〈Original Article〉

Altered substrate affinity of monomeric sarcosine oxidase by mutation of phenylalanine-103 or histidine-348

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Summary Monomeric sarcosine oxidase from *Arthrobacter* sp. TE1826 (SoxA) and its mutants are commercially used in the diagnostic analysis of creatinine and creatine. Understanding the substrate affinities and specificities of the enzymes for diagnostic reagents is desirable for clinical assays, since reactions to substrate analogs or derivatives may occur in clinical samples and interfere with the assay. In this study, altered substrate affinities and specificities of the four mutant SoxAs were investigated. The structure models constructed by homology modeling showed that the F103 residue of SoxA could interact with M248, which exerted a direct influence on the substrate. The substrate specificities of the F103L and F103Y mutants demonstrated the relationship between F103 and M248. On the other hand, H348 located around the active site and its side chain formed a part of the substrate-binding space. The Km value of H348A for sarcosine was markedly elevated because of the larger active site space. In contrast, H348K had no activity although its FAD-binding structure was normal. The side chain of H348K was most likely to inhibit the substrate binding. The substrate specificities of the wild-type SoxA and F103L are discussed in terms of the enzyme-substrate docking models constructed.

Key words: Sarcosine oxidase, Amino acid substitution, Substrate affinity, Substrate specificity, Molecular docking

1. Introduction

Monomeric sarcosine oxidase (mSox, 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, 2]. The mSox is industrially important and is used with creatininase and creatinase for the enzymatic assay of creatinine and creatine in clinical settings[3, 4].

We have previously screened an mSox from the genus *Arthrobacter* (SoxA) and cloned the gene[5]. We have also succeeded in alteration of the substrate specificity and affinity of the enzyme and stabilization of that by mutagenesis techniques[6-9]. The wild-type and mutant SoxAs are produced commercially and are being used for application to diagnostic reagents[1].

The enzymes for diagnostic reagents may react to

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substrate analogs or derivatives in clinical samples and interfere with the assay. Accordingly, understanding of the enzymatic properties, such as substrate specificities, affinities, or stereoselectivities, is desirable for clinical assays. In the previous report, we have investigated the stereoselectivity of mSox from the genus *Bacillus* (SoxB), and have indicated that the enzyme reacted to both L- and D-substrates although it was generally thought that the stereoselectivity of mSox is L-specific[10]. The result was discussed in terms of the models of enzyme-substrate complexes, which were constructed by computer aided docking study[10]. Formerly, we have also constructed a three-dimensional structure of SoxA using homology modeling based on the X-ray structure of SoxB[11]. Binding properties of SoxA with various substrate analogues could be observed by fitting in the active site[12].

In this report, altered substrate affinity and specificity of SoxA by the mutation of phenylalanine-103 (F103) or histidine-348 (H348) were structurally investigated. The enzymatic properties of the F103 and H348 mutants have been previously studied[6, 9]. However, the structural features of them were unknown. The result was discussed in terms of the enzyme-substrate docking models constructed. This must be also of assistance in the improvement of the enzyme using protein engineering techniques.

2. Materials and methods

1) Mutagenesis and enzyme preparation

Site-directed mutagenesis was performed by using the Transformer kit (Clontech Laboratories Inc., Mountain View, CA, USA) according to the manufacturer's instructions. The plasmid pSAOEP3[5] was used as the mutagenesis template, and *Escherichia coli* JM109 was used as a host strain. The F103Y mutant-producing strain was newly constructed in this study. Each recombinant strain producing wild-type or a mutant of SoxA was cultured, and was purified to homogeneity as reported previously[5-9].

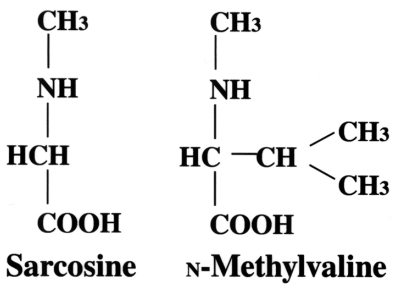

Fig. 1 Structural formulas for sarcosine and N-methylvaline

2) Enzyme assay

Compounds used as substrates were sarcosine and N-methylvaline (Fig. 1), which were purchased from Nacalai Tesque (Kyoto). The enzyme assay was based on the measurement of hydrogen peroxide produced during the oxidation of substrate. The 4-aminoantipyrine peroxidase system was used for the enzyme assay as described previously[5-10]. The assay mixture finally contained 100 mmol/L sarcosine or N-methylvaline, 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 at 500 nm against the blank by spectrophotometry. 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} (k_{cat}) values.

