA non-competitively antagonizes the α7-nicotinic acetylcholine receptor (α7nAChR) (Alkondon et al., 2004; Albuquerque and Schwarcz, 2013), modulating important neurophysiological processes. Moreover, KYNA plays a role as a direct reactive oxygen species (ROS) scavenger. On the other hand, abnormally high KYNA levels have been detected in biological samples from patients with schizophrenia (Erhardt et al., 2007; Sathyasaikumar et al., 2011), and the pharmacological elevation of KYNA concentrations in the CNS correlates with cognitive deficits (Chess et al., 2007). These observations point to KYNA as an important player in the onset and progression of neurological and psychiatric diseases that are associated with impaired glutamatergic and/or cholinergic neurotransmission (Stone and Darlington, 2013; Fujigaki et al., 2017). Moreover, KYNA is an agonist of the broadly expressed G protein-coupled receptor 35 (GPR35) (Wang et al., 2006) and the aryl hydrocarbon receptor (AhR) (DiNatale et al., 2010), which are involved in immunomodulation processes (Wirthgen et al., 2018). Taken as a whole, these studies disclose the multiplicity of biological actions of KYNA inside and outside the CNS (**Figure 1B**).

KYNA cannot easily cross the blood-brain barrier. For this reason, the local concentration of the compound is thought to mainly depend on (i) the absolute availability of tryptophan and/or L-kynurenine (KYN), (ii) the competition that exists between the kynurenine monooxygenase/kynureninase-dependent branches of the catabolic KP cascade (gray arrows in **Figure 1A**) and the direct transformation of KYN to KYNA, and (iii) the activity of KYNA-synthesizing aminotransferases (Schwarcz et al., 2012). KATs catalyze the irreversible transamination of KYN to KYNA and 3-hydroxykynurenine (3-HK) to xanthurenic acid (XA), thus controlling the routing of KYN and 3-HK to their corresponding branches of the KP (blue arrows in **Figure 1A**). KAT-dependent catalysis proceeds through a pyridoxal-5'-phosphate (PLP)-dependent transamination reaction, which has been studied in great detail (Bellocchi et al., 2009). More recently, a pathway for the non-enzymatic production of KYNA has been described and shown to be based on the spontaneous oxidation of KYN (Blanco Ayala et al., 2015). At present, information about the contribution of this mechanism to overall KYNA synthesis is scarce, although this pathway could represent a significant source of the molecule under specific circumstances (Ramos-Chávez et al., 2018). Inside the KP, the conversion of KYN to KYNA is the reaction for which the highest number of isozymes have been identified and arbitrarily named kynurenine aminotransferases (KATs). Human and rodent genomes encode four aminotransferases that have been demonstrated to be capable of using KYN as an amino group donor during the first half-reaction (**Figure 1A**, inset): KAT I (also known as glutamine transaminase K or cysteine conjugate beta-lyase), KAT II (also known as α-aminoadipate aminotransferase), KAT III (also known as glutamine transaminase L), and KAT IV (the mitochondrial aspartate aminotransferase). The interspecies comparison of each KAT reveals a high degree of primary sequence conservation (Yu et al., 2006), with KAT IV representing the most phylogenetically conserved among the four mammalian isozymes (**Figure 1C**). Conversely, the alignment of the primary sequences of the four KATs from a single species shows that KAT II and KAT IV are the most divergent isozymes, most likely as a consequence of their peculiar features and the different roles played by the N-terminal regions of the two proteins (Han et al., 2010b). Here, we will refer to human, mouse and rat KATs as hKATs, mKATs, and rKATs, respectively. As reviewed in great detail elsewhere (Han et al., 2010a,b; Passera et al., 2011), mammalian KATs are differentially expressed during ontogenesis and display peculiar tissue distribution profiles and sensitivity to the action of endogenous and exogenous modulators. In particular, although the four isozymes possess overlapping biochemical properties, they differ considerably in terms of substrate specificity as well as specific activity and catalytic efficiency toward KYN and 3-HK (**Figure 1D**). However, it must be noted that a full interspecies comparison of the catalytic properties and enzyme kinetics of KATs is precluded in part by the lack of uniform biochemical assay conditions for the characterization of the individual isozymes.

MAMMALIAN KAT STRUCTURES: *LEITMOTIV* AND UNIQUE TRAITS

The crystal structures of hKAT I (Rossi et al., 2004; Han et al., 2009b; Nadvi et al., 2017), hKAT II (Han et al., 2008a,b; Rossi et al., 2008a, 2010; Dounay et al., 2012, 2013; Tuttle et al., 2012; Nematollahi et al., 2016a), mKAT III (Han et al., 2009a; Wlodawer et al., 2018), and mKAT IV (Han et al., 2011) in their holo-forms, in different ligand-bound states and in complex with inhibitors, enormously expanded the ability to identify the structural determinants that are the basis for the common features and unique traits displayed by each KAT. More recently, the structure of the apo-form of mature human mitochondrial aspartate aminotransferase was solved (Jiang et al., 2016), however, considering the high percentage of sequence identity between human and mouse KAT IV (95%) and their structural conservation (root mean square deviation = 0.49 Å), only the murine isozyme will be discussed.

All of the KATs that have been studied thus far form homodimers both in solution and in their crystalline state (**Figure 2A**). This dimerization is required to build up two identical catalytic cavities, each hosting a co-factor molecule, that are located at the interdomain interface in each subunit and at the intersubunit interface in the dimer. Across-species comparison of KAT structures reveals the high degree of conservation of the monomer architecture, which consists of an N-terminal arm, a small domain and a large domain. As a general rule, the N-terminal arm is a crucial element in aminotransferases; it participates in the proper assembly of the functional dimer, controls enzyme subcellular localization, and regulates substrate access to the active site. The globular domains

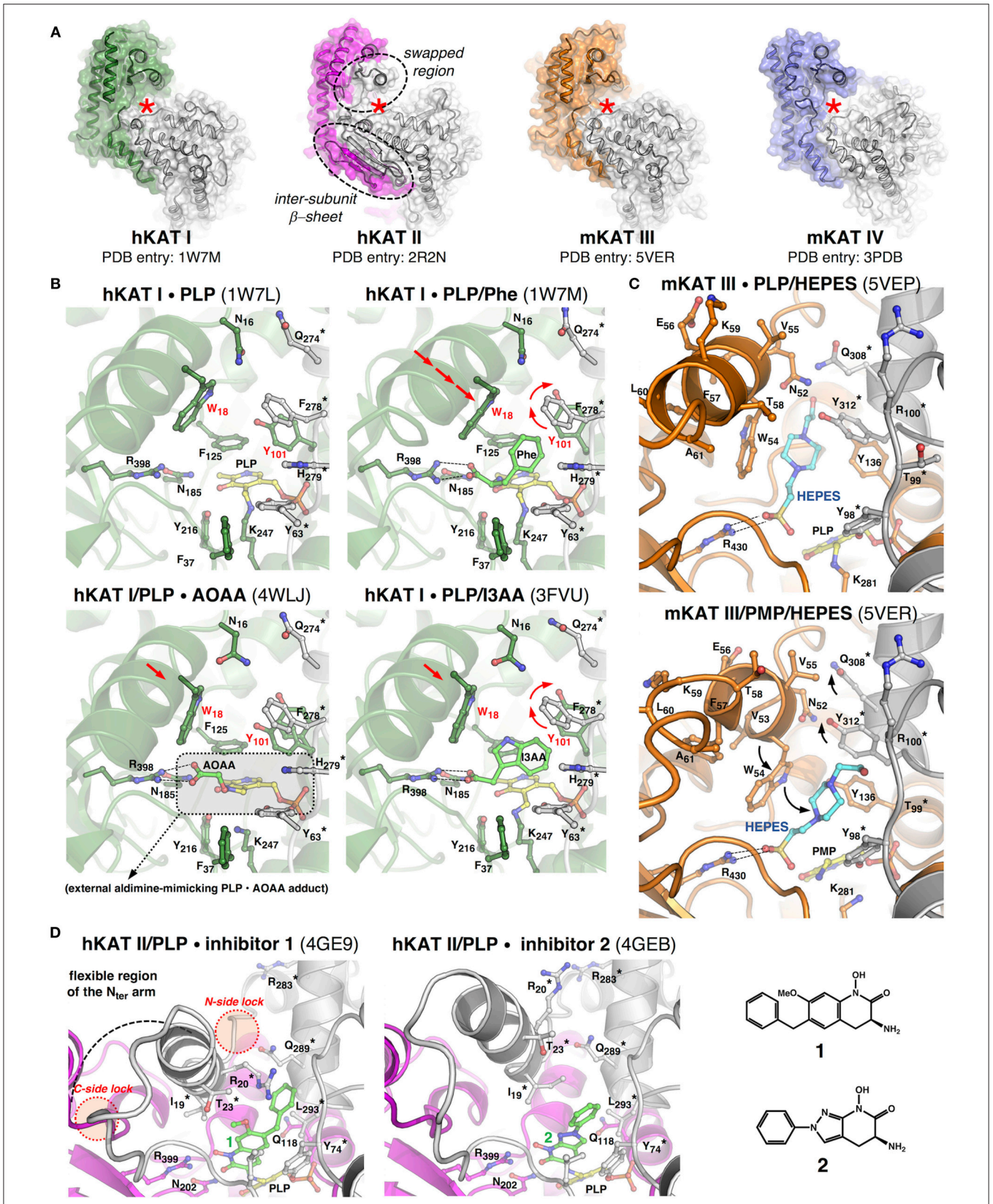


FIGURE 2 |

(Continued)

FIGURE 2 | Structural features and properties of mammalian KATs. **(A)** In each KAT dimer, the "A" subunit appears in color, the "B" subunit appears in gray, and the red star labels one of the two identical active sites, the dotted circles frame the peculiar structural features of hKAT II. **(B)** Close-ups of the catalytic cavity of hKAT I in different ligand-bound states. **(C)** The active site of mKAT III in complex with HEPES, which adopts two alternative conformations. **(D)** Zoomed views of the hKAT II active site in complex with two irreversible inhibitors (**1** and **2**). In each image, the protein backbone is depicted as a cartoon, the selected residue side chain is depicted as a ball-and-stick model, the asterisk labels residues belonging to one subunit of the dimer, and the arrows indicate the major rearrangements discussed in the text. The PDB codes appear in brackets. As a matter of clarity, the images, which correspond to optimally superimposed structures, are presented side by side. The figures have been generated by PyMol (www.pymol.org).

host the majority of the residues that are required for PLP co-factor binding and reactivity, and shaping the ligand binding cavity (Jansonius, 1998). The comparison of representatives of each KAT isozyme reveals peculiar features that characterize the ligand binding site architecture, the mode of dimer assembly, and remarkable differences in the conformational changes that accompany catalysis.

The structures of hKAT I in its PLP-bound pre-reaction state and in complex with PMP at the end of the first half-reaction (PDB entries: 1W7L and 1W7N, respectively) (Rossi et al., 2004) show that catalysis does not significantly alter the overall positioning of the cofactor inside the catalytic cavity, and this holds true for all KATs. Information about ligand binding can be obtained by comparing the structures of hKAT I in complex with phenylalanine (an amino group donor substrate) (PDB entry: 1W7M) (Rossi et al., 2004) or with the inhibitors indole-3-acetic acid (I3AA; PDB entry: 3FVU) (Han et al., 2009b) and 3-amino-oxyacetic acid (AOAA; PDB entry: 4WLJ) (Nadvi et al., 2017; **Figure 2B**). In the latter structure, a covalent PLP-AOAA adduct is observed, representing a snapshot along the catalytic cycle that catches the cofactor in a state mimicking an external aldimine. These analyses highlight the role of the coordinated action of the strictly conserved arginine and asparagine residues in fixing the α-carboxylic group of the substrate (or any structurally equivalent group from other ligands), which leads to the correct positioning of the α-amino group in close proximity to the PLP C4' catalytic center. Moreover, when a substrate or inhibitor molecule occupies the active site, the Tyr101 side chain undergoes a drastic repositioning to make room for the benzyl or indole group, respectively, while the presence of glycerol or tris(hydroxymethyl)aminomethane molecules in the active site (PDB entries: 3FVS; 3FVX) does not promote such a rearrangement (Han et al., 2009b). These studies confirm Trp18 in the N-terminal arm as a key residue for substrate/inhibitor binding to the active site. In fact, by approaching the ligand molecule, Trp18 completes the formation of the narrow, mainly aromatic ligand binding cavity and constrains sliding of the short region of the N-terminal arm (residues 14–34), which shields the active site during catalysis. Interestingly, in the structure of hKAT I in complex with I3AA, two ligand molecules (I3AA and glycerol) are simultaneously present in each active site. This observation allowed the authors to propose a possible pathway acting as the exit for KYNA at the end of the first half-reaction and/or for the incoming α-ketoacid that initiates the second half-reaction (Han et al., 2009b), shedding light on a still elusive aspect of KAT-mediated catalysis, that is, the series of events that brings the enzyme back to its starting state, ready for a new catalytic cycle.

As expected from the high sequence identity between hKAT I and mKAT III (49.8%) (Yu et al., 2006), comparison of their crystal structures confirmed that the two isozymes share the same fold and a similar mode of subunit assembly in the form of a functional dimer, while they differ in a few residues that build up the walls of the ligand-binding cavity. These peculiarities could represent the basis for the different substrate specificities and catalytic properties displayed by the two isozymes (Han et al., 2009a). Most recently, the original crystal structures describing mKAT III in PLP-bound form (original PDB entry: 3E2F), in complex either with L-KYN and PMP (original PDB entry: 3E2Z), or glutamine and PMP (original PDB entry: 3E2Y) (Han et al., 2009a) underwent a re-refinement process (Wlodawer et al., 2018). This new analysis suggests that the electron density signals initially assigned to two glycerol molecules, or to a molecule of KYN or glutamine non-covalently bound at the active site, should instead be interpreted as HEPES molecules, which were present in the crystallization buffer (Han et al., 2018). Notably, the observation of HEPES molecules in the re-refined structures highlights the possibility for the ligand to lay in two alternative sub-cavities in the ligand binding site (**Figure 2C**). In the PLP-bound holo-form of mKAT III (re-refined PDB entry: 5VEP), the ligand molecule occupies a narrow, elongated pocket underneath the region of the N-terminal arm spanning from Asp50-Trp54. In both the PMP-bound structures (re-refined PDB entries: 5VEQ and 5VER), the HEPES molecule appears to be rotated 45° toward the previously identified "canonical" ligand-binding cavity, keeping the sulfate group in contact with Arg430. The shift between the two ligand conformations seems to be facilitated by repositioning of the Trp54 side chain. Although serendipitously discovered, this information could be used for the better understanding of the mechanistic aspects and molecular dynamics of KAT-mediated catalysis.

The crystal structure of mKAT IV has been solved in its PLP-bound form (PDB entry: 3HLM), in complex with the amino group donor/substrate kynurenine, or with the amino group acceptor/co-substrate oxaloacetate (PDB entries: 3PD6 and 3PDB, respectively) (Han et al., 2011). These structures reveal an overall architecture that closely resembles the one described for mitochondrial aspartate aminotransferases from other species (Han et al., 2010b). Similarly, substrate binding does not appear to be accompanied by a significant reshaping of the catalytic cavity; instead, each monomer switches from an "open" to a "closed" form as a consequence of rotation of the small domain toward the large domain (Ford et al., 1980; McPhalen et al., 1992).

From a structural standpoint, KAT II is a "maverick" among the four KATs, and its uniqueness is made clear by comparing the numerous crystal structures of hKAT II in different ligand-bound

states that are currently present in the Protein Data Bank. This wealth of information is the result of recent efforts aimed at the selection and/or optimization of small molecules acting as KYNA synthesis attenuators, that is, molecules capable of lowering KYNA levels in pathological conditions without completely suppressing KYNA-dependent neuroprotective actions. Since KAT II is thought to be the main factor responsible for KYNA synthesis in the mammalian brain (Guidetti et al., 1997; Schwarcz et al., 2012; Chang et al., 2018), and considering that the peculiar KAT II characteristics make it unique among the functionally validated KATs (see below), the rational design of isozyme-specific inhibitors appeared feasible and indeed was successful [comprehensive reviews on progress in KP pharmacological manipulation and in the development of KAT inhibitors appear in Dounay et al. (2015), Zádori et al. (2016), and Nematollahi et al. (2016b)].

The most striking feature of the hKAT II structure is the swapped conformation adopted by a discrete region of the N-terminal arm of each subunit (residues 16–39) (**Figure 2D**). This region acts as a dynamic element that simultaneously shields and shapes the ligand binding cavity of the other monomer in the dimer to an extent that depends on the nature of the bound molecule. The functional role of the intrinsic conformational plasticity displayed by the swapped region of hKAT II can be fully appreciated upon superposition of the entire panel of protein crystal structures -in complex with PLP (PDB entries: 2VGZ, 2QRL, 5EUN) (Han et al., 2008b; Rossi et al., 2008a; Nematollahi et al., 2016a), substrates (PDB entries: 3DC1, 2R2N) (Han et al., 2008a,b) or inhibitors (PDB entries: 2XH1, 3UE8, 4GE4, 4GE7, 4GE9, 4GEB) (Rossi et al., 2010; Dounay et al., 2012, 2013; Tuttle et al., 2012). As observed in the other KAT structures, anchoring of the α-carboxylic group of the physiological substrate (or of any structurally equivalent group from the inhibitor molecule) is invariably provided by Arg399 regardless of whether there is a covalent bond between the ligand and cofactor. The noteworthy broad ligand binding potential of hKAT II seems to rely on the chemical nature of the residues lining the catalytic cavity and, most importantly, the possibility to modulate the available space and protein/ligand bonding networks, which largely depends on the dynamics of the swapped region. This is clearly exemplified by the evolution of compounds **1** and **2** in **Figure 2D**, which are the most potent isozyme-specific brain penetrant inhibitors of hKAT II currently available (Tuttle et al., 2012; Dounay et al., 2013).

The structural information on KATs summarized here strengthens the initially hypothesized fundamental role of a discrete region of the N-terminal arm of KATs in controlling substrate/inhibitor access into and binding inside the active site (Rossi et al., 2008b). In hKAT I and mKAT III, the equivalent regions slide toward the preformed catalytic cavities as rigid bodies, which places the invariant Trp18 or Trp54 residues in an optimal position to entrap the ligand. In hKAT II, the same region is characterized by a higher degree of conformational freedom, which is apparently required to better adapt to a broader array of ligands that differ in size, chemical nature and steric hindrance, thus resulting in a more pronounced reshaping of the active site. However, both situations highlight the need for anchoring points at both sides of the region of the N-terminal arm that moves (**Figure 2D**) to limit its unrestrained flexibility. Notably, a *kat1* gene variant isolated from spontaneously hypertensive rats (SHR) is characterized by a missense mutation that leads to a Glu-to-Gly amino acid substitution at the C-terminal side of the sliding α-helix in the KAT I N-terminal arm (Kwok et al., 2002). The elimination of this "distal lock" could hamper proper active site shielding upon substrate binding and ultimately translate into suboptimal catalysis.

FUTURE DIRECTIONS FOR KAT STRUCTURAL INVESTIGATIONS

The analysis of KYNA synthesis from an integrated structural biology/neuropharmacology standpoint will drive the lead optimization process aimed at further improving the pharmacodynamics, bioavailability and specificity of action for the most potent hKAT II inhibitors developed thus far, as a prerequisite to their safe use in pathological situations characterized by abnormally elevated brain KYNA levels. However, due to the potential adverse effects associated with the irreversible inhibition of KATs, the future challenge for the field is the identification and/or the structure-based design of molecules capable of reversibly associating with specific KAT targets (Nematollahi et al., 2016b). Among the different compounds that behave as competitive KAT ligands, one potential source of such innovative hKAT II-specific reversible inhibitors is a recently described group of sulfated oestrogens and their derivatives (Jayawickrama et al., 2017, 2018).

At the same time, by revealing the determinants of substrate specificity in specific KAT isozymes, a combined structural biology/biochemistry approach could provide new small molecule tools to better characterize the interplay between the alternative branches of the KP in physiological conditions and in human diseases. A paradigmatic example of the applicability of such an approach is its recent use to study the roles of XA in the CNS. In light of the pro-apoptotic and neurotoxic potential associated with 3-HK (Okuda et al., 1998), it is somewhat surprising that the KAT-dependent transformation of 3-HK to XA had not been analyzed at the same level of detail as the transamination of KYN to KYNA, although the label "KAT" explicitly tags the XA-producing reaction in schematic representations of the KP. Once considered a mere by-product of the KP, research on XA came back in focus only recently following the description of its roles in invertebrate biology (Savvateeva et al., 2000; Cerstiaens et al., 2003). In most insect species, the central KP branch abruptly ends with the synthesis of the free radical generator 3-HK. The production of XA is therefore a way to protect the organism from the accumulation of 3-HK when it is not required for the biosynthesis of eye and body pigments or tissue remodeling during (neuro)metamorphosis (Han et al., 2007). Notably, in haematophagous insects, XA acts as a scavenger for free iron derived from *heme* demolition, and XA deficiency has been associated with direct oxidative damage to insect midgut epithelial cells (Lima et al., 2012). Moreover, XA produced by *Anopheles gambiae* 3-hydroxykynurenine transaminase (Rossi et al., 2005, 2006) is a trigger for *Plasmodium*

male gametogenesis, which takes place in the mosquito upon a parasite-infected blood meal (Billker et al., 1998). XA also exerts multiple actions in the mammalian brain (Sathyasaikumar et al., 2017; Schwarcz and Stone, 2017). Of particular significance is the observation that levels of XA are reduced in the brain and serum of schizophrenic patients, which is opposite to what has been reported for KYNA (Fazio et al., 2015). By using different approaches, including *in vivo* analyses, it has been proposed that KAT II could be the main determinant of XA synthesis in the CNS (Sathyasaikumar et al., 2017). Although XA and KYNA production appears to be sustained by distinct brain cell populations (Roussel et al., 2016), these studies highlight the need to analyse the whole complement of KAT isozymes for their role in XA synthesis and to re-evaluate the impact of specific KAT inhibitors on the local balance of these two fundamental products of the KP.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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MINI REVIEW
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Modulation of Enzyme Activity in the Kynurenine Pathway by Kynurenine Monooxygenase Inhibition

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The kynurenine pathway is the major route for tryptophan metabolism in mammals. Several of the metabolites in the kynurenine pathway, however, are potentially toxic, particularly 3-hydroxykynurenine, 3-hydroxyanthranilic acid, and quinolinic acid. Quinolinic acid (QUIN) is an excitotoxic agonist at the NMDA receptor, and has been shown to be elevated in neurodegenerative diseases such as Alzheimer's Disease and Huntington's Disease. Thus, inhibitors of enzymes in the kynurenine pathway may be valuable to treat these diseases. Kynurenine monooxygenase (KMO) is the ideal target for an inhibitor, since inhibition of it would be expected to decrease the toxic metabolites and increase kynurenic acid (KynA), which is neuroprotective. The first generation of KMO inhibitors was based on structural analogs of the substrate, L-kynurenine. These compounds showed reduction of QUIN and increased KynA *in vivo* in rats. After the determination of the x-ray crystal structure of yeast KMO, inhibitor design has been facilitated. Benzisoxazoles with sub-nM binding to KMO have been developed recently. Some KMO ligands promote the reaction of NADPH with O_2 without hydroxylation, resulting in uncoupled formation of H_2O_2. This potentially toxic side reaction should be avoided in the design of drugs targeting the kynurenine pathway for treatment of neurodegenerative disorders.

Keywords: kynurenine, kynurenine monooxygenase, kynurenine pathway, quinolinate, NMDA - receptor, inhibitor

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THE KYNURENINE PATHWAY

The kynurenine pathway (KP) is the major pathway for tryptophan catabolism in mammals (**Figure 1**). In fact, as much as 95% of dietary tryptophan is catabolized via the KP (Botting, 1995). The KP has been implicated to play a major role in many diseases and disorders. These illnesses range from cancer to infectious diseases, such as HIV, neurological disorders such as schizophrenia (Erhardt et al., 2017) and depression (Réus et al., 2015); autoimmune diseases such as multiple sclerosis (Lovelace et al., 2016) and rheumatoid arthritis (Cribbs et al., 2014); peripheral conditions such as cardiovascular disease (Song et al., 2017) and acute pancreatitis (Mole et al., 2016); neurodegenerative diseases such as Huntington's disease (Sathyasaikumar et al., 2018), Alzheimer's disease (Giil et al., 2017), and Parkinson's disease (Lim et al., 2017).

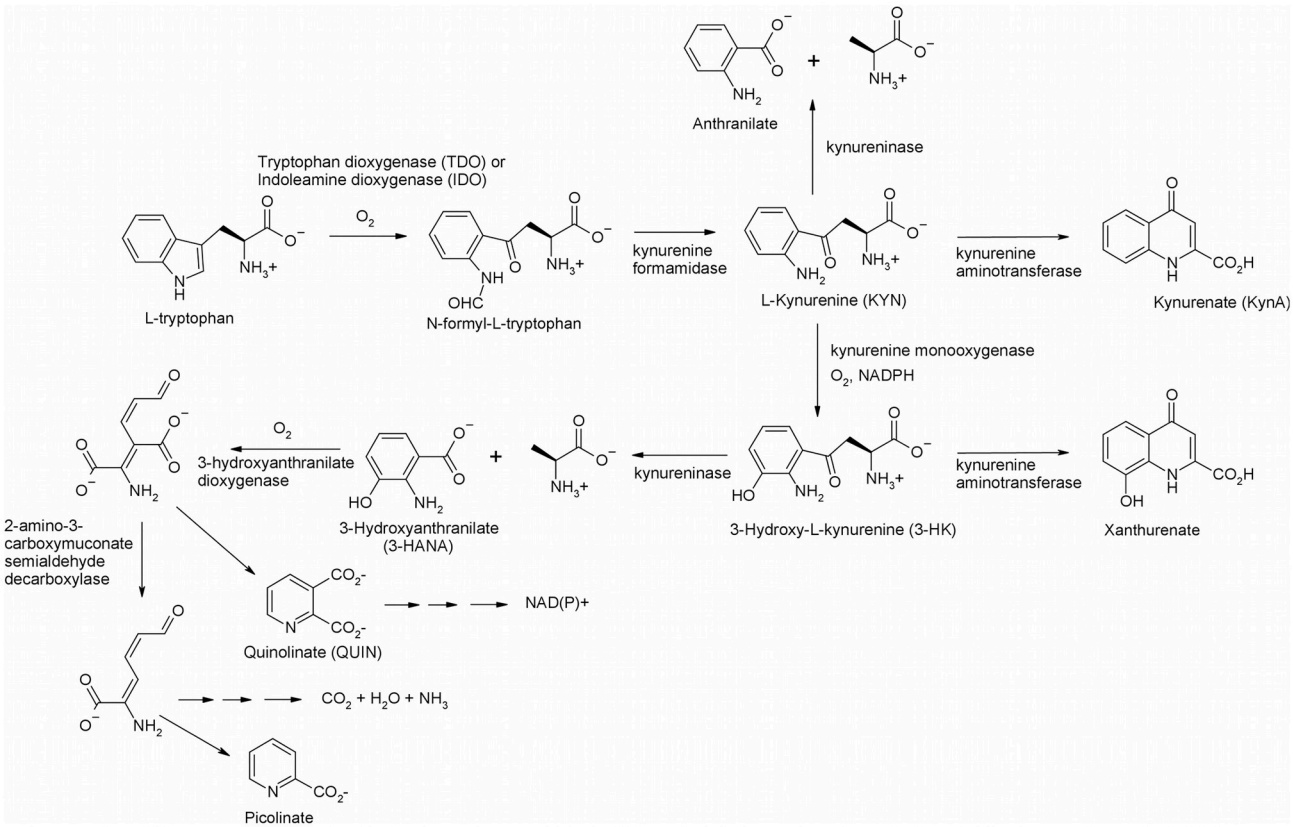


FIGURE 1 | The kynurenine pathway in mammals.

The KP begins with oxygenation of L-tryptophan, catalyzed by one of two heme-containing dioxygenases, indoleamine 2, 3-dioxygenase (IDO) or tryptophan 2, 3-dioxygenase (TDO), to yield N-formyl-L-kynurenine. IDO exists ubiquitously in the body (Théate et al., 2015), and, while low quantities of TDO have been found in the brain, it is primarily expressed in the liver (Larkin et al., 2016). N-Formyl-L-kynurenine is rapidly converted by a formamidase to L-kynurenine (KYN), the eponymous intermediate of the KP. KYN can act as a substrate for three enzymes, kynurenine aminotransferase (KAT), kynureninase, and kynurenine 3-monooxygenase (KMO). When KYN undergoes metabolism via KAT it forms kynurenic acid (KynA), a known neuroprotective agent due to its binding to nicotinic acetylcholine receptors and antagonism on the NMDA, AMPA and kainite glutamate receptors (Perkins and Stone, 1982; Schwarcz et al., 2012; Vécsei et al., 2013). KYN can also react with kynureninase forming anthranilic acid, although it is a poor substrate (Lima et al., 2008). Kynurenine monooxygenase (KMO) converts KYN to 3-hydroxykynurenine (3-HK), which gives 3-hydroxyanthranilate (3-HANA) by the action of kynureninase. Another oxygenase, 3-hydroxyanthranilate dioxygenase, subsequently produces 2-amino-3-carboxymuconate semialdehyde, which undergoes spontaneous cyclization to quinolinate (QUIN) (Colabroy and Begley, 2005), ultimately leading to $NAD(P)^+$ via quinolinate phosphoribosyltransferase. Decarboxylation of 2-amino-3-carboxymuconate semialdehyde initiates the pathway leading to complete catabolism and a branch leads to picolinate. It is important to note that there are several neuroactive intermediates in this pathway: KynA, QUIN, 3-HK, and 3-HANA. KynA is a known neuroprotective agent since it is an antagonist of the N-methyl D-aspartate (NMDA) receptor (Stone, 2000). The other neuroactive intermediates, QUIN, 3-HK, and 3-HANA, are known neurotoxic agents. 3-HK and 3-HANA serve as free-radical generators (Goldstein et al., 2000), while QUIN is an excitotoxic NMDA agonist (Stone and Perkins, 1981). Thus, modulation of enzyme activity in the KP will have effects on the NMDA receptor, which may be useful for treatment of neurodegenerative diseases that result from excessive

QUIN. The best drug target in the pathway is KMO, since blockage at this point will likely increase the neuroprotective KynA and decrease the neurotoxic metabolites, 3-HK, 3-HANA, and QUIN. The structure and inhibition of KMO has been reviewed recently (Dounay et al., 2015; Smith et al., 2016). In this review, we present the historical development of KMO inhibitors and review more recent developments.

In healthy tissue, the concentration of QUIN in the brain is low compared to blood and systemic tissues. An immune response, however, causes levels of QUIN to rise dramatically (Heyes et al., 1992). Macrophages, microglia and dendritic

cells are the major generators of QUIN under inflammatory conditions. Astrocytes and neurons are capable of up taking and catabolizing QUIN. In this case, however, the catabolic system is easily saturated further resulting in the toxic accumulation of QUIN within the cells (Chen and Guillemin, 2009).

The toxicity caused by QUIN has been attributed to its ability to activate the neuronal NMDA subtype of glutamate receptors. While this remains true, additional mechanisms have also been shown to contribute to this complex neurotoxicity. QUIN is not only capable of potentiating it's own toxicity but also other excitotoxins such as glutamate, while inhibiting the reuptake of glutamate by astrocytes. QUIN compromises the integrity of the BBB, generates reactive oxygen intermediates, and depletes endogenous antioxidant and peroxidation of lipid molecules (Guillemin, 2012).

Increased production of nitric oxide has been shown in rodents and human neurons and astrocytes following induction of neuronal nitric oxide synthase by QUIN (Aguilera et al., 2007). Dysregulation of astroglial function and gliotoxicity is also proposed to augment QUIN's ability to kill neurons, redefining the cellular connection between neurons and glia in both physiological processes and pathological conditions (Lee et al., 2010). QUIN increases the phosphorylation of cellular structural proteins damaging the cytoskeleton of neurons and astrocytes (Pierozan et al., 2010). This destruction of cellular structure has brought significant interest to QUIN's role in hyperphosphorylated tau in Alzheimer's disease (AD) (Rahman et al., 2009).

Several studies have confirmed the pathological role QUIN plays in the development of many diseases. Elevated concentrations of QUIN have proven to directly contribute to HD, AD, AIDs related dementia (Chen and Guillemin, 2009), poliovirus brain infection (Allegri et al., 2012), multiple sclerosis (Aeinehband et al., 2016), cerebral ischemia (Saito et al., 2006), cerebral malaria (Dobbie et al., 2000), and epilepsy (Heyes et al., 1994).

THE THREE DIMENSIONAL STRUCTURE OF KYNURENINE MONOOXYGENASE (KMO)

KMO belongs to a family of NADPH dependent flavin monooxygenases (Okamoto et al., 1967). It is encoded by one gene, has an FAD coenzyme, utilizes either NADPH or NADH, releases $NADP^+/NAD^+$ after flavin reduction, and has one Rossmann fold dinucleotide binding domain, which categorizes it as a Class A flavoprotein aromatic hydroxylase (van Berkel et al., 2006; Crozier and Moran, 2007). Human KMO (hKMO) is 486 amino acids in length with a molecular weight approximately 50 kDa (Alberati-Giani et al., 1997; Breton et al., 2000). Eukaryotic sequences of KMO exhibit a C-terminal transmembrane helix about 50 residues long, which is responsible for binding to the outer membrane of mitochondria. As discussed above, due to its proposed involvement in a number of diseases, KMO has been purified and studied from several sources. Tissue distribution studies have shown that mammalian KMO is highly expressed in the liver and kidney, and in small amounts it has also been found in endothelial, macrophages, microglial, and monocytic cells. While expressed in a wide array of cell types, very low levels of KMO have been found in brain cells (Courtney and Scheel, 2010). The mammalian enzyme has been difficult to express and purify due to its membrane binding properties. Unfortunately, since it makes recombinant expression difficult, the C-terminal membrane anchor helix was found to be essential for activity of pig KMO (Hirai et al., 2010).

The proposed catalytic mechanism of KMO from *Pseudomonas fluorescens* is shown in **Figure 2** (Crozier-Reabe et al., 2008). Like many oxidoreductases, the catalytic cycle of KMO can be divided into two half reactions, a reductive half and an oxidative half. The binding of KYN to KMO is relatively slow, making the reduction half of this reaction KYN dependent. Once kynurenine and NADPH bind to KMO, the FAD cofactor is reduced by NADPH, and $NADP^+$ dissociates from the enzyme. The enzyme complex then reacts with molecular oxygen, forming a 4a-peroxyflavin intermediate that transfers an oxygen atom to the substrate. The resulting 4a-hydroxyflavin is rapidly dehydrated prior to product release. The oxidized enzyme complex subsequently undergoes a conformational change, facilitating the release of the product 3-HK, in the rate-limiting step of this mechanism. As a result of this conformational change, there is a change in the visible spectrum of the oxidized enzyme on product release.

The first crystal structure of KMO, published in *Nature* in 2013, was of the *Saccharomyces cerevisiae* enzyme (ScKMO) (PDB 4J36 and 4J33), truncated at the C-terminus (Amaral et al., 2013). The structure was determined not only in the free form, but also in complex with the tight-binding inhibitor, UPF648. Both structures were solved as a dimer with PDB 4J33 at a resolution of 1.82 Å and PDB 4J36 at a resolution of 2.13Å. The KMO structure, similar to other flavin-dependent hydroxylase structures, features a Rossmann fold domain for flavin adenine dinucleotide (FAD) binding that interacts with a part of the β-domain holding five β-sheets and four α-helices (Huijbers et al., 2014). It was found that UPF-648 binds closely to this domain, initiating a conformational change, precluding L-Kyn binding and therefore inhibiting KMO activity. Conserved residues, Arg83 and Tyr97, bind the UPF-648 carboxylate and conserved hydrophobic residues, Leu221, Leu234, Met230, Ile232, Phe246, Phe322, and Pro321, flank the aromatic dichlorobenzene moiety. Mutagenesis and functional assays have found these residues to be conserved across different organisms, allowing the translation of this work to hKMO. ScKMO and human KMO share 38% identity and 51% similarity. Thus, the structure of ScKMO has been a useful template for docking screens using virtual compound libraries and aiding in the development of novel inhibitor scaffolds.

