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Inhibition of serine and proline racemases by substrate-product analogues

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ABSTRACT

D-Amino acids can play important roles as specific biosynthetic building blocks required by organisms or act as regulatory molecules. Consequently, amino acid racemases that catalyze the formation of D-amino acids are potential therapeutic targets. Serine racemase catalyzes the reversible formation of D-serine (a modulator of neurotransmission) from L-serine, while proline racemase (an essential enzymatic and mitogenic protein in trypanosomes) catalyzes the reversible conversion of L-proline to D-proline. We show the substrate-product analogue α-(hydroxymethyl)serine is a modest, linear mixed-type inhibitor of serine racemase from *Schizosaccharomyces pombe* (K_i = 167 ± 21 mM, K_i' = 661 ± 81 mM, cf. K_m = 19 ± 2 mM). The bicyclic substrate-product analogue of proline, 7-azabicyclo[2.2.1]heptan-7-ium-1-carboxylate is a weak inhibitor of proline racemase from *Clostridium sticklandii*, giving only 29% inhibition at 142.5 mM. However, the more flexible bicyclic substrate-product analogue tetrahydro-1*H*-pyrrolizine-7a(5*H*)-carboxylate is a noncompetitive inhibitor of proline racemase from *C. sticklandii* (K_i = 111 ± 15 mM, cf. K_m = 5.7 ± 0.5 mM). These results suggest that substrate-product analogue inhibitors of racemases may only be effective when the active site is capacious and/or plastic, or when the inhibitor is sufficiently flexible.

Although L-amino acids predominate in nature, D-amino acids may be employed as specific metabolites, regulators, or used as specific biosynthetic building blocks required by organisms. D-Amino acids may be generated or degraded through the action of racemases, which catalyze the inversion of stereochemistry at the α-carbon. That some of these molecules of unusual stereochemistry play unique roles in organisms or lead to particular pathologies if under- or overproduced, has led to the recognition of racemases (and epimerases) as potential therapeutic targets.[1–8] Our studies on mandelate racemase[9–12] revealed that mimicking the structure of both enantiomeric substrates (substrate-product analogues) may serve as a useful design strategy for developing inhibitors of racemases. For example, benzilate competitively inhibits mandelate racemase with K_i = 0.7 mM (cf. K_S = 0.7 mM).[11] Inspection of the X-ray crystal structures of serine racemase[13] and proline racemase[14] suggested that these two enzymes might be susceptible to inhibition by substrate-product analogues.

Serine racemase (SR, EC 5.1.1.18) is a pyridoxal-5′-phosphate (PLP)-dependant enzyme that catalyzes both the racemization and α,β-elimination reaction of the amino acid serine (Scheme 1A).[15] SR is activated by ATP and divalent metal ions such as Mg^{2+} and Ca^{2+}, and utilizes a two-base mechanism wherein one enantiospecific Brønsted base abstracts the proton from the L-Ser-PLP aldimine and the conjugate acid of a second enantiospecific Brønsted base protonates the intermediate to form the D-Ser-PLP aldimine, and vice versa (Scheme 1A). SR also catalyzes the α,β-elimination of L- and D-serine to yield pyruvate and ammonia.

SR activity in the brain serves as the only endogenous mechanism for generating D-serine, a co-agonist of the *N*-methyl-D-aspartate receptor (NMDAR) which mediates glutamatergic neurotransmission.[16] Inhibition of SR has been suggested as a means of controlling D-serine levels to limit the extent of NMDAR-mediated neurotoxicity[17] in Alzheimer's disease[18–20] and amyotrophic lateral sclerosis,[21] to ameliorate neuropathic pain,[22] and to protect against cerebral ischemia.[23] Indeed, a number of SR racemase inhibitors have been developed including dicarboxylic acids,[24] small peptides containing a 3-phenylpropanoic acid moiety,[25] and hydroxamic acids.[26] However, the development of potent and selective SR inhibitors remains a challenge.[7]

The X-ray crystal structures of human,[16] rat,[16] and *Schizosaccharomyces pombe* (fission yeast)[13,27] SRs have been solved. The amino acid sequence of SR from *S. pombe* (*Sp*SR) shares 35% identity with human SR, and the overall structure appears to be conserved for

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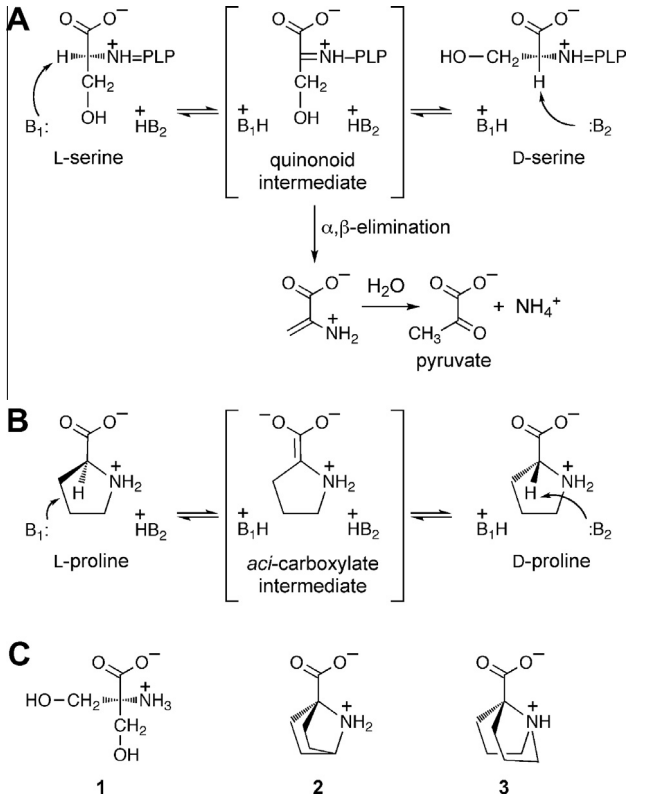