3) Molecular modeling

A three-dimensional model structure of SoxA was constructed by homology modeling based on the structure of SoxB (PDB ID: 1b3m), as previously described[11, 12]. The amino acid sequences were aligned (DDBJ accession numbers of SoxA and SoxB are AB007122 and D16521, respectively), and the structure was built by automatic segment matching method using the software MOE (Chemical Computing Group Inc., Montreal, Quebec, Canada). Energy minimization calculations were conducted

using the steepest descent method followed by the conjugated graduent routine using the software MOE. The enzyme-inhibitor complexes were also constructed by modeling based on the structural data of SoxB (PDB ID: 1el5). The program Pymol was used for molecular visualization.

4) Molecular docking

Molecular docking studies were performed using the software Autodock ver. 4.2[13] on the basis of a grid-based docking procedure. The ligand structures were obtained from Protein Data Bank (sarcosine; PDB ID: 3qse) and PubChem database (N-methyl-L-valine). For the ligands, Gasteiger charges were calculated using the software Autodock Tools. The enzyme model obtained from the homology modeling was prepared with Autodock Tools, deleting all water molecules, adding polar hydrogens, and loading charges. The hydrogen atoms were added and optimized from the software Reduce[14]. The program AutoGrid settings with 30 × 30 × 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 by Lamarckian genetic algorithm. The best docked conformers with the lowest free energies conformations were taken for discussion.

3. Results and discussion

The four SoxA mutants investigated were F103L, F103Y, H348A, and H348K. F103L and F103Y had an amino acid substitution of phenylalanine at position 103 with leucine and tyrosine, respectively. H348A and H348K had an amino acid substitution of histidine at position 348 with alanine and lysine, respectively. The K_m and k_{cat} values of wild-type and mutant SoxAs for substrates were estimated from Lineweaver-Burk plots (Table 1). The K_m value of the wild type for N-methylvaline was approximately 31 times higher than that for sarcosine. In contrast, F103L shows quite a different tendency. The K_m of F103L for N-methylvaline is much smaller than that of the wild type. The K_m for sarcosine is, however, increased remarkably. It appears that the binding affinity is much higher for the larger than for the smaller substrate. As a result, the substrate specificity of F103L was considerably changed from that of the wild type. The catalytic efficiency (k_{cat}/K_m) for N-methylvaline is approximately 64 times higher than that of the wild type because of a corresponding decrease in the K_m, although the catalytic efficiency for sarcosine is approximately 1/24th that of the wild type. F103Y also shows the same tendency as F103L, except that the catalytic efficiency for N-methylvaline is much lower than that of F103L. The amino acid residue at position 103 influences the SoxA-substrate interaction.

The K_m value of H348A for sarcosine was approximately 56 times higher than that of the wild type (Table 1). The catalytic efficiency of H348A was 1/60th that of the wild type due to the lower value of the binding affinity ($1/K_m$). Moreover, H348K had no activity, although its FAD-binding structure was normal as described previously[9]. The residue at

Table 1 Kinetic parameters of wild type and its mutants

Enzyme	Sarcosine (N-Methylglycine)			N-Methylvaline		
	K_m (mmol/L)	k_{cat} (s^{-1})	k_{cat}/K_m ($L \cdot mmol^{-1}s^{-1}$)	K_m (mmol/L)	k_{cat} (s^{-1})	k_{cat}/K_m ($L\text{-}mmol^{-1}s^{-1}$)
Wild type	3.6	14	3.9	110	7.9	0.072
F103L	62	9.9	0.16	1.2	5.5	4.6
F103Y	41	11	0.27	5.0	2.9	0.58
H348A	200	13	0.065		NT	
H348K		ND			NT	

ND: enzyme activity was not detected.