Tryptophan catabolism via the KP has been identified in a number of bacteria, including *P. fluorescens, Cytophaga hutchinsonii* and *Ralstonia metallidurans* (Kurnasov et al., 2003). Soluble KMOs have been found in bacteria, *P. fluorescens* (Crozier and Moran, 2007) and *C. hutchisonii* (Kurnasov et al., 2003), which have facilitated mechanistic and structural studies. The enzyme from *P. fluorescens* (PfKMO) is a soluble enzyme

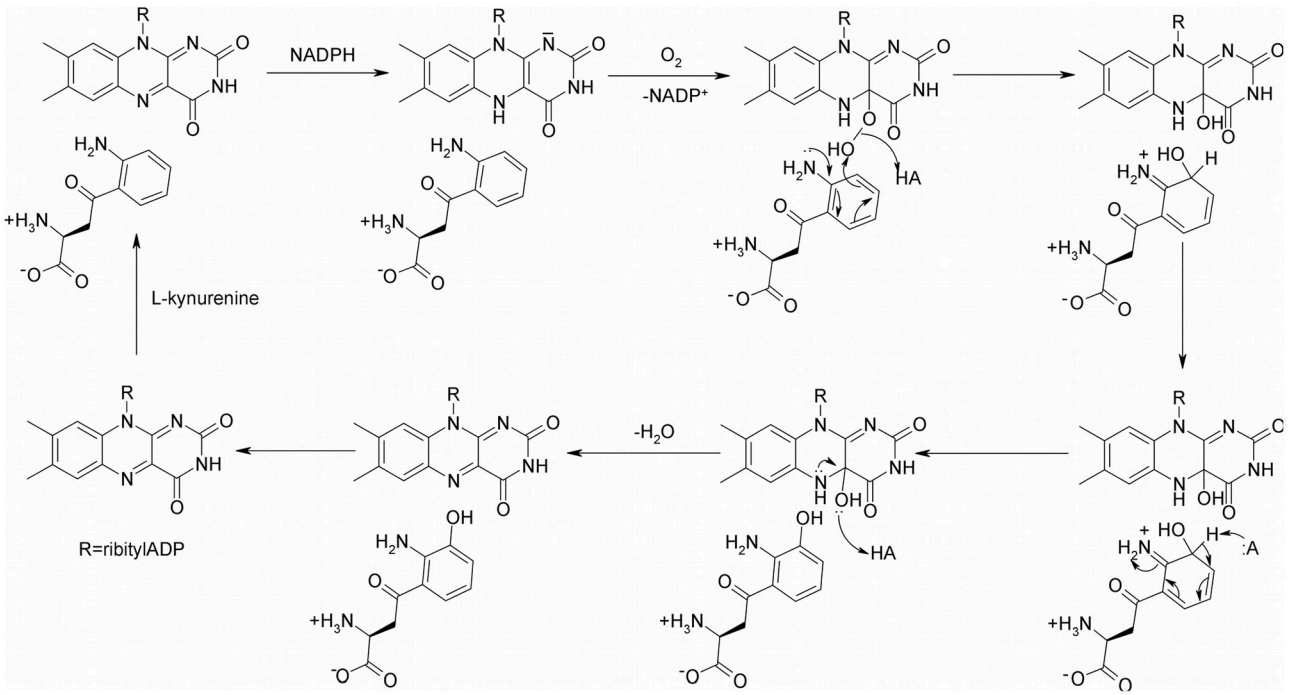


FIGURE 2 | The proposed catalytic mechanism of KMO.

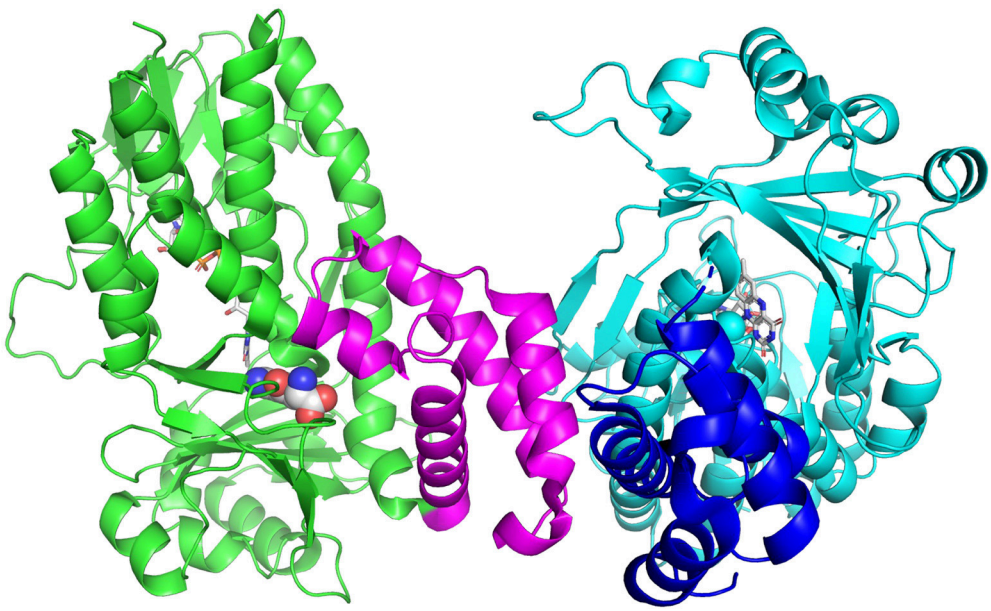


FIGURE 3 | Structure of PfKMO with L-kynurenine bound (PDB 5NAK). L-Kynurenine (spheres) and FAD (sticks) are bound to the A-chain (green), while the B-chain (cyan) has only FAD bound (sticks). The C-terminal domain is shown in magenta (A chain) and blue (B-chain). This figure was prepared with Pymol (Version 2.1, Schrödinger, LLC).

with 37% identity to human KMO that can be expressed heterologously in *Escherichia coli* (Crozier and Moran, 2007). The crystal structures of PfKMO with a number of inhibitors and L-kynurenine bound have been solved recently (Hutchinson et al., 2017; Gao et al., 2018; Kim et al., 2018). The structure of PfKMO (**Figure 3**) is very similar to that of ScKMO. PfKMO

contains two domains, with the main domain holding the Rossmann fold, the active site, the FAD cofactor and a C-terminal domain. Hydrophilic residues. Arg84, Tyr98, Tyr404, and Asn404, are close to the carboxylate groups of the substrate, and hydrophobic residues, Leu213, Leu226, Ile224, Phe238, and Met373, are close to the aromatic ring of the substrate. When L-kynurenine is in the active site, interactions between the carboxylate group and Arg84, Tyr98, Tyr404, and Asn369 are also present, revealing key interactions between PfKMO and substrates. These residues present in the active site of this enzyme are thought to be important in substrate binding and recognition. A significant conformational change was seen in the position of the C-terminal domain with substrate binding. For this reason, it was concluded that the C-terminal domain must play an integral role in the binding of substrates (Wilkinson, 2013; Gao et al., 2018). When PfKMO is not binding a substrate or inhibitor, the enzyme is said to be in an "open" conformation. It is theorized that this open conformation allows for accelerated binding of substrate and product release. Once a substrate binds to PfKMO, the C-terminal domain then moves to give a "closed" conformation, observed in the structure with L-kynurenine or inhibitors bound. Understanding the structural changes associated with substrate binding will be valuable in the development of effective KMO inhibitors.

The first structure of hKMO was reported recently (Kim et al., 2018). The crystal structure was solved to a resolution of 2.1 Å after engineering the deletion mutant, hKMO-374 (residues 1-374), in which the transmembrane domains were deleted, in order to obtain a human KMO protein suitable for crystallization. As described earlier (Hirai et al., 2010), hKMO, located in the mitochondrial outer membrane, contains two transmembrane domains (TMDs) and a C-terminal region responsible for mitochondrial targeting. As expected, the structure of hKMO is very similar to that of PfKMO and ScKMO. The first domain contains the FAD binding region, while the second contains the small N-terminal domain, consisting of alpha helices and an antiparallel beta sheet. However, hKMO-374 is inactive, in agreement with previous studies in which the transmembrane domains in pig and recombinant hKMO enzymes were required for enzymatic activity (Breton et al., 2000; Hirai et al., 2010). The findings from this study further provide insight into the KMO enzyme and will likely facilitate the development of KMO inhibitors.

KMO INHIBITORS

Design of Inhibitors Before the KMO Crystal Structure

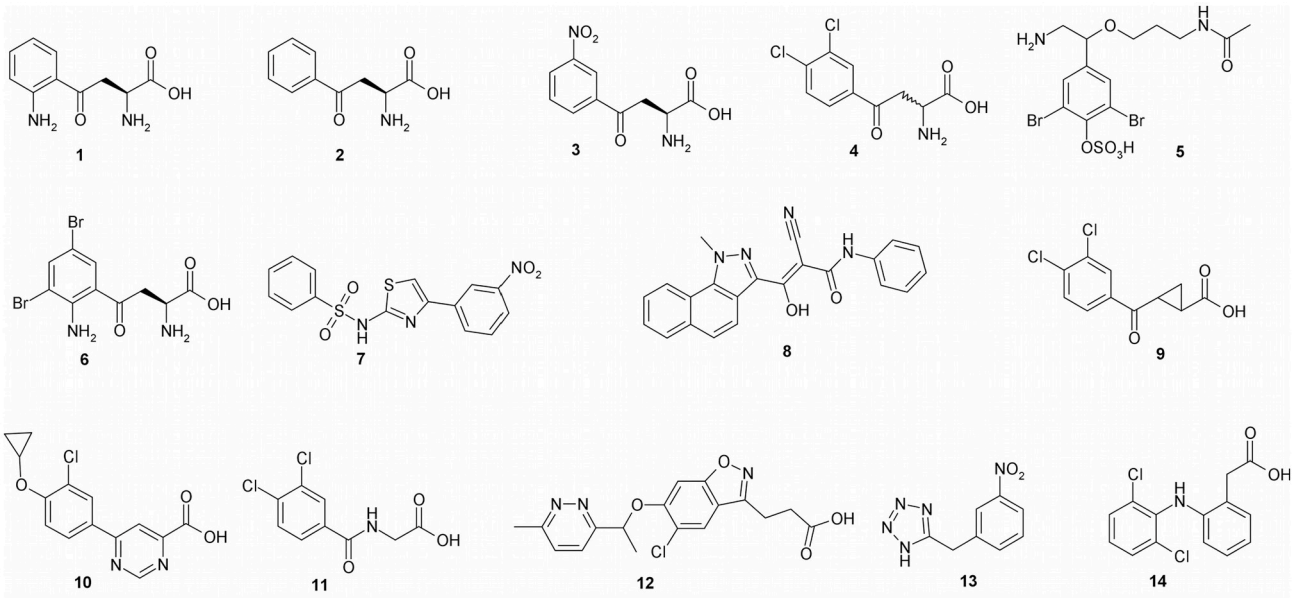
Two decades ago, when the first KMO inhibitors were evaluated, the crystal structure of KMO was still unknown, and therefore, inhibitor design was based on the structure of the KMO substrate, L-kynurenine (**1**), as a lead compound (**Table 1**). The desamino analog of L-kynurenine, β-benzoyl-L-alanine (**2**), was found to be a competitive inhibitor, with a K_d of 7.4 μM for PfKMO (Crozier-Reabe et al., 2008). Several of the first generation of KMO inhibitors have shown promising results in regulating NMDA receptor agonism and antagonism homeostasis. Among these compounds, (*m*-nitrobenzyl)alanine (*m*-NBA) (**3**) was the most potent inhibitor (IC_{50} = 0.9 μM) (Chiarugi et al., 1996). When 400 mg/kg of **3** was administered to rats, there was an increased level of both L-kynurenine and KYNA up to 10 times and 5 times, in the brain and blood, respectively. Inspired by those results, *m*-NBA was used as a lead compound to synthesize more potent inhibitors. (R,S)-3,4-Dichlorobenzoylalanine (FCE 28833A) (**4**), with an IC_{50} of 0.2 μM, was the most potent of a series of compounds prepared at Farmitalia Carlo Erba by adding substitutents to the benzene ring of **2**. **4** was found to have increased inhibition by almost 40-fold over **2** and 4.5-fold over **3**. *In vivo* studies with **4** were performed on rats with 400 mg/kg orally, and this showed an increase in both L-kynurenine and KYNA levels in brain tissue (Speciale et al., 1996). Ianthellamide A (**5**, IC_{50} = 1.5 μM) was isolated from the Australian sponge *Ianthella quadrangulata*. *In vivo* studies showed an increase of KYNA levels in rat brains following a systematic injection of 200 mg/kg (Feng et al., 2012). Kynurenines substituted at the 3-position were found to be competitive inhibitors with K_i values in the low μM region (Phillips et al., 2017). The most potent of these, 3,5-dibromo-L-kynurenine (**6**), is a competitive inhibitor with a K_i of 1.2 μM.

Structurally different sulfonamides were then examined and exhibited much stronger inhibitory potency compared to the previously examined scaffolds. Ro61-8048 (**7**) was the most active in this group, with inhibition in the nanomolar range (IC_{50} = 37 nM) (Röver et al., 1997). This compound has been shown to raise both L-kynurenine and KYNA levels in the brain through peripheral KMO inhibition, since it does not cross the blood brain barrier. JM6, a prodrug of Ro61-8048, was shown to reduce neurodegeneration in an Alzheimer's mouse model, despite not crossing the blood-brain barrier (Zwilling et al., 2011). This suggests that modulation of NMDA receptor activity can be achieved by compounds that do not cross the blood brain barrier. A series of tricyclic 3-oxo-propanenitriles compounds were patented, and one member of this series, PNU-168754 (**8**), has an IC50 of 40 nM (Pevarello et al., 1999). After determining that the α-amino group is not required for inhibition, and that the acid moiety is indeed essential for inhibition, a number of 4-aryl-4-oxobutanoic acids derivatives were prepared, with UPF-648 (**9**) being the best among them (IC_{50} =20 nM). Treatment of mice with 100 μM UPF-648 has been shown to shift the KP to the synthesis of KYNA (Sapko et al., 2006). This compound was later used in the first KMO crystal structure to be solved (Amaral et al., 2013). This achievement marked the beginning of structure-based drug design of KMO.

Design of Inhibitors After the KMO Crystal Structure

After the KMO structure determination, it became possible to design KMO inhibitors computationally with more precision. The first class of KMO inhibitors designed using the structural data was arylpyrimidine carboxylic acids. These can be considered as cyclic rigid analogs of kynurenine. The N3 is

TABLE 1 | Inhibitors of KMO.



Compound	Name	IC_{50} or K_d (μM)	Tested for neurodegeneration	Peroxide production
1	L-Kynurenine (substrate)	17.4	–	No
2	β-Benzoyl-L-alanine	7.6	–	Yes
3	*m*-NBA	0.90	Yes	Yes
4	FCE28833A	0.20	Yes	–
5	Ianthellamide A	1.5	–	–
6	3,5-Dibromo-L-kynurenine	1.2	–	–
7	Ro-61-8048	0.037	Yes	Yes
8	PNU-168754	0.040	–	–
9	UPF-648	0.020	Yes	–
10	CHDI-340246	0.0005	–	–
11	3,4-Dichlorohippuric acid	34	–	–
12	GSK 366	0.0007	–	–
13	5-(3-nitrobenzyl)-1H-tetrazole	6.3	–	–
14	Diclofenac	13.6	–	–

thought to mimic the L-kynurenine carbonyl oxygen, whereas the N1 mimics the amine group that is very essential for the inhibition. Compound **10** (IC_{50} = 0.5 nM) was found to be the best compound. It is highly selective for KMO over other enzymes in the KP, and has shown potency both *in vivo* and *in vitro*. When 10 mg/kg of **10** was administered orally in rats, an increase in L-kynurenine and KYNA, and a decrease of both QUIN and 3HK, in the brain was observed (Toledo-Sherman et al., 2015). Computational studies have been performed on arylpyrimidine carboxylic acids, predicting new derivatives with possible high inhibitory activity for KMO (Amin et al., 2016)

Using the first ScKMO structure (Amaral et al., 2013) and an already known KMO inhibitor, UPF 648 (9), another set of compounds were proposed using a pharmacophore (**Figure 4**). This pharmacophore allowed prediction of a set of compounds that fit the active site of the KMO as **7** does. Among several predicted inhibitors, 3,4-dichlorohippuric acid (**11**) showed greatest inhibitory potency *in vitro* (K_i = 34 μM) (Phillips et al., 2017).

Benzisoxazoles are the most potent KMO inhibitors found so far. They were examined for inhibitory potency against KMO, with the goal of targeting acute pancreatitis and multiple organ dysfunction syndrome (Hutchinson et al., 2017). They are similar in structure to L-kynurenine and have inhibitory potency in the nanomolar range. GSK 366 (**Table 1**, **Compound 12)** is by far the most potent among them, with IC_{50} values of 2.3 nM and 0.7 nM for hKMO and PfKMO, respectively. This series of compounds contain chlorine, which was previously determined to play an important role in inhibiting KMO in **4**, **8**, and **10** (Breton et al., 2000; Hutchinson et al., 2017). This compound binds to the active

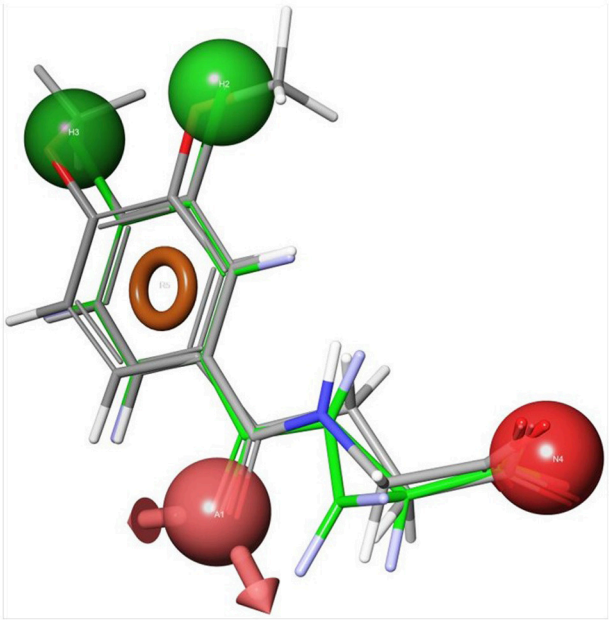


FIGURE 4 | Receptor-based pharmacophoric models of 3,4-dimethoxyhippuric acid (**9**) aligned with the conformation of UPF648 as bound to ScKMO (pdb code: 4J36) (Phillips et al., 2017). The green spheres are hydrophobic centers, the red spheres are acidic centers, the pink spheres with arrows are H-bond acceptors, and the orange rings are aromatic planes.

site, and the methylpyridazine ring on the benzisoxazole tilts the flavin, which is thought to promote the potency and residence time of the inhibition. Though they have not been studied yet for their potency against neurodegeneration, notably the ability to cross the blood brain barrier, they still provide exciting insights and serve as great lead compounds for the future drug design and discovery for neurodegenerative disorders.

High throughput screening of a series of tetrazoles was performed with RapidFire mass spectrometry. The best compound identified was 5-(3-nitrobenzyl)-1H-tetrazole (**13**), with an IC_{50} of 6.3 μM *(*Lowe et al., 2014). Recently, another group proposed a molecular similarity and drug repurposing approach. Known drugs were computationally tested for KMO inhibitory capacities. Through this ligand-based approach, diclofenac (**Table 1**, **Compound 14**), a known anti-inflammatory drug, has been identified as a KMO inhibitor (IC_{50} = 13.6 μM) (Shave et al., 2018). This study sets the stage for future studies using molecular similarities studies as well as drug repurposing. The properties of various types of KMO inhibitors are summarized in **Table 1**.

Limitations

Though structural analogs of L-kynurenine show inhibition, some of them have shown the potential of causing life threatening side effects, since they were found to generate cytotoxic hydrogen peroxide through futile cycles of flavin reduction and oxidation. Upon binding, NADPH reduces FAD and leaves as $NADP^+$, then the oxygen molecule binds and forms an L-Kynurenine-FAD-hydroperoxide complex intermediate (**Figure 2**). The hydroxylation of L-kynurenine then proceeds to make water and 3-HK. Since these inhibitors have structural similarities to L-kynurenine, but are not capable of hydroxylation, some potential KMO inhibitors are uncouplers of NADPH oxidation (Crozier-Reabe et al., 2008). In the presence of *m*-NBA, and UPF-648, there is an accumulation of the hydroperoxyflavin, which decays to yield hydrogen peroxide. A recent study provided a better understanding on how the uncoupling happens (Kim et al., 2018). The authors proposed the flavin reduction in KMO is associated with conformational changes, via π- π interactions between a substrate or an NADPH uncoupler, and the loop above the *re*-side of the flavin. They determined that the substrate binding precedes flavin reduction. Ro61-8048 has shown interesting results where it acts as a simple competitive inhibitor in ScKMO and hKMO, and an NADPH uncoupler in PfKMO. This is is due to different binding modes this compound has in those two species. It binds in the active site of ScKMO but does not induce conformational changes in the protein the same way it does in PfKMO. Thus, it is important in the design of KMO inhibitors for treatment of neurodegenerative diseases to avoid compounds that will act as uncouplers of NADPH oxidation, generating highly toxic reactive oxygen species.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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The Energy Landscape of Human Serine Racemase

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Human serine racemase is a pyridoxal 5′-phosphate (PLP)-dependent dimeric enzyme that catalyzes the reversible racemization of L-serine and D-serine and their dehydration to pyruvate and ammonia. As D-serine is the co-agonist of the N-methyl-D-aspartate receptors for glutamate, the most abundant excitatory neurotransmitter in the brain, the structure, dynamics, function, regulation and cellular localization of serine racemase have been investigated in detail. Serine racemase belongs to the fold-type II of the PLP-dependent enzyme family and structural models from several orthologs are available. The comparison of structures of serine racemase co-crystallized with or without ligands indicates the presence of at least one open and one closed conformation, suggesting that conformational flexibility plays a relevant role in enzyme regulation. ATP, Mg^{2+}, Ca^{2+}, anions, NADH and protein interactors, as well as the post-translational modifications nitrosylation and phosphorylation, finely tune the racemase and dehydratase activities and their relative reaction rates. Further information on serine racemase structure and dynamics resulted from the search for inhibitors with potential therapeutic applications. The cumulative knowledge on human serine racemase allowed obtaining insights into its conformational landscape and into the mechanisms of cross-talk between the effector binding sites and the active site.

Keywords: pyridoxal 5′-phosphate, enzyme catalysis, allosteric regulation, conformational landscape, D-serine, N-methyl-D-aspartate receptor, neuropathologies

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INTRODUCTION

The N-methyl-D-aspartate (NMDA) receptors are ligand-gated ion channels involved in synapse formation, synaptic plasticity, learning and memory potentiation (Paoletti et al., 2013). They are the only neurotransmitter receptors whose activation requires two distinct agonists, glutamate and either glycine or D-serine, with the latter ones sharing the same binding site. Intermediates of the kynurenine pathway also bind the NMDA receptors: quinolinic acid is a strong agonist, whereas kynurenic acid acts as an antagonist (Németh et al., 2006; Lugo-Huitrón et al., 2013).

Increased levels of D-serine are associated with neuronal excitotoxicity caused by excess influx of calcium ions, as observed in several pathological conditions, including Parkinson and Alzheimer diseases, stroke and amyotrophic lateral sclerosis. On the other hand, low levels of D-serine are associated with schizophrenia. These pathological conditions prompted the search for the enzyme responsible for the production of D-serine in the brain. Serine racemase (SR) was first localized in astrocytes and later in neurons. Nowadays, the prevalent view is that the main production of

D-serine takes place in neurons (Wolosker et al., 2016). Then, D-serine is exported via the ASC-1 transporter to astrocytes, where it is stored and subsequently released in the synaptic space (Wolosker et al., 2016). Other D-amino acids, such as D-aspartate and D-alanine, were detected in the brain (Hashimoto et al., 1992). The racemases involved in their production have not yet been identified (Conti et al., 2011). SR itself was proposed to be responsible for the production of D-aspartate, an agonist of the NMDA receptors (Ito et al., 2016).

SR activity depends on the coenzyme pyridoxal 5′-phosphate (PLP), in contrast with other racemases that are PLP-independent (Conti et al., 2011). A unique feature of human SR (hSR) is the modulation of its activity by several ligands, protein interactors and post-translational modifications (PTMs), suggesting significant conformational plasticity. In this review, we will present SR structure, dynamics, function and regulation, with special emphasis on the human ortholog, discussing them in the frame of the enzyme energy landscape.

SR STRUCTURE

PLP-dependent enzymes are classified in seven fold types (Grishin et al., 1995; Jansonius, 1998; Mehta and Christen, 2000; Schneider et al., 2000; Percudani and Peracchi, 2009). Traditional classification of PLP-dependent enzymes assigns fold-type I to the aspartate aminotransferase family, the largest and best characterized family (Bruno et al., 2001; Phillips et al., 2002; Kaiser et al., 2003; Storici et al., 2004; Spyrakis et al., 2014). Fold type II group includes tryptophan synthase, *O*-acetylserine sulfhydrylase, threonine deaminase and serine dehydratase (Bettati et al., 2000; Campanini et al., 2003; Raboni et al., 2003, 2005, 2007, 2009, 2010; Spyrakis et al., 2006, 2013). Bacterial alanine racemase is the archetypal protein of fold type III and the D-amino acid aminotransferase family is the most representative example of the fold type IV subgroup. Glycogen phosphorylase corresponds to fold type V. Fold types VI and VII were more recently introduced and include D-lysine 5,6-aminomutase and lysine 2,3-aminomutase, respectively. SR exhibits the type II fold, which consists of a large and a small domain with similar α/β architecture, constituted of a central β-sheet surrounded by helices. The PLP cofactor, covalently bound to a lysine of the large domain, lies in a cleft between the two domains (**Figures 1A,B**).

At present, 10 SR X-ray crystallographic structures have been deposited in the PDB, and, among these, three structures are of the human enzyme (**Table 1**). In hSR numbering, the large domain is formed by residues 1–68 and 157–340, while the small domain comprises residues between 78 and 155. The longer connecting region, residues 69–77, forms a flexible hinge that is only partially detected by X-ray crystallography for hSR (Smith et al., 2010; Takahara et al., 2018). In yeast *Schizosaccharomyces pombe* SR (SpSR), this region is folded to form a short α-helix (Goto et al., 2009; Yamauchi et al., 2009), while in rat SR (rSR) it forms a loop (Smith et al., 2010). In the small domain of hSR, three α-helices surround the four β-strands (S3–S6) of the β-sheet. Two of these helices (H4 and H5) are on the same side with respect to the β-sheet and lie toward the interface with the large domain. The third helix (H6) is on the opposite site, forming a solvent-exposed surface. The large domain is formed by six β-strands, forming a twisted β-sheet (S1, S2, S7–S10) and 11 flanking α-helices (H1–H3, H7–H14) (**Figures 1A,B**).

The structural investigation of SpSR (Goto et al., 2009; Yamauchi et al., 2009), which exhibits 35.1% sequence identity with hSR, allowed the detection of an open-closed conformational shift occurring upon binding of the substrate. This mechanism was described previously for fold type I PLP-dependent aspartate aminotransferases (Jansonius and Vincent, 1987; Jäger et al., 1994; Okamoto et al., 1994) and for several fold-type II enzymes such as OASS and tryptophan synthase (Raboni et al., 2009; Mozzarelli et al., 2011). The structure of SpSR without any ligand at the active site (PDB code: 1V71), is in an open conformation (Goto et al., 2009), whereas SpSR modified at the active site with a lysino-D-alanyl group—which mimics the substrate—was found to be in a closed conformation (PDB code: 2ZPU) (Yamauchi et al., 2009). Co-crystallization of this modified form with serine, which could still be accommodated at the active site despite the modification, also stabilized a closed conformation (PDB code: 2ZR8) (Goto et al., 2009). In the observed closed conformation, the small domain undergoes a 20° rotation toward the large domain to close the active site (**Figure 2A**). A large conformational change occurs to the asparagine loop Ser-Ser-Gly-Asn (residues 81–84 for SpSR, 83–86 for rat and human SR), at the N-terminal part of α-helix H5 (H4 in rat and human SR, since in SpSR an extra helix is present after helix H3), which forms the binding site for the carboxylate moiety of the substrate serine. Moreover, the carboxylate is involved in a salt bridge with the N-terminal Arg133 of α-helix H7 (H6 in rat and human SR) (Goto et al., 2009). An analogous open-closed conformational change was described for rSR by Smith and coworkers (Smith et al., 2010). The structure of rSR converts from an open conformation (PDB code: 3HMK) of the enzyme to a closed conformation upon formation of a complex with the competitive inhibitor malonate (PDB code: 3L6C). Arg135 (corresponding to Arg133 in SpSR) and the asparagine loop move toward the ligand bound to the active site, similarly to the behavior observed for SpSR (**Figure 2B**). The structure of hSR bound to malonate was also reported (PDB code: 3L6B) (Smith et al., 2010). Human and rat SR are 90% identical in sequence and their structures are almost indistinguishable (Smith et al., 2010). A comparison of the structure of hSR bound to malonate with a recently published structure of hSR in the free form (PDB code: 5X2L) (Takahara et al., 2018) confirmed that also hSR undergoes an open-closed conformational change when a ligand is present at the active site (**Figure 2C**). The structure of a plant SR from maize was also solved (PDB code: 5CVC), showing a fold similar to the other SR structures, apart from the C-terminal helix, which protrudes outside the core of the monomer (Zou et al., 2016).

All structures of SR deposited so far in the PDB share two common features: the presence of a site for the binding of divalent cations and a similar spatial arrangement of the PLP-binding site. The binding site for divalent cations is in the large domain and is physiologically occupied by Mg^{2+}. Binding

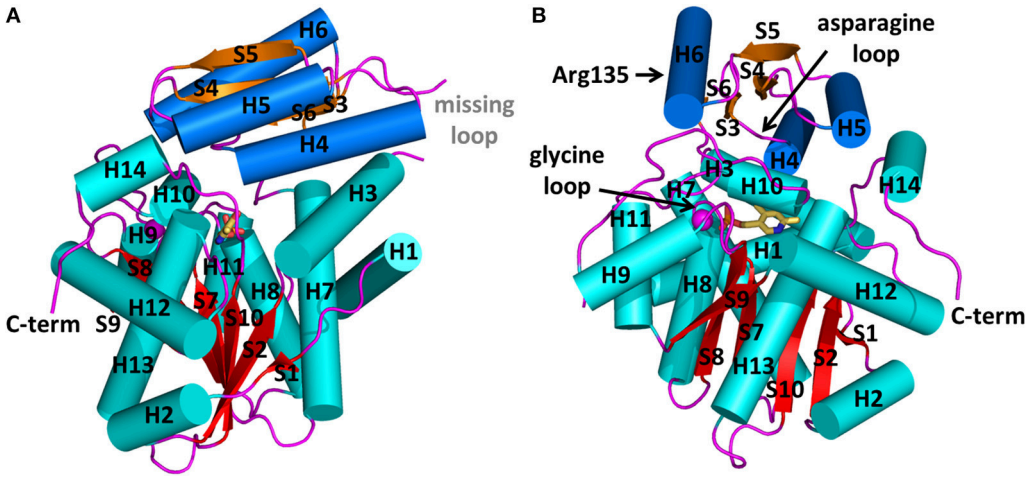


FIGURE 1 | Structure of human serine racemase (PDB code 3L6B) shown in two orthogonal views **(A,B)**. α-helices and β-strands belonging to the large domain are colored in cyan and red, respectively, while those belonging to the small domain are reported in blue and orange, respectively. The divalent cation is represented as a pink sphere. All loops are colored in pink. The positions of the asparagine and glycine loops and of Arg135 are indicated by arrows.

TABLE 1 | Structures of serine racemase available in the PDB.

PDB code	Species	Ligands	Length from FASTA	Missing a.a.	Mutations	Open or closed conformation	References
5X2L	*Homo sapiens*	PLP, Mg^{2+}	348	1,2, 67–76, 318–348	–	Open	Takahara et al., 2018
3L6B	*Homo sapiens*	Malonate, PLP, Mn^{2+}	348	1,2, 69–73, 330–348	C2D, C6D	Closed	Smith et al., 2010
3L6R	*Homo sapiens*	Malonate, PLP, Mn^{2+}	348	1,2, 69–75, 330–348	C2D, C6D. selenomethionine labeled	Closed	Smith et al., 2010
3HMK	*Rattus norvegicus*	PLP, Mn^{2+}	339	1,2, 324–339	C2D, C6D	Open	Smith et al., 2010
3L6C	*Rattus norvegicus*	Malonate, PLP, Mn^{2+}	339	1, 324–339	C2D, C6D	Closed	Smith et al., 2010
1V71	*Schizosaccharomyces pombe*	PLP, Mg^{2+}	323	1–5	–	Open	Goto et al., 2009
2ZR8	*Schizosaccharomyces pombe*	Serine, PDD, Mg^{2+}	323	1–4	–	Closed	Goto et al., 2009
1WTC	*Schizosaccharomyces pombe*	PLP, Mg^{2+}, Mg·AMP–PCP	323	1–5	–	Open	Goto et al., 2009
2ZPU	*Schizosaccharomyces pombe*	PDD, Mg^{2+}	323	1–4	–	Closed	Yamauchi et al., 2009
5CVC	*Zea mays*	PLP, Mg^{2+}	346	1–15, 345–346	–	Open	Zou et al., 2016

The length of 3L6B on the PDB file is reported to be 346 a.a., but the FASTA sequence contains 348 a.a. Hence, the length of the FASTA sequence is shown in the table. Missing amino acids are those not present in the X-ray electron density.

of divalent cations is essential for the enzyme correct folding, stability and activity (see below) (Cook et al., 2002; Ito et al., 2012; Bruno et al., 2017). In the structures of rat and human SR (all but 5X2L), a Mn^{2+} ion is present instead of Mg^{2+}, since $MnCl_2$ was used in the crystallization buffer. This ion has no physiological relevance and the presence of Mn^{2+} does not alter the structure of the enzyme. The metal binding site is formed by ionic interactions with the carboxylate groups of Glu208 and Asp214 (Glu210 and Asp216 in rSR and hSR), the backbone carbonyl group of Gly212 (Ala214 in rSR and hSR) and three water molecules (Goto et al., 2009; Smith et al., 2010; **Figure 3A**). The metal ion is coordinated with an octahedral geometry. This site is not directly involved in catalysis, although it is connected through water molecules to a tetra-glycine loop (Gly 183-184-185-186 for SpSR and 185-186-187-188 for rSR and hSR) at the N-terminal of α-helix H9 (in SpSR numbering, H8 in rSR and hSR), which forms a series of H-bonds with the phosphate group of PLP, contributing to the correct positioning of the cofactor

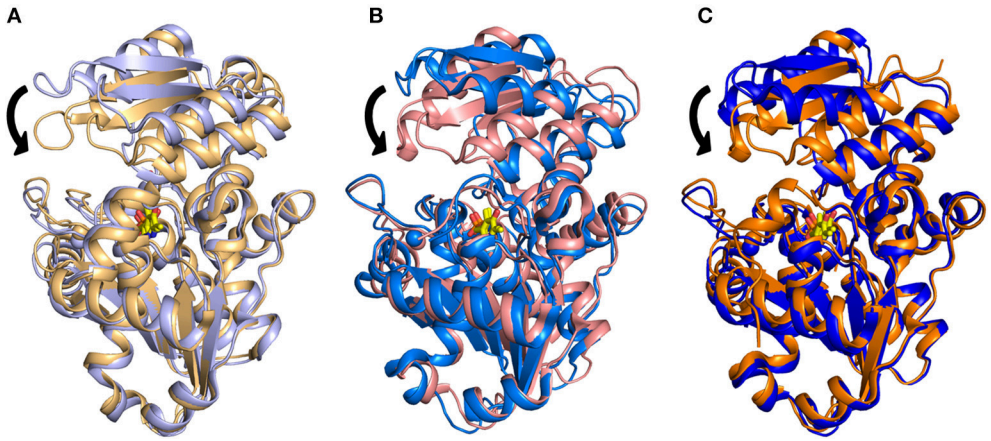


FIGURE 2 | Overlay of **(A)** the open (PDB 1V71, light purple) and closed (PDB 2ZR8, light orange) structures of SpSR; **(B)** the open (PDB 3HMK, light blue) and closed (PDB 3L6C, light pink) structures for rat SR; **(C)** the open (PDB 5X2L, blue) and closed (PDB 3L6B, orange) structures of hSR. PLP is in yellow sticks. The divalent cation is reported as a sphere with the same color code of the cartoon representation. The major conformational change occurring upon ligand binding, i.e., a 20° hinge movement of the small domain toward the large domain, is indicated by arrows.

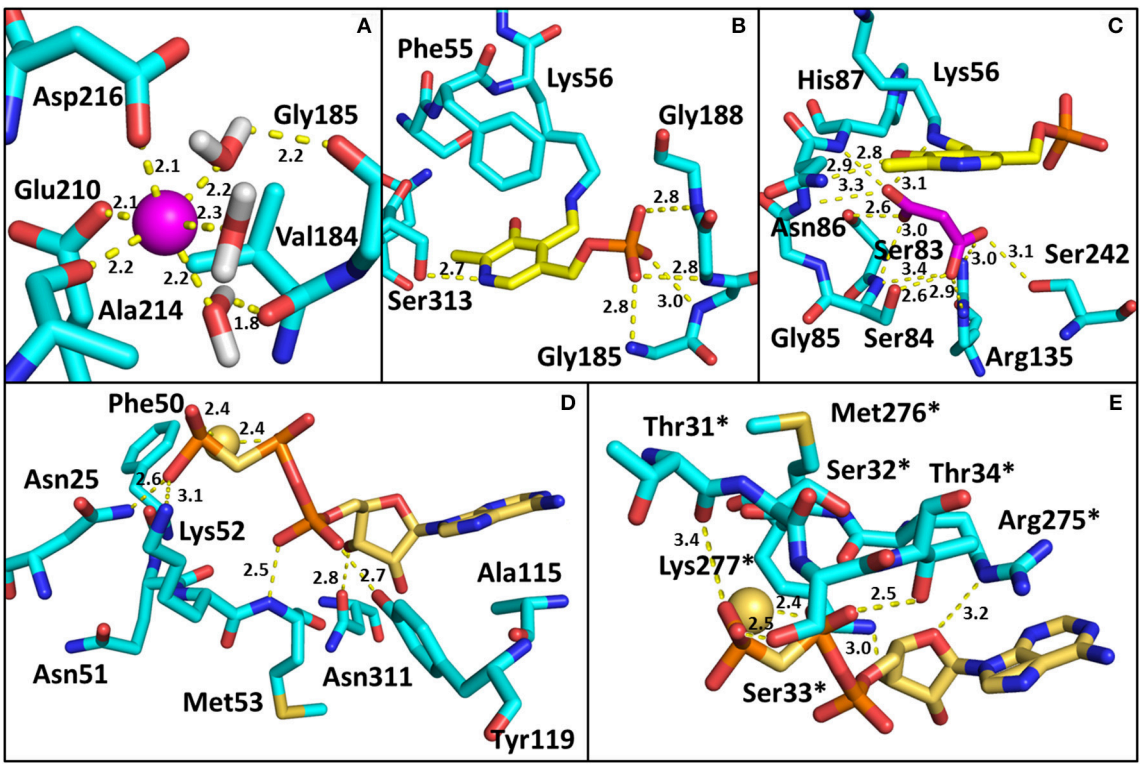


FIGURE 3 | Binding sites in SR. The amino acids involved in the interactions are reported as cyan sticks, and polar interactions are highlighted by yellow dotted lines. The PDB used are 3L6B (hSR, closed form) for panels **(A–C)**, and 1WTC (spSR with AMP-PCP) for **(D,E)**. **(A)** Divalent cation binding site in hSR. The cation (Mn^{2+}) is represented as a pink sphere; **(B)** PLP binding site in hSR; **(C)** Malonate binding site in hSR; **(D)** AMP-PCP binding site in spSR. The residues of the monomer in closer contact with the allosteric effector are reported. The positions of Asn25, Phe50, Asn51, Lys52, Met53, Ala115, Tyr119, and Asn311 in spSR correspond to His24, Phe49, Asn50, Lys51, Thr52, Ala117, Tyr121, and Asn316 in hSR, respectively; **(E)** residues of the second monomer involved in the interaction with AMP-PCP are reported. Asterisks indicate that the residues belong to the monomer on the opposite side of AMP-PCP. The positions of Thr31, Ser32, Ser33, Thr34, Arg275, Met276, and Lys277 in spSR correspond to Thr30, Ser31, Ser32, Ile33, Arg277, Met278, and Lys279 in hSR, respectively. Water molecules involved in the binding of SR with ligands are omitted for the sake of simplicity in all panels except **(A)**. All distances are within 3.4 Å.

