

Quantitative *In silico* analysis of mammalian serine racemase activity

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Abstract— Serine racemase (SR) is a pyridoxal 5'-phosphate (PLP)-dependent enzyme catalyzing the racemization of *S*-serine to *R*-serine, and also oxidizes *R*-serine to pyruvate; however, the enzymatic racemization reaction is not fully understood. Therefore, quantitative *in silico* analysis of mammalian SR was performed followed by D-amino acid oxidase (DAO) analysis. The downloaded stereo structures of *Rattus norvegicus* (PDB ID: 3hmk) and mammalian (PDB ID: 316r and 316b) SR were optimized using molecular mechanic (MM2) calculation. Furthermore, the stereo structure of mammalian SR was constructed using 3hmk and the amino acid sequence data. The atomic partial charge (*apc*) of the target atoms was calculated using MOPAC-PM5. The atomic distances, bond angles, and *apc* of the mutants were used to study the enzyme reaction mechanism. The serine carboxyl group contacted lysine-56, where its amino group contacted the oxygen molecules of PLP aldehyde. The $\square apc$ of $\square C$ indicated that *S*-serine was selectively oxidized. Manganese located at another side of PLP is directly involved in the serine racemization by forming bonding with the hydroxyl group of serine. The enzyme racemization activity was quantitatively related to the bond angle of the substituted serine rather than to that of alanine. However, 316r and 316b lack several amino acid residues; therefore, the selective oxidation of S84 mutants was not clearly demonstrated.

Keywords— Mammalian serine racemase, Quantitative analysis, Enzyme activity, *in silico*.

I. INTRODUCTION

Substantial levels of *R*-serine in the mammalian brain were discovered in 1992 [1]. Mammalian SR (hSR) was first purified from rat brain and functionally characterized in 1999 [2]. The serine racemase synthesizes *R*-serine from *S*-serine, and controls the *R*-serine level by oxidation of *R*-serine to pyruvate. *R*-Serine is the main endogenous coagonist of the *N*-methyl-D-aspartate (NMDA)-type glutamate receptor (NMDAR) involved in important

functions such as synaptic plasticity, learning, memory, and excitotoxicity. The x-ray crystal structure of mammalian and rat serine racemase were determined to provide a molecular basis for rational drug design. The residues 69-73 of hRS were found to be disordered; therefore, these residues were not included in the final model. The associated reaction mechanisms have been reported and the structure described in detail. The domains of SR were connected by a flexible linker and could adopt different orientations with respect to each other. The crystal structures corresponded to biophysical data and confirmed the dimeric state of the enzyme. The racemization reaction was critically dependent on the proximity of the catalytic S82 to the substrate as shown by the closed ligand-bound structures. [3]. SR was most active as a noncovalent dimer containing one or more free sulfhydryls in the enzyme's active center or a modulatory site. The recombinant hSR was sensitive to oxidative stress *in vivo*, perhaps consistent with a scenario in which such modification plays a role in feedback or other forms of regulation [4]. A decrease in *R*-serine concentration was reported in psychiatric diseases such as schizophrenia and bipolar disorder: association studies had linked DAO and the primate-specific gene 72 (pLG72) with schizophrenia susceptibility. The opportunity to regulate DAO activity opened the way to *R*-serine level *in vivo* and thus to act on different pathological states involving the alteration in the NMDAR function. *R*-serine levels were decreased in cells over expression hDAO, while cotransfection with pLG restored *R*-serine levels and decreases the hDAO activity. The results highlight the cellular function of hDAO in *R*-serine catabolism and confirm the role of pLG72 as a negative effector of the flavoenzyme. The interaction of pLG72 to hDAO on the cytosolic side of the outer mitochondrial membrane induced enzyme inactivation and speeded up its degradation. In this way cytosolic *R*-serine was preserved and cells were protected from oxidative stress by hydrogen peroxide generated by the flavoprotein in

cytosol. pLG72 inhibited hDAO and highlighted the specificity of class C compounds as they did not inhibit glucose oxidase or D-aspartate oxidase [5]. The potential involvement of *R*-serine in neurodegenerative diseases pertains to the impaired activity of DAO, which has potential for development as a therapeutic agent in Amyotrophic lateral Sclerosis. An up-regulation of *R*-serine caused either by impaired metabolism of *R*-serine by DAO or increased biosynthesis by SR [6]. Serine racemase was a PLP-dependent enzyme catalyzing the racemization of *S*-serine to *R*-serine; however, the enzymatic racemization reaction was not fully understood [7]. Therefore, the bi-functional reaction mechanisms of mammalian SR were quantitatively analyzed *in silico* as was the case for *R*-amino acid oxidase [8-10] and alanine racemase [11].