NT: enzyme activity was not tested.

position 348 also influences the SoxA-substrate interaction.

The effects of F103 and H348 on the substrate affinities and specificities were studied by using the structure models constructed by homology modeling (Figs. 2, 3). It was shown that the F103 residue of SoxA could interact with M248 (methionine at position 248), which directly exerted an influence on the substrate (Fig. 2). The side chain of F103 was close to that of M248, and the shortest distance was estimated to be 3.7Å. When F103 was substituted with leucine, the distance between L103 and M248 was extended as far as 7.7Å (Fig. 2). The side chain of M248 in F103L was expected to be more flexible than that in the wild type, and thus F103L was suitable for the larger substrate. The side chain of Y103 might forcibly move that of M248, since the distance to M248 was reduced by the F-to-Y substitution at

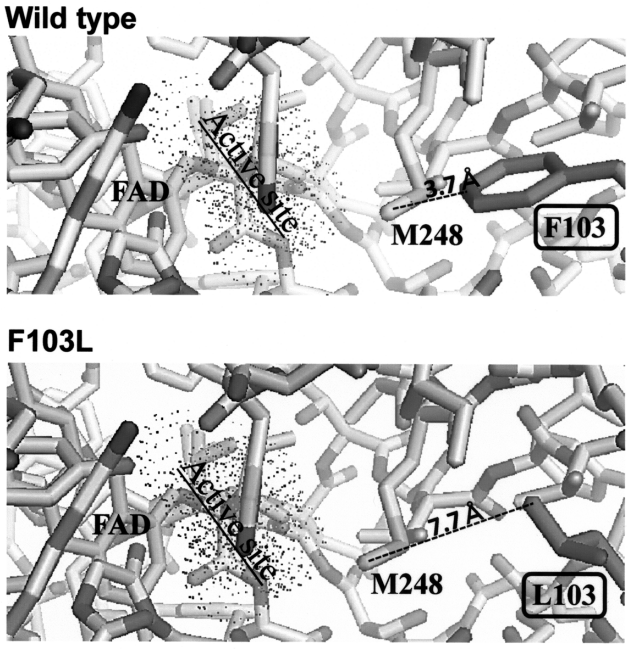

Fig. 2 Localized SoxA structure of wild type or F103L around the active site. Each structure was constructed by molecular modeling, as described in the materials and methods section. Amino acid residues and the coenzyme, flavin adenine dinucleotide (FAD), are shown by stick drawings. Dots of the active site represents the occupied space of a substrate analog, dimethylglycine (DMG).

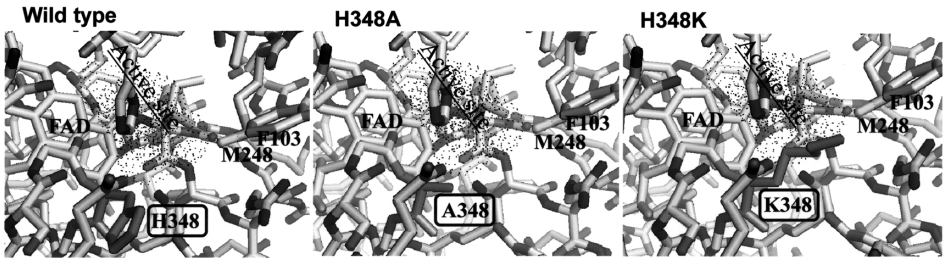

Fig. 3 Localized SoxA structure of wild type, H348A, or H348K around the active site. Each structure was constructed by molecular modeling, as described in the materials and methods section. Amino acid residues and FAD are shown by stick drawings. Dots of the active site represents the occupied space of DMG.