(**Figure 3B**). The PLP ring is covalently linked as an internal aldimine to a Lys residue in the active site (Lys57 in SpSR and Lys56 in rSR and hSR). PLP binds with the *re* face toward the solvent, in the same orientation as in aspartate aminotransferases (Goto et al., 2009). Considering hSR numbering, conserved residues in the PLP active site are motifs formed by residues 54–59 (Ser-X-Lys-Ile-Arg-Gly), 313–316 (Ser-X-Gly-Asn) and the tetra-glycine loop (Smith et al., 2010). Ser84 (hSR numbering), a highly conserved residue, was proved to be essential for racemase and D-serine dehydratase activities because it is involved in the binding of ligands to the active site (see below) (Yoshimura and Goto, 2008; Goto et al., 2009; Smith et al., 2010; **Figure 3C**). SR is present in solution as a symmetric dimer, as confirmed by X-ray crystallography, size-exclusion chromatography and glutaraldehyde cross-linking (Goto et al., 2009; Smith et al., 2010; **Figure 4**). Most residues at the dimer interface are conserved among different species (Goto et al., 2009). The dimer was found in both open and closed conformations. The analysis of the buried monomer-monomer surface area for rSR in the open and closed form indicated that the dimer interface has a high degree of flexibility (Smith et al., 2010), probably corresponding to a rearrangement of the interactions between the two monomers upon ligand binding to the active site, as a consequence of the open-closed conformational switch. An equilibrium between dimer and tetramer has been described (Wang and Barger, 2011), and found to depend on the presence of ligands and metal ions (Bruno et al., 2017).

The dimeric structure of SR is crucial for the regulation of enzyme activity. The structure of SR bound to a stable analog of ATP, 5′-adenylyl methylene diphosphonate (AMP-PCP), in the absence of ligands bound to the active site, i.e., in the open form, was solved for SpSR (Goto et al., 2009). AMP-PCP in complex with Mg^{2+} ions binds into a cleft at the interface between the subunits at two symmetry-related sites. AMP-PCP interacts with the small and large domains of one subunit, and with the large domain of the other subunit. The binding of Mg·AMP-PCP to the open form changes the relative orientation between the two subunits, increasing the width of the groove between the two monomers (**Figure 5**). The residues involved in AMP-PCP binding include Ala115 (SpSR numbering) and Tyr119, interacting within the small domain. The large domain binds AMP-PCP with Asn25, Phe50, Asn51, Lys52, Met53, and Asn311, all residues present in loop regions (**Figure 3D**). On the opposite side, the ligand interacts with Thr31, Ser32, Ser33, Thr34, and Arg275, Met276, Lys277, both at a terminal part of α-helices of the large domain of the second monomer in the dimer (**Figure 3E**). Water molecules are also involved in the binding. The position of Mg·AMP-PCP has been docked into the structure of hSR in the presence of malonate (PDB code: 3L6B), suggesting that the overall interaction with ATP is similar between yeast and mammalian SR (Jirásková-Vanícková et al., 2011). Although the AMP-PCP site and the substrate binding site are 15 Å away, and the two symmetric ATP sites are 24 Å apart, an allosteric communication occurs, likely by rearrangement of a H-bond network, which connects the O3′ group of PLP with the

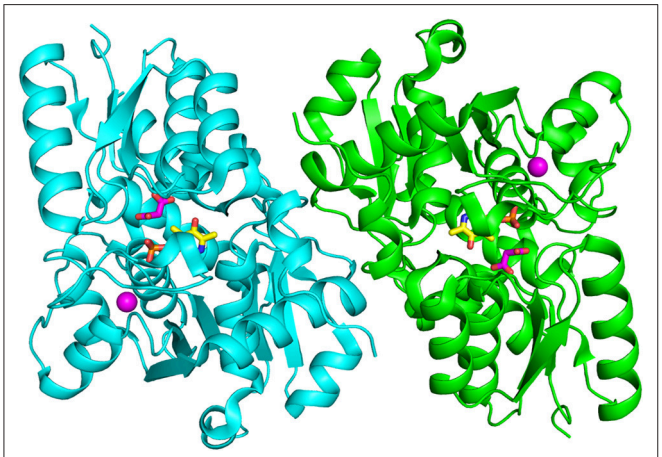


FIGURE 4 | Dimeric structure of hSR (PDB code: 3L6B). The two monomers are represented in cyan and green. PLP and malonate are in sticks, and colored in yellow and pink, respectively. The divalent cation is represented as a pink sphere.

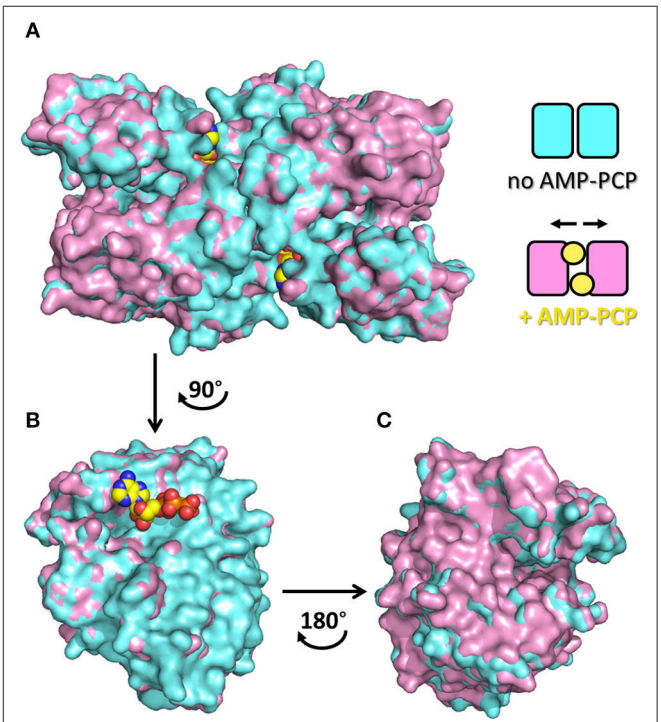


FIGURE 5 | Quaternary rearrangement of spSR upon binding of AMP-PCP with enlargement of the groove between the two monomers. **(A)** Surface representation of spSR in the open form in the presence (PDB 1WTC, pink) and absence (PDB 1V71, cyan) of AMP-PCP bound to two symmetric sites at the dimer interface. The scheme indicates the changes in the relative position of the monomers in the two conditions, i.e., without or with AMP-PCP. In order to highlight the quaternary changes caused by AMP-PCP, the superposition of only one monomer is shown **(B)** facing the direction of the dimer interface, upon rotation of 90° of the dimer, and **(C)** from the opposite side of the subunit, upon rotation of 180° of the monomer, i.e. rotation of the dimer of 90° in the opposite direction. AMC-PCP is in yellow. In **(B)**, only the molecule of AMP-PCP bound to the monomer is shown.

γ-phosphate of AMP-PCP. This network involves Thr52, Asn86, Gln89, Glu283 and Asn316 in hSR (Met53, Gln87, Glu281, Asn311 in SpSR) and two water molecules (Goto et al., 2009; Marchetti et al., 2013; Canosa et al., 2018).

SR CATALYSIS

SR catalyzes two reactions, the reversible racemization of L-Ser or D-serine and the irreversible dehydration of L-Ser and D-Ser to pyruvate and ammonia (De Miranda et al., 2002; Foltyn et al., 2005).

Racemases have been classified as PLP-dependent or PLP-independent (Conti et al., 2011), with SR belonging to the first group. Amino acid racemization is a two-steps reaction where alpha proton abstraction is followed by re-protonation from the opposite side of a planar intermediate. Since the pK_a of alpha proton is considerably high, pK about 21 or higher (Yoshimura and Esak, 2003), racemases have evolved to face the problem of increasing the acidity of this proton. In hSR, PLP is covalently bound to Lys56 and, differently to fold type I PLP-dependent enzymes (Griswold and Toney, 2011), presents a deprotonated pyridine nitrogen (Goto et al., 2009). This feature is shared with fold type II PLP-dependent enzymes, like bacterial alanine racemase (Toney, 2005), tryptophan synthase (Raboni et al., 2009) and *O*-acetylserine sulfhydrylase (Mozzarelli et al., 2011). Lys56 is not only involved in the formation of the internal aldimine with PLP, but, together with Ser84, is also a catalytic residue and participates in proton abstraction/reprotonation of serine in a classical two-base mechanism (Foltyn et al., 2005; Goto et al., 2009; **Scheme 1**). In the deprotonated form, the substrate binds to SR active site and undergoes a transaldimination reaction with PLP with formation of an external aldimine. The protonation state of Ser84 and Lys56 depends on the enantiomer of serine that binds to the enzyme. When L-Ser is the substrate, a deprotonated Lys56 can abstract the alpha proton leading to an anionic quinonoid intermediate. When D-Ser binds, Lys56 is protonated and Ser84 is deprotonated, thus allowing proton abstraction from the opposite side of the amino acid. The existence of a true quinonoid species is questioned by the lack of any experimental observation of this intermediate and by the structural evidence that a negative charge on the pyridine nitrogen could not be stabilized by a positively charged residue, as observed for transaminases (Griswold and Toney, 2011). On the other hand, the formation of a metastable quinonoid would favor reaction specificity, as already observed for alanine racemase (Toney, 2005). The final step of the reaction is reprotonation by either Ser84 or Lys56. Human SR does not racemize L-Thr, whereas archaeal SR does, albeit with lower efficiency with respect to serine (Ohnishi et al., 2008). However, very recent works have demonstrated that mouse SR (mSR) can catalyze the racemization of L-Asp (Ito et al., 2016). The efficiency of D-Asp production is more than 500-fold lower than that of D-Ser production ($k_{cat}/K_m = 12\ min^{-1}\cdot mM$ for L-Ser racemization vs. $0.022\ min^{-1}\cdot mM$ for L-Asp racemization). Over-expression of SR in cultivated cells increases the concentration of D-Asp, thus suggesting another relevant role *in vivo* for this enzyme. Phylogenetic analyses indicated that animal Asp and Ser racemases form a serine/aspartate racemase family cluster (Uda et al., 2016) and the aspartate racemase activity evolved from the SR activity by acquisition of a triple serine loop facing the substrate binding site (Uda et al., 2016, 2017).

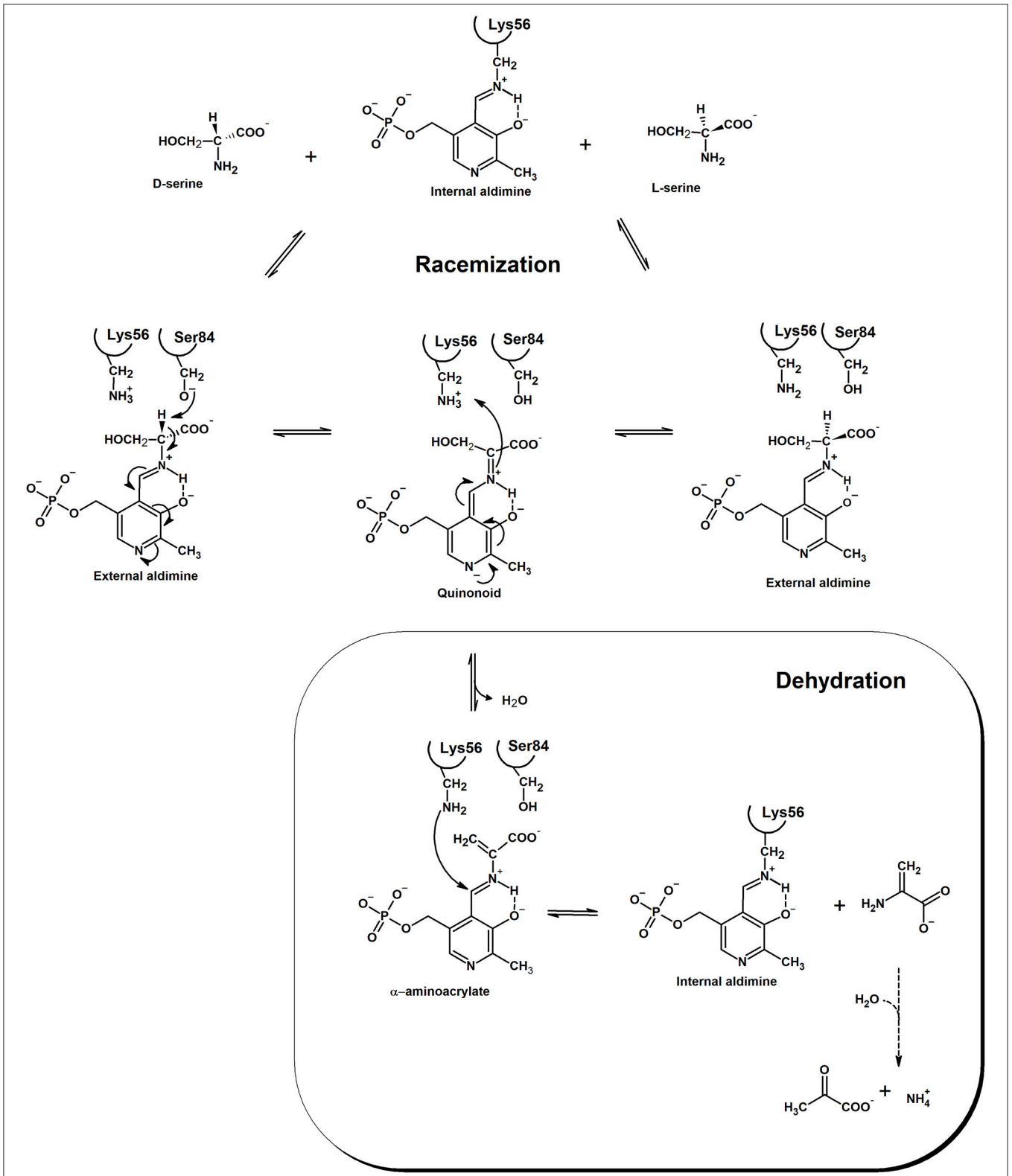
The anionic intermediate that forms upon alpha proton abstraction can undergo a β-elimination reaction with formation of the α-aminoacrylate, an unstable intermediate that is readily hydrolyzed to pyruvate and ammonia with restoration of the internal aldimine. The β-elimination reaction is also catalyzed on β-chloroalanine, L-threonine, L-Ser-*O*-sulfate and L-threo-3-hydroxyaspartate (Panizzutti et al., 2001; Strísovský et al., 2005) and does not lead to syncatalytic inactivation of the human enzyme, an event often observed in transaminases (Morino et al., 1979; Cooper et al., 2002). Both racemization and dehydration reactions are activated, to a different extent, by ATP (see below), the dehydration of L-Ser being the most affected, with a 31-fold increase in catalytic efficiency upon nucleotide binding (De Miranda et al., 2002; Canosa et al., 2018; **Table 2**). Under physiological conditions, i.e., SR fully saturated with ATP, the dehydration reaction is several folds more efficient than the racemization reaction. The physiological relevance of D-Ser production by SR has been demonstrated with knock-out mice, which show a D-Ser concentration in the brain that is less than 10% compared to normal mice (Labrie et al., 2009; Balu et al., 2013). On the other hand, the role of dehydration *in vivo* is still debated and was suggested to be relevant for D-Ser degradation, especially in those brain areas lacking D-amino acid oxidase (De Miranda et al., 2002). Since SR has evolved from serine dehydratases (De Miranda et al., 2000), it is likely that the dehydration reaction is the vestige of the enzyme ancestor and was maintained during evolution for its contribution to D-Ser homeostasis. Overall, the efficiency of SR in D-Ser production is very low, likely due to the low metabolic requirement for this amino acid and the need for a strict regulation of its production. However, there is evidence that localization and/or interaction with other proteins might play a role in increasing the rate of D-Ser production under physiological conditions (see below).

SR SMALL LIGANDS AND PROTEIN INTERACTORS

hSR activity is finely regulated by several physiological effectors, including ATP, cations, anions and interacting proteins, whose fluctuations contribute to the homeostasis of D-serine.

Small Ligands

The stimulation of SR activity by ATP was first described by Neidle and Dunlop (Neidle and Dunlop, 2002) on the murine ortholog, when they observed a five-fold increase in the catalytic activity in the presence of small quantities of yeast extract. Successive experiments demonstrated that SR activity was triggered by either magnesium or calcium and nucleotides, the most active being ATP, ADP and GTP. In the same year, other two research groups independently confirmed the activation role of divalent cations and nucleotides on mSR activity



SCHEME 1 | Mechanism of the racemization and dehydration reactions catalyzed by SR. Electron movement is shown in the direction of D-Ser racemization. The product of the dehydration reaction, α-aminoacrylate, is spontaneously and rapidly hydrolyzed to pyruvate and ammonia. Residue numbering refers to the human ortholog. Modified from Goto et al. (2009) and Smith et al. (2010).

(Cook et al., 2002; De Miranda et al., 2002). The effectiveness of ADP and non-hydrolyzable ATP analogs (De Miranda et al., 2002; Neidle and Dunlop, 2002) further demonstrated that the nucleotides do not provide an energetic contribution to catalysis.

ATP is one of the principal modulators of hSR catalysis and takes part in a fine-tuning of the enzymatic activity (**Table 2**). The catalytic efficiency of dehydration on L-serine is deeply enhanced by ATP, which exerts a 31-fold increase in activation, with a k_{cat}/K_M of 8.1 ± 1.1 and 253.0 ± 15.0 s^{-1} M^{-1} in the absence and presence of ATP, respectively (Marchetti et al., 2013). Otherwise, under the same conditions, the efficiency of dehydration activity on D-serine increases 4-fold, with only a small effect of ATP on D-serine degradation rate (0.6 and 2.4 s^{-1} M^{-1}, respectively). The racemization of L-serine occurs with about a two-fold increased efficiency in the presence of ATP (from 9.2 to 17.5 s^{-1} M^{-1}), but the net effect of ATP binding is a strong stimulation of L-serine degradation, with an increment from 0.9 to 14.5 of the ratio between the two activities in the absence and presence of ATP, respectively (Marchetti et al., 2013). As shown above, the comparison of the structure of SpSR in the absence and presence of the ATP analog AMP-PCP (PDB 1V71 and 1WTC) revealed a change in the relative orientation of the major and minor domain (Goto et al., 2009), resulting in a wider back groove. Moreover, in the presence of ATP and either glycine or malonate, hSR active site appears less accessible than in the absence of ATP, as demonstrated by PLP fluorescence quenching (Marchetti et al., 2015). Moreover, by monitoring PLP fluorescence in the absence and presence of ATP, it was demonstrated that ATP binding to hSR promotes a decrease in the polarity of the active site, diminishing its accessibility (Marchetti et al., 2013). These observations are in line with the existence of a H-bond network that links the ATP binding site with the active site (Goto et al., 2009) and modulates the active site open-to-closed transition, promoting the correct orientation of the catalytic residues. Recently, Gln89 has been pointed out as a key residue in the allosteric communication between the active site and the ATP-binding site. This residue is specifically involved in the activation of the dehydration reaction by ATP and its mutation to either Ala or Met completely abolishes nucleotide-dependent modulation of serine dehydration (Canosa et al., 2018).

Based on the intracellular ATP concentration, hSR was thought to be always saturated *in vivo*. However, *in vitro* studies revealed that ATP binds with a strong cooperativity (Hill n close to 2) with calculated ATP K_Ds for high and low affinity states of 11.5 μM and 1.8 mM, respectively. These values fall within the physiological range (Marchetti et al., 2013), underlying a potential sensitivity of hSR activity on intracellular ATP fluctuations. The apparent K_Ds for ATP obtained through fluorescence measurements in the absence of active site ligands (0.26 ± 0.02 mM) and through activity assays (0.22 ± 0.01 mM and 0.41 ± 0.02 mM for L-serine and D-serine dehydration, 0.22 ± 0.05 mM for L-serine racemization, respectively), are very similar. This finding indicates that the intermediates that form during the catalytic cycle do not allosterically affect ATP binding site, in contrast with glycine that forms a stable Schiff base and affects ATP affinity (Dunlop and Neidle, 2005; Marchetti et al., 2013). Furthermore, when the active site is involved in the formation of a stable complex between PLP and a ligand, such as glycine, ATP binds to hSR in a non-cooperative fashion, with a 50-fold stronger affinity. In the same way, ATP influences the substrates affinity for the active site (as mirrored on K_M values) and strengthens the binding of covalent (i.e., glycine, 15-fold) or non-covalent (i.e., malonate, 10-fold) inhibitors (Marchetti et al., 2013, 2015). Based on these evidences, glycine, the alternative glutamate co-agonist to D-serine on NMDA receptors, may participate to the fine-tuning of the communication between active and allosteric sites.

As for ATP, also the residues involved in the metal binding site are highly conserved in the yeast, murine and human SR sequences, giving a similar coordination in all the three structures (see above) (Goto et al., 2009; Smith et al., 2010; Takahara et al., 2018). Although the mammalian enzyme can be activated both by calcium or magnesium (Cook et al., 2002; De Miranda et al., 2002; Neidle and Dunlop, 2002), the metal binding site has always been assumed as physiologically occupied by the latter because of its intracellular concentration and the stronger activation effect with respect to the former. Recently, investigations of hSR enzymatic activity *in vitro* in the presence of either magnesium or calcium support the absence of a relevant competition between the two ions at physiological level (Bruno et al., 2017). Racemization and dehydration activities in the presence of 1 mM L-serine and 2 mM ATP, concentrations close to intracellular conditions in

TABLE 2 | Catalytic parameters of hSR for serine racemization and dehydration in the presence and absence of ATP, at saturating concentrations of Mg^{2+}.

Reaction	K_M (mM)		k_{cat} (min^{-1})		k_{cat}/K_M ($s^{-1} \cdot M^{-1}$)		Fold increase
	–ATP	+ATP	–ATP	+ATP	–ATP	+ATP	
L-Ser racemization	35 ± 5	34 ± 4	19 ± 1	35 v1	9.2 ± 1.4	17.5 ± 2.1	1.9
L-Ser dehydration	76 ± 10	12 ± 1	37 ± 4	183 ± 3	8.1 ± 1.1	253.0 ± 15.0	31.0
D-Ser dehydration	46 ± 3	167 ± 16	1.7 ± 0.1	23 ± 1	0.6 ± 0.1	2.4 ± 0.1	4.0

Data are from Marchetti et al. (2013) and Canosa et al. (2018).

neurons (Gribble et al., 2000; Foltyn et al., 2005; Genc et al., 2011), unveiled a larger effect of magnesium on catalytic efficiency, with a 2.5-fold difference with respect to calcium. Magnesium and calcium bind to hSR with similar affinities both in the absence (EC_{50} of 28 ± 3 μM and 126 ± 7 μM, respectively) and presence of ATP (EC_{50} of 17 ± 1 μM and 194 ± 6 μM, respectively). Therefore, the two cations are able to elicit with different affinities similar conformational changes responsible for the activation of hSR, thus behaving as activators (Purich and Allison, 2002). During the neural transmission, Ca^{2+} concentration locally rises up to 100 μM in neurons, but cannot compete with magnesium for the binding to hSR and does not influence the enzymatic turnover number (Bruno et al., 2017). For these reasons, the impact on hSR regulation *in vivo* is likely to be insignificant.

It has been demonstrated that the activity of hSR can be further affected by halides (Marchetti et al., 2015). Among them, chloride, the only one with physiological relevance, behaves like an "uncompetitive activator" (Wild et al., 1976; Maruyama, 1990), since it influences both k_{cat} and K_M, but does not alter the catalytic efficiency (Marchetti et al., 2015). In mature neurons in the CNS, the intracellular concentration of chloride is maintained at about 5 mM through the co-transport of different ionic species (Doyon et al., 2016). During the action potential, the intracellular concentration of Cl^- can increase five-fold, to 20–25 mM, both in the presynaptic terminal and in the postsynaptic dendrites. This rapid change occurs due to the activation of $GABA_A$ and glycine receptors, and modulates both the release of glutamate neurotransmitter and the inhibitory post-synaptic current (Price and Trussell, 2006). It is interesting to note that *in vitro* studies show that in the presence of ATP and Mg^{2+}, hSR quaternary structure can be affected by the concentration of NaCl. In particular, hSR exists as a tetramer in the absence of NaCl, whereas at increasing salt concentration the equilibrium is shifted toward the dimeric form (Bruno et al., 2017). The half effect corresponds to 20–50 mM NaCl, suggesting a possible role of chloride in the oligomeric distribution of hSR.

Protein Interactors

In addition to small molecule effectors and post-translational modifications (see below), SR function is regulated by the interaction with specific proteins, particularly proteins associated with AMPA and NMDA receptors. Particularly, the C-terminal end, located near the ATP binding site, mediates the interaction with protein partners.

The Glutamate Receptor Interacting Protein (GRIP) enhances SR activity and D-serine release from glia (Kim et al., 2005) and in transfected mammalian cells (Baumgart et al., 2007). Furthermore, GRIP was shown to cause SR conformational changes (Baumgart et al., 2007). This conclusion was supported by molecular modeling of the interaction between SpSR and a GRIP-contained PDZ domain (Baumgart et al., 2007).

Protein Interacting with C-kinase (PICK1) is activated by the erythropoietin-producing hepatocellular receptor (Eph), which promotes its release in the cytosol of astrocytes, where it interacts with SR. Specifically, upon Eph receptor activation, there is a dissociation of PICK1 from Eph and an increased association with SR (Zhuang et al., 2010; Kiriyama and Nochi, 2016), accompanied with an increase in D-serine synthesis (Fujii et al., 2005; Hanley, 2008; Hikida et al., 2008). GRIP and PICK1 both contain a PDZ domain, which is recognized by three carboxyl-terminal amino acids of SR with a well characterized consensus sequence (Val-Ser-Val) (Baumgart et al., 2007). How PICK1 and GRIP interact to regulate SR is still unclear, but they are both dependent on the phosphorylation status of AMPA receptors (Wolosker et al., 1999; Fujii et al., 2005; Kim et al., 2005). Phosphorylation at Ser880 causes dissociation of GRIP, whereas PICK1 remains bound (Mustafa et al., 2004; Wang and Barger, 2011). Other accessory proteins, such as Stargazin and postsynaptic density proteins 95 (PSD-95), also regulate AMPA receptors (Ma et al., 2014). There is evidence of the formation of a ternary complex of SR with Stargazin and PSD-95, presumably affecting SR activity and, therefore, glutamate neurotransmission (Ma et al., 2013).

Another interactor of SR is Golga-3, a protein that binds to the cytosolic face of the Golgi apparatus and stabilizes SR levels through inhibition of its ubiquitination (Wolosker et al., 1999; Fujii et al., 2005; Dumin et al., 2006; Canu et al., 2014). Recently, it was also showed that the protein Disrupted in Schizophrenia 1 (DISC1), which is implicated in pathology of major psychiatric disorders, binds SR, preventing its ubiquitination and degradation. The C-terminus truncated form of DISC1 is unable to bind SR and induces its degradation and D-serine depletion (Ma et al., 2013). FBXO22, a component of the ubiquitin-proteasome system, also interacts with SR modifying its intracellular organization (see below) (Dikopoltsev et al., 2014).

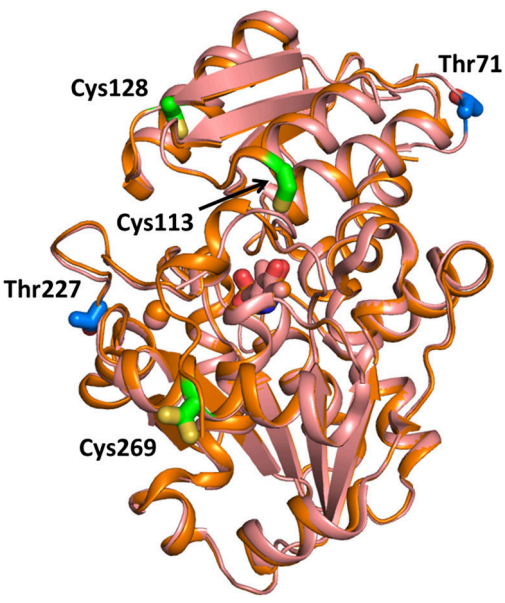


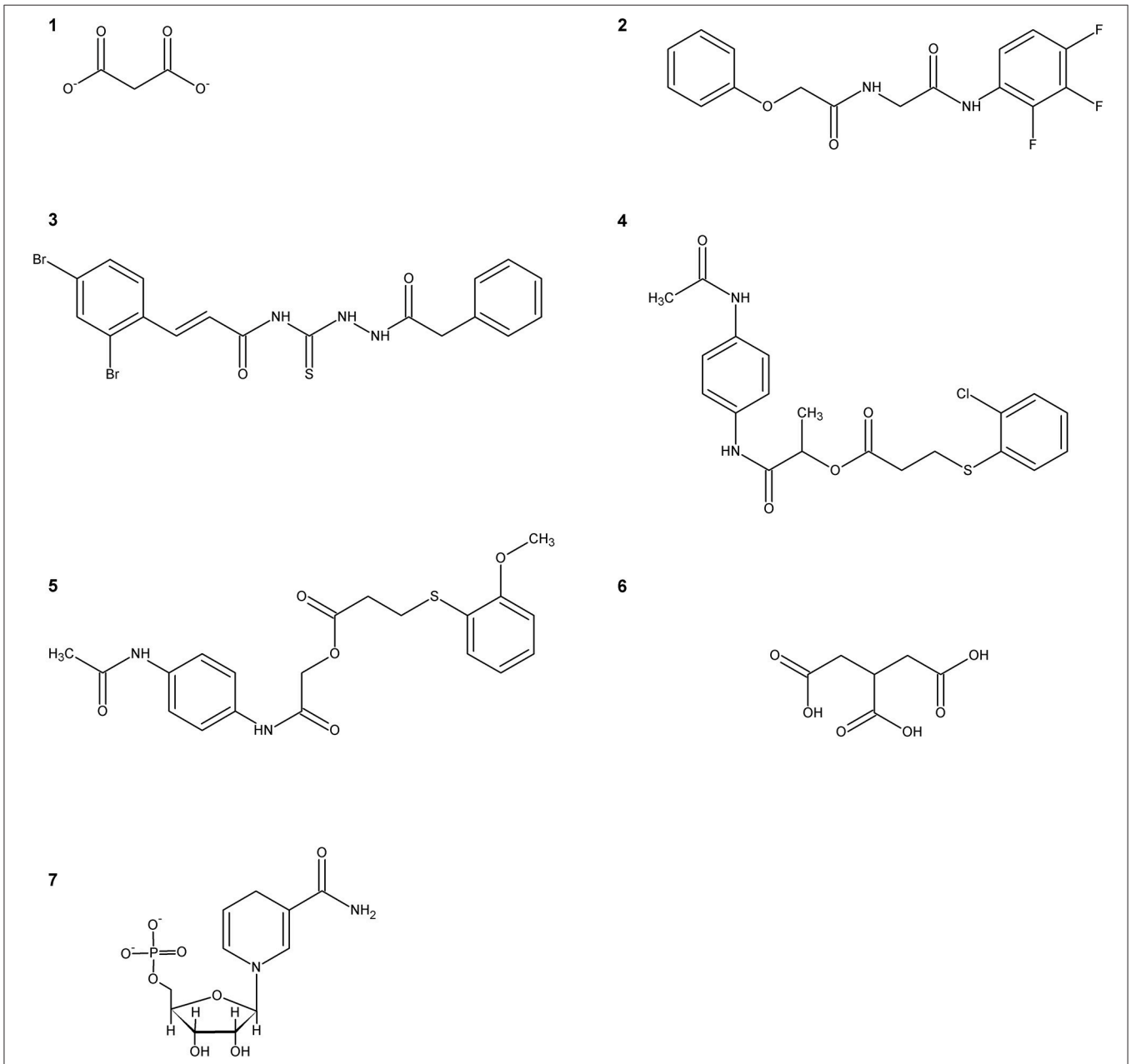
FIGURE 6 | Sites for SR nitrosylation and phosphorylation. The structure of hSR (PDB 3L6B, light pink) and rSR (PDB 3L6C, orange) are superimposed and the positions of the sites for S-nitrosylation (in green on the structure of hSR) and phosphorylation (in blue on the structure of rSR) are reported in sticks. The divalent cation is in pink. Cys269 has two possible positions with 0.5 occupancy based on the PDB file.

SR POST-TRANSLATIONAL MODIFICATIONS

SR was reported to be post-translationally modified by nitrosylation, phosphorylation and palmitoylation. SR levels are also regulated by ubiquitin-tagging for proteasomal degradation.

Nitrosylation

Experiments on a human glioblastoma cell line showed that the activity of SR was inversely regulated by nitric oxide, and addition of D-serine promoted denitrosylation of the murine purified ortholog (Shoji et al., 2006). These results suggested a regulation mechanism in which D-serine, besides being the substrate of the enzyme, is also an activator through denitrosylation (Shoji et al., 2006). The nitrosylation site in murine SR was determined to be Cys113, adjacent to the ATP binding site, whose S-nitrosylation was shown to inhibit the enzyme activity of about 10-fold (Mustafa et al., 2007). This regulation mechanism was interpreted as a feedback control of NMDA activation (Mustafa et al., 2007). Unlike the murine ortholog, human SR was shown to be S-nitrosylated at three cysteine residues, Cys113, Cys269, and Cys128, with Cys269 being unique to the human ortholog



SCHEME 2 | Representative inhibitors of SR.

(Marchesani et al., 2018; **Figure 6**). The inhibition kinetics was biphasic, indicating that at least two of these residues were responsible for enzyme inhibition, as confirmed by site-directed mutagenesis of Cys113 (Marchesani et al., 2018). When nitrosylated, hSR binds ATP with affinity and cooperativity similar to the native enzyme. However, nitrosylation does not affect enzyme activity in the absence of ATP, suggesting that nitrosylation alters the allosteric communication between the ATP binding site and the active site, possibly via the stabilization of a distinct conformation (Marchesani et al., 2018).

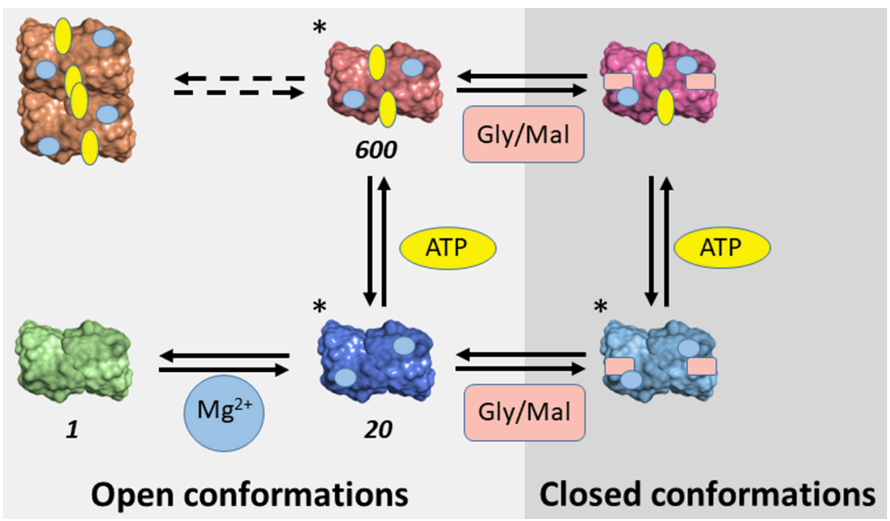
Phosphorylation

Phosphorylation of murine SR takes place at Thr71 in the cytosolic and in the membrane-bound SR. In the latter, an additional phosphorylation site was detected at Thr227 (Balan et al., 2009; Foltyn et al., 2010; **Figure 6**). Phosphorylation at Thr71 is the main phosphorylation site and promotes D-serine synthesis by increasing enzyme turnover rate. Indeed, Thr71Ala SR exhibits a 50% lower activity when compared to the wild type. However, this phosphorylation site is not conserved in hSR, indicating that it belongs to a rodent-specific regulation mechanism. Phosphorylation of Thr227 favors the association of SR with membranes. Indeed, the levels of membrane-bound SR under non-stimulated conditions are reported to decrease in Thr227Ala mutant. Prediction of the kinases mediating SR phosphorylation at Thr71 and Thr227 carried out by *in silico* analysis of the phosphorylation motif identified proline-directed kinases as the strongest candidates. However, treatment with inhibitors of the predicted kinases did not alter the phosphorylation profile nor changed SR binding to the membrane making the identification of the specific kinase still elusive. In addition, phosphorylation at Thr71 was reported to be regulated by phosphatases and stimulated by growth factors present in the serum, as expected for a dynamic event. Phosphorylation by protein kinase C (PKC) on serine residue of rodent SR was suggested by Mustafa and colleagues based on the proximity of the two proteins mediated by PICK1 binding (Mustafa et al., 2004). Later, Vargas-Lopes et al. demonstrated

TABLE 3 | Selected inhibitors of hSR.