II. EXPERIMENTAL

The stereo structure of hSR was constructed by homology modeling using *Rattus norvegicus* (PDB ID: 3hmk). The homology model of hSR and *R*- and *S*-amino acid complexes were optimized using MM2 force-field parameters of version 6.1 CAChE MM2 (Fujiitsu, Japan). The amino acid residues present within 3Å of a substituted amino acid group were extracted with the co-enzyme PLP, then locked the molecules, prior to being optimized using the MOPAC PM5 program. The differences in the ΔG values of key atoms and their relationships with enzyme activities were analyzed. Calculations were performed using a DCPIx86-based PC with an Intel Core™i7-2600 3.40 GHz CPU. The minimum energy level was 10^{-7} kcal mol⁻¹.

III. RESULTS AND DISCUSSION

The downloaded stereo structure of hSR (PDB, ID: 3l6r) [12] lacks seven residue amino acids P69-K75. Furthermore, some methionines were not identified. V68 was directly connected to P76. Therefore, the lack of these residues shortens the distance of these two loops, a long right-handed alpha and a short β -parallel loop. This results in R135 and PLP getting closer to each other. Another hSR (PDB ID: 3l6b) [12] lacks five amino acid residues (P69 – E73); in addition, C6 appeared as D6. The location of R135 is similar to that of 3l6r. The conformation of PLP lacks the carbonyl group, and the substrate is malonate. Therefore, the stereo structure of hSR was reconstructed using that of *Rattus norvegicus* SR (PDB, ID: 3hmk) [12]. The homology model of hSR was

defined as h3hmk. The conformation of the key molecules is shown in Figure 1, and that of 3l6b in Figure 2.

However, *Rattus norvegicus* SR lacks a substrate; therefore, malonate was inserted at the same location as the malonate (inhibitor) being held in hSR, and the complex was optimized using MM2 calculations prior to being replaced by serine. Alanine was used as a blank amino acid. The oxidation reaction of SR was first analyzed as with the case of DAO, where FAD is the co-enzyme and the carbonyl group of the flavin ring is the electron-transfer for the deamination of *R*-amino acids

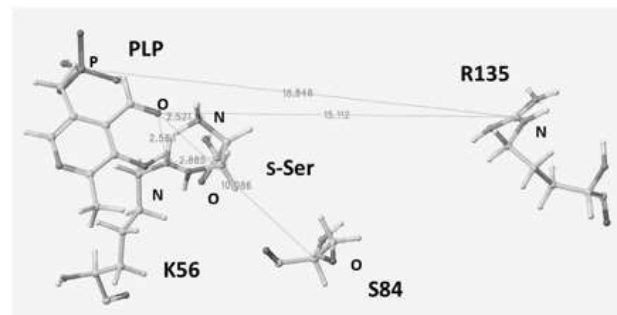


Fig. 1. Conformation of selected molecules in mammalian serine racemase h3hmk.

Black color balls: oxygen or phosphine, gray color balls: nitrogen, white color large and small balls: carbon and hydrogen. Indicated atomic distance: Å.

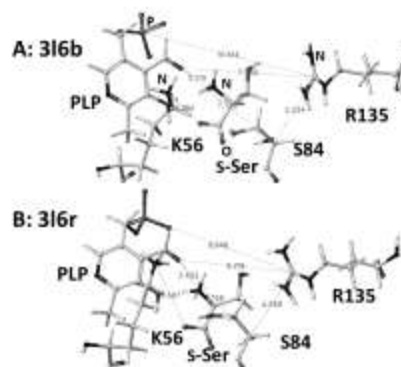


Fig. 2. A and B are conformation of selected molecules in mammalian serine racemase 3l6b and 3l6r. Symbols: see Figure 1.