Scheme 1. Two-base racemization mechanisms of serine racemase (A) and proline racemase (B); and substrate-product analogues (C). For *Sp*SR, B_1 and B_2 are Lys 57 and Ser 82, respectively.[13] For *Tc*PR, B_1 and B_2 are Cys 130 and Cys 300, respectively.[14]

mammalian SRs.[13] To delineate the binding of serine within the active site of *Sp*SR, Goto et al.[13] generated a model using the structure of lysino-D-alanyl-modified *Sp*SR and rat serine dehydratase with bound *O*-methyl-L-serine. This model suggested that the hydroxymethyl side chain of the substrate could occupy one of two binding pockets depending on which enantiomer was bound (Fig. 1A). Consequently, we anticipated that the substrate-product analogue α-(hydroxymethyl)serine (**1**) might be an inhibitor of SR.

We sub-cloned the open reading frame encoding *Sp*SR from *S. pombe* and expressed and purified the enzyme as a fusion protein bearing an N-terminal hexahistidine (His_6) tag (see Supplementary data). Inhibition studies with **1** (prepared by the copper-catalyzed condensation of formaldehyde with glycine)[28] were conducted by following the production of pyruvate (dehydratase activity) using a coupled assay with L-lactic dehydrogenase.[27] As shown in Fig. 2, **1** is a modest, linear mixed-type inhibitor of *Sp*SR. (We also verified the mode of inhibition by plotting the initial velocity data in the form of Cornish–Bowden plots.)[29] The inhibition constants for binding to the free enzyme (K_i) and the enzyme-substrate complex (K_i') are equal to 167 ± 21 mM and 661 ± 81 mM, respectively. Thus, **1** binds to the free enzyme with an ~8-fold weaker affinity relative to that of the substrate (i.e., K_m = 19 ± 2 mM). Although the model presented by Goto et al. (Fig. 1A) suggests that *Sp*SR should be able to accommodate the hydroxymethyl side chains of L- and D-serine simultaneously, as would be the case for binding of **1**, our results suggest that such a binding scenario is unfavorable. It is conceivable that the polar β-OH of serine is bound at only a single site and therefore an inhibitor bearing two hydroxyl functions is not tolerated. Alternatively, the packing arrangement within the active site is not flexible enough to accommodate the added steric bulk of **1**, or a conformational change occurs in the enzyme as it converts between the substrate-bound L- and D-forms such that only a single side chain can be bound at the active site.

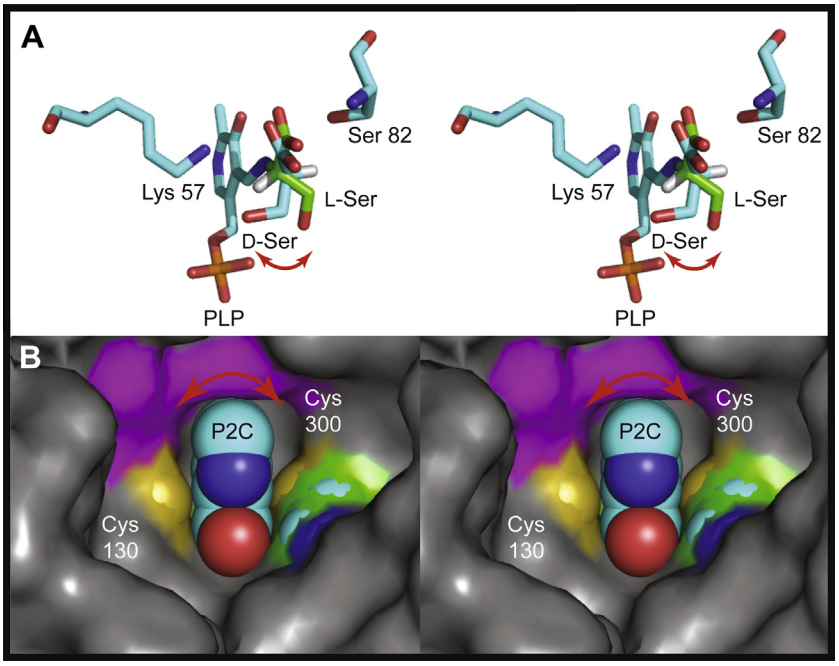

Figure 1. Stereoviews (wall-eyed) showing putative motion of amino acid side chains during racemization (red arrows). Panel A shows a model of *Sp*SR with bound PLP-L-serine aldimine (green) and PLP-D-serine aldimine (blue) superimposed at the active site.[13] This model was constructed using the X-ray structures of *Sp*SR (PDB 2ZR8) and rat serine dehydratase complexed with *O*-methyl-L-serine (PDB 1PWH).[30] Panel B shows the active site structure of *Tc*PR with the transition state analogue P2C bound (PDB 1W61) with space available on either side of the planar ring to accommodate the side chains of D- and L-proline.[14] The van der Waals surface corresponding to the active site Cys residues is shaded yellow, while surfaces corresponding to Phe 102 and Phe 290 are shaded magenta. This figure was prepared using MacPyMOL.[31]