Compound	K_i (μM)	Type of inhibition	Site of inhibition	References
Malonate	710 ± 33 (- ATP) 77 ± 9 μM (+ATP)	Reversible	Active site	Marchetti et al., 2015
Glycine	7000 ± 300 (-ATP) 470 ± 30 (+ATP)	Reversible, covalent	Active site	Marchetti et al., 2013
3	*4	Reversible	Active site	Mori et al., 2017
5	*207	Reversible	Active site	Takahara et al., 2018
6	1300	Reversible	Active site	Dellafiora et al., 2015
Dicarboxylic cyclopropane	900	Reversible	Active site	Beato et al., 2016
7	18 ± 7	Reversible	ATP site	Bruno et al., 2016

K_i calculated from IC50 using the equation $K_i = IC_{50}/([S]+K_M)+1$ that can be applied to reversible competitive inhibitors. Values of L-serine concentration and K_M were derived from data reported in the quoted references.



SCHEME 3 | Conformational space of SR. The conformations determined crystallographically are marked with (*). For conformations characterized in terms of catalytic activity, the relative value of catalytic efficiency in comparison with the metal-free form is reported. Gly and Mal indicate glycine and malonate, respectively.

TABLE 4 | SR interactors and effects on SR structure and function.

Interactors	Isoform	Site of interaction on SR	Effect	Experimental evidences	References
SMALL LIGANDS					
ATP	Human	Dimer interface (by similarity)	Structural rearrangement, activation	Activity assays, binding assays, molecular docking	Jirásková-Vanícková et al., 2011 Marchetti et al., 2013 Marchetti et al., 2015 Canosa et al., 2018
	Mouse	Dimer interface (by similarity)	Activation	Activity assays	Cook et al., 2002 De Miranda et al., 2002 Neidle and Dunlop, 2002
	S. pombe*	Dimer interface	Structural rearrangement, activation	Activity assays, X-ray crystallography	Goto et al., 2009
Mg^{2+}/Ca^{2+}	Human**	Glu210, Ala214, Asp216	Activation	X-ray crystallography, activity assays	Smith et al., 2010 Bruno et al., 2017
	Mouse	Glu210, Ala214, Asp216 (by similarity)	Activation	Activity assays, circular dichroism, radiolabelling	Cook et al., 2002 De Miranda et al., 2002 Neidle and Dunlop, 2002
	Rat**	Glu210, Ala214, Asp216	Not determined	X-ray crystallography	Smith et al., 2010
	S. pombe	ATP binding site Glu208, Gly212, Asp214	Activation	Activity assays, X-ray crystallography	Goto et al., 2009 Yamauchi et al., 2009
Cl^-	Human	Unidentified	Activation, oligomerization	Activity assays, gel filtration	Marchetti et al., 2015 Bruno et al., 2017
NADH	Human	Dimer interface	Inhibition	Activity assays	Bruno et al., 2016
Glycine	Human	Active site	Inhibition	Activity assays, binding assays	Hoffman et al., 2009 Marchetti et al., 2013
	Mouse	Active site	Inhibition	Activity assays	Dunlop and Neidle, 2005 Strísovský et al., 2005
Malonate	Human	Active site	Inhibition	Activity assays, binding assays, X-ray crystallography	Strísovský et al., 2005 Smith et al., 2010 Marchetti et al., 2015
	Rat	Active site	Inhibition	X-ray crystallography	Smith et al., 2010
PIP_2	Mouse	Lys70, Lys77, Lys96, Lys137, Leu168	Inhibition, association to intracellular membranes	Immunocytochemistry, activity assays, binding assays	Mustafa et al., 2009
POST-TRANSLATIONAL MODIFICATIONS					
Nitrosylation	Human	Cys113, Cys128, Cys269	Inhibition	Activity assays, binding assays, mass spectrometry	Marchesani et al., 2018
	Mouse	Cys113	Inhibition	Activity assays	Shoji et al., 2006 Mustafa et al., 2007
Phosphorylation	Mouse	Thr71, Thr227	Activation	Mass spectrometry, radiolabelling, activity assays	Balan et al., 2009 Foltyn et al., 2010
Acylation/palmitoylation	Mouse	Unidentified Ser or Thr	Translocation to membranes	Radiolabelling, immunocytochemistry	Balan et al., 2009
Ubiquitination	Mouse	Not determined	Degradation	Western blotting	Dumin et al., 2006
PROTEINS					
GRIP	Mouse	C-terminus	Activation	Western blotting, activity assays	Jirásková-Vanícková et al., 2011
	Rat	C-terminus	Activation	Yeast two-hybrid screening, immunoprecipitation	Kim et al., 2005
PICK1	Human	C-terminus	Activation	Yeast two-hybrid screening, binding assays, immunoprecipitation, immunocytochemistry	Fujii et al., 2005
	Mouse	C-terminus	Activation	Binding assays, siRNA, western blotting, immunoprecipitation, HPLC	Hikida et al., 2008 Zhuang et al., 2010
Stargazin/PSD-95	Mouse	C-terminus	Inhibition	Immunoprecipitation, western blotting, immunocytochemistry, HPLC	Ma et al., 2014

(Continued)

TABLE 4 | Continued

Interactors	Isoform	Site of interaction on SR	Effect	Experimental evidences	References
Golga-3	Mouse	N-terminus	Increased half-life	Yeast two-hybrid screening, immunoprecipitation, immunocytochemistry, binding assays	Dumin et al., 2006
DISC1	Human	Not determined	Increased half-life	Western blotting, immunoprecipitation, immunocytochemistry	Ma et al., 2013
	Mouse	Not determined	Increased half-life	Immunohistochemistry, western blotting, immunoprecipitation, immunocytochemistry, binding assays	Ma et al., 2013
FBXO22	Mouse	Not determined	Prevention of translocation to membranes	Activity assays, binding assays, immunoprecipitation	Dikopoltsev et al., 2014

**Co-crystallized with the ATP analog AMP-PCP.*
***Mn^{2+} in the crystallographic structure.*

that PKC phosphorylates SR on serine residues and decreases SR activity *in vitro* (Vargas-Lopes et al., 2011). Analogously, PKC activation increases SR phosphorylation and reduces D-serine levels in astrocyte and neuronal cultures and in rat frontal cortex. In particular, PKC-mediated phosphorylation regulates D-serine availability in the brain in NMDA-dependent memory-related processes. Alignment of the amino acid sequences of human, rat and mouse SR revealed five conserved consensus sequences for PKC phosphorylation but the site remains to be established.

Acylation/Palmitoylation

SR does not possess specific amino acid motifs required for prenylation, isoprenylation, or myristoylation. Interestingly, SR is a rare example of an *O*-palmitoylated protein at still unidentified serine or threonine residues (Balan et al., 2009). Palmitoylation contributes to membrane binding and plays a key role in SR translocation from the cytosol. In addition to [^{3}H]palmitic acid, [^{3}H]octanoic acid was also incorporated into SR in neuroblastoma cells, suggesting that *in vivo O*-acylation may involve fatty acids of different lengths (Balan et al., 2009).

Ubiquitination

SR has a relatively short half-life compatible with the existence of an efficient degradation/regulatory system. Indeed, it was demonstrated that SR undergoes poly-ubiquitination in an ATP-dependent manner both *in vitro* and *in vivo*, which leads to degradation by the ubiquitin-proteasome system. However, the E3 ubiquitin ligase that transfers ubiquitin chains to SR is still unidentified (Dumin et al., 2006). The ubiquitin system is a key regulator of SR and D-serine levels. Partner proteins have been reported to affect the rate of protein degradation by the ubiquitin-proteasome system, altering its half-life and modulating SR function (see above). In particular, interaction with Golga-3 both *in vitro* and *in vivo* stabilizes SR, prevents its ubiquitination and slows down its degradation by the ubiquitin-proteasome system. Consequently, the significant increase in its half-life and steady-state levels indirectly raises D-serine levels. Analogously, DISC1 diminishes SR ubiquitination acting as a scaffold that stabilizes SR. Truncation of the C-terminus in a mutant DISC1 disrupts the physiologic binding to SR and increases ubiquitination and degradation of SR in astrocytes with a consequent decrease in D-serine production (Ma et al., 2013). FBXO22, an F-box motif-containing protein and a component of the SCF ubiquitin ligase complex, does not promote SR ubiquitination nor its targeting to the proteasome system. Indeed, it is the free FBXO22a species, without the participation of the SCF-FBXO22a complex, which prevents the accumulation of membrane-bound SR species and regulates D-serine synthesis (Dikopoltsev et al., 2014).

SR INHIBITORS

High concentrations of D-serine in the brain are associated with high NMDA receptors activity, leading to severe excitotoxicity, as observed in Parkinson and Alzheimer diseases, amyotrophic lateral sclerosis and ischemia. Therefore, significant efforts have been directed toward the development of active-site inhibitors with potential therapeutic effects (Conti et al., 2011; Jirásková-Vanícková et al., 2011).

Active Site Ligands

The first SR inhibitors were identified by screening a series of dicarboxylic acids (Strísovský et al., 2005). Malonate (**compound 1**, **Scheme 2**) emerged as the most active compound, with a K_i of 27 μM for mSR, and 77 and 710 μM for hSR, in the presence and absence of ATP, respectively (Marchetti et al., 2015; **Table 3**). The structure of the malonate-hSR complex (**Figure 3C**) indicated that malonate is H-bonded to the protein backbone and to amino acid residues. Also three water molecules are involved in the binding (Goto et al., 2009; Smith et al., 2010). Attempts to improve the affinity led to 2,2-dichloromalonate, which exhibits a K_i of 19.3 μM for mSR (Vorlová et al., 2015). Other SR inhibitors were identified in small peptide libraries containing the 3-phenylpropanoic acid moiety (Dixon et al., 2006). Nonspecific hydroxamic acid derivatives were also identified as inhibitors of SR (Hoffman et al., 2009). However, the affinities of all these inhibitors were lower than 2,2-dichloromalonate.

By a combination of *in silico* screening and *de novo* synthesis, **compound 2** was identified (Mori et al., 2014), and further optimized to generate **compound 3**, which showed an IC_{50} *in vitro* of 14 μM for mSR, a calculated K_i of about 4 μM (**Table 3**)

and an *in vivo* activity on a mouse model, suppressing neuronal over-activation (Mori et al., 2017). An inhibitor with an IC_{50} of 1 mM (**compound 4**) was identified and optimized by structure-based drug design starting from hSR, leading to **compound 5,** with an IC_{50} of 0.84 mM (Takahara et al., 2018) and a calculated K_i of 0.207 mM (**Table 3**).

In order to expand the chemical space of hSR inhibitors, *in silico* structure-based screening using both the open and the closed conformations of hSR was carried out (Dellafiora et al., 2015). Compounds with very heterogeneous chemical scaffolds were identified, sharing the presence of carboxylate moieties or carboxylate isosters. They exhibit K_i values in the low millimolar range, such as **compound 6** that possesses three carboxylate moieties and exhibits a calculated K_i of 1.3 mM (**Table 3**).

A further effort was carried out to mimic malonate with a cyclopropane scaffold synthesizing a small library of substituted cyclopropane derivatives. These compounds were docked into hSR structures and evaluated *in vitro*. The most active compound was dicarboxylic cyclopropane, which binds with a K_i of 0.9 mM (Beato et al., 2016).

The goal of developing high affinity, competitive inhibitors based on analogs of the substrate L-serine was initially perceived as relatively easy, as structural models for both open and closed conformations in complex with a few ligands were available for computer-assisted drug design. However, the results obtained so far indicate that SR is a rather difficult target for drug discovery. More structures of hSR in the presence of ligands should be of help in mapping the conformational ensemble. Furthermore, the so far identified inhibitors exhibit a relatively low affinity making rather difficult to saturate the active site and thus to obtain a high-resolution structure of the enzyme-ligand complex, hampering a structure-based drug discovery approach. In addition, it should be pointed out that hSR does not easily crystallize.

Allosteric Ligands

It was reported that the reduced form of NADH inhibits SR activity (Suzuki et al., 2015). This finding hints for a potential link of D-serine concentration to the glycolytic flux. A well-defined metabolic link between D-serine concentration and the glycolytic flux is represented by the fact that L-serine is generated in three steps from phosphoglycerate that is a main glycolytic metabolite. The structural similarity of NADH with ATP, which acts as an allosteric effector activating hSR, suggested a potential competition for the same binding site. Indeed, it was found that the affinity of ATP for hSR in the presence of NADH decreased and the binding lost cooperativity (Bruno et al., 2016). NADH analogs based on the reduced N-substituted 1,4 dihydronicotinamidic ring, including 1-methyl-1,4-dihydronicotinamide (MNA-red) and β-1,4-dihydronicotinamide monucleotide (NMN-red) (**compound 7**, **Scheme 2**) were found to be partial inhibitors of hSR activity. In particular, NMN-red exhibited a mixed-type inhibition, with a K_i of $18 \pm 7\,\mu M$ (Bruno et al., 2016; **Table 3**). By molecular docking, the binding site for NADH, MNA-red and NMN-red was identified to be at the dimer interface, close to the ATP binding site (Bruno et al., 2016).

Surprisingly, no effort has been made so far to develop allosteric activators binding at the ATP site capable of increasing the levels of D-serine for the treatment of schizophrenia, which is characterized by low D-serine concentrations in the brain. This avenue possibly needs to await the full determination of the complex hSR interactome.

CONFORMATIONAL LANDSCAPE OF hSR

The evidence accumulated on SR suggests that the enzyme is partitioned among different conformations characterized by distinct functional properties depending on the bound ligands (**Table 4**). Some of these conformations were crystallographically detected and classified as open and closed enzyme states (**Scheme 3**). The transition involves the reorientation of the small domain with respect to the large one within each subunit, leading to a closure of the active site. Such dynamic events are common to many PLP-dependent enzymes, such as tryptophan synthase, *O*-acetylserine sulfhydrylase and aspartate aminotransferase. Further conformational states, such as the form present in the absence of divalent cations, were inferred to be open-like based on fluorescence experiments (Bruno et al., 2017). A further level of complexity is generated by observations suggesting the existence of equilibria between dimers and tetramers in solution experiments for mSR and hSR (Wang and Barger, 2011; Marchetti et al., 2015; Bruno et al., 2017). The different conformations are associated with significantly different catalytic properties, ranging from the lowest catalytic efficiency in the absence of divalent cations, to the intermediate –20-fold higher– when Mg^{2+} is added, to the highest when ATP is also present, with a further 30-fold increase (**Scheme 3**). Furthermore, ATP binds cooperatively, with a dissociation constant ranging from millimolar to low micromolar range depending on the ligation state of the active site. Conversely, the affinity of malonate and glycine increased several fold in the presence of ATP.

PERSPECTIVES

Overall, results obtained on hSR indicate that protein function is regulated by alterations of the conformational distribution (**Table 4**), i.e. effectors stabilize distinct tertiary and quaternary enzyme conformations. This finding might be exploited for the design of positive and negative allosteric effectors with therapeutic actions. However, in order to proceed along this pathway, the three-dimensional structures of hSR in the presence of ligands that bind to either the active site or the allosteric site should be determined. This aim might be challenging due to difficulties in hSR crystallization. Indeed, surprisingly, so far the structure of hSR in the presence of ATP or a stable ATP analog has not yet been determined. As cryo-EM methods for protein structural determination are approaching the resolution of X-ray crystallography, it might be feasible to collect structures of hSR complexed with ligands and protein interactors in order to obtain a more detailed map of the conformational landscape of SR. In turn, this information should improve the identification of hits *in silico* ligand screening campaigns.

AUTHOR CONTRIBUTIONS

SR, MMarc, SF, BC, SB, MMarg, FM and AM contributed by reviewing the literature and writing distinct sections of the manuscript. Specifically, SR and SB focused on PTM, SF on structure, BC on catalysis, MMarc and MMarg on effectors, AM and FM on inhibitors. AM designed the manuscript and coordinated the activities. All authors read and approved the manuscript.

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REVIEW
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D-3-Phosphoglycerate Dehydrogenase

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L-Serine is the immediate precursor of D-serine, a major agonist of the N-methyl-D-aspartate (NMDA) receptor. L-Serine is a pivotal amino acid since it serves as a precursor to a large number of essential metabolites besides D-serine. In all non-photosynthetic organisms, including mammals, a major source of L-serine is the phosphorylated pathway of L-serine biosynthesis. The pathway consists of three enzymes, D-3-phosphoglycerate dehydrogenase (PGDH), phosphoserine amino transferase (PSAT), and L-phosphoserine phosphatase (PSP). PGDH catalyzes the first step in the pathway by converting D-3-phosphoglycerate (PGA), an intermediate in glycolysis, to phosphohydroxypyruvate (PHP) concomitant with the reduction of NAD^+. In some, but not all organisms, the catalytic activity of PGDH can be regulated by feedback inhibition by L-serine. Three types of PGDH can be distinguished based on their domain structure. Type III PGDHs contain only a nucleotide binding and substrate binding domain. Type II PGDHs contain an additional regulatory domain (ACT domain), and Type I PGDHs contain a fourth domain, termed the ASB domain. There is no consistent pattern of domain content that correlates with organism type, and even when additional domains are present, they are not always functional. PGDH deficiency results in metabolic defects of the nervous system whose systems range from microcephaly at birth, seizures, and psychomotor retardation. Although deficiency of any of the pathway enzymes have similar outcomes, PGDH deficiency is predominant. Dietary or intravenous supplementation with L-serine is effective in controlling seizures but has little effect on psychomotor development. An increase in PGDH levels, due to overexpression, is also associated with a wide array of cancers. In culture, PGDH is required for tumor cell proliferation, but extracellular L-serine is not able to support cell proliferation. This has led to the hypothesis that the pathway is performing some function related to tumor growth other than supplying L-serine. The most well-studied PGDHs are bacterial, primarily from *Escherichia coli* and *Mycobacterium tuberculosis*, perhaps because they have been of most interest mechanistically. However, the relatively recent association of PGDH with neuronal defects and human cancers has provoked renewed interest in human PGDH.

Keywords: D-serine, L-serine, phosphoglycerate, dehydrogenase, biosynthesis

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INTRODUCTION

D-Serine is an agonist of the N-methyl-D-aspartate (NMDA) receptor and it is synthesized from L-serine by serine racemase (SR) (Fuchs et al., 2006; Ehmsen et al., 2013; Abe et al., 2014). For nearly all organisms, including mammals, L-serine is described as a non-essential amino acid because it is not required in the diet, but is produced by a biosynthetic pathway (**Figure 1**) (Sallach, 1956; Greenberg and Ichihara, 1957; Hanford and Davies, 1958; Willis and Sallach, 1962, 1964; Walsh and Sallach, 1966; Cheung et al., 1968; Nelson et al., 2009; Voet and Voet, 2011). In these organisms, L-serine is made from the glycolytic intermediate D-3-phosphoglycerate (PGA) and the first enzyme in the L-serine biosynthetic pathway is D-3-phosphoglycerate dehydrogenase (PGDH). It converts PGA to phosphohydroxypyruvate (PHP) with the concomitant reduction of NAD^+ to NADH. To complete the pathway, phosphoserine amino transferase (PSAT) converts PHP to L-phosphoserine (PS) with the concomitant conversion of glutamate to α-ketoglutarate (αKG), followed by the conversion of PS to L-serine with the loss of phosphate by phosphoserine phosphatase (PSP). The official designation of the genes coding for enzymes in this pathway differ with species. For instance, in *Escherichia coli*, *Mycobacterium tuberculosis*, and *Homo sapiens*, the gene coding for PGDH is designated as *serA1*, *Rv2996c*, and *PHGDH*, respectively. In this review, the designation "PGDH" will be used to refer to this enzyme from any species.

The use of the term "non-essential" to describe L-serine is unfortunate because it is a very important amino acid and is very essential as the precursor to many metabolites. In addition to being a precursor for D-Serine, L-serine is also a precursor for the production of such metabolites as glycine, cysteine, tryptophan, phosphatidyl L-serine, sphingolipids, purines, porphyrins, glyoxalate, and glycine (Nelson et al., 2009; Voet and Voet, 2011). As the major precursor to glycine, it also contributes the one-carbon unit (C1) that is the donor in methylation reactions mediated by derivatives of tetrahydrofolate and S-adenosyl methionine.

L-serine can also be produced from glycine by the action of serine hydroxymethyl transferase (SHMT) (Nelson et al., 2009; Voet and Voet, 2011). However, this is not a usual or major route to the production of L-serine because it depletes glycine levels as well as methylene tetrahydrofolate which is the source of one-carbon units used for methylation reactions. Many organisms, including mammals, also contain a pathway that is often mistaken for a second pathway for the production of L-serine. This so-called "un-phosphorylated" pathway actually functions in L-serine degradation (Snell, 1986). The enzyme that is a counterpart to PGDH in this pathway is a D-glycerate dehydrogenase (GDH usually designated *serA2*) and is located in the mitochondria in mammals (Snell, 1975) and the peroxisomes in plants (Voet and Voet, 2011). The existence of a catabolic pathway is necessary because the dephosphorylation of PS to produce L-serine is irreversible. The "phosphorylated" pathway, located in the cytosol and utilizing PGDH, is therefore the only anabolic source of L-serine in non-photosynthetic organisms. In addition to being a precursor for many other metabolites, L-serine, particularly when present at high levels, can also be converted into pyruvate and ammonia by L-serine dehydratase (LSD). The glycolytic precursor of L-serine, PGA can be generated from glucose by glycolysis or from pyruvate by an abbreviated gluconeogenesis pathway that produces phosphoenolpyruvate and then PGA. In *E. coli*, ∼15% of the carbon assimilated when it is grown on glucose passes through L-serine before incorporation into biosynthetic products (Pizer and Potochny, 1964). In humans, ∼75% of the L-serine that appears during fasting comes from *de novo* serine synthesis (Kalhan and Hanson, 2012). In mammals, under normal dietary conditions, most of the L-serine is synthesized in the kidney. However, when dietary protein is limiting, a marked increase in L-serine synthesis occurs in the liver (Kalhan and Hanson, 2012). In the central nervous system, L-serine is predominately synthesized in astrocytes rather than neurons (Tabatabaie et al., 2010).

From a structural and mechanistic point of view, the most studied PGDH is that from *E. coli* (Pizer, 1963; Pizer and Potochny, 1964; Rosenbloom et al., 1968; Sugimoto and Pizer, 1968a,b; Winicov and Pizer, 1974; Dubrow and Pizer, 1977a,b; McKitrick and Pizer, 1980; Tobey and Grant, 1986; Schuller et al., 1995; Al-Rabiee et al., 1996a,b; Grant et al., 1996, 1999a,b, 2000a,b, 2001a,b, 2002, 2003, 2004, 2005; Zhao and Winkler, 1996; Grant and Xu, 1998; Bell et al., 2002, 2004; Grant, 2004, 2011, 2012, 2018; Thompson et al., 2005; Dey et al., 2007; Burton et al., 2008, 2009a), followed by that from *M. tuberculosis* (Grant et al., 1999c; Dey et al., 2005a,b, 2008; Burton et al., 2007, 2009b; Xu and Grant, 2014; Xu et al., 2015). There are also reports from various animal tissues (Pizer, 1964; Walsh and Sallach, 1965; Cheung et al., 1969; Pizer and Sugimoto, 1971; Grant and Bradshaw, 1978; Grant et al., 1978; Lund et al., 1986; Fell and Snell, 1988; Achouri et al., 1997), other eukaryotes (Ulane and Ogur, 1972; Ali et al., 2004; Singh et al., 2014), other bacteria (Umbarger and Umbarger, 1962; Umbarger et al., 1963; Saski and Pizer, 1975; Peters-Wendisch et al., 2002, 2005), and plants (Hanford and Davies, 1958; Cheung et al., 1968; Slaughter and Davies, 1968a,b; Rosenblum and Sallach, 1970). More recently, investigations of PGDH from another bacterial species (Zhang et al., 2017) and humans (Grant, 2012; Fan et al., 2015; Xu et al., 2015; Unterlass et al., 2017) have been reported. PGDH has also been implicated in abnormal neural development in humans and as a potential cancer therapy target. These topics will be referenced and discussed later in this review.

PGDH TYPES

Although all PGDH enzymes (EC 1.1.1.95) catalyze the same reaction, they exhibit certain mechanistic differences and they can be divided into three structural types based on domain structure (Grant, 2012) (**Figure 2**). Type 1 enzymes are composed of four domains, the substrate binding domain, the nucleotide binding domain, the ASB domain (where ASB stands for allosteric substrate binding), and the regulatory domain which is an ACT domain (Aravind and Koonin, 1999; Grant, 2006) (where ACT stands for the first letters in Aspartate kinase, Chorismate mutase, and TyrA). As will be discussed later, the regulatory

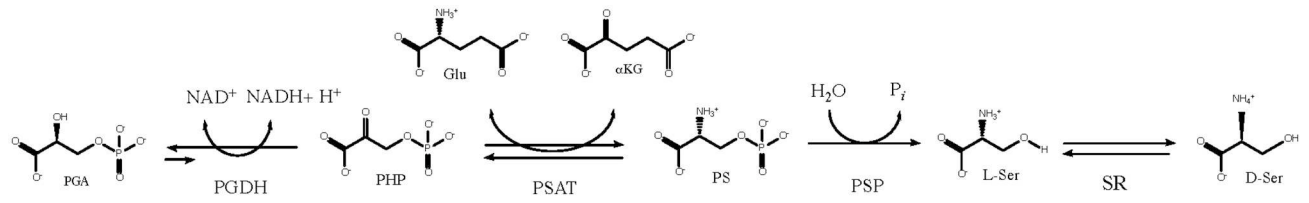

FIGURE 1 | The L-serine biosynthetic pathway uses D-3 phosphoglycerate from glycolysis in the first step. D-serine is produced from L-serine by serine racemase.

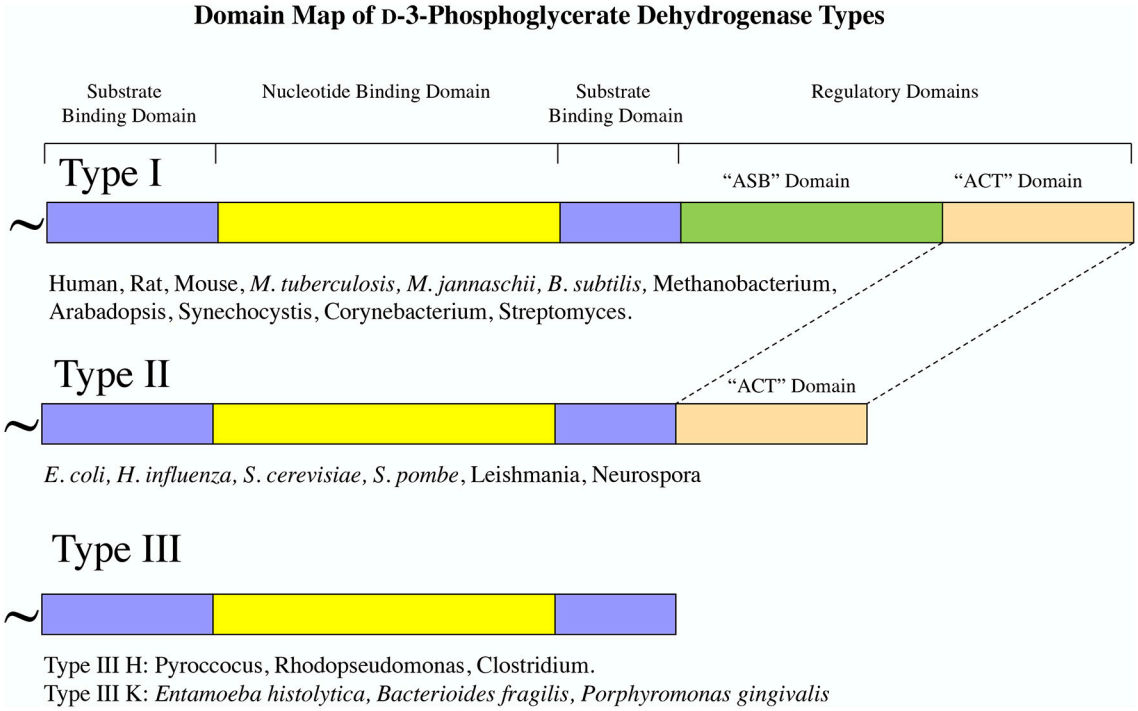

FIGURE 2 | PGDH exists as three different types distinguished by domain structure. The domain structure, going from amino terminus to carboxyl terminus, is illustrated for each type along with representative species containing each type. The "∼" symbol signifies that the enzyme from different species can contain different lengths of amino acid sequence at the amino terminus.

domain designation is based on its role in the regulation of enzyme activity by L-serine. Although it is often reported, particularly in introductory textbooks, that PGDH in general is feedback inhibited by L-serine (Walsh and Sallach, 1965; Slaughter and Davies, 1968a; Rosenblum and Sallach, 1970; Fell and Snell, 1988; Achouri et al., 1997), all mammalian enzymes so far studied as well as those from many other species have lost this ability. The ASB domain is so named because it functions as a substrate binding regulatory site in PGDH from some species (Dey et al., 2005a; Burton et al., 2007, 2009b). The function of the other two domains corresponds to their designation, namely that they function mainly in the binding of substrate and coenzyme. Type 2 enzymes are missing the ASB domain and consist of a substrate binding domain, a nucleotide binding domain, and a regulatory (ACT) domain (Schuller et al., 1995). Type 3 enzymes consist of only a substrate binding domain and a nucleotide binding domain (Ali et al., 2004). In addition, as demonstrated in **Figures 2**, **3**, some, but not all, Type 3 enzymes utilize lysine as the catalytic residue (Ali et al., 2004; Singh et al., 2014) instead of the histidine that is found in all other PGDH types. Interestingly, there does not appear to be a pattern of domain type associated with type of organism. For instance, Type 1 enzymes containing all four domains are found in mammals, plants, and bacteria and Type 2 enzymes are found in eukaryotic organisms as well as bacteria. It is important to note that the presence of a homologous structural domain does not necessarily mean that it is functional.

PGDH HOMOLOGY

The amino acid sequence alignment of representative PGDHs is shown in **Figure 3**. The numbering refers to position in the figure rather than in any particular sequence. The types are shown

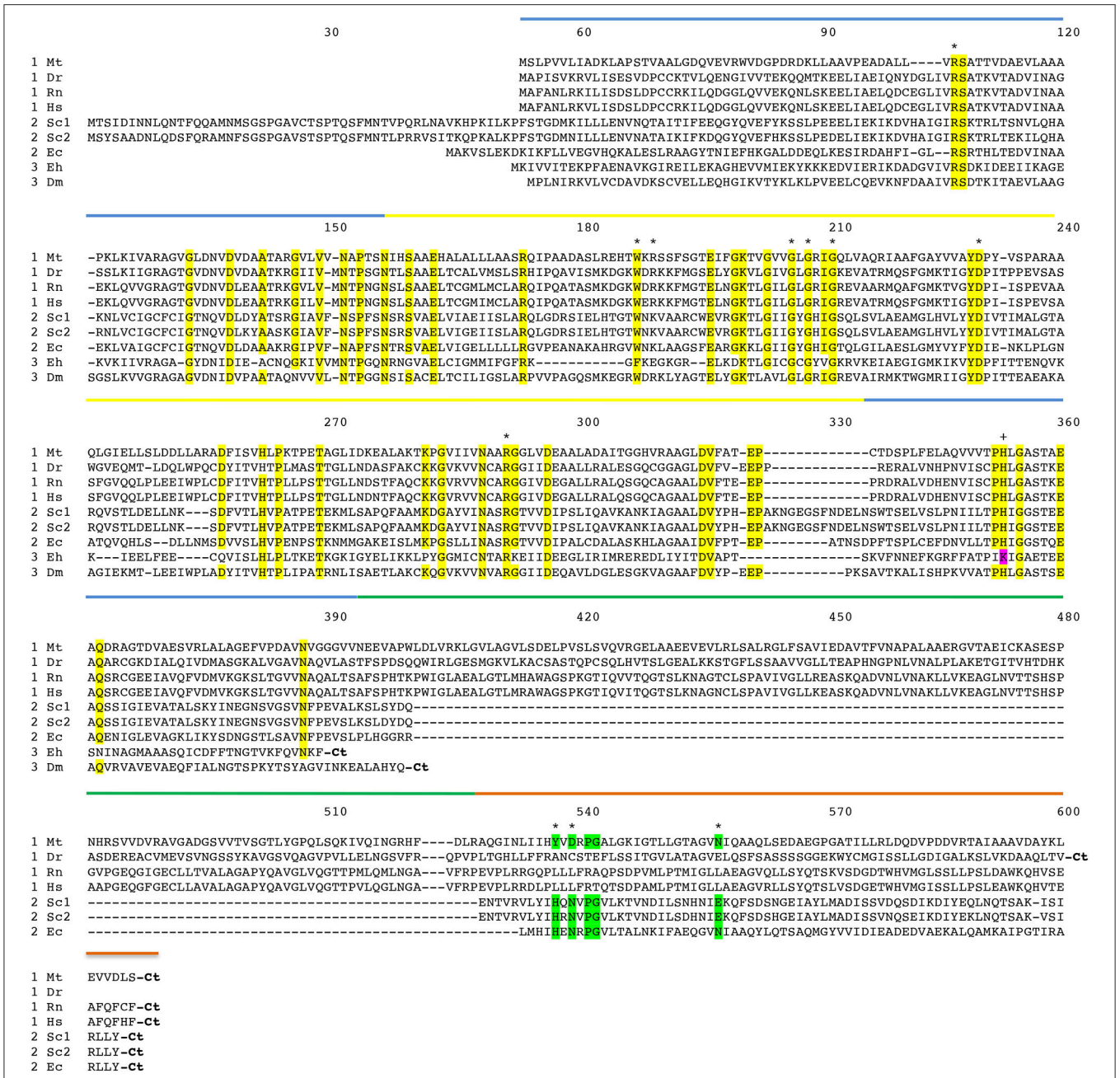

FIGURE 3 | Amino acid sequence alignment of PGDH form representative species. Mt, *Mycobacterium tuberculosis*; Dr, *Danio rerio* (zebrafish); Rn, *Rattus norvegicus*; Hs, *Homo sapiens*; Sc1 and Sc2, two variants from *Saccharomyces cervisiae*; Ec, *Escherichia coli*; Eh, *Entamoeba hystolytica*; Dm, *Drosophila melanogaster*. The numbering designates residue position in the figure rather than the sequence of a particular PGDH. The PGDH type is shown as a numeral in front of the species abbreviation. The domains are highlighted with colored lines. Substrate binding domain, blue; nucleotide binding domain, yellow; ASB domain, green; and ACT domain, orange. Conserved residues are highlighted in yellow. Residues involved in L-serine binding at the ACT site are highlighted in green. The active site lysine in some type 3 enzymes is highlighted in magenta. Asterisks designate residues involved in substrate and effector binding and the plus sign identifies the active site histidine or lysine. The carboxyl termini are designated "Ct".

before the species abbreviation and the domains are highlighted with colored lines. Residues known to be involved in catalysis or ligand binding are shown with an asterisk or a plus sign (see Figure legend). The arginine residues at positions 106 and 189 are involved in interaction with the substrate phosphoryl group. The aromatic amino acid at position 187 forms the bottom of the active site cleft. The glycine residues at positions 205, 207, and 210 and the aspartic acid residue at position 230 are conserved residues in the Rossman fold involved in coenzyme binding. The arginine residue at position 291 anchors the substrate into

the active site by interaction with the substrate carboxyl group. The active site histidine (or lysine) that donates a proton is at position 353. The residues that participate in binding L-serine at the regulatory site are at positions 537, 539, and 557.

A comparison of the conserved amino acid residues shows that there is a relatively low degree of identity between human PGDH and those of the non-mammalian PGDHs. There is 36.8, 31.9, and 24.5% identity of the nucleotide binding and substrate binding domains combined, between Human and *M. tuberculosis*, *E. coli*, and *Entamoeba histolytica*, respectively. There is 30.7, 27.6, and 24.5% identity of all common domains between Human and *M. tuberculosis*, *E. coli*, and *E. histolytica*, respectively. There is only 8.4% identity of the ASB domains between Human and *M. tuberculosis*, and 3.9 and 8.2% identity of the ACT domains between Human and *M. tuberculosis*, *E. coli*, respectively. Among mammalian species, such as between human and rat PGDH, there is a 94.6% identity.

PGDH STRUCTURE

Oligomeric Conformations

The structure of PGDH differs depending on its domain makeup or Type (**Figures 4–6**). The only complete PGDH structures that have been determined and published are a Type I from *M. tuberculosis* (Dey et al., 2005a, **Figure 4**), a Type II from *E. coli* (Schuller et al., 1995, **Figure 5**), and a Type III from *E. histolytica* (Singh et al., 2014, **Figure 6**). A partial structure of human PGDH has been published (Unterlass et al., 2017) showing the substrate and nucleotide binding domains but without the ASB and ACT domains which were proteolytically removed to facilitate crystallization. The structure of a Type II PGDH from *Brucella melitensis* and Type III PGDHs from *Lactobacillus plantarum*, *Pyrococcus horokoshi*, *Sulfolobus tokodaii*, *Ralstonia solanacearum*, and *Vibrio cholera* have been deposited in the RCSB protein data bank but have not been described in a publication.

The one structure of a Type I PGDH that has been determined is from *M. tuberculosis* (**Figure 4**). It is a tetramer with identical subunits with a molecular weight of 54,522. However, the subunits are identical with respect to amino acid composition but not with respect to domain orientation (Dey et al., 2005a). Interestingly, the subunits adopt two different conformations, designated syn- and anti- (**Figure 7**), arranged in the tetramer as shown in **Figure 4** and which results in an internal asymmetry. The syn- and anti-conformations differ by a rotation of ∼180° at a position composed of three consecutive glycine residues (position 389–391 in **Figure 3**). This results in two different sets of inter-subunit interactions between the nucleotide binding and ASB domains resulting in different portions of the respective subunits being exposed to solvent. A diagram of the subunit domain orientations is provided in **Figure 4** showing that the two syn-conformers are in the middle and the two anti-conformers are at the ends of the elongated tetramer.