Arginine R275 is the key amino acid residue holding the *R*-amino acid carboxyl group in the DAO reaction chamber [8-10]. However, there is no arginine close to the PLP site of 3hmk and h3hmk. R135 is far from PLP in 3hmk and h3hmk (see Figure 1).

The atomic distance between the oxygen of PLP aldehyde and the center carbon of the R135 guanidyl group is 15.1 Å; corresponding distance in 3l6b and 3l6r

were 7.76 and 6.72 Å (see Figure 2), respectively. In addition, the atomic distance between the aldehyde oxygen and \square C of S84 were 10.1, 7.78, and 7.14 Å for h3hmk, 3l6b, and 3l6r, respectively. Indeed, the conformation of h3hmk is very different from the stereo structure of the downloaded hSR 3l6b and 3l6r. In the case of the latter, R135 is located close to PLP; this should facilitate the trapping of substrate serine carboxyl groups. The atomic distances between PLP phosphine and the R135 guanidyl group center carbon were 18.8 Å, 10.5, and 9.85 Å for h3hmk, 3l6b, and 3l6r, respectively. The carboxyl group of the serine substitute was superimposed on the carboxyl group of the original substrate malonate; then, the complex structure was optimized to analyse the oxidation activity. The carboxyl group of the serine substrate was located near the amino group of K56.

The apc values of amino acids were calculated using these downloaded structures containing the fragmental library of the CAChe program. The downloaded structures were locked prior to the MOPAC calculation. The *R*-form amino acids were converted from the original *S*-form amino acids. The apc values of *S*- and *R*-alanines were the same, but those of *R*-serine were a little different. The \square apc value of *S*-serine \square C was 0.20 au, while those of *R*-serine, *S*- and *R*-alanines were 0.20, 0.17, and 0.18 au, respectively. However, the amino group of *S*-alanine contacted with G239, and that of *R*-alanine did not face to the oxygen of PLP aldehyde. When the original apc value (-0.384 au) of *S*-serine were used, the \square apc of *R*-serine was smaller (0.19 au) than that of *S*-serine. The result supported the fact that SR relatively oxidized *S*-serine at the location. Normally hSR favors *S*-serine elimination activity over SR activity [13].

The oxidation activity was further studied using hSR mutants, S84A, S84D, S84N, and S84T [13]. The K_{cat}/K_m values of WT and S84N were about five times higher than those of S84A, S84D, and S84T. However, such reaction selectivity was not obtained from the \square apcs of the \square Cs of *R*- and *S*-serines. \square apc was key to explaining the selective oxidation of *R*-amino acids in DAO [10]. However, such a change was observed while the apc was calculated using the 3l6r model. The difference should be the atomic distance between the \square C of S84 and the aldehyde oxygen of PLP. The distances in h3hmk, 3l6b, and 3l6r were 10.1, 7.78 and 7.14 Å, respectively. S84 is located near PLP in 3l6b and 3l6r and may contribute to the selective reaction of mutants, but it is far from the reaction center of the aldehyde oxygen in h3hmk as shown in Figure 3.

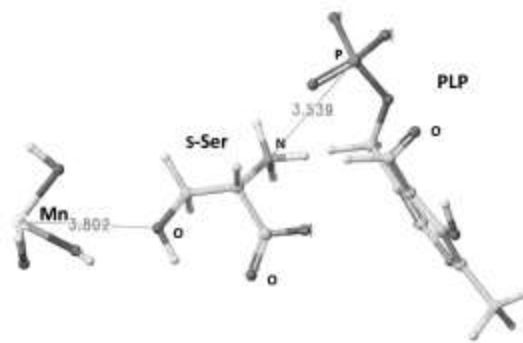


Fig. 3: Conformation of selected molecules in mammalian serine racemase h3hmk at manganese PLP site. Symbols: see Figure 1.