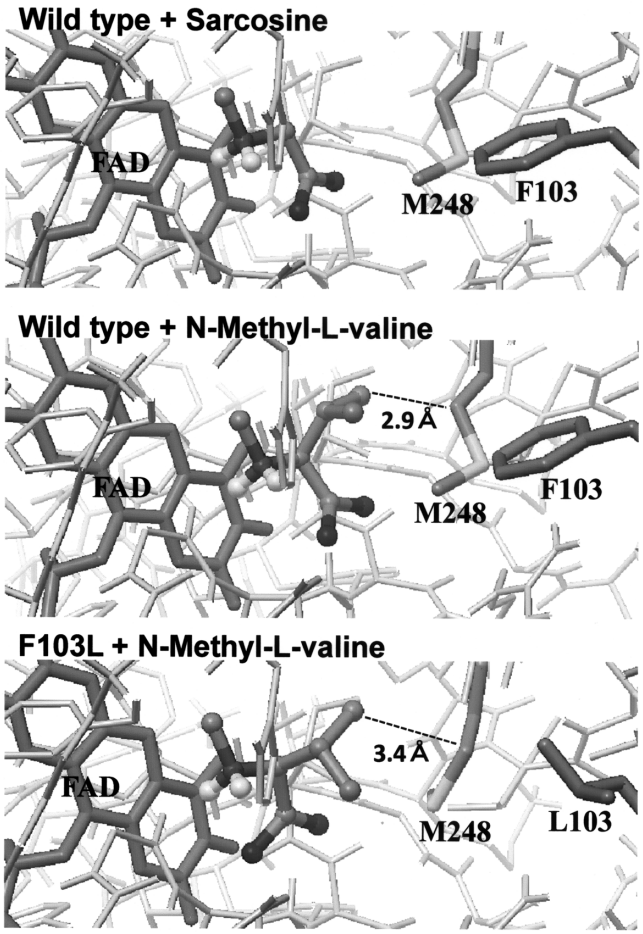

Fig. 4 Localized SoxA structure of wild type or F103L around each substrate. The enzyme-substrate complexes were constructed by molecular docking, as described in the materials and methods section. The substrates, sarcosine and N-methyl-L-valine, are represented by ball and sticks. Amino acid residues and FAD are shown by stick drawings.

position 103. The substrate specificities of the F103L and F103Y mutants demonstrated the relationship between F103 and M248.

On the other hand, H348 located around the active site and the side chain formed a part of the substrate-binding space (Fig. 3). The K_m value of H348A for sarcosine was markedly elevated because of the larger active site space. The side chain of H348K was most likely to inhibit the substrate binding.

For the purpose of discussing the interaction between SoxA and each substrate, molecular docking models were constructed using the software Autodock (Fig. 4). 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 the docking models also help to enhance our understanding of the enzyme-substrate interactions. It was expected that a molecular docking study would be useful for understanding enzyme reactions in the enzymatic assay field.

The binding configuration of SoxA-sarcosine predicted from molecular docking (Fig. 4A) was almost the same as that of SoxB-sarcosine in the previous report[10]. The configuration of N-methyl-L-

valine in the active site of SoxA (Fig. 4B), particularly that of carboxylate, was also close to that of sarcosine. The binding energy scores of sarcosine and N-methyl-L-valine were -2.9 and -3.2 kcal/mol, respectively. The energy scores were similar to each other. Hence, the Autodock prediction indicated that N-methyl-L-valine was able to effectively bind to SoxA. The binding energy of N-methyl-L-valine was the same level as that of sarcosine, whereas the binding affinity was much lower (Table 1). This finding suggests that the formation frequency of the enzyme-substrate complex might be extremely low. In fact, the distance of N-methyl-L-valine to the side chain of M248 was too close to yield stable formations, as compared with that of sarcosine (Fig. 4A, B).

The binding configuration of F103L with N-methyl-L-valine was also predicted from molecular docking, in which M248 was designated as a residue of the flexible side chain, and its conformational search was performed with a grid spacing of 0.247 Å (Fig. 4C). The side chain of M248 underwent a conformational change and, as a result, the distance of N-methyl-L-valine to the side chain of M248 increased (Fig. 4B, C). The binding energy score of F103L and N-methyl-L-valine was -3.7 kcal/mol. Since the side chain of M248 can be moved more easily by the F-to-L mutation at position 103, it is most likely that the reactable form between F103L and N-methyl-L-valine can be more stable in the active site.

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