M. tuberculosis PGDH has also been shown to exhibit multiple oligomeric equilibrium states (Xu and Grant, 2014) modulated by phosphate and polyphosphates (**Figure S1**). In the absence of these ions, the enzyme is in equilibrium between an inactive dimer and an active tetramer that is relatively insensitive to inhibition by L-serine. However, in the presence of phosphate ion, a conversion to active tetramers and active octamers occurs. These two species are in equilibrium and both are very sensitive to inhibition by L-serine. Small polyphosphates, such as pyrophosphate and triphosphate, induce a conversion to an active dimer that is insensitive to L-serine. A similar dependency on phosphate ion for oligomeric state is also observed for human PGDH (unpublished) which is also a Type I enzyme.

The dependency of activity on phosphate ion concentration is further illustrated when the activity of *M. tuberculosis* PGDH is monitored as a function of time (Dey et al., 2005b, **Figure S2**). When the enzyme is diluted 500-fold in the presence of 200 mM KPO_4 buffer, the enzyme retains activity. However, a 500-fold dilution in 20 mM KPO_4 buffer results in a time dependent loss of activity that fits best to a double exponential function. This demonstrates that there are at least two enzyme forms that lose activity with discernable dissociation constants. These forms most likely correlate with the observed changes in oligomeric state.

The mammalian enzymes are also Type I PGDHs. The subunit molecular weight of human PGDH is 56,651, but it has been crystallized only after removal of the ASB and ACT domains. Therefore, it is not known if mammalian PGDHs adopt the same internal asymmetry of the tetramer found in the *M. tuberculosis* PGDH. Since they do not contain the triple glycine sequence that presumably allows for the domain rotation found in *M. tuberculosis* PGDH, it is possible that they do not share the same tetrameric configuration. Furthermore, it is not known whether *M. tuberculosis* PGDH actually adopts the asymmetric conformation in solution. Since *M. tuberculosis* PGDH could only be crystallized in the presence of 1 M tartrate, and a tartrate molecule is found between adjacent ASB domains, it has been speculated that the crystal structure may represent only one of at least two alternative conformations and that these alternative conformations may be responsible for the peculiar phosphate dependent sensitivity to L-serine (see section Feedback Inhibition) and the variations in oligomeric states.

A representative structure of a Type II PGDH is from *E. coli* (**Figure 5**). It is a tetramer of identical subunits with respect to amino acid composition and domain orientation with a subunit molecular weight of 44,044. The tetramer is a dimer of dimers, with one dimer interface at the nucleotide binding domains and the other at the ACT or regulatory domains.

A representative structure of a Type III PGDH is from *E. histolytica* (**Figure 6**). Since it lacks ASB and ACT domains, the only subunit interface is at the catalytic domains resulting in a dimeric configuration. Its subunit molecular weight is 33,469.

Catalytic Sites

The basic mechanism of all PGDHs is the same. The substrate, PGA, is oxidized by the transfer of a proton to the active site histidine or lysine and a hydride ion to NAD^+, to yield PHP. The catalytic site of human PGDH with NAD and malate bound is depicted in **Figure 8**. The three cationic residues that interact with the acidic ends of the substrate are shown and are common to all PGDH active sites. In **Figure 3**, Arg 53 corresponds to position 107, Arg 134′ to position 189, Arg 235 to position 291, and His 282, which is the proton donor, to position 353. Arg

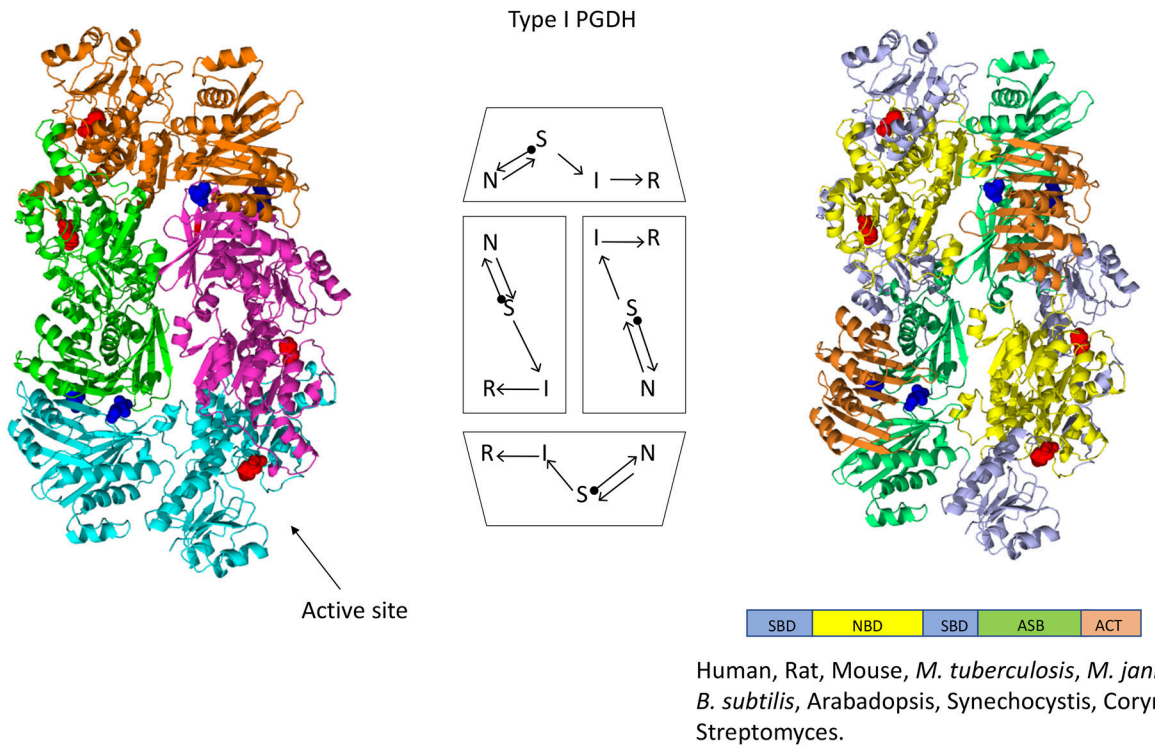

FIGURE 4 | Crystal structure of a Type I PGDH from *M. tuberculosis*. On the left, each subunit is colored differently. In the center is a diagram of the subunits showing the course of the polypeptide chain from amino terminus, designated by a black dot, to the carboxyl terminus. On the right, the individual domains are colored according to the scheme shown below the structure. Representative species with Type 1 enzymes are listed.

235 interacts with the carboxyl of PGA/PHP while the other two arginine residues interact with the phosphate group. In this depiction, the active site is closed, but since malate is a shortened version of the substrate, the spatial configuration of the residues is not optimal. The active sites in the crystal structures of *E. coli* and *M. tuberculosis* PGDH (**Figure 8**) are in a more open configuration, as if they are poised just prior to closing of the active site cleft. The open conformation of the active site may be due to crystal packing constraints that aren't in play with the human structure since the ASB and ACT domains are missing in the crystallized form reported. In *M. tuberculosis* PGDH, Arg 51 corresponds to position 107, Arg 132′ to position 189, Arg 233 to position 291, and His 280 to position 353.

The order of substrate and coenzyme binding in *E. coli* PGDH (Grant et al., 2002) and *Pseudomonas* species PGDH (Zhang et al., 2017) is coenzyme before substrate. This is also apparently the case for human PGDH (Unterlass et al., 2017) since the crystal structure contains NADH (Unterlass et al., 2017). However, this has not been determined conclusively from kinetic analysis of the complete enzyme. The order in *M. tuberculosis* PGDH is substrate before coenzyme, opposite of that for the others. The crystal structures of *M. tuberculosis* PGDH show that the coenzyme sites have restricted access to solvent. In the syn-conformation, the coenzyme site is covered by the ASB domain, resulting in a narrow channel leading to solvent. In the anti-conformation, the ASB is rotated away from the coenzyme site but is blocked by a long flexible loop. Therefore, significant domain and loop movements are likely related to coenzyme binding in solution. Superimposing the human and *M. tuberculosis* PGDH catalytic sites shows that the latter has a more open cleft, requiring a rotation for closure, but the PHP is in approximately the same position as the malate in the human structure. In *E. coli* PGDH, Arg 60 corresponds to position 107, Lys 141′ to position 189, Arg 240 to position 291, and His 292 to position 353. Note that in *E. coli* PGDH a lysine residue is present at position 189 instead of an arginine. NAD is not present in the *M. tuberculosis* structure because it binds after a conformational change induced by substrate binding. For both *E. coli* and *M. tuberculosis* PGDH, a rotation of the substrate binding domain relative to the nucleotide binding domain is necessary to close the cleft and move the various elements into place for catalysis.

Effector Binding at the ACT and ASB Domain Interfaces

In the Type II PGDH from *E. coli*, feedback inhibition of catalytic activity occurs when L-serine binds at the ACT domain interface as shown in **Figure 9**. The serine carboxyl group hydrogen bonds to the imidazole nitrogen of His 344 (position 537 in **Figure 3**), and the amino group of serine bonds to the amide side chains of Asn 346 (position 539) and Asn 364′ (position 557). The hydroxyl group of serine does not form hydrogen bonds directly with an amino acid but rather interacts through a water molecule with the main chain carbonyl groups of Thr

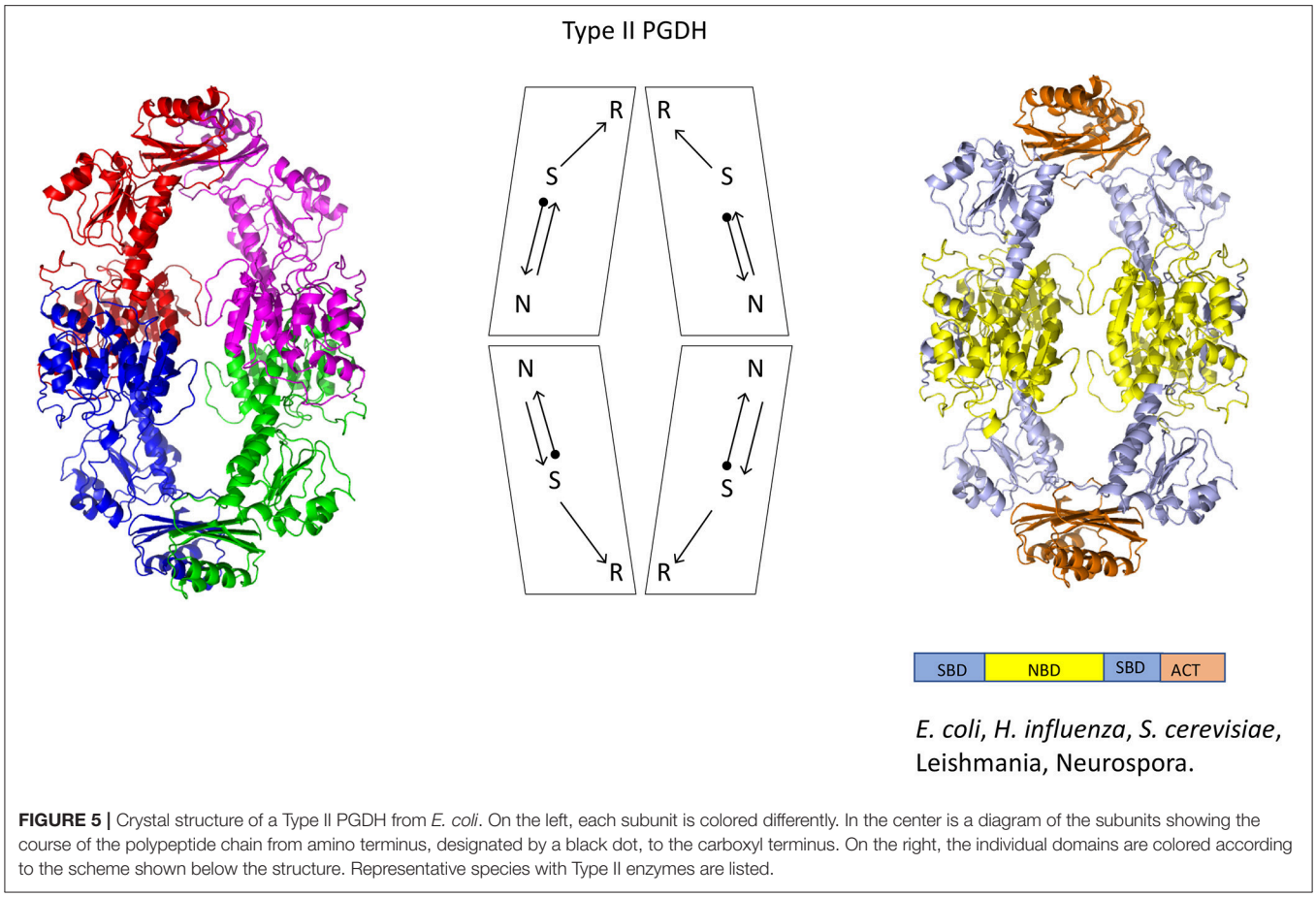

FIGURE 5 | Crystal structure of a Type II PGDH from *E. coli*. On the left, each subunit is colored differently. In the center is a diagram of the subunits showing the course of the polypeptide chain from amino terminus, designated by a black dot, to the carboxyl terminus. On the right, the individual domains are colored according to the scheme shown below the structure. Representative species with Type II enzymes are listed.

352 (position 545) and Val 363′ (position 556). When serine binds, there is a rotation of the substrate binding domain-ACT domain relative to the nucleotide binding domain. This has a significant effect on the conformation of the active site and is likely responsible for the inhibition of activity caused by serine binding. L-serine enters the effector site through an opening at the surface around Asn 364′. Upon binding, the opening is covered by movement of Asn 364′ and Pro 348 (**Figure 9**). The two binding sites at each ACT domain interface assume different conformations in the absence of serine. One is open and one is closed similar to the structure seen when serine is bound. Since all four sites are occupied in the crystal structure (pdb 1psd), the binding of L-serine to the open site apparently induces the other site to open. Binding studies (Grant et al., 2001b) show that serine binding is positively cooperative for the first two sites in the tetramer, located at opposite ACT domain interfaces, and negatively cooperative for the last two sites to be occupied.

In *M. tuberculosis* PGDH, L-serine is bound at the interface of the ACT domains (**Figure 9**) as it is in *E. coli* PGDH. However, the interaction with serine is somewhat different. The serine carboxyl group is hydrogen bonded to the hydroxyl group of Tyr 461 (position 537 in **Figure 3**) and the side chain oxygen of Asp 463 (position 539). The amino group of serine hydrogen bonds to the side chain of Asn 481′ on the opposite subunit (position 557) and the serine hydroxyl is hydrogen bonded directly to main chain amide of Leu 468 (position 544). As is the case with *E. coli*, the pocket closes when serine binds as a result of Asn 481′ moving over the pocket opening. This is accompanied by a movement of Asp 463 and Arg 464 (positions 539 and 540) with Asp 463 forming an additional hydrogen bond with serine.

The crystal structure of *M. tuberculosis* PGDH contains a molecule of tartrate bound at the interface of adjacent ASB domains (**Figure 10**). This site is populated with a number of cationic residues that interact with the tartrate, including Lys 439, Arg 446, His 447, Arg 451, and Arg 501 (positions 512, 519, 520, 524, and 576, respectively, in **Figure 3**). Note that Arg 501 is actually in the ACT domain and produces the largest decrease in serine inhibition when it is mutated to alanine (Burton et al., 2009b). Since tartrate is an analog of the substrate, it was originally proposed that this may be an allosteric substrate binding site. Alteration of the residues at the ASB interface by mutagenesis, demonstrated that this site appeared to bind substrate which resulted in an attenuation of catalytic activity (Burton et al., 2009b; Grant, 2012). It has also been proposed, but not definitively shown, that this is also the phosphate binding site and that interplay between substrate and phosphate ion at this site results in phosphate dependent inhibition of catalytic activity when serine binds to the ACT domain (Xu and Grant, 2014).

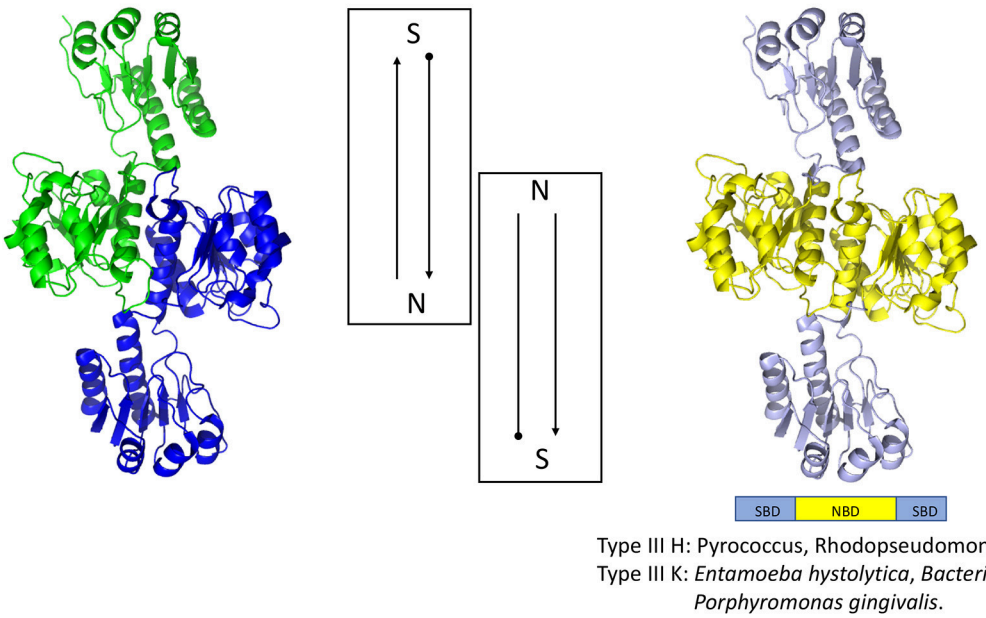

FIGURE 6 | Crystal structure of a Type III PGDH from *E. hystolytica*. On the left, each subunit is colored differently. In the center is a diagram of the subunits showing the course of the polypeptide chain from amino terminus, designated by a black dot, to the carboxyl terminus. On the right, the individual domains are colored according to the scheme shown below the structure. Representative species with Type III enzymes are listed.

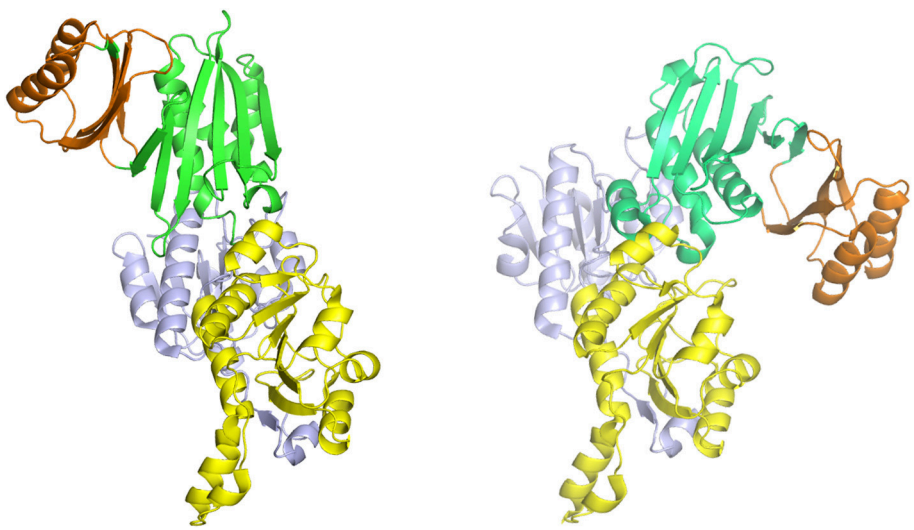

FIGURE 7 | The syn- **(right)** and anti- **(left)** configurations of the subunits of *M. tuberculosis* PGDH. The subunits are pictured with their substrate (blue) and nucleotide (yellow) binding domains in essentially the same orientation. The ASB (green) and ACT (orange) domains are rotated to the left in the anti-configuration and to the right in the syn-configuration.

REGULATION OF CATALYTIC ACTIVITY

The production of L-serine is regulated at the level of enzyme activity of PGDH by three main mechanisms: the equilibrium of the reaction, substrate inhibition, and feedback inhibition. However, as noted previously, all three are not necessarily operative in PGDH from all organisms.

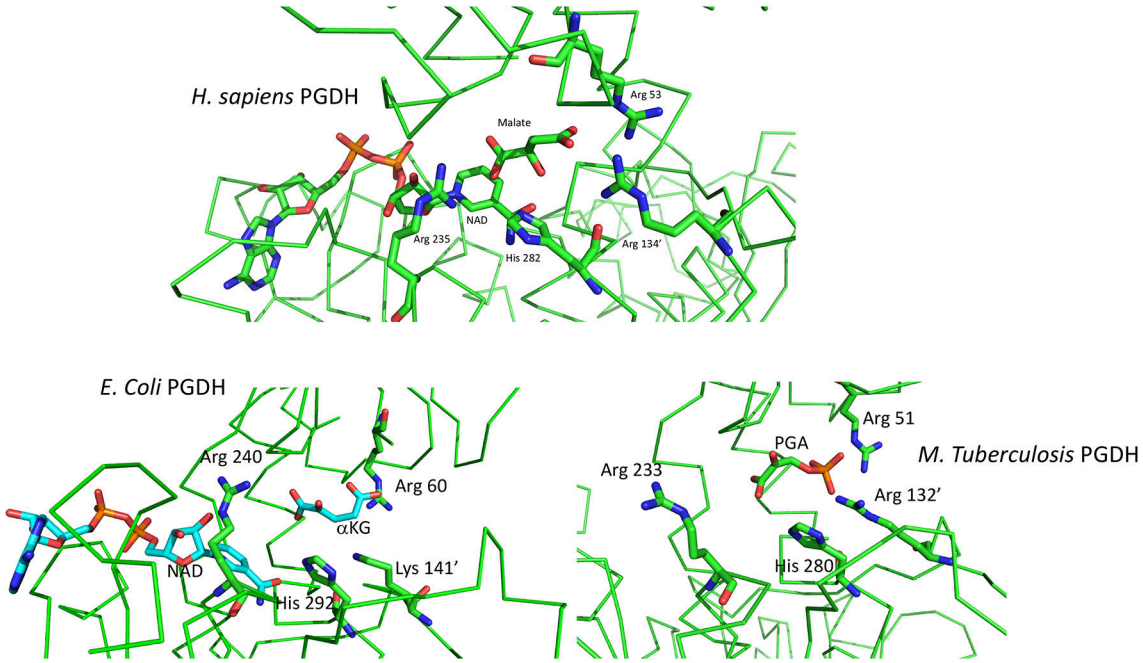

FIGURE 8 | (Top) Depiction of the active site of human PGDH from pdb 2g76 with malate and NAD bound. Some of the alpha chain in the background has been cut away for clarity. **(Bottom)** Depiction of the active site of *E. coli* PGDH from pdb 1yba (left) and of *M. tuberculosis* PGDH from pdb 2ddn (right).

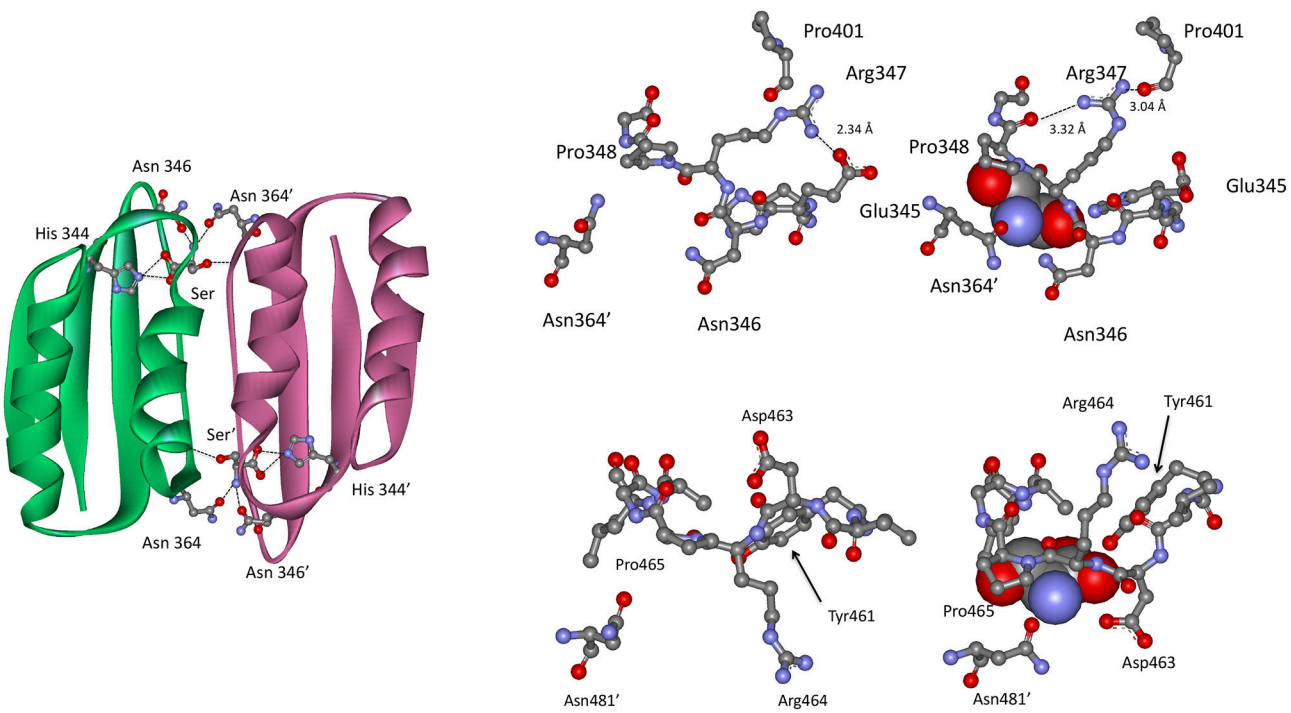

FIGURE 9 | (Left) The ACT domain interface in *E. coli* PGDH showing the interaction of L-serine with specific residues. Reprinted from Grant (2012). **(Top Right)** The serine binding site in *E. coli* PGDH. The serine atoms are shown as spheres in the right-hand structure. Reprinted from Grant (2012), with permission from Elsevier © 2011. **(Bottom Left)** The serine binding site in *M. tuberculosis* PGDH. The serine atoms are shown as spheres in the left-hand structure. Reprinted from Grant (2012), with permission from Elsevier © 2011.

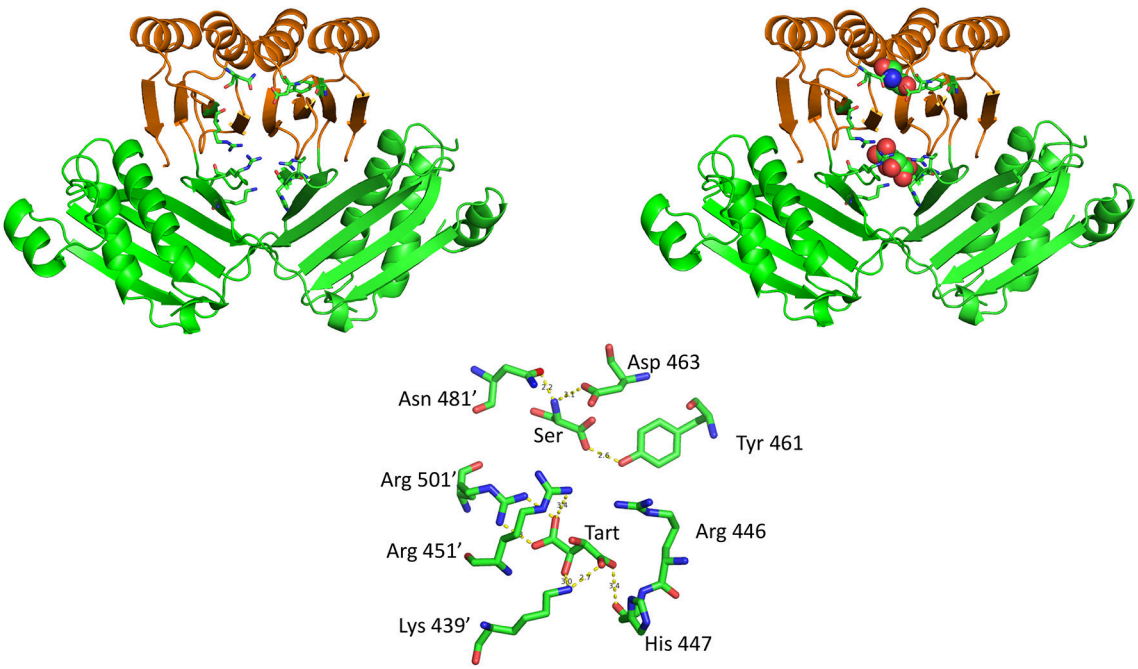

FIGURE 10 | The relationship of the ACT (orange) and ASB (green) domains in *M. tuberculosis* PGDH. The depiction on the left shows the domains in the absence of bound ligands and the depiction on the right shows L-serine and tartrate bound at their respective sites. The stick model at the bottom shows the specific residue interactions seen in the crystal structure.

Catalytic Activity and the Equilibrium State of the Reaction

The equilibrium of the reaction catalyzed by PGDH lies far in the direction of PGA, opposite of that leading to serine synthesis (Sugimoto and Pizer, 1968b). At equilibrium, <5% of the substrates and products are in the form of PHP. The reaction is pulled in the forward direction by the downstream enzymes despite the strong tendency for PGDH to act in the opposite direction. The reaction catalyzed by PSAT is freely reversible, but the de-phosphorylation of L-serine by PSP is not reversible and is the point of no return. This latter reaction, in effect, acts like a sink, keeping serine from being depleted by reaction in the reverse direction and assuring that the flux of the pathway continues in the direction of L-serine synthesis.

As a result of the equilibrium state of the reaction, it is difficult to assay PGDH in the forward direction and historically it has been assayed mostly in the reverse direction using PHP as the substrate. Forward assays using PGA as the substrate usually employ fluorescence monitoring for sensitivity and with hydrazine added, presumably to trap the PHP produced (Sugimoto and Pizer, 1968b), but they are difficult to perform accurately. In order to measure the activity of PGDH in the forward direction, it is best to use a coupled assay that depletes either PHP or NAD^+ in order to drive the reaction forward. However, as noted below (see section Alternate Substrates), the activity of *E. coli* PGDH cannot be measured by monitoring the formation of NADH when coupled with PSAT because of its conversion back to NAD^+ by αKG generated by PSAT. This is not the case for *M. tuberculosis* and human PGDH, where the production of NADH can be measured in the coupled reaction. The kinetic constants for PGDH from several species are listed in **Table 1**.

Substrate Inhibition

In addition to the equilibrium of the PGDH catalyzed reaction favoring PGA, human and *M. tuberculosis* PGDH exhibits significant substrate inhibition by PHP (Xu et al., 2015, **Figure S3**). Therefore, theoretically, if PHP were to accumulate to a certain level, its conversion to PGA would be inhibited, counteracting to some extent the equilibrium tendency of the reaction. However, there are no definitive studies of pathway flux that demonstrate that this is functional physiologically. PHP is inherently unstable and the investigations of metabolite levels that are available (in bacteria, Bennett et al., 2009) don't list PHP as a measurable metabolite. Substrate inhibition by PHP is also less pronounced in phosphate buffer than other buffers that have been reported (Xu et al., 2015).

Feedback Inhibition

As noted earlier, textbooks often refer to PGDH as being inhibited by the end-product of the pathway, L-serine. While this is true for PGDH from some organisms, it does not pertain to that from all species. When it does occur, it results from the interaction of L-serine with the regulatory or ACT domain. However, the sensitivity of PGDH to L-serine concentration varies greatly. PGDH from *Bacillus subtilis* (Saski and Pizer, 1975) and *Corynebacterium glutamicum* (Peters-Wendisch et al.,

TABLE 1 | Catalytic properties of D-3-phosphoglycerate dehydrogenase[a].

Reaction	$K_{m,substrate}$ (mM)	$K_{m,coenzyme}$ (mM)	k_{cat} (s^{-1})	$k_{cat}/K_{m,substrate}$ ($M^{-1}s^{-1}$)	L-Serine IC_{50} (Hill coefficient) (μM)
E. coli					
PHP reduction[e]	0.0032[b]	<0.01	28[b]	~9×10^{6}[b]	2–13[d] (~2)
αKG reduction[e]	0.088[b]	<0.01	12–33[d]	~4×10^{5}[b]	2–8[d]
PGA oxidation	1.2[b]	ND[c]	0.6[b]	~5×10^{2}[b]	44
HGA oxidation	0.4[b]	ND[c]	0.7[b]	~2×10^{3}[b]	36
PGA oxidation[h] (PGDH/PSAT)	NDS[c]				
M. tuberculosis					
PHP reduction[e]	0.17	0.06[f]	2,400	~1×10^{7}	36(~2)
αKG reduction	DNR[c]				
PGA oxidation	ND[c]				
HGA oxidation	ND[c]				
PGA oxidation MOPS[h]	0.54	0.06	1.4	2.6×10^{3}	
(PGDH/PSAT) $PO_4^{f,h}$	9.2	5.2	3	3.3×10^{2}	13 (1.5)
HUMAN					
PHP reduction[e]	0.10	0.02	300	~3×10^{3}	DNI[c]
KG reduction	DNR[c]				
PGA oxidation[h] MOPS (PGDH/PSAT)	0.48	0.49	0.4	0.8×10^{3}	DNI[c]
RAT[e]					
PHP reduction[e]	~0.015[g]	0.025	ND[c]	ND[c]	DNI[c]
PGA oxidation	0.1	0.027	ND[c]	ND[c]	DNI[c]
αKG reduction	DNR[c]				

[a] *PHP, phosphohydroxypyruvic acid; αKG, α-ketoglutarate; PGA, D-3-phosphoglycerate; HGA, D-hydroxyglutaric acid. Values are per tetramer.*
[b] *From Zhao and Winkler [29].*
[c] *ND, not determined; DNI, does not inhibit; DNR, does not react; NDS, not detectable spectrophotometrically at 340 nm.*
[d] *Value varies slightly from different groups. Note that in all cases, all of the kinetic parameters can vary depending on the buffer used.*
[e] *Exhibits substantial substrate inhibition in phosphate buffer, pH 7.0.*
[f] *Unpublished.*
[g] *Estimated from Achouri et al. (1997).*
[h] *From Grant (2018).*

2002, 2005) are Type I enzymes that are relatively insensitive to L-serine, with IC_{50}-values of ~5 and 10 mM, respectively. On the other hand, the Type I PGDH from *M. tuberculosis* has an IC_{50} for L-serine of ~30 μM, but only in the presence of phosphate ion (50–100 mM). In the absence of phosphate, the IC_{50} is in the low mM range (Xu and Grant, 2014) similar to that for *B. subtilis* and *C. glutamicum*. The available evidence shows that L-serine will bind to the ACT domain in the absence of phosphate ion but it is the interaction of phosphate at the ASB domain that allows L-serine to inhibit activity in the micromolar range (Burton et al., 2009b; Xu and Grant, 2014). However, the phosphate dependent sensitivity to L-serine does not occur with all mycobacteria (Xu et al., 2015) although it seems to be consistent among pathogenic mycobacteria to the extent that it has been studied. PGDH from all mammalian sources that have been studied show no sensitivity to serine at all, even though they contain ACT domains (Achouri et al., 1997; Dey et al., 2008; Grant, 2012). In these cases, the ACT domain residues are altered so that they no longer bind serine (see **Figure 3**). The classical feedback regulation by L-serine occurs with the Type II PGDH from *E. coli* which has an IC_{50} for L-serine of 2–10 μM, depending on the buffer used in the assay (Grant et al., 2000a, 2005). Since Type III PGDH enzymes do not contain an ACT domain, they are not feedback regulated by L-serine.

E. coli PGDH contains a Gly–Gly sequence (Gly 336–Gly 337 corresponding to positions 397–398 in **Figure 3**) between the substrate binding domain and the regulatory domain that acts like a hinge and plays a role in the conformational change that takes place upon serine binding and that is responsible for inhibition of activity (Grant, 2011). A similar sequence is found in *M. tuberculosis* PGDH (Gly 316–Gly 317–Gly 318, corresponding to positions 389–391 in **Figure 3**) but not in human PGDH. The absence of this hinge region in human PGDH is a contributing factor to its lack of feedback regulation by L-serine.

ALTERNATE SUBSTRATES

In 1996, Zhao and Winkler discovered that *E. coli* PGDH could use α-KG as a substrate in the reverse direction in place of PHP (Zhao and Winkler, 1996) to produce α-hydroxyglutarate (α-HG) with the concomitant oxidation of NADH. The ability to

use αKG as a substrate has also been reported for *Pseudomonas stutzeri* (*ps*PGDH) (Zhang et al., 2017), *Pseudomonas aeruginosa* (*pa*PGDH) (Zhang et al., 2017), *Saccharomyces cerevisiae* (*sc*PGDH) (Becker-Kettern et al., 2016), and human PGDH (*hs*PGDH) (Fan et al., 2015). However, the level of activity of human PGDH for α-KG is relatively low with a k_{cat} of ∼0.08 s^{-1} compared to that for *E. coli* PGDH of 33 s^{-1} (Fan et al., 2015). PGDH from *Mycobacterium tuberculosis* (*mt*PGDH) (Dey et al., 2005b) and *Rattus norvegicus* (Achouri et al., 1997) are reported to not use α-KG as a substrate. Zhang et al. (Zhang et al., 2017) have shown in *Pseudomonas* species, that the coupling of this reaction with d-2-hydroxyglutarate dehydrogenase can serve to drive L-serine synthesis. Grant (2018) has shown that in *E. coli*, there is a process that conserves coenzyme in the production of L-serine by utilizing an intrinsic cycle of NAD^+/NADH interconversion coupled with the conversion of αKG to αHG. Interestingly, this cycle can be maintained *in vitro* by production of αKG by the second enzyme in the pathway, PSAT, and does not require any additional enzymes (**Figure 11**). However, there is probably an ample pool of αKG available *in vivo* so that this is not strictly required. Note also that the kinetic mechanism shown in **Figure S4** is for the reverse direction where NADH can displace NAD^+. Therefore, the NADH conversion cycle is likely not functional in this direction.