The apc values of C are similar for *R*- and *S*-serines, about 0.2 au, and did not indicate the selectivity of these mutants. The reason is R135 and S84 are located far from the PLP in the h3hmk structure. This conformation differs from those of 3l6r and 3l6b. In 3l6b, the \square apcs of the \square Cs of *R*- and *S*-serines in S84N, S84A, S84D, and S84T were 0.20, 0.20, 0.18, 0.16, and 0.18, respectively. *S*-serine amino group did not face the aldehyde oxygen. Similar results were obtained for 3l6r. In addition, S83 and S84 are connected to the G82 of \square -parallel and G85 right-handed alpha helix, respectively; therefore, S84 should cause a conformation change in the mutants. R135 is a part of the right-handed alpha loop; therefore this residue has no flexibility of movement on its own. Consequently, the flexibility of these serine residues is limited. One of the reasons why no clear selectivity among the mutants was observed is that the K56 \square amino group contacts with both the serine amino group and PLP aldehyde in these conformations. This structure differs from that observed in DAO, where the key amino acid residue guanidyl group was far from the flavin carbonyl group. Here, the flexible conformation of PLP did not clearly demonstrate the localization of electron. Unfortunately, the amino acid sequence data of these mutants were not presented to justify this speculation.

Deprotonation of the \square C and the formation of imine are the basic mechanisms underlying alanine racemase reactivity. The atomic distance between \square H and \square C, and the apc of \square H changed little in the conformation of the molecular forms of PLP. The apc of the alanine \square C was well correlated with the reactivity, and the value was high for *R*-alanine. The alanine carboxyl and amino groups

contact the oppositely charged residue's cation and anion for the electron transfer. The bond angles of the substitute alanine were well correlated with mutant reactivity. The bond angle, therefore, can be used for quantitative analysis of the reactivity [11]. The observation was applied to analyze the racemization mechanism of serine racemase.

Metals often function as efficient and selective promoters of catalysis. Why does this enzyme require manganese as an essential element? Does it function to maintain a suitable stereo structure or is directly involved in the reaction? Another side of PLP is the oxidation reaction center with the K56 amino group. Manganese forms a complex using the hydroxyl group as a ligand [14]. The possible conformation indicates that the hydroxyl group of serine may indeed contribute as a ligand. Serine was non-enzymatically oxidized to pyruvate at the sites where metal ions exist. However, the metal ion is located near the PLP carbonyl group according to the schematic explanation. Manganese is located at the site opposite to the oxidation reaction center in the enzymatic reaction. Furthermore, the catalytic activity of manganese is weak compared to aluminum, copper, and iron, where the metals are involved in the deamination of serine, but not racemization [15]. Manganese forms multi-ligands with water molecule [16-18]. Therefore, the manganese site may not be the oxidation reaction center in the enzymatic reaction; the stereo structure was then optimized using the MM calculations. The structure change in serine was measured as with the case of alanine in an alanine racemase [11]. Alanine was used as a blank substrate. Therefore, serine and alanine were located between manganese and PLP phosphate in h3hmk to study the contribution of manganese. The atomic distance between manganese and phosphine was 9.1 Å for both 3hmk and h3hmk. The atomic distance was a little longer for the mutants, 9.4 Å for S84D and 9.7 Å for S84A, S84N, and S84T. The hydroxyl group of serine may conjugate with manganese as a ligand, but such a phenomenon should not be expected in the case of alanine. The amino group of serine and alanine should face the PLP phosphate; then, the complexes were optimized. The calculated dihedral angle and apc of key atoms are summarized in Table 1 to demonstrate the flatness of amino acids especially for \square -hydrogen. The combined dihedral angle (DA)1 = $\text{HOH}_2\text{C}^* - \square\text{C}^* - \text{C}^*\text{O}_2^- + \text{HOH}_2\text{C}^* - \square\text{C}^* - \square\text{H}^* + \square\text{H}^* -$

$\square\text{C}^* - \text{C}^*\text{O}_2^-$; DA2 = $\text{HOH}_2\text{C}^* - \square\text{C}^* - \text{N}^*\text{H}_3^+ + \text{HOH}_2\text{C}^* - \square\text{C}^* - \square\text{H}^* + \square\text{H}^* - \square\text{C}^* - \text{N}^*\text{H}_3^+$; DA3 = $\text{O}_2\text{C}^* - \square\text{C}^* - \text{N}^*\text{H}_3^+ + \text{O}_2\text{C}^* - \square\text{C}^* - \square\text{H}^* + \square\text{H}^* - \square\text{C}^* - \text{N}^*\text{H}_3^+$. The target atoms were C^* , H^* , and N^* . In the case of aniline, H_3C^* was used instead of HOH_2C^* .

