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〈Original Article〉

Monomeric sarcosine oxidase acts on both L- and D-substrates

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Summary The stereoselectivity of monomeric sarcosine oxidase (mSox), an enzyme used for creatinine determination, was assayed by using N-methylalanine and proline. As a result, mSox reacted to both L- and D-substrates, although heterotetrameric sarcosine oxidase has already been known as an L-specific enzyme. The catalytic efficiency of mSox for N-methyl-L-alanine was higher than that for N-methyl-D-alanine, while the efficiency for L-proline was lower than that for D-proline. Those substrate specificities were discussed in terms of the enzyme-substrate docking models constructed.

Key words: Sarcosine oxidase, Stereoselectivity, Catalytic efficiency, Substrate specificity, Molecular docking

1. Introduction

Sarcosine oxidase (EC 1.5.3.1; sarcosine:oxygen oxidoreductase) is a flavoprotein that catalyses the oxidative demethylation of sarcosine (N-methylglycine) to yield glycine, formaldehyde, and hydrogen peroxide. This enzyme is involved in the bacterial metabolism of creatinine with the related enzymes, creatininase and creatinase[1)]. Two types of sarcosine oxidases, monomeric sarcosine oxidase (mSox) and heterotetrameric sarcosine oxidase (hSox), have been known and well-studied[2, 3)]. The mSox is industrially important and is used with creatininase and creatinase for the enzymatic assay of creatinine in clinical settings[4, 5)]. We have previously screened an mSox from the genus *Arthrobacter* and cloned the gene[6)]. We have also succeeded in altering the substrate specificity of the enzyme and its stabilization by using mutagenesis techniques[7-9)]. The wild-type and mutant mSox enzymes are produced commercially and are being used for application to diagnostic reagents[1)].

Understanding the substrate specificities of the enzymes for diagnostic reagents (including the stereoselectivities) is desirable for clinical assays, since reactions to substrate analogs or derivatives may occur in clinical samples and interfere with the assay. It is generally thought that the stereoselectivity of Sox is L-specific[10, 11)], because hSox only reacts to L-substrates[12)]. However, to our knowledge, the stereoselectivity of mSox remains unknown.

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In this report, we investigated the stereoselectivity of mSox, and found that mSox reacted to both L- and D-substrates. Our results were discussed in terms of the enzyme-substrate docking models constructed. These findings also provide information for further improvements to the functionality of the enzyme.

2. Materials and methods

The mSox used was from *Bacillus* sp. (Asahi Kasei Pharma, Tokyo). We have developed the *Arthrobacter* mSox for application to creatinine assay reagents, and have improved both the stability and substrate specificity of this enzyme. In this study, we used the *Bacillus* mSox, the X-ray crystal structure of which has been solved[2, 10, 13], because the models of enzyme-substrate complexes can be constructed by a computer aided docking study.

Compounds used as substrates were sarcosine, N-methyl-L-alanine (NML-Ala), N-methyl-D-alanine (NMD-Ala), L-proline, D-proline, and L-hydroxyproline. They were purchased from Nacalai Tesque (Kyoto). Possible reactions of mSox to sarcosine, N-methylalanine, and proline are shown in Fig. 1.

The enzyme assay was based on the measurement of hydrogen peroxide produced during the oxidation of a substrate. The 4-aminoantipyrine peroxidase system was used for the enzyme assay as described previously[9]. The assay mixture finally contained 100 mmol/L sarcosine, NML-Ala, NMD-Ala, or one of the other substrates, 0.49 mmol/L 4-aminoantipyrine, 2.1 mmol/L phenol, 50 mmol/L Tris-HCl (pH 8.0), and 5 U of horseradish peroxidase per ml. An enzyme solution (0.05 ml) was incubated with an assay mixture (1.0 ml) at 37℃, and the appearance of quinoneimine dye formed by coupling with 4-aminoantipyrine, phenol, and horseradish peroxidase was measured by spectrophotometry at 500 nm against the blank. One unit of activity was defined as the formation of 1 micromole of hydrogen peroxide (0.5 micromole of quinoneimine dye) per minute at 37℃ and pH 8.0. Reaction mixtures containing several concentrations of substrate solution were used to determine the K_m and V_{max} values.