No other compounds have been found to display significant activity as substrates. However, several NAD analogs have been demonstrated to be able to substitute for the coenzyme (Walsh and Sallach, 1965; Sugimoto and Pizer, 1968b; Rosenblum and Sallach, 1970; Winicov and Pizer, 1974; Unterlass et al., 2017). These include thionicotinamide adenine dinucleotide in wheat germ and human PGDH, 3-acetylpyridine adenine dinucleotide in wheat germ, *E. coli*, chicken liver, and human PGDH, 3-acetylpyridine deamino adenine dinucleotide in wheat germ and chicken liver PGDH, deamino nicotinamide adenine dinucleotide in *E. coli* and chicken liver PGDH, and 3-pyridinealdehyde adenine dinucleotide in chicken liver and human PGDH. Note, however, that not all coenzyme analogs have necessarily been tested with each species. In all cases, $NADP^+$ displays <10% of the activity of NAD^+.

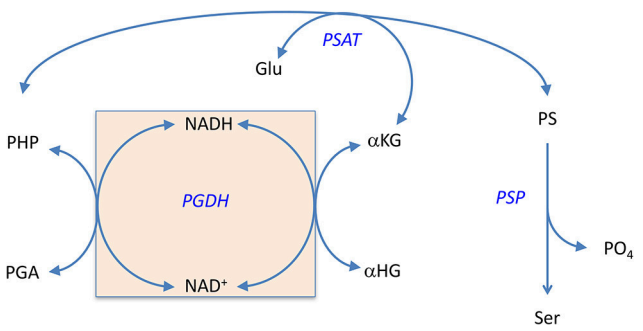

FIGURE 11 | The self-sustaining cycle that regenerates coenzyme bound to PGDH during biosynthesis of L-serine in *E. coli*. The NADH that is produced during the conversion of PGA to PHP is converted back to NAD^+ *in situ* by conversion of αKG to αHG. The αKG is formed from glutamate by the second enzyme in the pathway, PSAT. Reprinted from Grant (2018) with permission from the American Chemical Society.

MECHANISM

Only *E. coli* (Grant et al., 2003, 2004; Burton et al., 2008, 2009a; Grant, 2018) and *M. tuberculosis* (Grant et al., 1999c; Dey et al., 2005a,b, 2008; Burton et al., 2007, 2009b; Xu and Grant, 2014; Xu et al., 2015) PGDHs have been studied extensively with regard to mechanism. A previous review (Grant, 2012) summarized the findings of these studies in detail so they will only be briefly summarized here.

E. Coli PGDH

The mechanism of *E. coli* PGDH was studied in the reverse direction using α-KG and exhibits an ordered Bi Bi mechanism where NADH must be present before substrate binds (Burton et al., 2008). The k_{cat} of *E. coli* PGDH in the reverse direction is relatively slow at 7 s^{-1} per subunit with PHP as substrate. In the forward direction, the k_{cat} is ∼0.6 s^{-1} in an uncoupled reaction with PGA as substrate. It has not been measured in the forward direction by coupling it to PSAT and monitoring the reduction of NAD^+ because of the rapid regeneration of NAD^+ from NADH described above (**Figure 11**). *E. coli* PGDH is isolated with tightly bound NADH and, for a long time, it was thought that only NADH was tightly bound to the resting enzyme. However, recent evidence (Grant, 2018) has shown that NAD^+ is also rather tightly bound to the enzyme although it can be displaced by NADH. Both coenzyme forms appear to remain bound to the enzyme during continuous turnover when coupled to PSAT and participate in a cycle where they are continuously regenerated *in situ* (Grant, 2018). The sites that lie across the nucleotide binding domain interface appear to operate in a "flip-flop" manner (**Figure S5**), with each site trading places functionally after each turnover (Grant et al., 2003, 2004; Grant, 2018).

When enzyme catalysis is studied in the reverse direction with enzyme in which all of the NADH has been converted to NAD^+, there are two kinetically distinguishable NADH binding phenomena (Burton et al., 2008, **Figure S4**). One where coenzyme is very tightly bound and induces a conformational change that increases the dissociation constant for coenzyme even more, and one that is less tightly bound (Burton et al., 2008). The former, found in the resting enzyme, can turn over in the presence of substrate but the latter is the one that is functional during continuous turnover. That is, the conformational change that occurs with the binding of NADH in the absence of substrate does not occur to an appreciable extent during continuous turnover. Note that these "sites" are distinguished kinetically rather than positionally. Also note that this was determined for *E. coli* PGDH and is likely not the same for human and *M. tuberculosis* PGDH.

A type of half-of-the-sites activity appears to be functional for inhibition of activity by serine binding (Grant et al., 2004). Although all four effector sites in the ACT domains eventually bind serine, only two, on opposite sides of the nucleotide binding domain, need to be occupied for optimal inhibition. Binding to

these two sites displays positive cooperativity. Binding of serine to the last two sites is weaker due to negative cooperativity across the ACT domain interfaces.

The catalytic activity of *E. coli* PGDH can be inhibited in a reversible manner by cross-linking adjacent regulatory (ACT) domains with a disulfide bridge (Al-Rabiee et al., 1996a). Furthermore, this inhibition can be completely reversed by reduction of the bridge with dithiothreitol. This suggests that the regulatory domains move in some manner relative to each other during the transition from active to inhibited state. The disulfide bridge appears to mimic the binding of inhibitor since L-serine binds across the regulatory domain interface linking the two domains. Therefore, the mechanism of inhibition is one where serine binding eliminates a conformational change resulting from substrate binding and forms a dead-end quaternary complex consisting of enzyme, coenzyme, substrate, and effector (Burton et al., 2009a). Thus, the mechanism is a V-type that results in the reduction of active species rather than in a graded modulation of the velocity of the active enzyme.

Tryptophan 139 (position 187 in **Figure 3**), which sits at the bottom of the catalytic site of the adjacent subunit, plays a critical role in the oligomeric state of the enzyme as well as the integrity of the catalytic site (Grant et al., 2000b). When it is converted to a glycine residue, the enzyme dissociates into dimers and is 600-fold less active. However, it is just as sensitive to inhibition by L-serine as the native enzyme, although the cooperativity of inhibition is lost. It is quite surprising that a single residue can have such a drastic effect on the oligomerization state given that there is extensive subunit-subunit contact in this region, but this result demonstrates its critical importance. That this tryptophan is conserved in all other tetrameric PGDHs, suggests it plays a similar role in their catalytic and structural integrity.

Other than L-serine, which is a very potent inhibitor of *E. coli* PGDH, no other natural inhibitors have been identified. However, several heterocyclic organic inhibitors have been reported from studies involving predicted allosteric sites (Qi et al., 2012; Wang et al., 2014).

M. tuberculosis PGDH

The k_{cat} of 600 s^{-1} per subunit in the reverse direction for *M. tuberculosis* PGDH is much faster than that of *E. coli* PGDH. However, when coupled to PSAT in the forward direction, it is only 1.4 and 3 s^{-1} at pH 7.0 in MOPS and phosphate buffer, respectively. The kinetic mechanism is also ordered Bi Bi, but in this case, substrate binds before coenzyme. Although the order was determined with a reverse direction assay, it is consistent with the observation that the *E. coli* enzyme crystallizes with coenzyme and shows strong affinity for 5′-AMP Sepharose, an NAD analog, while the *M. tuberculosis* enzyme does not crystalize with coenzyme bound to the active site and has no affinity for 5′-AMP Sepharose. Interestingly, pre-steady state analysis showed that NADH could in fact bind to the enzyme in the absence of substrate but that the binding constants were too slow to account for the catalytic reaction (Dey et al., 2008, **Figure S6**). Subsequent investigation (Burton et al., 2009b; Grant, 2012) indicated that NADH bound at or near the ASB site and reduced the amount of substrate inhibition due to substrate interaction at the ASB site. It is not known what the exact nature of the interaction of NADH with the ASB site is, but it does not have an inhibitory effect on the rate of catalysis. The dissociation constants for substrate binding to the catalytic and ASB sites are 0.09 and 8 μM, respectively.

Other than L-serine, which effectively inhibits *M. tuberculosis* PGDH only in the presence of substantial concentrations of phosphate ion, no other inhibitors, natural or synthetic, have been identified for PGDH from mycobacteria. However, our unpublished work has demonstrated that CBR5884, a potent inhibitor of human PGDH (Mullarky et al., 2016), does not inhibit *M. tuberculosis* PGDH. This limited evidence suggests that specific inhibitors could be found for PGDH from different species, and therefore, in light of the essential metabolic role of L-serine in metabolism, this is an area in need of further exploration.

Human PGDH

No detailed studies of the mechanism of human PGDH have been reported but the kinetic constants for human PGDH are similar to those for *M. tuberculosis* PGDH when coupled to PSAT, with the exception that the K_m for NAD^+ is ∼8-fold higher for the human enzyme. Like *E. coli* PGDH, the enzyme appears to contain bound cofactor when isolated and the binding order appears to be coenzyme before substrate (Unterlass et al., 2017). Although the human enzyme has been reported to slowly convert αKG to αHG (Fan et al., 2015), the measured rate of conversion is very slow (Fan et al., 2015) and it does not sustain a coenzyme conservation cycle like that reported for *E. coli* PGDH (Grant, 2018). As mentioned earlier, while human PGDH is a Type 1 enzyme, it is not regulated by L-serine. In fact, human PGDH, as well as other mammalian PGDHs, have not been found to be regulated by any small molecule. Therefore, any regulation of human PGDH occurs either at transcription or translation or in the case of genetic mutations of amino acid residues as described below.

CONGENITAL DEFECTS ASSOCIATED WITH THE LACK OF PGDH IN MAMMALS

Serine deficiency disorders, which are caused by defects in the pathway leading to the synthesis of L-serine, were first reported in 1996 (Jaeken et al., 1996) and are mostly neurological in nature (Yoshida et al., 2004; Furuya, 2008; van der Crabben et al., 2013; El-Hattab et al., 2016). Although defects in the expression or catalytic activity of any of the three biosynthetic enzymes in the pathway can lead to L-serine deficiency (Tabatabaie et al., 2010; van der Crabben et al., 2013), those related to PGDH deficiency are the most common. The important role of PGDH was shown with a knock-out mouse model. The phenotype was associated with embryonic lethality and clearly demonstrated the L-serine biosynthetic pathway was critical (Yoshida et al., 2004).

That L-serine synthesized in the brain was the source of D-serine in the mature brain was shown using a conditional knock-out of PGDH in the brain that bypassed the embryonic lethal phenotype resulting from systemic deletion (Yang et al., 2010). The study found that both L-serine and D-serine levels were

significantly decreased in the cerebral cortex and hippocampus without altering levels of SR and NMDA receptor subunits. The study concluded that "in mature neuronal circuits, L-serine availability determines the rate of D-serine synthesis in the forebrain and controls NMDA receptor function at least in the hippocampus."

Infantile, juvenile, and adult onset phenotypes have all been reported for PGDH deficiency (Tabatabaie et al., 2010; van der Crabben et al., 2013). In the infantile phenotype, damage to the brain has already occurred prior to birth and usually manifests itself with congenital microcephaly, intractable seizures, and severe psychomotor defects. In general, oral supplementation with L-serine is very effective in reducing seizures but has little effect on psychomotor function. The juvenile phenotype was found in two siblings and traced to a single amino acid mutation. These patients first showed symptoms after 5 and 9 years of age and were not diagnosed as such until they were teenagers. The symptoms were much milder than in the infantile phenotype, consisting of absence seizures and moderate developmental delay without microcephaly. Both responded well to oral supplementation with L-serine. The adult phenotype in a single individual consisted of congenital cataracts, mental retardation in childhood, and progressive polyneuropathy as an adult.

One mutation, in particular, has been found as a common cause of PGDH deficiency, having been found in at least seven different individuals (Tabatabaie et al., 2010). This mutation, Val490Met, is found in the regulatory or ACT domain of PGDH. It is not known how it results in PGDH deficiency and the literature contains conflicting conclusions. One group (Pind et al., 2002) reports that the mutation results in a decrease in expression and an increase in degradation of PGDH, while another group (Tabatabaie et al., 2009) concluded that the mutation was without effect on expression and degradation, but rather produced an enzyme with low residual activity. It is interesting to note that the mutation is in the ACT domain and if the human structure is the same as that reported for PGDH from *M. tuberculosis*, the side chain of residue 490 would be found at the subunit interface between the ASB/ACT domains from adjacent subunits. Although far from the catalytic site, this mutation could result in an interference of structural integrity of the oligomer that could result in reduced activity and enhanced susceptibility of enzyme to degradation, which could be consistent with both literature reports.

PGDH AND CANCER

In 1970, Davis et al. (1970) published the first indication that changes in serine biosynthesis may be related to cancer. This work showed that PGDH activity was greater in rat hepatoma cell lines compared to normal liver cells, and correlated the fastest growth rate with the highest PGDH activity. Subsequent work (Snell, 1984) showed that this was true in many other tumors as well, and that among the pathway enzymes, tumor growth was most consistently correlated with an increase in PGDH activity. Renewed interest in the link between serine biosynthesis and cancer has occurred within the last 10 years or so with the observation that an increased level of expression of PGDH has been found in human cancers such as breast (Possemato et al., 2011), cervical (Zhang et al., 2015), glioma (Liu et al., 2013), melanoma (Ou et al., 2015), colon (Yoon et al., 2015), pancreatic (Zhiwang et al., 2018), liver (Shanshan et al., 2017), kidney (Yoshino et al., 2017), and others as well. Based on multiple lines of evidence (Mattaini et al., 2016), the phosphorylated pathway of serine biosynthesis, which utilizes PGDH, is the sole source of serine synthesis in non-photosynthetic organisms, including humans. In most cases, tumor cell proliferation is associated with increased levels of PGDH and decreased cell proliferation is seen when PGDH is knocked out, even though exogenous L-serine is supplied. In addition, inactive PGDH, due to site-specific mutation, cannot support proliferation of PGDH-dependent cells (Mattaini et al., 2015). It has also been shown that increased levels of PGDH synthesis, as well as the other serine biosynthetic enzymes, correlates with patient survival outcome and may be used as a prognostic factor for some cancers (Antonov et al., 2014). In some cases, extracellular serine seems sufficient to promote tumor cell proliferation, whereas in other cases, extracellular serine is not able to support cell proliferation when PGDH is absent. For instance, adding excess serine to the growth medium of PGDH knockdown human breast cancer cells was not able to rescue cell proliferation (Possemato et al., 2011; Chen et al., 2013). This suggests that flux through the serine biosynthetic pathway is providing something other than just a source of serine. In those cases where extracellular serine seems sufficient, additional reactions may promote flux through the pathway (**Figure S7**). It is still unknown just what the link is, but the requirement for pathway flux suggests that increased levels of the pathway intermediates, such as PHP or phosphoserine, may be critical. This has become a very active area of investigation in recent years and more detailed information on the link between cancer and PGDH can be found in several reviews (Luo, 2011; Mullarky et al., 2011; Zogg, 2014; Mattaini et al., 2016).

As a result of the correlation between high PGDH expression levels and cancer, there has been interest in finding inhibitors of the enzyme activity as potential starting points for drug development. Several recent articles have reported advancements in this area (Mullarky et al., 2016; Pacold et al., 2016; Wang et al., 2016; Ravez et al., 2017; Unterlass et al., 2018).

SUMMARY AND PROSPECTIVE

D-3-Phosphoglycerate dehydrogenase catalyzes the same reaction in all known organisms where it is found. That is, the NAD^+/NADH dependent interconversion of D-phosphoglycerate and PHP. However, there are significant differences among organisms as to how or whether PGDH activity is regulated by other factors. In all organisms where it has been studied, the equilibrium of the PGDH reaction lies far in the direction away from serine synthesis. Therefore, the downstream enzymes play a large role in keeping the pathway flux moving in the direction of serine synthesis by depleting PHP and then phosphoserine. The last reaction of the pathway is

irreversible, so once L-serine is produced it cannot be converted back to PS by simple reversal of the pathway. This presumably provides a relatively stable pool of L-serine that is available for conversion into other metabolites like D-serine. In *E. coli*, for example, there exists a very sensitive regulation of PGDH activity by L-serine that provides for fine tuning of the L-serine pool. A similar feedback mechanism exists in *M. tuberculosis* PGDH, except that the enzyme's sensitivity to L-serine is modulated by the available phosphate ion content. Yet, in other organisms, such as *C. glutamicum* and some plants, the serine sensitivity is as much as three orders of magnitude less. In humans, inhibition of PGDH by L-serine does not occur.

Very little is known about the regulation of PGDH or the serine biosynthetic pathway itself in mammalian cells or organisms, including *H. sapiens* especially. The attraction to the bacterial enzymes for detailed investigation may be partly due to the perception that the bacterial enzymes are more interesting from an enzymological point of view in that the human enzyme does not appear to be regulated by an effector molecule such as L-serine. This lack of feedback control in human PGDH may provide for a relatively large pool of available serine for conversion into other metabolites as well as protein synthesis. Although the literature is very sparse in this regard, it does not seem that the other enzymes in the serine biosynthetic pathway in mammals display any significant level of regulation by small molecule effectors. Since the available literature clearly points to the availability of L-serine as being critical to the synthesis of D-serine, this would seem to be a particularly relevant area of investigation in regards to the function and physiology of the NMDA receptor.

One very important facet of the study of the human enzyme that is missing is a complete picture of its structure which would aid in any conclusions about its mechanism that might be made relative to the *M. tuberculosis* enzyme, another Type I PGDH. The availability of a complete structure of human PGDH will also be necessary in the evaluation and fine tuning of inhibitors that may eventually be developed into drugs targeting PGDH activity in malignant cells and tumors.

From a physiological viewpoint, a thorough analysis of the flux of the serine biosynthetic pathway in mammalian astrocytes, in response to different stimuli, will be useful. An even broader question in this respect concerns the interplay between glycolysis, gluconeogenesis, and serine synthesis. If the L-serine biosynthetic pathway in mammals lacks any type of specific regulatory mechanisms to control the production of L-serine, what factors govern consumption of the common metabolite, PGA? Asked another way, what keeps the serine pathway in these organisms from consuming an excess of PGA and adversely affecting energy production from glucose?

The studies conducted with malignant mammalian cells demonstrate that the expression of PGDH can be modulated, but the precise factors leading to changes in expression levels are not well-understood. This would also seem to be an area requiring further investigation that may be relevant not only to cancer but to neurological function as well.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmolb.2018.00110/full#supplementary-material

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REVIEW
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Changes in Serine Racemase-Dependent Modulation of NMDA Receptor: Impact on Physiological and Pathological Brain Aging

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The N-methyl-D-Aspartate glutamate receptors (NMDARs) are pivotal for the functional and morphological plasticity that are required in neuronal networks for efficient brain activities and notably for cognitive-related abilities. Because NMDARs are heterogeneous in subunit composition and associated with multiple functional regulatory sites, their efficacy is under the tonic influence of numerous allosteric modulations, whose dysfunction generally represents the first step generating pathological states. Among the enzymatic candidates, serine racemase (SR) has recently gathered an increasing interest considering that it tightly regulates the production of D-serine, an amino acid now viewed as the main endogenous co-agonist necessary for NMDAR activation. Nowadays, SR deregulation is associated with a wide range of neurological and psychiatric diseases including schizophrenia, amyotrophic lateral sclerosis, and depression. This review aims at compelling the most recent experimental evidences indicating that changes in SR-related modulation of NMDARs also govern opposite functional dysfunctions in physiological and pathological (Alzheimer's disease) aging that finally results in memory disabilities in both cases. It also highlights SR as a relevant alternative target for new pharmacological strategies aimed at preventing functional alterations and cognitive impairments linked to the aging process.

Keywords: NMDA receptors, serine racemase, aging, Alzheimer's disease, D-serine, long term potentiation, glutamate

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INTRODUCTION

Through the fine regulation of neurotransmitters/neuromodulators availability at their respective binding sites, enzymatic activities are critical for normal brain functions and are generally targeted by pathophysiological processes. In this context, the modulation of the N-methyl-D-Aspartate subtype of glutamate receptors (NMDARs) certainly represents a school case, which actually focuses the attention of a large proportion of the scientific community as illustrated by the almost 5,000 review articles referenced in pubmed. In fact, based on their large distribution throughout the nervous system and their diversity in subunit composition associated with regional specificity

in the brain and even with segregated localization at synapse level (see Paoletti et al., 2013; Zhu and Paoletti, 2015), NMDARs thus appear as a perfect example to evaluate the impact of specific allosteric regulation of selective brain activities and notably of cognitive capacities, in normal and pathological conditions. These receptors are complex entities under the modulation of a wide range of regulatory processes driven by magnesium, polyamines and histamine environments as well as levels of redox state (Johnson and Ascher, 1990; Kleckner and Dingledine, 1991; Lipton et al., 1998; Choi and Lipton, 2000; Brown et al., 2001; Haas et al., 2008; Zhu and Paoletti, 2015). Beside these salient regulation features, NMDAR activation is also characterized by the obligatory fixation in addition to the main agonist glutamate of a co-agonist at a specific binding site (Traynelis et al., 2010; Paoletti, 2011; Paoletti et al., 2013). Attributed initially to glycine (Johnson and Ascher, 1987, 1992; Kleckner and Dingledine, 1988), this role of co-agonist in much brain area and particularly in those involved in cognitive functions, is now devoted to D-serine (Schell et al., 1997; Mothet et al., 2000; Snyder and Kim, 2000; Shleper et al., 2005; Billard, 2008, 2012; Henneberger et al., 2012; Bardaweel et al., 2014; Wolosker, 2018), a D-amino acid produced by the racemisation of L-serine by the enzyme serine racemase (SR) (Wolosker et al., 1999). Like the degradation of D-serine (Mothet et al., 2000; Shleper et al., 2005; Strick et al., 2011; Papouin et al., 2012; Rosenberg et al., 2013; Le Bail et al., 2015), the genetic deletion of SR impairs the connectivity and the functional plasticity of neuronal networks and has been associated with cognitive impairments (Inoue et al., 2008, 2018; Basu et al., 2009; Labrie et al., 2009; Balu and Coyle, 2012; Bai et al., 2014; Puhl et al., 2017; Balu et al., 2018). Consequently, changes in SR-dependent modulation of NMDAR activation through alterations of synaptic availability of D-serine, have been postulated to contribute to pathophysiological mechanisms governing several neurological diseases [reviewed in Billard (2013) and Coyle and Balu (2018)]. Thus, weaker NMDAR activation linked to down regulation of SR activity is now viewed as a critical synaptic dysfunction in schizophrenia, addictions, anxiety disorders, and depression (Coyle, 2006; Benneyworth and Coyle, 2012; Gómez-Galán et al., 2012; Coyle and Balu, 2018). On the opposite, up regulation of NMDAR activity due to increased production of D-serine by SR is viewed as a central mechanism for neurodegenerative processes underlying the amyotrophic lateral sclerosis (Sasabe et al., 2007; Lee et al., 2017; Kondori et al., 2018).

In the last decades, the role of SR-dependent regulation of NMDAR activity in cognitive aging has also been investigated, that is the focus of the present review. After recapitulating our knowledge that now considers NMDAR modulation by SR as an essential mechanism involved in learning and memory, currently available information related to its deregulation in physiological aging and Alzheimer's disease (AD) will be presented, with the main conclusion that a strict regulation of SR activity is required for a successful cognitive aging. This review could also offer new opportunities for considering new relevant pharmacological strategies specifically targeting the SR-associated pathway to treat memory deficits linked to age-related brain disorders.

NMDA RECEPTORS: STRUCTURE AND FUNCTIONAL REGULATION

NMDARs are part of a large multiprotein complex at glutamatergic synapses, that have received much attention over the last decades, due to their role in many types of neural plasticity on the one hand, and their involvement in neurotoxicity on the other hand. They are hetero-tetramers generally formed by two GluN1 subunits associated with the combination of two other partners including either four distinct GluN2 (GluN2A-D) or a mixture of GluN2 with two different GluN3 (GluN3A and 3B) subunits (Ulbrich and Isacoff, 2008; Traynelis et al., 2010; Paoletti, 2011; Paoletti et al., 2013) (**Figure 1**). The GluN1 subunit is expressed throughout the brain since it is mandatory for NMDAR activation through the necessary binding of a co-agonist at the amino-terminal domain of the extracellular region (Ballard et al., 2002; Paoletti et al., 2013). Besides, GluN2 subunits specifically bind the main agonist glutamate and differ from each other by their pharmacological profiles and also by providing distinct functional properties to NMDARs (Nakanishi and Masu, 1994; Dingledine et al., 1999; Hofmann et al., 2000; Paoletti et al., 2013). Although the wide range of subunit associations predicts a large diversity within the NMDARs family, preferential combinations have been regionally detected in the brain that is also observed at synaptic levels where GluN2A and GluN2B subunits are enriched at postsynaptic densities and extrasynaptic zones respectively (Traynelis et al., 2010; Paoletti, 2011; Paoletti et al., 2013). Important in the context of aging, GluN1 expression remains elevated throughout lifespan (Laurie and Seeburg, 1994; Monyer et al., 1994) whereas a progressive decrease in the GluN2B/GluN2A ratio generally occurs with age at cortical synapses (Monyer et al., 1994; Stocca and Vicini, 1998; Liu et al., 2004; Swanger and Traynelis, 2018), that have suggested the interest of pharmacologically targeting the GluN2B subunit to treat or prevent age-related memory decline (Wang et al., 2014).

In contrast to their diversity in subunit composition, all NMDARs are structurally homogenous (**Figure 1**) and characterized by three helices (M1, M3, M4) and a hairpin (M2) that form a transmembrane domain allowing the ion selectivity of the receptors. While this domain is subjected to tonic modulation, notably by magnesium (Mg^{2+}), this is not the case for the cytoplasmic carboxy-terminal intracellular domain that controls the coupling to different intracellular signaling cascades and the receptor trafficking (Traynelis et al., 2010; Paoletti, 2011; Paoletti et al., 2013).

Compared to the other subtypes of ionotropic glutamate receptors, NMDARs display distinct functional properties identified by slow gating and deactivating kinetics associated with high calcium permeability, which depend on the subunit composition (Dunah et al., 1999; Paoletti, 2011; Wyllie et al., 2013; Zhang and Luo, 2013; Sun et al., 2017). In addition to their specific voltage-dependent blockade by Mg^{2+} (Johnson and Ascher, 1990; Kleckner and Dingledine, 1991), another impressive functional feature of NMDARs consists in their activation processes which require not only the binding of

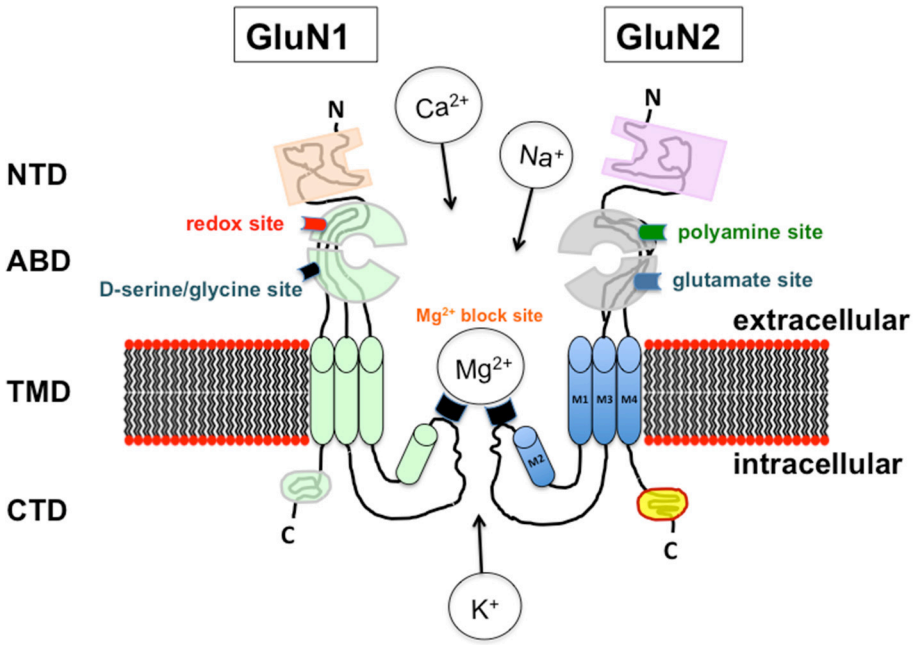

FIGURE 1 | Schematic representation of the assembly and modular organization of a N-Methyl-D-Aspartic acid receptor (NMDAR). The extracellular segment includes the N-terminal domain (NTD) and the agonist binding domain (ABD) where D-serine /glycine and glutamate bind to the GluN1 and GluN2 subunit respectively. ABD also contains the redox and polyamine regulatory sites. The ion channel is localized in the transmembrane domain (TMD) that contains the site for the magnesium blockade while the C-terminal domain (CTD) is included in the intracellular segment.

glutamate on GluN2 subunits but in synergy, the fixation of a co-agonist on a specific site present on the GluN1 components (Traynelis et al., 2010; Paoletti, 2011). This necessity of a dual binding was initially characterized in the late 80s when the induction magnitude of inward currents through native or NMDARs expressed in oocytes (Kleckner and Dingledine, 1988) or in cultured mouse neurons (Johnson and Ascher, 1987) was found to closely rely on glycine levels present in the external medium, thus revealing the existence of the so-called glycine-binding site. After more than 20 years of biochemical, immunohistochemical and electrophysiological investigations [reviewed in Billard (2012)], the initial view of glycine as the endogenous NMDAR co-agonist has then been progressively substituted by the concept assigning this role to the amino acid D-serine, though the most recent emerging view now considers that D-serine rather cooperates with glycine in a complex interplay to control NMDAR activation following time and space constraints (Mothet et al., 2015). D-serine is directly converted from its precursor enantiomer L-serine by the activity of the pyridoxal 5-phosphate (PLP)-dependent enzyme serine racemase (SR) (Wolosker et al., 1999). Interestingly, this enzyme is also able to metabolize D-serine into pyruvate and ammonia by catalyzing an α,β elimination of water (De Miranda et al., 2002; Foltyn et al., 2005). This reaction may represent an alternative route to degrade D-serine in forebrain regions where the endogenous degrading enzyme D-amino acid oxidase DAAO (Pollegioni et al., 2007), is poorly expressed (Bendikov et al., 2007; Verrall et al., 2007; Jagannath et al., 2017). However, since the efficacy of the racemisation process of L-serine is five times higher than the reaction of α, β elimination (Strísovský et al., 2005), one generally considers that SR preferentially governs D-serine synthesis.

SERINE RACEMASE: LOCALIZATION, REGULATION AND CONTRIBUTION TO FUNCTIONAL PLASTICITY AT SYNAPSES

Nowadays, the question to know if SR is expressed in a specific cellular population at synapses is heavily discussed and has broadened to the larger debate asking if D-serine may be considered as a gliotransmitter like glutamate and ATP (Wolosker et al., 2016, 2017; Papouin et al., 2017). Indeed, the initial characterization of SR expression in astrocytes (Wolosker et al., 1999) and the view that different NMDAR-dependent functions could be driven by a vesicular release of D-serine from this subtype of glial cells (Yang et al., 2005; Panatier et al., 2006; Williams et al., 2006; Martineau et al., 2008; Papouin et al., 2012; Martineau, 2013; Lalo et al., 2018; Robin et al., 2018) are now strongly questioned. This is mainly due to the development of more selective SR antibodies and improved immunohistochemical protocols, to the lack in those pre-cited experiments of negative controls with SR knock-out ($SR^{-/-}$) mice which display a 90% decrease in brain D-serine without significant changes in levels of the other amino acids except D-aspartate (Miya et al., 2008; Basu et al., 2009), and finally because the use of mice with disrupted SNARE-dependent exocytosis in astrocytes to specifically assess glio-transmission is still under debate (Fiacco and McCarthy, 2018; Savtchouk

and Volterra, 2018). When rigorous experimental conditions are achieved *in vivo*, SR is mainly expressed in excitatory neurons and GABAergic inhibitory interneurons of the human and rodent brains with only a weak if any detection in astrocytes (Kartvelishvily et al., 2006; Miya et al., 2008; Benneyworth et al., 2012; Ehmsen et al., 2013; Balu et al., 2014; Perez et al., 2017). Nowadays, an emerging concept of a serine shuttle gathers increasing interest (Wolosker, 2011; Wolosker and Radzishevsky, 2013) in which it is viewed that through orchestrated activities of neutral amino acid transporters including at least alanine-serine-cysteine 1 (Asc-1) and ASCT1 subtypes (Rosenberg et al., 2013; Sason et al., 2017; Kaplan et al., 2018), the astrocyte-derived precursor L-serine fuels the neuronal SR to produce D-serine, which is then released to bind NMDAR before to be subsequently removed from synapses by either neurons or astrocytes (**Figure 2**). Although this shuttle sounds attractive to account for the synaptic turnover of D-serine in the healthy brain though it needs to be definitively validated, it fails to work when pathological conditions associated with astrogliosis prevail, such as those occurring in traumatic brain injury for example. Indeed, a controlled cortical brain insult results in a down-regulation of neuronal SR expression and a parallel increase in reactive astrocytes (Perez et al., 2017), that thus devotes a major role *in vivo* to glia-derived D-serine only when pathological mechanisms inducing excitotoxic damages and neuronal death are promoted.

In addition to help for a better determination cellular localization of SR, lessons from $SR^{-/-}$ mice have also provided information for a pivotal role of the SR-associated processes in controlling functional plasticity at synapses. This has been particularly investigated using the electrophysiological paradigm of long-term potentiation (LTP) of synaptic transmission, a form of long lasting form of synaptic plasticity now viewed as a major functional requirement for memory formation (Izquierdo, 1991; Bear and Malenka, 1994; Collingridge and Bliss, 1995; Izquierdo and Medina, 1995; Lisman and McIntyre, 2001; Kim and Linden, 2007). Indeed, LTP is significantly reduced *ex vivo* in slice preparations isolated from mice with specific deletion of SR in neurons using the calmodulin kinase II promoter or *in vivo* using the Thy1-mediated Cre recombination, the deficits being rescued in both cases by exogenous D-serine (Benneyworth et al., 2012; Perez et al., 2017). On the contrary, similar designs but selectively targeting astrocytes using the GFAP promoter has no significant impact on LTP expression (Benneyworth et al., 2012). These results provide additional functional evidences that SR-induced D-serine from glia plays a minor role in synaptic plasticity in healthy conditions, in opposition to what is claimed (Panatier et al., 2006; Henneberger et al., 2010; Papouin et al., 2012; Lalo et al., 2018). However, it is worth noting that glia-derived D-serine could impact functional plasticity when pathological conditions prevail as recently reported after traumatic brain injury where the induction of SR expression in reactive astrocytes associated with an excessive release of D-serine, impairs LTP expression (Perez et al., 2017) and behavior (Liraz-Zaltsman et al., 2018). Whether similar deleterious effects of glia-derived D-serine on synaptic plasticity also occur in other astrogliosis-associated brain injuries remains to be determined.

The SR-dependent modulation of functional plasticity involves changes in NMDAR activation in response to altered D-serine availability. Indeed, isolated NMDAR-dependent excitatory postsynaptic currents (EPSCs) show slower decay kinetics in $SR^{-/-}$ mice (Basu et al., 2009; Balu et al., 2013) while the amplitude of miniature NMDAR-EPSCs are significantly reduced in mice with selective neuronal SR deletion (Benneyworth et al., 2012). Providing exogenous D-serine to SR-deleted animals not only rescues these functional deficits but also increases the amplitude of NMDAR-dependent currents more extensively than in wild-type animals, consistent with lower occupancy of the NMDAR glycine-binding site when SR is invalidated.

SR is functionally modulated by a wide range of regulatory mechanisms including changes in cofactors likely to be present in the vicinity of the enzyme, protein interactions, dynamic changes in subcellular localization and posttranslational processes (recently reviewed and detailed in Wolosker, 2018). An increase in SR activity, due to activation or the prevention of its degradation, may be promoted by the small ligands ATP and Mg^{2+} (De Miranda et al., 2002; Strísovský et al., 2003; Foltyn et al., 2005), multiple protein interactors including GRIP, Golga3, Disc-1 and FBXO22 (Kim et al., 2005; Dumin et al., 2006; Ma et al., 2013; Dikopoltsev et al., 2014), by O-palmitoylation-related processes (Balan et al., 2009) and also possibly through phosphorylation at different residues (Balan et al., 2009; Foltyn et al., 2010). On the other hand, nicotinamide adeninedinucleotide (NADH) (Suzuki et al., 2015; Bruno et al., 2016), protein interactions with Pick-1 (Fujii et al., 2006), PSD-95 (Ma et al., 2014; Lin et al., 2016), SAP102 and stargazin (Ma et al., 2014), membrane or nuclear translocations (Balan et al., 2009; Kolodney et al., 2015) and S-Nitrosylation-related oxidative processes (Mustafa et al., 2007) inhibit SR activity. Therefore, the SR activity itself appears to be modulated in a complex manner by a large mosaic of mechanisms, which can be targeted by the aging process.