After optimization *S*-serine became flat, and the total angle increased from 328.3 to 341.87° at site B. However, the angle was changed a little for *S*-alanine. The conformation is such that the amino acid amino group is held by the PLP phosphate, and the carboxyl group of alanine faced manganese, while the hydroxyl group of serine faced the metal. When the carboxyl group of serine faced the phosphate, and the amino group is faced the metal, the change in angle was only 1.9°. The angle change in the case of *S*-alanine was -0.1°. The changes in angle for *R*-serine and *R*-alanine were 11.9 and 9.5°. Since serine became flat at site A. However, the oxidation changes serine to pyruvate at site A. If serine racemase handles both reactions simultaneously, racemization may occur at site B where the flatness of serine is high compared to that of alanine.

IV. CONCLUSION

Deprotonation of the C and the formation of imine are the basic mechanisms underlying racemase reactivity. The serine carboxyl and amino groups contact the oppositely charged cation and anion of the residue for electron transfer. The bond angles of the serine substitute indicated the location of oxidation and racemization. The bond angle, therefore, can be used for the quantitative analysis of racemization. The substrate serine formed a complex with PLP. Calculations were performed without considering the solvent (water). The results may not directly compare with those *in vivo*. The Lewis acid-base interaction is the strongest; however, because water molecules cannot interrupt this interaction, they may function as a bridge to fill the gap. Here, we performed a quantitative analysis of the selective reactivity of serine racemase. The *S*-form of serine was more stable in complexes containing this enzyme due to the favorability of the enzyme structure. The probability depends on the reality of the stereo structure. The \square apc values among the mutants were small, and the downloaded stereo structures lacked several amino acid residues; therefore, the selectivity could not be justified without their amino acid sequence data.

Table.1: Observed and change of dihedral angle (DA/ \square DA) and atomic partial charge (apc)

| Amino acid | DA/ \square DA ($^{\circ}$) | | | apc (au) | |
|---------------------------------|---------------------------------|--------------|---------------|----------|--------|
| | DA1 | DA2 | DA3 | N | O(OH) |
| S-Serine (site A) | 341.53/13.20 | 331.81/0.99 | 323.60/-2.85 | -0.102 | -0.030 |
| R-Serine (site A) | 341.18/12.85 | 332.39/4.41 | 324.72/-1.73 | -0.083 | 0.005 |
| S-Alanine (site A) | 338.14/8.15 | 332.71/5.40 | 319.28/-8.02 | -0.076 | - |
| R-Alanine (site A) | 336.79/6.81 | 334.95/7.66 | 319.68/-7.61 | -0.075 | - |
| S-Serine (site B) | 341.87/13.54 | 327.36/-3.46 | 323.62/-2.83 | -0.037 | -0.010 |
| S-Serine (site B [#]) | 338.29/9.96 | 326.15/-4.67 | 328.72/0.74 | -0.049 | 0.002 |
| R-Serine (site B) | 333.60/5.27 | 335.09/7.11 | 325.76/-0.69 | -0.041 | -0.052 |
| R-Serine (site B [#]) | 335.53/7.20 | 332.12/4.14 | 314.40/-16.42 | -0.017 | -0.081 |
| S-Alanine (site B) | 334.04/3.75* | 322.12/-5.18 | 322.12/-5.18 | 0.001 | - |
| R-Alanine (site B) | 332.86/2.88* | 333.74/6.45 | 322.19/-0.51 | 0.015 | - |

Site A: oxidation reaction site; site B: manganese-phosphate site. Site B[#]: Serine carboxyl and ammonium groups contacted with manganese and phosphate, respectively.

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