Molecular docking studies were performed using the software Autodock ver.4.2[14] on the basis of a grid-based docking procedure. The ligand structures were obtained from the Protein Data Bank (sarcosine; PDB ID: 3qse, L-proline; PDB ID: 2eiw, D-proline; PDB ID: 2ej6), and the PubChem database (NML-Ala, NMD-Ala). For the ligands, Gasteiger charges were calculated using the software Autodock Tools. The

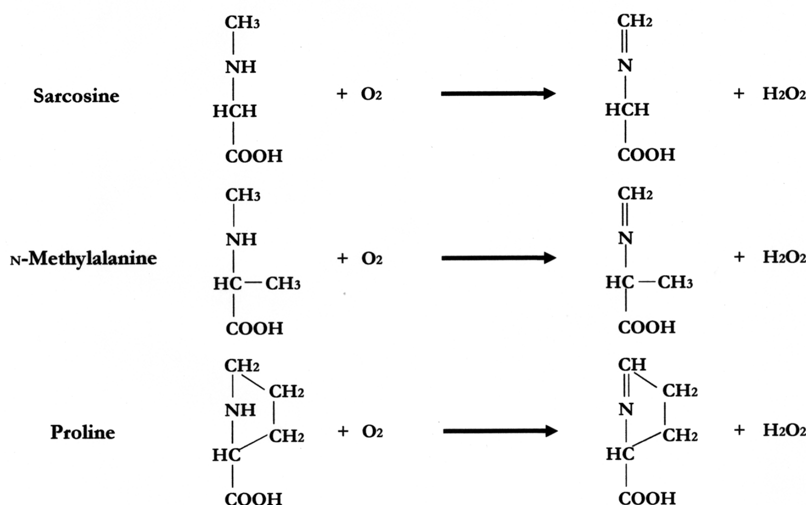

Fig. 1　Reactions of sarcosine oxidase to sarcosine, N-methylalanine, and proline.

enzyme model obtained from the X-ray crystal structure (PDB ID: 1el5, resolution: 1.80 Å) was prepared with Autodock Tools, deleting all water molecules, adding polar hydrogens, and loading charges. The hydrogen atoms of histidine residues were predicted from the software Reduce[15]. The program AutoGrid settings with a 30 x 30 x 30 grid size and a grid spacing of 0.375 Å were used for preparing each grid, which was localized at the active site. Five billions of conformations were evaluated using the Lamarckian genetic algorithm. The best docked conformers with the lowest free energy conformations were selected for discussion.

3. Results and discussion

The stereoselectivity of mSox was assayed by using sarcosine, NML-Ala, NMD-Ala, L-proline, D-proline, and L-hydroxyproline, respectively. As a result, mSox reacted to NMD-Ala and D-proline as well as to sarcosine, NML-Ala, and L-proline (Table 1). In contrast, L-hydroxyproline was not a substrate. Accordingly, it was demonstrated that mSox acted on both L- and D-substrates, although hSox has already been known as an L-specific enzyme.

The K_m and V_{max} values of mSox for substrates were estimated from Lineweaver-Burk plots (Table 1). The kinetic parameters estimated from Eadie-Hofstee