DOWN REGULATION OF SR-RELATED ACTIVITY IN PHYSIOLOGICAL AGING

Changes in neurologic functions generally occur with physiological aging that may substantially interfere with everyday activities (Craik and Bialystok, 2006). Indeed, older adults experience deficits in learning and memory while the speed of cognitive processing is frequently slowed down, that have initially been associated with neuroanatomical changes (Brunso-Bechtold et al., 2000; Driscoll et al., 2003; Finch, 2003; Geinisman et al., 2004; Hayakawa et al., 2007; Burke and Barnes, 2010). However, lessons from numerous preclinical investigations now rather support the view that impaired expression of NMDAR-dependent functional plasticity at synaptic connections is the major cellular substrate of physiological cognitive aging (Lynch, 1998; Barnes, 2003; Billard, 2006; Foster, 2012). A decrease in NMDAR density, and notably in GluN2B subunits, was initially suspected to underlie LTP deficits in the aging brain (Magnusson, 1998, 2000; Clayton et al., 2002a,b; Magnusson et al., 2002; Bai

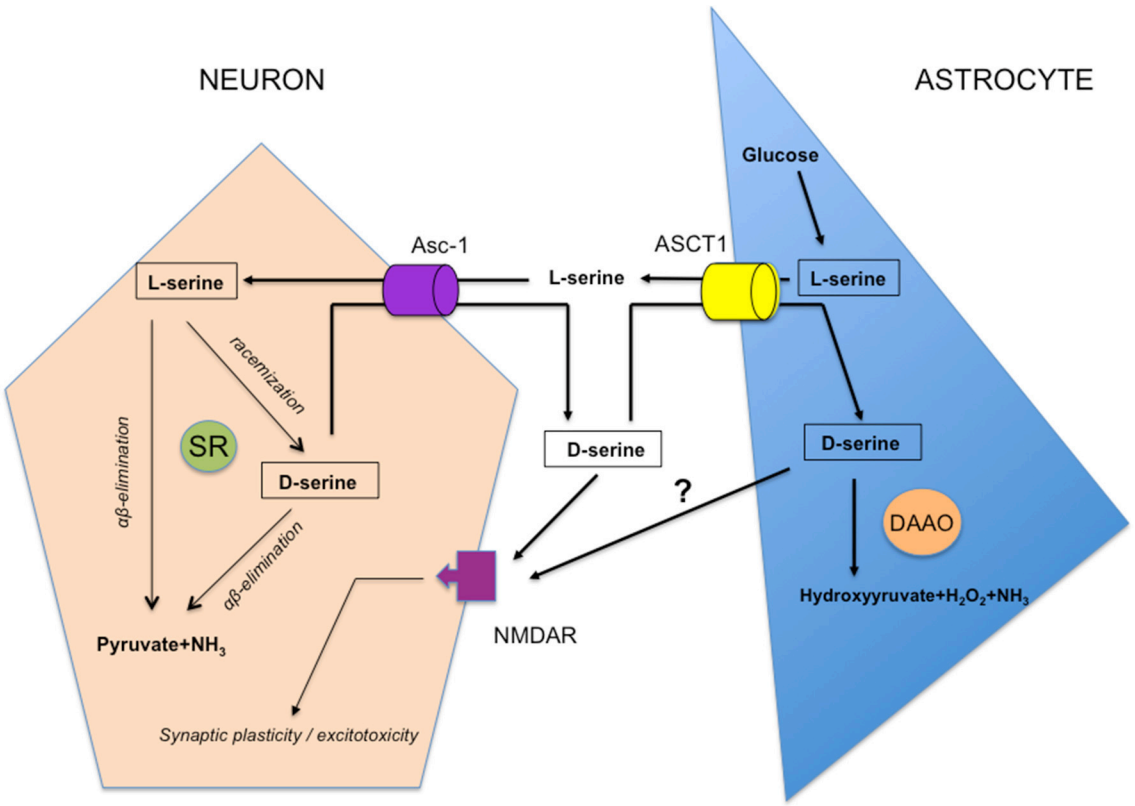

FIGURE 2 | Schematic representation of the serine shuttle. L-serine specifically synthesized from glucose in the astrocyte subtype of glial cells, is released in external medium through the Alanine, serine, cysteine, threonine (ASCT1) subtype of neutral amino acid transporters. It is then taken-up by neurons through the Asc-1 subtype where it is converted into D-serine by serine racemase (SR) while part of the amino acid may be degraded into pyruvate and NH_3 by α,β elimination of water. D-serine is delivered back to extracellular space through Asc-1 hetero-exchange with L-serine to act on NMDAR thus promoting functional plasticity at synapses or neurotoxicity in pathological conditions. D-serine is taken-up from the synaptic cleft through ASCT1 hetero-exchange with L-serine in astrocytes where it is degraded by D-amino acid oxidase (DAAO) activity. Whether part of D-serine-derived astrocytes may be released to impact NMDAR is under debate.

et al., 2004; Brim et al., 2013) but defects affecting the functional modulation of the receptor have also been later characterized including deregulation at the redox site (Kuehl-Kovarik et al., 2003; Bodhinathan et al., 2010; Yang et al., 2010; Kumar et al., 2017), changes in non-competitive blockade (Norris and Foster, 1999) and even altered lipid composition of postsynaptic membranes (Lynch and Voss, 1994; McGahon et al., 1999; Latour et al., 2013). In the search of such functional deficits, changes in SR-modulation of NMDAR activation has also been postulated to develop with age (Billard, 2013). According to this possibility, aged humans with impaired memory capacities in the Groton maze computer test improve their performances if they previously receive a D-serine-enriched drink (Avellar et al., 2016) while learning deficits in aged drosophila in an olfactory conditioning is rescued by feeding the flies with the amino acid (Yamazaki et al., 2014). Subsequent analyses in aged rodents indicate that a reduced SR expression is a prominent feature of hippocampal aging (**Figure 3A**), which decreases D-serine levels within neuronal networks and promotes NMDAR hypofunction (Mothet et al., 2006; Potier et al., 2010; Turpin et al., 2011). Providing the amino acid to the "aged" tissues then restores NMDAR activation and LTP induction at synapses (Yang et al., 2005; Mothet et al., 2006; Turpin et al., 2011). In animal models of successful cognitive aging such as the LOU/C strain of rats (Alliot et al., 2002; Kappeler et al., 2004), the potent memory abilities and NMDAR-dependent LTP displayed by aged individuals correlate with preserved SR expression and D-serine production (Kollen et al., 2010; Turpin et al., 2011). One characteristic of aged LOU/C rats is to present high resistance to oxidative stress (OS) induced by the accumulation of free radical damages that progressively take place in the course of aging (Sohal and Weindruch, 1996; Golden et al., 2002; Ali et al., 2006; Dröge and Schipper, 2007). Increased oxidation of sulfydryl groups of SR (Mustafa et al., 2007) and/or changes in its dimer active conformation (Wang and Barger, 2012) could then be viewed as critical mechanisms driven by OS to impact SR activation in the aging brain. Accordingly, long-term treatment with the reducing agent N-acetyl cysteine in aged rats to prevent from OS development, protects SR expression and activity and preserves a potent NMDAR activation in the animals (Haxaire et al., 2012). In addition, weaker SR activity promoted by OS could also be managed through an hypermethylation in the

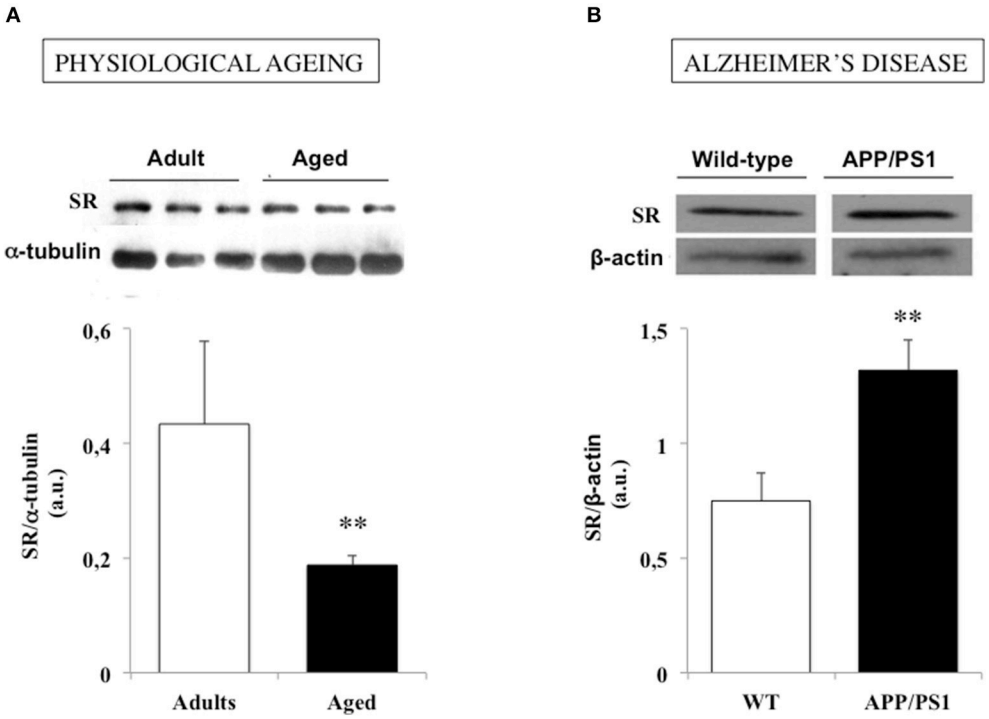

FIGURE 3 | Serine racemase (SR) expression is down and up regulated in physiological and pathological brain aging respectively. **(A)** Examples of immunoblots for serine racemase (SR) and α-tubulin in adult and aged rats (up) and bar graphs depicted the mean SR expression determined for each group when normalized to α-tubulin (down). **(B)**. Examples of immunoblots for SR and β-actin in a wild-type (WT) and an APP/PS1 mouse model of Alzheimer's disease (up) and bar graphs depicted the mean SR expression determined for each group when normalized to β-actin (down). (**$P < 0.01$). Modified with permissions from (Potier et al., 2010) and (Madeira et al., 2015).

promoter of SR gene (Zhang et al., 2015) that could explain the age-related decreased levels of SR transcripts (Mothet et al., 2006; Potier et al., 2010). These results therefore reinforce the idea of preventing oxidative stress as a major strategy to alleviate cognitive aging (Sohal and Weindruch, 1996; Liu et al., 2003; Dröge and Schipper, 2007).

Besides the OS-dependent dysfunctions of SR activation, a down-regulation of its enzymatic activity could also be viewed in the aging brain as resulting from a reduced synaptic availability of L-serine (postulated in Ivanov and Mothet, 2018). However, though the expression of PHGDH, one of the enzymes predominantly involved in the synthesis pathway of the D-serine precursor (Yamasaki et al., 2001), is reduced in acutely isolated astrocytes from aged mice (Orre et al., 2014; Holtman et al., 2015), overall levels of the amino acid are not altered in the aging hippocampus (Mothet et al., 2006; Turpin et al., 2011; Haxaire et al., 2012) and providing L-serine does not help in preventing the age-related decrease in NMDAR activation (Junjaud et al., 2006). On the other hand, recent evidence reports that the D-serine shuttle, and notably the potency of the Asc-1 transporters to release D-serine from neurons, is not affected by age (Billard and Freret, 2018). These results further indicate that changes in SR-related modulation of NMDAR represent a critical mechanism associated with physiological brain aging and that boosting SR activation could thus be viewed to represent an alternative strategy to alleviate age-related memory impairment. Among different possibilities, a strategy based on SR stimulation by Mg^{2+} could be hypothesized considering that Mg^{2+} has been shown to enhance learning and memory (Ozturk and Cillier, 2006; Slutsky et al., 2010).

UP REGULATION OF SR-RELATED ACTIVITY IN ALZHEIMER'S DISEASE

Compared to other neurological disorders such as schizophrenia, depression or amyotrophic lateral sclerosis (Goltsov et al., 2006; Labrie and Roder, 2010; Mitchell et al., 2010; Gómez-Galán et al., 2012; Balu and Coyle, 2015; Coyle and Balu, 2018), our current knowledge on the role of the SR-related pathway in the pathophysiology of Alzheimer's disease (AD) is so far limited. One reason for this weaker interest probably comes from the initial biochemical observations indicating that free D-serine levels were not consistently altered in the brain of AD patients, although the percentage of D-serine in the total (D + L) serine was significantly lower than that of aged-matched controls (Chouinard et al., 1993; Kumashiro et al., 1995; Nagata et al., 1995; Hashimoto et al., 2004; Biemans et al., 2016) but see (Fisher et al., 1998). Nevertheless, the absence of a clear-cut contribution of SR to AD-related pathophysiology could reflect the fact that the levels of D-serine in those experiments were determined in patients at late stages of the pathology whereas the

most recent preclinical studies suggest that the amino acid could rather be involved in the very early steps of the disease (Madeira et al., 2015). Indeed, a significant increase in D-serine levels has recently been characterized in the cerebrospinal fluid (CSF) of subjects with only mild cognitive impairment that will probably evolve into dementia (Madeira et al., 2015). This observation has suggested that a deregulation of the SR-related activity could serve as a new biomarker of the entry into the pathology [see also (Hashimoto et al., 2004)], although this postulate has not recently been confirmed (Biemans et al., 2016). Nevertheless, several preclinical data strongly argue for the involvement of SR in the pathophysiological processes underlying AD. Thus, two major soluble factors involved in AD pathogenesis, the amyloid ß-peptide (Aß) and the secreted form of ß-amyloid precursor protein (APP) (Cline et al., 2018), stimulate SR expression and promote D-serine release in microglial cell cultures whereas these subtypes of glial cells do not normally produce the amino acid (Wu et al., 2004, 2007). The Aß peptide evokes D-serine synthesis and efflux also from neurons, in synergy with the release of glutamate (Brito-Moreira et al., 2011; Madeira et al., 2015) that drives over-stimulation of NMDAR and promotes neurotoxicity, a typical picture of the pathophysiology of AD (Harkany et al., 2000; Butterfield, 2002; Hynd et al., 2004). Several other preclinical observations fit well with a contribution of D-serine in AD-related neurotoxicity: neuronal cell death induced by NMDA is strongly reduced in cerebral tissues depleted in D-serine after a pre-treatment with DAAO (Katsuki et al., 2004) as well as in organotypic hippocampal slices pre-treated with the recombinant D-serine deaminase, an enzyme 100 fold more active than DAAO in degrading the amino acid (Shleper et al., 2005). *In vivo*, both NMDAR and Aß-induced neurotoxicity are largely attenuated in $SR^{-/-}$ mice (Inoue et al., 2008). Through the binding of inducible proto-oncogenes *c*-fos and JunB to the activator protein-1 sequence present on the first intron of the SR gene, Aß promotes the transcriptional induction of SR (Wu and Barger, 2004), an observation which fits with the increase in SR messenger RNAs in the brain of AD patients (Wu et al., 2004). Post-transcriptional mechanisms may also contribute such as an increase in intracellular calcium levels by Aß (Wu et al., 2004) knowing that calcium overload in neurons is able to boost SR activity (Cook et al., 2002; De Miranda et al., 2002).

Besides, a significant increase in SR expression and D-serine levels also occur *in vivo* in a mouse model of AD with a transgene for APP associated with a mutant form of presenilin 1 (APP/PS1 mice) (**Figure 3B**) (Madeira et al., 2015). Finally, recent preliminary data indicate that in the 5xFAD model of AD which expresses high levels of soluble Aß oligomers (Oakley et al., 2006; Giannoni et al., 2013; Lee and Han, 2013), the impaired functional plasticity reported at hippocampal synapses (Kimura and Ohno, 2009; Crouzin et al., 2013) was rescued after deleting the SR gene, that further points out a major role of an altered SR-dependent modulation of NMDAR functions in the Aß-related pathophysiology of AD (Billard et al., 2018).

Considering the current state of knowledge summarized above, the elevated SR expression and the subsequent increase in D-serine levels in the extracellular space could be viewed as pro-death signals in AD that promotes, in conjunction with the release of glutamate, the neurotoxicity exhibited by inflammatory processes (Barger et al., 2007; Vesce et al., 2007). Although this view clearly remains to be definitively characterized and notably if the glia-derived SR could contribute to mechanisms of the insult, the up-regulation of the SR-related pathway in AD therefore appears as a perfect example of how a deregulation of allosteric modulation of NMDAR may drive the onset of pathological conditions.

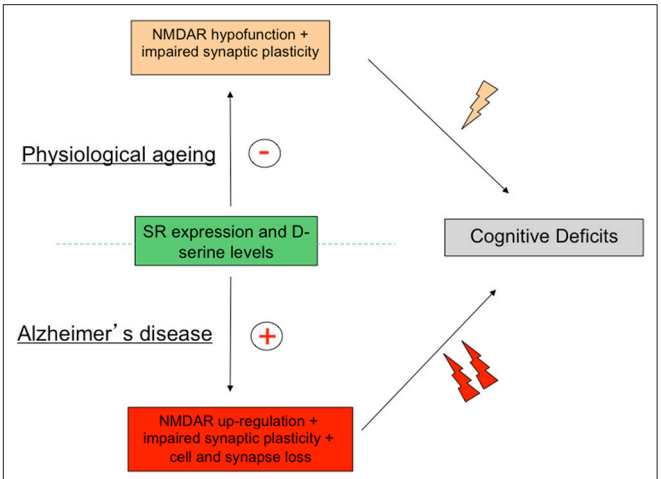

FIGURE 4 | Activity of serine racemase (SR) must be strictly regulated to avoid age-related memory deficits. Schematic diagram outlying the concept that although changes in SR expression and activity are opposite in physiological and pathological brain aging brain through down- and up-regulation of N-Methyl-D-Aspartic acid receptor (NMDAR) activity respectively, cognitive deficits, and notably memory impairments, represent the ultimate syndrome in both conditions.

CONCLUSION

Nowadays, a wealth of preclinical and clinical evidences argues for a critical role of SR throughout lifespan in the regulation of functional plasticity through the synaptic availability of the NMDAR co-agonist D-serine. Such modulation impacting NMDAR activation allows the enzyme to control many brain functions in healthy conditions while being a preferential target for pathophysiological insults (Coyle and Balu, 2018). When interest is focused on age-related memory disabilities, a down- and up-regulation of the SR-associated pathway are specifically associated with physiological aging and AD respectively. Although these alterations show striking opposite directions, they both result *in fine* in memory deficits indicating that a strict control of SR expression and activity is required to achieve a successful cognitive aging (**Figure 4**). These results therefore highlight SR as a potent target for the development of alternative pharmacological interventions aimed at relieving cognitive impairments linked to the aging process. Protection of SR to the age-related oxidative stress is already suggested to represent such an alternative procedure to rescue memory deficits associated with physiological aging (Haxaire et al.,

2012). In preclinical studies, SR antagonists such as Phenazine Ethosulfate (Phen-Et) and erythro-β-Hydroxy L-aspartate have been used to investigate SR involvement in specific NMDAR-dependent processes (De Miranda et al., 2002; Kim et al., 2005; Strísovský et al., 2005; Stevens et al., 2010), that could represent other pharmacological alternatives to prevent the onset of pathological conditions in which SR activity is facilitated such as ALS, AD or brain trauma (Sasabe et al., 2007; Madeira et al., 2015; Lee et al., 2017; Perez et al., 2017; Kondori et al., 2018), though the specificity of these pharmacological tools have recently been questioned. However, there is no doubt now that increasing our knowledge of SR-dependent regulation of NMDAR activation certainly represents a key route that will help people keeping potent cognitive abilities throughout lifespan.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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REVIEW
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Human D-Amino Acid Oxidase: Structure, Function, and Regulation

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D-Amino acid oxidase (DAAO) is an FAD-containing flavoenzyme that catalyzes with absolute stereoselectivity the oxidative deamination of all natural D-amino acids, the only exception being the acidic ones. This flavoenzyme plays different roles during evolution and in different tissues in humans. Its three-dimensional structure is well conserved during evolution: minute changes are responsible for the functional differences between enzymes from microorganism sources and those from humans. In recent years several investigations focused on human DAAO, mainly because of its role in degrading the neuromodulator D-serine in the central nervous system. D-Serine is the main coagonist of N-methyl D-aspartate receptors, i.e., excitatory amino acid receptors critically involved in main brain functions and pathologic conditions. Human DAAO possesses a weak interaction with the FAD cofactor; thus, *in vivo* it should be largely present in the inactive, apoprotein form. Binding of active-site ligands and the substrate stabilizes flavin binding, thus pushing the acquisition of catalytic competence. Interestingly, the kinetic efficiency of the enzyme on D-serine is very low. Human DAAO interacts with various proteins, in this way modulating its activity, targeting, and cell stability. The known properties of human DAAO suggest that its activity must be finely tuned to fulfill a main physiological function such as the control of D-serine levels in the brain. At present, studies are focusing on the epigenetic modulation of human DAAO expression and the role of post-translational modifications on its main biochemical properties at the cellular level.

Keywords: D-amino acid oxidase, D-serine, substrate specificity, structure-function relationships, NMDA receptor

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INTRODUCTION

Using FAD as cofactor, D-amino acid oxidase (DAAO, EC 1.4.3.3) catalyzes with strict stereoselectivity the oxidative deamination of neutral D-amino acids. DAAO has been discovered in pig kidney in 1935 (Krebs, 1935) and during the years it has been investigated as a prototype of FAD-dependent oxidases and has been the object of a plethora of studies: 96,325 publications concerning DAAO have appeared over the years (Scopus, 1 October 2018) with a significant increase from 2000 onward. D-Amino acids are dehydrogenated by DAAO into imino acids that spontaneously hydrolyzed to the corresponding α-keto acids and ammonia; the reoxidation of $FADH_2$ on molecular oxygen generated hydrogen peroxide (**Figure 1**).

The reaction catalyzed by DAAO is of biotechnological relevance since it can be used in biocatalysis (to produce α-keto acids from D-amino acids or 7-aminocephalosporanic acid from

Abbreviations: ALS, amyotrophic lateral sclerosis; CBIO, 5-chloro-benzo[d]isoxazol-3-ol; fALS, familial amyotrophic lateral sclerosis; DAAO, D-amino acid oxidase; hDAAO, human DAAO; NMDAR, N-methyl-D-aspartate type glutamate receptor; SR, serine racemase.

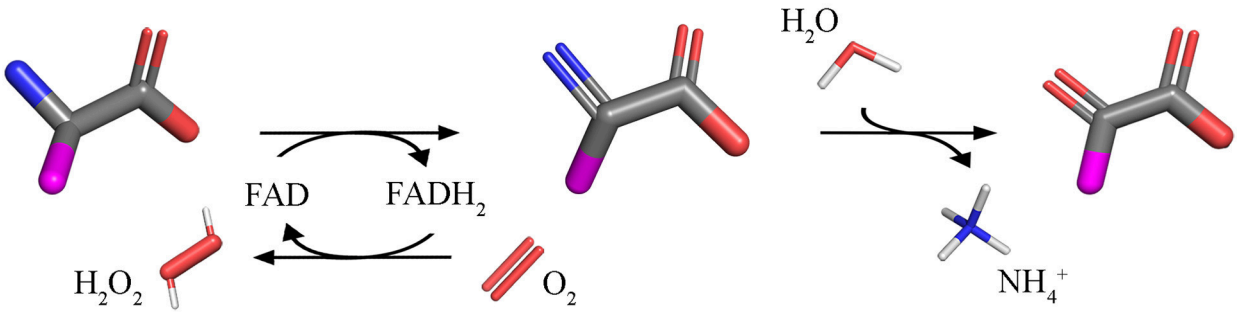

FIGURE 1 | Reaction catalyzed by DAAO.

cephalosporin C, to resolve racemic mixtures of natural and synthetic amino acids, etc.), in biosensors, and in cancer therapy, to mention only the main applications (Pilone and Pollegioni, 2002; Caligiuri et al., 2006a,b; Pollegioni and Molla, 2011). For such a use, DAAO was isolated from microorganisms: those from *Trigonopsis variabilis* and *Rhodotorula gracilis* have been investigated in depth (Pollegioni et al., 2002, 2008; Arroyo et al., 2007).

The investigations on DAAO from higher organisms started in the 1980s. The enzyme's physiological role was long debated largely because the levels of D-amino acids were barely detectable and their presence in many tissues was questioned. Later on, appreciable levels of various D-amino acids were determined in brain and other tissues based on improved analytical methods (mainly high-performance liquid chromatography) (Nagata, 1992; Nagata et al., 1992; Hashimoto et al., 1993; Hamase et al., 1997). This cleared the path to identifying specific physiological roles for D-amino acids (Wang et al., 2000; Wolosker et al., 2002; Fuchs et al., 2005) and to propose for DAAO a key role in their metabolic control. Here, the ability of D-serine to act in the central nervous system as a coagonist of N-methyl D-aspartate receptors (NMDAR), excitatory amino acid receptors critically involved in learning and memory, stimulated the field.

D-Serine is mainly synthesized in neurons by racemization of the L-enantiomer catalyzed by the pyridoxal phosphate-dependent enzyme serine racemase (SR, EC 5.1.1.18) (Wolosker et al., 1999). L-Serine is provided by astrocytes possessing a specific metabolic pathway, referred to as the "phosphorylated pathway," the primary route for the net synthesis of L-serine in the brain, considering the low permeability of the amino acid at the blood-brain barrier (Furuya et al., 2000). SR can also catabolize D- and L-serine through an α,β-elimination reaction to give pyruvate (Foltyn et al., 2005). From a cellular point of view, SR is a "complex" enzyme since its activity is modulated by energy level (ATP), metal ions, post-translational modifications, and protein interactors; for details, see (Pollegioni and Sacchi, 2010; Conti et al., 2011; Dellafiora et al., 2015; Beato et al., 2016). Once released by neurons, D-serine is rapidly taken up and stored in astrocytes (Wolosker, 2011; Wolosker and Radzishevsky, 2013). SR is poorly expressed in astrocytes, which instead produce DAAO; in these cells the flavoenzyme indirectly controls its availability at the synapse by regulating D-serine cellular concentrations and affects the activation level of NMDAR by modulating the occupancy of the co-agonist site.

ROLE OF DAAO IN PHYSIOLOGICAL AND PATHOLOGICAL CONDITIONS

DAO gene is present in a single copy in human chromosome 12 (12q23-24 region) (Konno, 2001, AA): its structure has been detailed in Figure 3 of Pollegioni et al. (2007). A definite report of the *DAO* gene and protein expression in human tissues, with a particular focus on the brain regions, was recently reported, see Table 1 in Sacchi et al. (2018) and Figure 2 of Molla (2017). The highest amount of DAAO protein is observed in liver and kidney: in the latter organ, the enzyme is expressed in proximal tubule cells (Koibuchi et al., 1995; Sasabe et al., 2014a). DAAO was associated with chronic, pathologic renal damage, e.g., D-serine and D-propargylglycine induced nephrotoxicity due to DAAO-mediated generation of H_2O_2 (Konno et al., 2000; Maekawa et al., 2005; Krug et al., 2007).

The elucidation of the physiological functions of DAAO was accelerated by investigating the mutant ddY/DAAO$^{-/-}$ mice strain expressing the inactive G181R enzyme variant (Konno and Yasumura, 1983): large amounts of D-amino acids were excreted in the urine of these animals. Accordingly, in liver and kidney (as well as in the urinary tract and in colon) DAAO eliminates D-amino acids originating in the cell walls of intestinal bacteria, from endogenous racemization, or from the diet. Indeed, increased D-serine levels were apparent in brain regions normally characterized by high DAAO expression in wild-type animals (Morikawa et al., 2001; Miyoshi et al., 2009).

In kidney and brain, the flavoenzyme is a component of the DAAO/3-MST pathway related to hydrogen sulfide (H_2S) generation (Shibuya et al., 2013). Within peroxisomes, DAAO metabolizes D-cysteine (mostly provided by food) to 3-mercaptopyruvate, which is then imported into mitochondria where it is converted to H_2S by 3-mercaptopyruvate sulfurtransferase (3MST). H_2S regulates kidney excretory

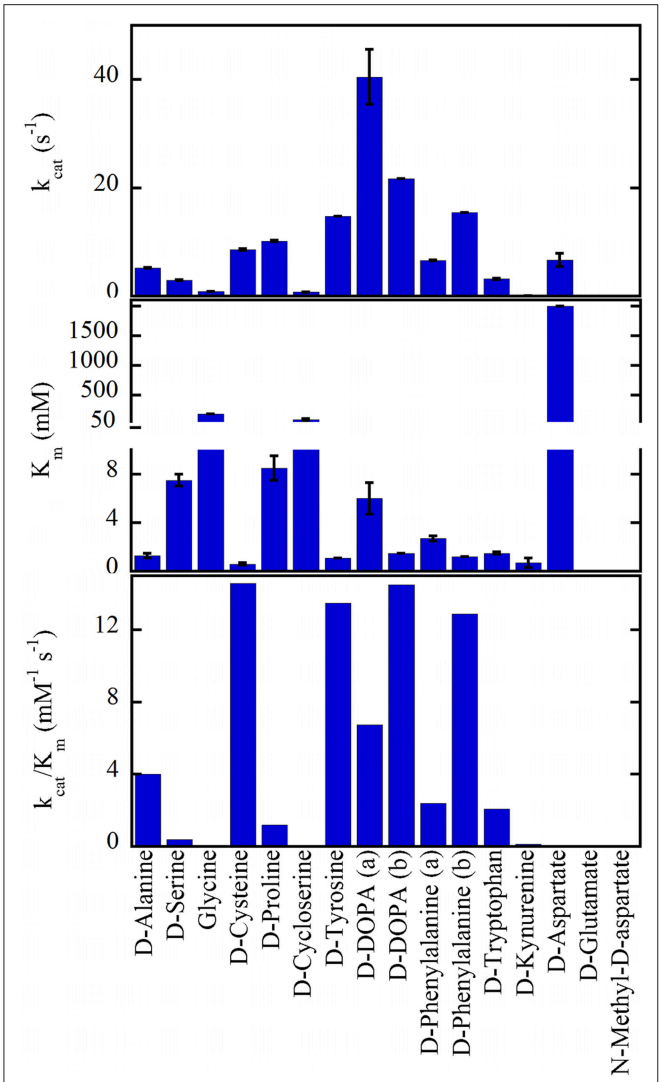

FIGURE 2 | Substrate preference of hDAAO. The apparent kinetic properties have been determined at 21% oxygen saturation, pH 8.5, and 25°C. All values were from Molla et al. (2006) with the exception of (a) Murtas et al. (2017b), Frattini et al. (2011); (b) Kawazoe et al. (2007b).

function and modulates blood pressure by affecting the release of renin.

The expression of DAAO was also reported in the granule fraction of mature human granulocytes (specifically on the cell surface), where it was proposed to participate in recognizing and counteracting foreign, phagocytosed microorganisms (Cline and Lehrer, 1969; Robinson et al., 1978). Within the phagosome, DAAO metabolizes D-alanine (derived from the peptidoglycan of the bacterial cell wall) producing H_2O_2, which in turn is the oxidant substrate for myeloperoxidase that converts chloride to hypochlorous acid, a strongly microbicidal compound. Compared to the wild-type strain, the aforementioned ddY/DAAO$^{-/-}$ mice show a higher susceptibility to *S. aureus* infection (Nakamura et al., 2012).

It was recently reported that DAAO plays a role in controlling the homeostasis of gut microbiota (Sasabe et al., 2016): DAAO (protein and activity) was identified in the proximal and middle small intestine of mice and humans, associated to the villus epithelium. A proteolyzed form of mouse DAAO was reported to be secreted in the lumen by goblet cells: this extracellular form is likely secreted by an N-terminal signal peptide and cleaved at the level of a putative cleavage site also located at the N-terminus (Sasabe et al., 2016). The H_2O_2 generated by DAAO during the catabolism of free D-amino acids of microbial origin represents an important factor in host defense (it protects the mucosal surface from the cholera pathogen) and in modifying microbiota composition (Sasabe et al., 2016).

In the central nervous system, DAAO is the enzyme mainly responsible for catabolism of D-serine: notably, in rodents and humans DAAO expression mirrors D-serine distribution. DAAO (activity and immunoreactivity) was mainly detected in cerebellum and, at lower levels, in the forebrain (Verrall et al., 2007; Madeira et al., 2008). A quite recent investigation confirmed DAAO expression in human forebrain regions and, at the same time, also highlighted that its activity is present in the white matter, throughout the corticospinal tract, and in the spinal gray matter, where it is localized in astrocytes mainly situated in the motor pathway (Sasabe et al., 2014b). The significant hDAAO activity assayed in spinal cord and brain stem is coherent with the proposed function in preventing excitotoxic cell death.

Morover, hDAAO activity was identified in dopaminergic neurons of the nigrostriatal system (Sasabe et al., 2014b): hDAAO is known to efficiently metabolize D-DOPA (see below); thus, the enzyme could affect the metabolism of dopamine, norepinephrine, and epinephrine.

In spinal cord neurons, NMDARs are expressed and are involved in the development of ongoing pain states via central sensitization (Latremoliere and Woolf, 2009). The tonic, pain-related behavior was amplified in the ddY/DAAO$^{-/-}$ mice strain (Wake et al., 2001): the boost in the second phase of the formalin response is due to the potentiated NMDAR activation by the ensuing increased D-serine concentration. Later on, the role of DAAO as a pronociceptive factor in the spinal cord was confirmed (Zhao et al., 2010; Gong et al., 2011). Notably, the administration of DAAO inhibitors in rat models of tonic and chronic pain reversed pain-related behaviors and decreased the electrophysiological activity in spinal cord dorsal horn neurons and peripheral afferent inputs (Hopkins et al., 2013a). Among the putative ways in which DAAO is involved in chronic pain, a change in local levels of reactive oxygen species has been reported for formalin-induced pain (Lu et al., 2012). In this case, by inhibiting DAAO activity, a decrease in the production of spinal H_2O_2 levels is apparent (Lu et al., 2012; Gong et al., 2014). Interestingly, spinal DAAO has been also involved in pain hypersensitivity induced by perturbing sleep-regulating circuitries in the central nervous system through the deprivation of sleep, a process that generates pain hypersensitivity with no nerve or tissue injury (Wei et al., 2013). The H_2O_2 generated by DAAO could target the pronociceptive TRPA1 channel expressed by central terminals of primary afferent nerve fibers in the spinal dorsal horn.

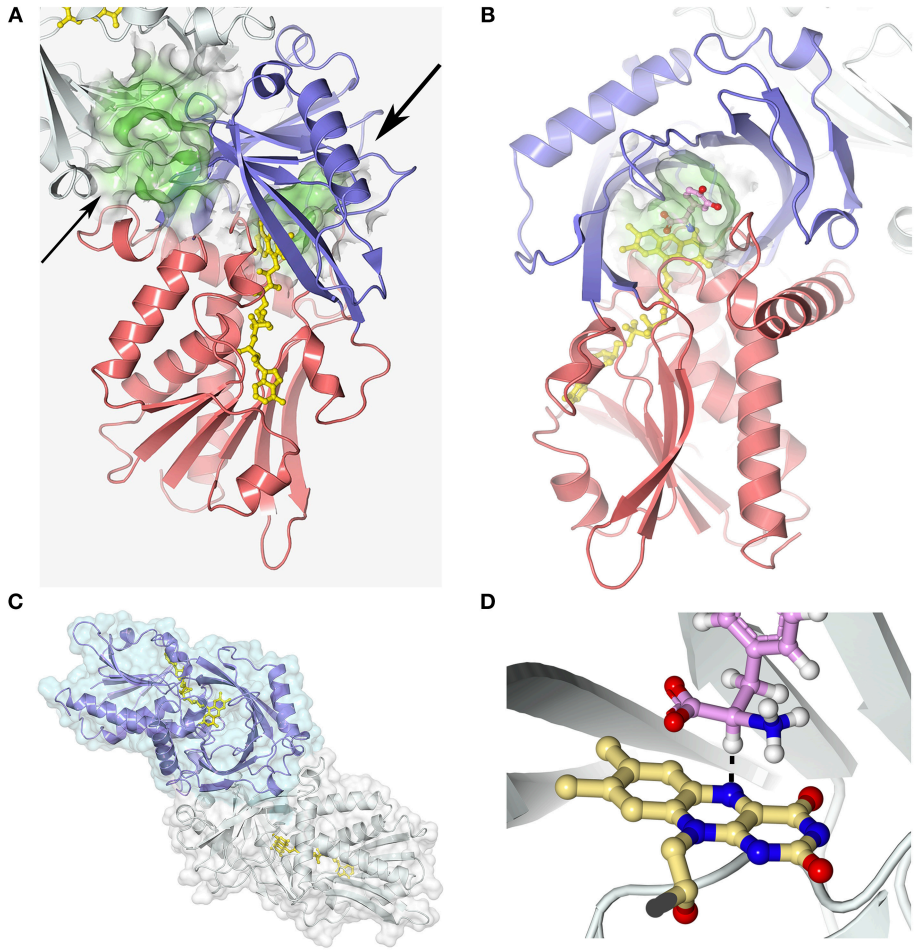

FIGURE 3 | hDAAO three-dimensional structure (pdb codes 2e49). **(A)** The hDAAO protomer is constituted by two domains: the substrate and the FAD-binding domain (in blue and red, respectively). The entrance to the active site is indicated by a large arrow. The thin arrow indicates the putative additional ligand-binding site (Kohiki et al., 2017). **(B)** The substrate is located above the *re*-side of the isoalloxazine ring of FAD, in a cavity of ~220 $Å^3$. **(C)** hDAAO is a stable homodimer, characterized by a head-to-head mode of monomer interaction (Kawazoe et al., 2006). **(D)** The substrate dehydrogenation ensues by the direct hydride transfer of the α-H from the α-C of the D-amino acid to the flavin N(5), see dotted line (Umhau et al., 2000). Following hydride transfer, a negative charge is generated on the reduced flavin, which is stabilized by the positive charge generated on the imino group of the product. This figure has been generated by modeling a D-Tyr molecule instead of the original ligand imino serine. Figure prepared with 3dproteinimaging.com.