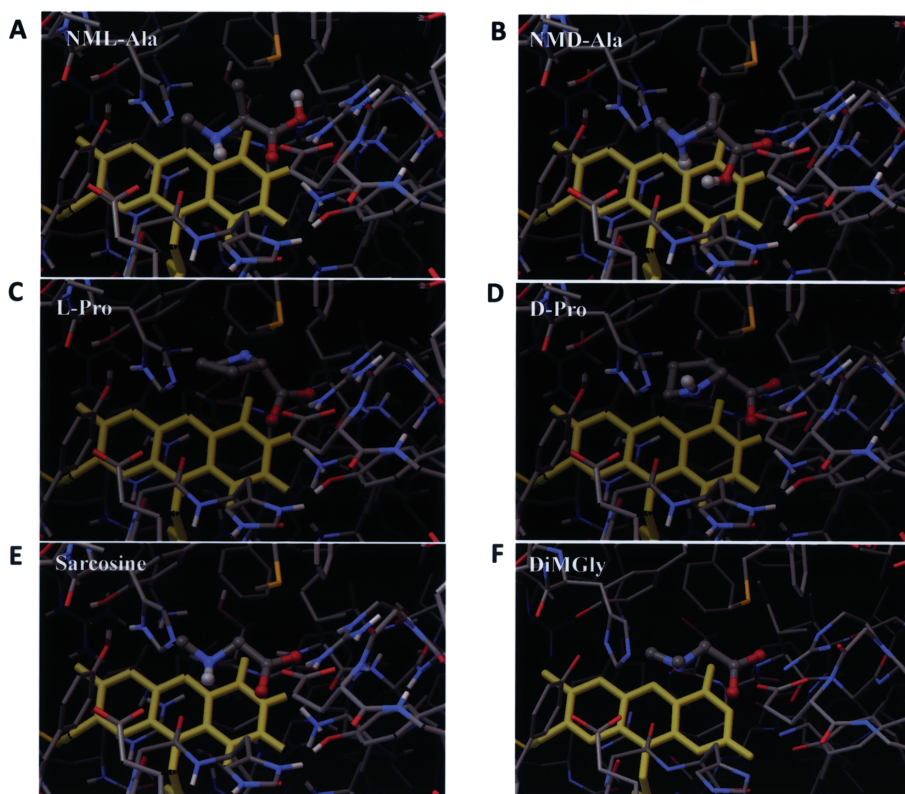

Fig. 2 Localized mSox structure around each substrate. The mSox-substrate complexes were constructed by molecular docking, as described in the materials and methods section. The substrates are represented by ball and stick drawings. Amino acid residues are shown by stick drawings. The coenzyme, flavin adenine dinucleotide, is designated by yellow sticks. Hydrogen (white), carbon (gray), nitrogen (blue), and oxygen (red) atoms are indicated. (A-E) View of mSox with NML-Ala, NMD-ALa, L-proline, D-proline, and sarcosine, respectively; (F) View of mSox with the substrate analog, dimethylglycine, based on its X-ray crystal structure.

Table 1 Kinetic parameters of mSox for substrates

Substrate	K_m (mmol/L)	V_{max} (U/mg)	V_{max}/K_m (%)
Sarcosine	17±2.6	34±4.0	100
N-Methyl-L-alanine	5.8±0.30	8.8±0.20	76
N-Methyl-D-alanine	88±4.0	2.4±0.30	1.4
L-Proline	240±39	0.25±0.031	0.052
D-Proline	37±4.2	0.18±0.015	0.24

plots and Hanes-Woolf plots were almost the same as those of Lineweaver-Burk plots (data not shown). The K_m value for NMD-Ala was approximately 15 times higher than that for NML-Ala. The catalytic efficiency (V_{max}/K_m) for NMD-Ala was approximately 1/50th that for NML-Ala due to the lower values of both its binding affinity ($1/K_m$) and V_{max}. On the other hand, the K_m for D-proline was approximately 1/6th that for L-proline. The catalytic efficiency for D-proline was approximately 5 times higher than that for L-proline, corresponding to the different binding affinity.

For the purpose of discussing the interaction between mSox and each substrate, we constructed molecular docking models using the software Autodock (Fig. 2). Molecular docking is a computational method that predicts how a ligand interacts with a protein, and plays an essential role in drug design. It is thought that docking models also help to enhance our understanding of the enzyme-substrate interactions. We expected that a molecular docking study would be useful for better understanding the enzyme reactions in an enzymatic assay field.