Amyotrophic lateral sclerosis (ALS) is a rapidly progressing, adult-onset, neuromuscular disease distinguished by the selective loss of motor neurons. A recent, comprehensive, exome-sequencing study revealed that the only DNA variants associated with clinical outcome of ALS and with lower rates of survival are located on the *DAO* gene (Cirulli et al., 2015). Actually, the R199W DAAO substitution was identified in a three-generational familial ALS (fALS) kindred (Mitchell et al., 2010). This substitution impaired DAAO activity, boosted the formation of ubiquitinated protein aggregates, promoted autophagy activation, and increased apoptosis when the protein was overexpressed in motor neuron cell lines or primary motor neuron cultures (Paul and de Belleroche, 2012; Paul et al., 2014). The transgenic mouse lines expressing R199W DAAO (DAO^{R199W}) were unaffected in survival although they exhibited the features common to several ALS mice models, i.e., decreased body weight, marked kyphosis, and loss of motor neurons in spinal cord (Kondori et al., 2017). Recently, it was reported that the most significant and robust splicing change after depletion of hnRNP A2/B1 in the mouse spinal cord was the skipping of exon nine within *DAO* gene, yielding a reading frameshift and early termination of the protein, predicted to lack 2 α-helices and 3 β-sheets and to generate a highly unstable and inactive variant (Martinez et al., 2016).

Impaired NMDAR signaling pathways are known to occur in the hippocampus and cerebral cortex of aging brains (Billard, 2008); in aged tissues a hypoactivation of NMDAR is related to decreased D-serine levels (Junjaud et al., 2006; Mothet et al., 2006). Neurodegeneration induced by NMDAR hypoactivity was also proposed to contribute to AD and to be related to the progression of aging brain from mild cognitive impairment to AD (Olney et al., 1997; Wozniak et al., 1998). Compared to

healthy individuals, the serum levels of DAAO are increased in patients affected by mild cognitive impairment and mild and severe AD (Lin et al., 2017), and DAAO levels correlate with the severity of cognitive deficit and with the D-serine level.

Alterations in D-serine levels have been observed in Alzheimer's disease (AD) and have been suggested to represent a pro-death signal (Billard, 2008; Madeira et al., 2015).

An NMDAR hypofunction was also related to schizophrenia (Coyle et al., 2003; Coyle, 2006; Stone and Pilowsky, 2007): the altered activation state of the receptor was proposed to depend on a deficiency in D-serine signaling (Hashimoto et al., 2003, 2005; Verrall et al., 2010). The protein and activity levels of hDAAO were altered in post-mortem brain tissues from schizophrenic patients in cerebral cortex (Madeira et al., 2008), cerebellum (Kapoor et al., 2006; Verrall et al., 2007; Burnet et al., 2008), medulla oblongata, and choroid plexus (Ono et al., 2009). Further support comes from the discovery that the *G72* gene, encoding the small protein pLG72, the main hDAAO-specific binding protein (see below), has been linked to schizophrenia (Chumakov et al., 2002; Sacchi et al., 2008, 2016; Pollegioni et al., 2018). Additional meta-analyses supported a genetic association between *DAO, G72,* and schizophrenia: they have been classified as schizophrenia susceptibility genes (Sacchi et al., 2016).

CELLULAR PROPERTIES OF hDAAO

DAAO is considered a marker of peroxisomes since it contains a classical PTS1 signal at the C-terminus (Horiike et al., 1994; Moreno et al., 1999). Notably, an active DAAO form has been reported in the cytosol, both in glial cells and neurons (Sacchi et al., 2008, 2011; Popiolek et al., 2011). In astrocytes overexpressing hDAAO, the cytosolic form seems to transiently accumulate in this compartment before targeting peroxisomes (Sacchi et al., 2011). Recent reports on rats demonstrated that DAAO is present both in the cytosol and nuclei of proximal tubule epithelial cells following treatment with the drug propiverine (Luks et al., 2017a,b) and that intestinal epithelial cells in mice secrete the flavoenzyme into the lumen (Sasabe et al., 2016).

The degradation pathway of hDAAO was investigated in U87 glioblastoma cells stably expressing the flavoenzyme fused to the C-terminus of the enhanced yellow fluorescent protein (EYFP, thus generating a peroxisomal chimeric protein) or at the N-terminus (thus producing a cytosolic chimeric protein since the PTS1 signal is masked). hDAAO is a long-lived protein showing a half-life > 60 h. The peroxisomal EYFP-hDAAO is degraded via the lysosomal/endosomal pathway, whereas the cytosolic hDAAO-EYFP protein is ubiquitinated and targets the proteasome. Overexpression of the interacting protein pLG72 (showing a rapid turnover, half-life in the 25–40 min range) increases the turnover of DAAO (half-life ∼6 h) (Sacchi et al., 2011): hDAAO-pLG72 complex formation seems to represent a means to play a protective role against excessive D-serine depletion by the active, cytosolic enzyme (see below).

BIOCHEMICAL PROPERTIES

General Properties

A comparison of the main biochemical properties of mammalian DAAOs is reported in Sacchi et al. (2012). Recombinant hDAAO is produced in fairly large amounts in *E. coli* cells (Kawazoe et al., 2006; Molla et al., 2006; Romano et al., 2009). It is purified as active holoenzyme by adding exogenous FAD to the purification buffers: hDAAO shows the classical properties of flavoprotein oxidases, such as a quick reaction with O_2 in the reduced form and stabilization of the anionic red flavin semiquinone.

In the 6–10 pH range, hDAAO shows a good activity and stability (Murtas et al., 2017b). From the fitting of the activity values determined at different pH values, two dissociations were apparent: a pK_a of 2.5 and 11.1, respectively. Notably, the enzyme is fully stable after 60 min of incubation at 4°C at pH values ≥3.0 and ≤10.0. The flavoenzyme is stable up to 45°C, a temperature corresponding to the optimum for the enzymatic activity. The melting temperature determined following the loss of activity was ∼55°C (Murtas et al., 2017b), higher than the values determined using the changes in protein fluorescence intensity (Caldinelli et al., 2010): this result suggests that the alteration in protein conformation brings forward the loss of enzymatic activity.

hDAAO activity is not affected by the presence of divalent ions (Ca^{2+} and Mg^{2+}) and/or nucleotides. Similarly, the reducing agent N-acetyl-cysteine, a derivative of L-cysteine, acting as antioxidant and anti-inflammatory agent and that is able to modulate NMDAR activity (Kumar, 2015), does not affect the activity of hDAAO (Murtas et al., 2017b).

Substrate Specificity

hDAAO shows a wide substrate acceptance: the best substrates are hydrophobic and bulky D-amino acids (D-DOPA > D-Tyr > D-Phe > D-Trp, **Figure 2**). The highest k_{cat} value was determined for D-3,4-dihydroxy-phenylalanine (D-DOPA) (Kawazoe et al., 2007a,b; Murtas et al., 2017a,b), also showing a high affinity due to two additional H-bonds between the OH-groups of the substrate and His217 and Gln53 (Kawazoe et al., 2007a). However, the oxidation of D-DOPA is hindered by the substrate inhibition effect, K_i of 0.5 (Murtas et al., 2017b) or 41 mM (Kawazoe et al., 2007b).

hDAAO is also active on small, uncharged D-amino acids (D-Cys > D-Ala > D-Pro > D-Ser) (Molla et al., 2006; Kawazoe et al., 2007b; Frattini et al., 2011; Murtas et al., 2017b). Purified recombinant hDAAO shows a low catalytic efficiency on what is known as the main physiological substrate, D-serine. Whether *in vivo* (and especially in glial cells) an increase in kinetic efficiency is achieved by the binding with a cellular compound (i.e., a protein or a small size ligand) or by a post-translational modification is still unknown: this issue deserves further investigations. The highest catalytic efficiency was determined for D-cysteine, a compound involved in H_2S generation (see above) (Shibuya et al., 2013).

hDAAO is not active on glycine and acidic D-amino acids (NMDA and D-Glu) while the activity on D-Asp is hampered by the high apparent K_m (in the molar range) (Molla et al., 2006; Murtas et al., 2017b). hDAAO also oxidizes D-kynurenine

with an apparent K_m value (0.7 mM) resembling that determined for D-cysteine. Kynurenic acid, the product of D-kynurenine oxidation, binds to the modulatory glycine site of the NMDAR resulting in an inhibitory effect. Furthermore, hDAAO is also active on D-cycloserine, an NMDAR modulator (Kumar, 2015).

The substrate promiscuity of hDAAO supports the hypothesis that this flavoenzyme might play a role in different tissues and cells.

The activity of hDAAO on D-serine is partially inhibited by the L-enantiomer (Murtas et al., 2017b). L-Serine acts as competitive inhibitor (K_i of 26.2 mM). Under anaerobic conditions L-serine, as well as L-alanine or L-valine, are able to reduce FAD. However, a physiological concentration of L-serine (corresponding to $\leq$2 mM in brain tissues and in blood) (Weatherly et al., 2017) should not modify the oxidation of D-serine by hDAAO.

Kinetic Mechanism

For all known DAAOs, the oxidative deamination of D-amino acids follows a ternary-complex mechanism (Pollegioni et al., 1993; Umhau et al., 2000; Molla et al., 2006). The substrate dehydrogenation ensues by the direct hydride transfer of the α-H from the α-C of the D-amino acid to the flavin N(5): please see below and **Figure 3D**. The distance between these atoms is 3.6 Å in the hDAAO-imino serine complex: owing to the tetrahedral geometry of the substrate α-C, the mentioned atoms should be closer in the Michaelis complex (∼3.2 Å). Following hydride transfer, the reduced flavin is negatively charged: the positive charge of the product imino group electrostatically stabilizes the reduced cofactor.

For mammalian DAAOs, and especially for hDAAO, the first half of the reaction (the reductive half-reaction), namely, the conversion of the tetrahedral D-amino acid into the planar imino acid coupled to the flavin reduction is fast (117 ± 6 s^{-1} on D-serine), significantly faster than turnover (6.3 ± 1.4 s^{-1}). The rate-limiting step in hDAAO catalysis is the product release (Molla et al., 2006; Molla, 2017). The rate of imino acid release from the reduced enzyme is $< 1\ s^{-1}$, too slow to allow the reoxidation step to start from the free, reduced enzyme. Accordingly, reoxidation must start from the corresponding reduced enzyme-imino acid complex: the second-order reaction corresponds to $1.25 \times 10^5\ M^{-1}s^{-1}$.

hDAAO STRUCTURAL-FUNCTIONAL PROPERTIES

Overall Structure

Each hDAAO protomer contains 347 amino acids (40.3 kDa), harbors one molecule of FAD, and is composed of 11 α-helices and 14 β-strands. hDAAO is constituted by two interconnected regions: an FAD-binding domain containing the dinucleotide binding motif (Rossman fold) and a substrate-binding domain in which a large, twisted, antiparallel β-sheet forms the active-site roof and part of the oligomerization interface (**Figures 3A,B**). hDAAO is a stable homodimer: the two monomers interact via a head-to-head geometry (**Figure 3C**; Kawazoe et al., 2006).

In the active site, the substrate is located above the *re*-side of the isoalloxazine ring of FAD, in a cavity of ∼220 $Å^3$ (**Figure 3B**). The substrate is bound via several hydrogen bonds in the correct orientation with respect to the flavin N(5) position for catalysis to proceed: the α-carboxylic group of the substrate electrostatically interacts with Arg283 and Tyr228, whereas the α-amino group interacts with Gly313 and the C(4)=O of FAD. The side chain of the substrate is placed in a pocket made up of hydrophobic residues (Leu51, Gln53, Leu215, and Ile230), named the substrate-specificity pocket (Kawazoe et al., 2006). The active-site "roof" is shaped by the side chain of Tyr224, a residue belonging to a mobile loop (216–228): the product/substrate exchange during catalysis is facilitated by the switch of this residue from a closed to an open conformation. This conformational change significantly influences the enzyme properties: limiting the turnover, increasing the hydrophobicity of the active site, and allowing hDAAO to bind large substrates (Molla et al., 2006; Kawazoe et al., 2007b).

The strict stereoselectivity of DAAO for the D-enantiomer of the amino acids is rationalized by the four-location model for enantioselectivity (Mesecar et al., 2000; Umhau et al., 2000; Mörtl et al., 2004). According to this model, the substrate establishes three interactions—using the α-carboxylic group, the α-amino group and the side chain—with the active site residues indicated above: the exact binding produces a "functional direction" exemplified by the alignment of the α-H of the substrate and the N(5) of FAD, which allows hydride transfer (**Figure 3D**).

Oligomeric Structure

Different from other DAAOs (Mattevi et al., 1996; Pollegioni et al., 2007; Frattini et al., 2011), an 80 kDa homodimer is generated by both the holo- and the apoprotein form of hDAAO (Molla et al., 2006). This results from a distinguishing charge distribution at the dimer interface (a region corresponding to ∼1,500 $Å^2$, i.e., the 9.8% of the overall solvent accessible surface, **Figure 3C**), where a significantly higher amino acidic substitution frequency was observed than for the overall protein (33 vs. 15%, respectively) (Kawazoe et al., 2006). Notably, the urea-induced dissociation of dimeric hDAAO generates protein conformers prone to aggregation (Caldinelli et al., 2009).

FAD Binding

In hDAAO the FAD cofactor shows an elongated conformation and it is buried in the protein core: the isoalloxazine ring is located at the interface between the two subdomains with the *re*-side facing the interior of the active site (Kawazoe et al., 2006; Molla, 2017). At this side of the flavin ring, the conformation of the surrounding residues is highly conserved among mammalian DAAOs. Conversely, at the *si*-face, the conformation of the hydrophobic stretch (47-VAAGL-51, a structurally ambivalent peptide) differs between the human and porcine enzymes, causing loss of the H-bond between Ala49 and N(5) of the cofactor and likely decreasing the strength of the interaction of the flavin cofactor and the rate of flavin reduction (Kawazoe et al., 2006).

hDAAO possesses the weakest binding of the FAD cofactor (K_d = 8.0 μM) among known DAAOs (K_d = 0.2 and 0.02 μM for pig and yeast DAAOs, respectively). Accordingly, hDAAO exists in solution as an equilibrium of holo- and apoprotein forms (Caldinelli et al., 2010). The presence of an active-site ligand increases the affinity of the flavin to the protein moiety, K_d = 0.3 μM (Molla et al., 2006; Caldinelli et al., 2010). Quenching of protein fluorescence intensity during titration of the apoprotein with the cofactor, in the presence or absence of sodium benzoate, is a biphasic process (Murtas et al., 2017b), suggesting that the apoprotein form exists in two conformations with differing cofactor binding affinity: the higher intensity amplitude associated with the first phase observed in the presence of benzoate indicates that binding of an active-site ligand favors the protein conformation with the higher avidity for FAD (Murtas et al., 2017b). A second possibility is the presence of two binding sites. Here, a recent investigation based on computational and labeling analyses suggests that an additional ligand-binding site is located at the monomer-monomer interface (**Figure 3A**; Kohiki et al., 2017).

The holoenzyme reconstitution is a sequential process: in the first step, FAD binds the apoprotein moiety and recovers the catalytic activity; in the second step, a slow, secondary conformational change generates the final holoenzyme conformation (Caldinelli et al., 2009). Notably, the first step is 20-fold faster when benzoate is present (Caldinelli et al., 2010).

The melting temperature for the unfolding of the holoenzyme is 6–9°C higher than for the corresponding apoprotein (Caldinelli et al., 2009).

The observed increase in cofactor binding affinity in the presence of benzoate suggested that, in addition to the different conformation of the hydrophobic VAAGL sequence observed in the hDAAO-benzoate complex (Kawazoe et al., 2006), an alternative conformation of the substrate-free enzyme should exist that binds the cofactor less efficiently (Murtas et al., 2017b). In any case, the structure of the free enzyme form (PDB 2e48) overlaps that of the hDAAO-benzoate complex (PDB 2du8).

Based on the *in vivo* concentration of FAD (∼5 μM), it is conceivable that in the cell an equilibrium between the hDAAO holoenzyme (active) and the apoprotein (inactive) form exists, with the latter one being predominant in the absence of an active-site ligand.

Ligand Binding

hDAAO inhibitors can essentially be grouped into substrate-competitive and cofactor-competitive inhibitors (Molla et al., 2006; Sacchi et al., 2012; Terry-Lorenzo et al., 2014; Molla, 2017). Among the active-site ligands, small aromatic (aryl) carboxylic acids or acid isosteres are powerful hDAAO inhibitors used as scaffolds for developing novel drugs (see below). These compounds, such as benzoate, *o*-aminobenzoate, substituted quinilinones, or 4H-furo[3,2-*b*]pyrrole-5-carboxylic acid (**Figure 4**), bind the flavoenzyme similarly to the substrate: the inhibitor COOH group (or the corresponding C=O or OH substituents) interacts with Arg283 and Tyr228, an H-bond donor binds Gly313, and the remaining part interacts with the hydrophobic region of the active site (that can accommodate molecules containing 12–13 atoms) (Duplantier et al., 2009). In the hDAAO-inhibitor complex, the side chain of Tyr224 is shifted toward the inner part of the active site and forms a strong π-π stacking interaction, "sandwich," between its aryl chain and the *re*-side of the isoalloxazine ring of the cofactor. The strongest interaction is observed when the aromatic rings are slightly displaced [i.e., with 3-hydroxyquinolin-2(1*H*)-one] (**Figure 4**) and not perfectly stacked (i.e., with benzoate). When saturated analogs of these compounds are used, a drop in the binding affinity is apparent, indicating the relevance of the π-π stacking interaction.

The binding of aromatic carboxylic acids to the hDAAO holoenzyme inhibits the flavoenzyme and perturbs its absorbance spectrum in the visible region. For example, benzoate yields a shoulder at ∼497 nm (K_d = 7 μM and K_i = 9 7 μM) (Kawazoe et al., 2006; Molla et al., 2006); anthranilate binding generates a spread classical charge transfer band at ∼580 nm (K_d = 40 μM) (Molla et al., 2006). The ligand-binding site is present in the apoprotein form, too, as made apparent by the alteration in protein fluorescence and in thermostability of the latter hDAAO form when the substrate D-serine or the substrate analog trifluoro-D-alanine is added (Caldinelli et al., 2009, 2010).

Conversely to benzoate, for the binding of the inhibitor 6-chloro-benzo[d]isoxazol-3-ol (CBIO) (**Figure 4**) to hDAAO a single-step binding process is evident and the K_d value estimated following the quenching of protein fluorescence intensity corresponds well to the K_d, IC_{50}, and K_i values determined using different methods.

ADP and CPZ (**Figure 4**) (IC_{50} of 580 and 5 μM, respectively) behave as FAD-competitive inhibitors for binding to hDAAO (Iwana et al., 2008; Sacchi et al., 2008; Terry-Lorenzo et al., 2014). In particular, CPZ binding generates a protein conformation more sensitive to proteolysis and thermal unfolding than the native holoenzyme (Caldinelli et al., 2010). The near-UV CD spectra show that the tertiary structure of hDAAO-CPZ complex differs from that of the hDAAO-FAD: the former more closely resembles that of the apoprotein (Caldinelli et al., 2010).

Notably, the ligands D-serine, FAD, benzoate, and CPZ did not affect the formation of the hDAAO-pLG72 complex (see below).

MODULATION OF hDAAO ACTIVITY

By Protein Interaction

Human flavoenzyme function is modulated by interacting with various proteins. hDAAO, through the PTS1-type peroxisomal-targeting signal, interacts with the Pex5p receptor, a protein involved in protein import and in the assembly of peroxisomes (Ghosh and Berg, 2010).

Genome-wide association studies and meta-studies in different populations have linked polymorphisms in the gene encoding pLG72 protein with schizophrenia and other psychiatric diseases (Drews et al., 2013; Sacchi et al., 2016; Pollegioni et al., 2018). In particular, hDAAO specifically binds to the primate-specific protein pLG72: two hDAAO homodimers interact with two pLG72 molecules, yielding a 200-kDa protein complex (K_d = 0.08–0.53 μM); for a recent

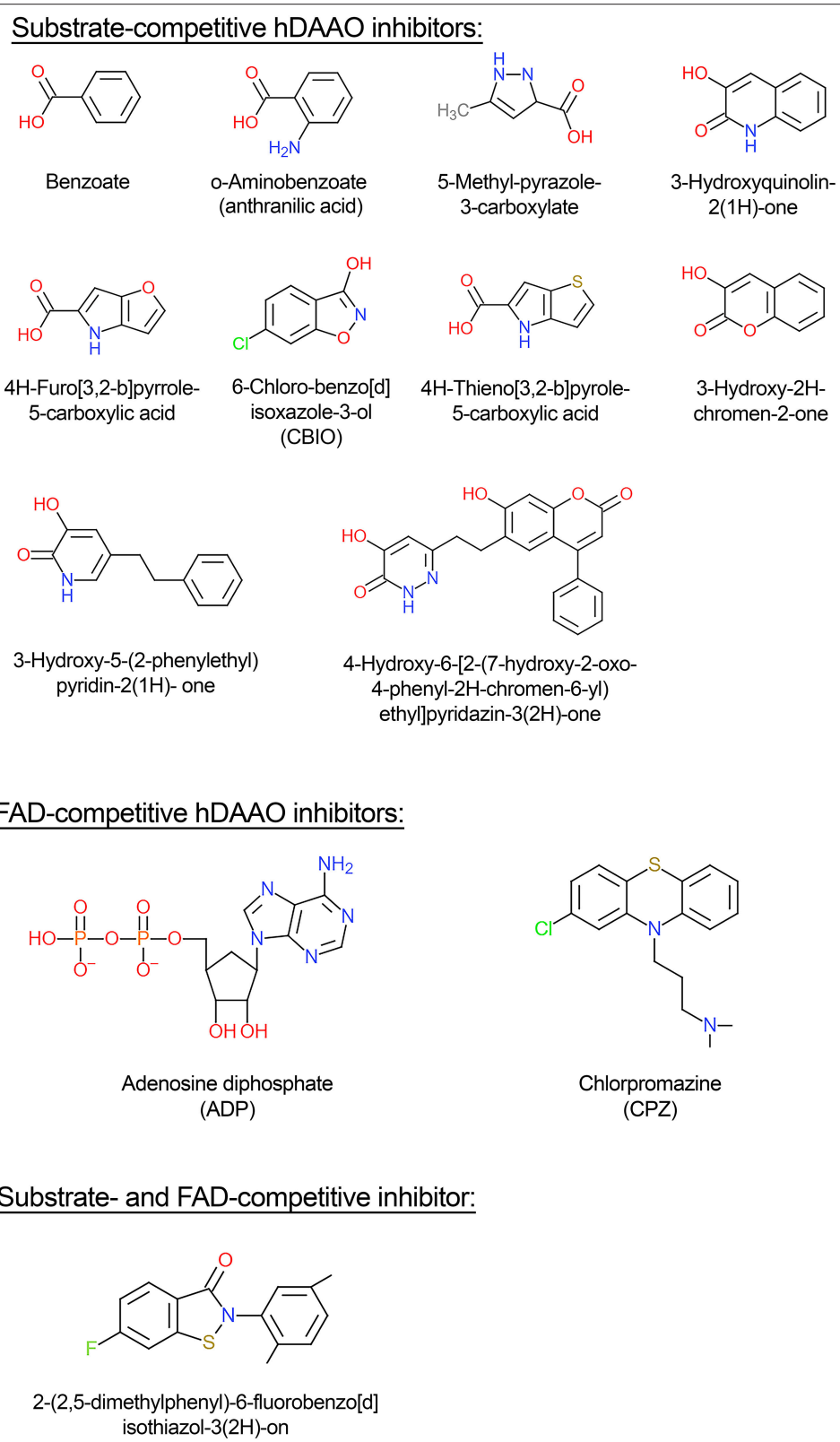

FIGURE 4 | Structural formula of selected hDAAO inhibitors classified based on their mechanism of enzyme inhibition.

review (see Pollegioni et al., 2018). *In vitro*, the formation of the 200-kDa complex does not alter the kinetic parameters or the binding with the FAD cofactor of hDAAO, but rather induces a change in its overall tertiary structure, causing a time-dependent inactivation (Sacchi et al., 2008). By using low-resolution techniques (i.e., limited proteolysis coupled to mass spectroscopy and cross-linking experiments) structural elements involved in forming the interface surface in the hDAAO-pLG72 complex have been identified, highlighting the role of the N-terminal region of pLG72 in forming the oligomerization interface (Birolo et al., 2016; Sacchi et al., 2017). hDAAO in transiently transfected glial cells (i.e., the U87 human glioblastoma cell line) is largely localized in peroxisomes but also present in cytosol (Sacchi et al., 2011) while pLG72 shows a mitochondrial localization. We proposed that, in this model cell system, newly synthesized hDAAO interacts with pLG72 on the cytosolic side of the outer mitochondrial membrane (Sacchi et al., 2011). Such an interaction increases the D-serine/total serine ratio and decreases hDAAO activity and half-life, see above (Sacchi et al., 2008, 2011; Cappelletti et al., 2014). We recently proposed that pLG72 (itself or recruiting further proteins) might target the cytosolic form of hDAAO to the ubiquitin-proteasome system, thus starting its degradation (Cappelletti et al., 2014). This mechanism could represent a further process to regulate the D-serine levels in the hindbrain where the flavoenzyme is expressed in glial cells.

Analogously, the activity of hDAAO is negatively regulated by bassoon, a component of the cytoskeletal matrix, mainly located at the presynaptic active zone. The hDAAO-bassoon complex formation has been proposed to prevent D-serine depletion acting on the active, extraperoxisomal enzyme form located at presynaptic terminals (Popiolek et al., 2011). The inhibitory effect of bassoon may account for the difficulties in detecting hDAAO activity in the forebrain (Verrall et al., 2007), a region where the enzyme is mostly expressed in neurons.

By hDAAO Inhibitors

Abnormal changes in hDAAO activity yielding locally decreased D-serine levels have been correlated with neurological disorders (e.g., schizophrenia); therefore, the identification of hDAAO inhibitors (to slowing down the neuromodulator degradation process) to be used as drugs has garnered growing interest. This treatment has beneficial effects on cognition and learning functions (Hopkins et al., 2013b).

More than 500 substrate-competitive inhibitors have been identified so far (Gilson et al., 2016). Analogously to the substrate, their chemical structure contains a planar moiety which interacts with the active-site residues close to the FAD cofactor isoalloxazine ring and a second portion which is positioned in the substrate side-chain binding pocket. The "core" of the planar moiety is usually formed by one or two fused rings (one of which might be aromatic) and contains at least a carboxylic group to establish the H-bond interaction with Arg283. The second part of the inhibitor molecule corresponds to the side chain of the substrate: this portion, depending on the size and chemical features, forms further interactions with residues belonging to the substrate specificity pocket and/or to the active site entrance.

A comprehensive review about the details of inhibitor binding to hDAAO has been published recently (Molla, 2017). It ranks classical and novel compounds in four classes:

(i) classical inhibitors: typically single-ring ligands, i.e., benzoate, anthranilate, and improved variations (Sacchi et al., 2012; Katane et al., 2013). The compound 5-methylpyrazole-3-carboxylic (**Figure 4**) acid is a prototype of this class of compounds in which two C atoms of the ring are substituted by N: the optimized H-bonds network allows a high affinity for hDAAO ($K_i = 0.39\,\mu M$). This compound crosses the blood-brain barrier in rats, thus raising the D-serine level in certain brain regions (Adage et al., 2008);

(ii) second-generation inhibitors: larger compounds than classical inhibitors since they are characterized by two substituted, heterocyclically fused rings, which form additional H-bonds and van der Waals interactions with residues forming the active site, i.e., compounds derived from 3-hydroxyquinolin-2(1H)-one and CBIO (Ferraris et al., 2008; Katane et al., 2013). The main drawback of the last compound is the low passage through the blood-brain barrier: such a compound does not increase D-serine levels in the brain (Ferraris et al., 2008);

(iii) third-generation inhibitors: bulky and flexible compounds whose side chain binds to an additional "subpocket" at the entrance of the active site generated by a conformational change in Tyr224 induced by ligand binding (Raje et al., 2013; Terry-Lorenzo et al., 2014);

(iv) novel-generation inhibitors: molecules that can interact with the hDAAO-pLG72 complex since they contain the "ebsulfur" (2-phenyl-2,3-dihydro-1,2-benzothiazol-3-one) substructure that forms S-S thiol bonds with the cysteines of hDAAO, when the protein is partially unfolded due to pLG72 binding (Terry-Lorenzo et al., 2015). Compound [2-(2,5-dimethylphenyl)-6-fluorobenzo[d]isothiazol-3(2H)-on (**Figure 4**) inhibits hDAAO, acting as both FAD- and D-serine-competitive inhibitor. The so-called "compound 22," classified as a class C compound by Terry-Lorenzo et al. (2015), only acts as hDAAO inhibitor under oxidizing conditions. This compound does not dissociate from the flavoenzyme in jump-dilution experiments and stably inactivate the enzyme: recovery of the DAAO activity is obtained only by adding a reducing agent.

By Single Point Substitutions

Based on biochemical properties, hDAAO variants corresponding to known single nucleotide polymorphisms or sequence conflicts have been grouped into two classes: hypoactive and hyperactive; for a recent review (see Sacchi et al., 2018). The conditions and levels of recombinant expression of seven variants of hDAAO are reported in Table 2 of Sacchi et al. (2018).

The G183R, R199W, and R199Q hDAAOs show significantly decreased enzymatic activity (or fully abolished for the latter variant, see **Figure 5**), and a perturbation of the conformation: (a) in G183R hDAAO, corresponding to the coding mutation occurring in the ddY/DAAO$^{-/-}$ mice strain expressing the inactive G181R DAAO (Konno and Yasumura, 1983), alterations

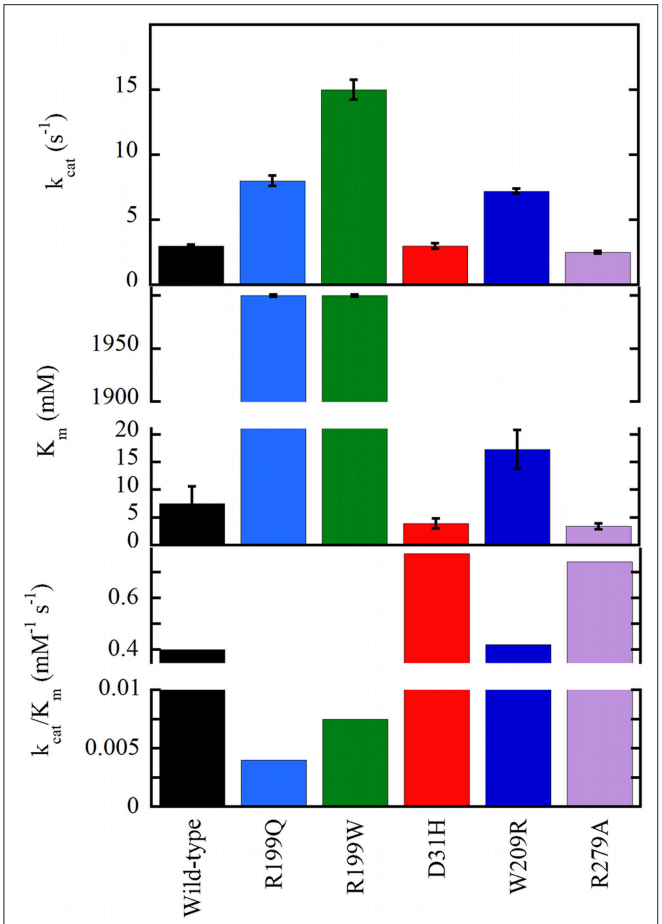

FIGURE 5 | Apparent kinetic parameters of wild-type and hDAAO variants, determined at air saturation (0.25 mM oxygen), 25°C, and pH 8.5 (Molla et al., 2006; Caldinelli et al., 2013; Cappelletti et al., 2015).

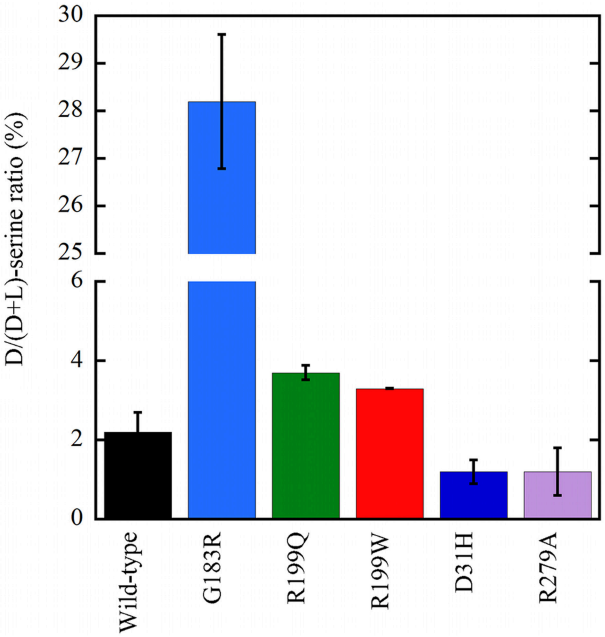

FIGURE 6 | Effect on the cellular D/(D+L) serine ratio in U87 human glioblastoma cells stably expressing EYFP-hDAAO variants (Caldinelli et al., 2013; Cappelletti et al., 2015; Murtas et al., 2017b). The effect of W209R substitution was established on transiently transfected cells (not shown): a figure of 4.2 ± 0.1 vs. 5.8 ± 0.4 was determined at 24 h for the variant and wild-type hDAAO, respectively.

in secondary structure elements likely alter the conformation of the flavin binding domain and thus negatively affect the cofactor binding (Murtas et al., 2017a); (b) the tertiary structure of R199W (the variant associated with the onset of fALS) and R199Q variants is significantly altered: this favors aggregation propensity but does not modify the interaction with pLG72 (Cappelletti et al., 2015; Murtas et al., 2017a); (c) in G331V hDAAO the change in the C-terminal α-helix promotes protein aggregation, strongly affecting the variant solubility (Caldinelli et al., 2013). At the cellular level, both G183R and G331V variants were partly mistargeted: they formed cytosolic protein aggregates, which largely colocalized with ubiquitin, and resulted in increased apoptosis (Caldinelli et al., 2013; Murtas et al., 2017a).

On the other hand, the D31H, W209R, and R279A substitutions have the opposite effect on hDAAO activity, resulting in slightly or significantly improved catalytic efficiency (**Figure 5**) and FAD affinity. For example, a 2-fold increased turnover number was apparent for the W209R hDAAO, which was more active than the wild-type hDAAO using 0.3 mM D-serine and 5 μM FAD, i.e., concentrations resembling physiological conditions (Cappelletti et al., 2015).

Following overexpression in U87 cells, all the investigated hDAAO variants significantly altered the cellular levels of D-serine (**Figure 6**; Caldinelli et al., 2013; Cappelletti et al., 2015; Murtas et al., 2017a). The expression of inactive variants of hDAAO could produce susceptibility to neurodegenerative disorders due to augmented D-serine levels which, when paralleled by elevated glutamate levels, could lead to hyperactivation of NMDAR and thus to excitotoxicity. In contrast, a deficit in NMDAR-mediated transmission might be related to the expression of hyperactive variants due to an abnormal decrease in D-serine at the synapses, as proposed in schizophrenia onset. Furthermore, hDAAO hyperactive variants produce nonphysiological levels of H_2O_2: this process could contribute to the molecular mechanism of the central sensitization typical of chronic pain.

To delve into the structure-function relationships in mammalian DAAOs, alanine-scanning analysis of first and second shell residues of the enzyme from pig prompted the focus on active-site lid residues (region 221–225) and on the positions 55 and 56 in hDAAO (Subramanian et al., 2018). Molecular dynamics simulations identified a narrow tunnel that could provide access to the active site of hDAAO, named tunnel T1. The Y55 residue was suggested to be involved in anchoring the lid loop in the closed conformation (its dynamics are hampered by Y314), modulating the solvent access and substrate/product exchange at the active site and separating T1 from an additional, putative tunnel. The Y55A substitution facilitated accessibility of

the active site: a 2-fold increase in specific activity on D-Trp was observed.

BY POST-TRANSLATION MODIFICATIONS

The molecular mechanisms by which hDAAO expression and acquisition of catalytic activity are achieved inside the cell are still largely unknown: a fine and careful regulation through post-translational modification(s) is expected. Actually, hDAAO was proposed to be regulated by nitrosylation (Shoji et al., 2006). In detail, the activity of DAAO, in a membrane fraction of U87 glioblastoma cells, was enhanced by NO in a dose-dependent manner. The authors proposed that, in astrocytes, NO may inhibit SR and enhance hDAAO activities thus accelerating D-serine degradation. Following D-serine supply from astrocytes to neurons, synthesis of nitric oxide in neurons may temporarily be increased, yielding a feedback regulation of the neuromodulator.

CONCLUSIONS

With the final aim to use hDAAO in different tissues responding to several needs, evolution adopted complicated regulatory strategies to modulate the activity of the flavoenzyme. In human brain tissues, hDAAO should be mainly present in the apoprotein, inactive form considering the physiological concentration of FAD and its weak interaction with the apoprotein moiety. Conversion of the inactive hDAAO apoprotein into the active holoenzyme is facilitated by the presence of an active-site ligand, such as the substrate: this represents an efficient way to maintain the level of selected D-amino acids in the physiological range.

We are conscious that, despite the important role played by hDAAO in main physiological processes, the modulation of its functional properties is still largely unknown. A main issue is the modulation of the activity by post-translational modifications (as known for serine racemase) and by further interacting proteins. A second matter is the role of hDAAO activity in important human diseases. Here, a way to elucidate links with cell functions is represented by the investigation of the role of epigenetic modifications on *DAO* gene expression in different cells and tissues during development and pathological conditions. In this regard, a CpG methylation analysis of the *DAO* promoter was performed recently and brain region-specific epiallelic profiles were detected in schizophrenic patients and healthy controls (Keller et al., 2018). These different methylation signatures have been proposed to be indicative of cell populations containing the *DAO* gene in different functional states.

The known properties of hDAAO strengthen our belief that the flavoenzyme activity must be finely tuned to fulfill a main physiological function such as the control of D-serine levels in the brain.

AUTHOR CONTRIBUTIONS

LP designed the review. All authors analyzed the literature and wrote the manuscript.

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