The binding configuration of sarcosine predicted from molecular docking (Fig. 2E) was almost the same as that of the substrate analog, dimethylglycine, based on its X-ray crystal structure (Fig. 2F). The configurations of other substrates (Fig. 2A-D), particularly those of carboxylates, were also close to that of sarcosine. The binding energy scores of sarcosine, NML-Ala, NMD-Ala, L-proline, and D-proline were -4.9, -4.5, -5.2, -6.0, and -6.0 kcal/mol, respectively. The energy scores of D-substrates were similar to those of L-substrates and were not significantly different from that of sarcosine. Hence, the Autodock prediction indicated that all substrates were able to effectively bind to mSox. The binding energies of NMD-Ala and L-proline were at the same levels as that of sarcosine, whereas the binding affinities were much lower (Table 1). This suggests that the enzyme-substrate complexes might be transferred from the substrate-binding forms to the reactable forms at an extremely low frequency. In fact, the predicted configuration of L-proline (Fig. 2C) unlike that of D-proline (Fig. 2D), is obviously unreactive to the flavin ring of mSox. In order to form a reactive complex, L-proline is at least required to be inverted for being approached its nitrogen atom to the flavin ring. A further computational study about transferring the substrate configurations to the reactable forms is now in progress.

References

1) Nishiya Y, Yamamoto K, Kawamura Y, Emi S: Development of creatinine-degrading enzymes for application to clinical assays.[Jpn] Nippon Nogeikagaku Kaishi, 75: 857-862, 2001.

2) Trickey P, Wagner MA, Jorns MS, Mathews FS: Monomeric sarcosine oxidase: Structure of a covalently flavinylated amine oxidizing enzyme. Structure, 7: 331-345, 1999.

3) Ida K, Moriguchi T, Suzuki H: Crystal structure of heterotetrameric sarcosine oxidase from *Corynebacterium* sp. U-96. Biochem Biophys Res Commun, 333: 359-366, 2005.

4) Kinoshita T, Hiraga Y: A fluorophotomeric determi-

nation of serum creatinine and creatine using a creatinine amidohydrolase-creatine amidinohydrolase-sarcosine oxidase-peroxidase system and diacetyldichlorofluorescein. Chem Pharm Bull, 28: 3501-3506, 1980.

5) Fossati P, Prencipe L, Berti G: Enzymic creatinine assay: A new colorimetric method based on hydrogen peroxide measurement. Clin Chem, 29: 1494-1496, 1983.

6) Nishiya Y, Imanaka, T: Cloning and sequencing of the sarcosine oxidase gene from *Arthrobacter* sp. TE1826. J Ferment Bioeng, 75: 239-244, 1993.

7) Nishiya Y, Imanaka T: Alteration of substrate specificity and optimum pH of sarcosine oxidase by random and site-directed mutagenesis. Appl Environ Microbiol, 60: 4213-4215, 1994.

8) Nishiya Y, Zuihara S, Imanaka T: Active site analysis and stabilization of sarcosine oxidase by the substitution of cysteine residues. Appl Environ Microbiol, 61: 367-370, 1995.

9) Nishiya Y, Kishimoto T: Alteration of L-proline oxidase activity of sarcosine oxidase and a structural interpretation. J Anal Bio-Sci, 33: 161-166, 2010.

10) Wagner MA, Jorns MS: Monomeric sarcosine oxidase: 2. Kinetic studies with sarcosine, alternate substrates, and a substrate analogue. Biochemistry, 39: 8825-8829, 2000.

11) Dodt G, Kim DG, Reimann SA, Reuber BE, McCabe K, Gould SJ, Mihalik J: L-Pipecolic acid oxidase, a human enzyme essential for the degradation of L-pipecolic acid, is most similar to the monomeric sarcosine oxidases. Biochem J, 345: 487-494, 2000.

12) Zeller H-D, Hille R, Jorns MS: Bacterial sarcosine oxidase: Identification of novel substrates and a biradical reaction intermediate. Biochemistry, 28: 5145-5154, 1989.

13) Wagner MA, Trickey P, Chen ZW, Mathews FS, Jorns MS: Monomeric sarcosine oxidase: 1. Flavin reactivity and active site binding determinants. Biochemistry, 39: 8813-8824, 2000.

14) Morris GM, Huey R, Lindstrom W, Sanner MF, Belew RK, Goodsell DS, Olson AJ: AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. J Comput. Chem, 30: 2785-2791, 2009.

15) Word JM, Lovell SC, Richardson JS, Richardson DC: Asparagine and glutamine: Using hydrogen atom contacts in the choice of side-chain amide orientation. J Mol Biol, 285: 1735-1747, 1999.