〈Original Article〉

Alteration of L-proline oxidase activity of sarcosine oxidase and a structural interpretation

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Summary Sarcosine oxidase, an enzyme used for creatinine determination, acts slightly on L-proline, which may interfere with the assay. The sarcosine oxidase gene was randomly mutated for the purpose of altering L-proline oxidase activity. Several mutants showing increased or decreased L-proline oxidase activity were isolated, and their amino acid substitutions were identified. Two of these mutants, V94G and K322R, were suitable for practical use, because they exhibited lower L-proline oxidase activities than the wild type without decreasing the sarcosine oxidase activities. Furthermore, the L-proline oxidase activity of a multiple mutant constructed by site-directed mutagenesis, V94G+K322R+Y224T+E250Q, was considerably changed from that of the wild type. The catalytic efficiency of the mutant for L-proline was approximately 1/8th that of the wild type, whereas that for sarcosine was at the same level. These mutational effects were discussed in terms of a three-dimensional structure model.

Key words: Mutagenesis, Protein engineering, Proline, Sarcosine, Creatinine

1. Introduction

Sarcosine oxidase (EC 1.5.3.1; sarcosine:oxygen oxidoreductase [demethylating]) is a flavoprotein that catalyses the oxidative demethylation of sarcosine to yield glycine, formaldehydethe, and hydrogen peroxide. This enzyme is involved in the bacterial metabolism of creatinine with the related enzymes, creatininase and creatinase[1-3)]. It is industrially important and is used with creatininase and creatinase for the enzymatic assay of creatinine in clinical settings[4, 5)]. Recently, the metabolite sarcosine has been identified as a potential urine-based biomarker for prostate cancer progression[6)]. Sarcosine oxidase might prove useful in the determination of prostate cancer.

We have previously screened a sarcosine oxidase from the genus *Arthrobacter* (SoxA) and cloned the *soxA* gene[7)]. We have also altered the substrate specificity and optimum pH of SoxA by random and site-directed mutagenesis[8)], and succeeded in active-site analysis and stabilization of this enzyme by the substitution of cysteine residues[9)]. The wild-type and stable

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mutant SoxAs are produced commercially and being used for application to diagnostic reagents.

The enzymes for diagnostic reagents must exhibit an extremely low reactivity to substrate analogs or derivatives that may occur in clinical samples and interfere with the assay. Proline, however, is known to react slightly with sarcosine oxidase[10] (Fig. 1). Therefore, improving the substrate specificity of sarcosine oxidase, especially for weakening L-proline oxidase activity, is desirable for minimizing interference with clinical assays.

Here, we report on the successful alteration of the L-proline oxidase activity of SoxA by means of random and site-directed mutagenesis techniques. We are able to weaken the L-proline oxidase activity of SoxA without decreasing sarcosine oxidase activity. We also describe the mutational effects on the basis of the tertiary structure model of SoxA previously constructed[11, 12]. These findings provide information for further improvements in the functionality of the enzyme.

2. Materials and methods

1. Culture conditions

Escherichia coli JM109 used as a host for the isolation and maintenance of clones was grown on L agar containing ampicillin (50 mg/L) for the selection of a plasmid carrier. Recombinant strains were grown in Terrific broth at 37℃.

2. Mutagenesis

Random mutagenesis of the *soxA* gene was carried out with error-prone PCR techniques as described previously[8]. The plasmid pSAOEP3 carrying the *soxA* gene[7] was used as the mutagenesis template, and successful mutations were identified by DNA sequencing. Site-directed mutagenesis was performed using the Transformer site-directed mutagenesis kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions.

3. Enzyme purification and analysis

Each recombinant strain was grown to the stationary phase and harvested by centrifugation. A crude extract was prepared by sonication of the cells. The enzyme was then purified to homogeneity as described previously[7-9]. The protein was analysed by the standard method using SDS-PAGE.

4. Assays of sarcosine and L-proline oxidase activities

The production of hydrogen peroxide was measured using the 4-aminoantipyrine peroxidase system[7-9]. An enzyme solution (0.05 ml) was incubated with a mixture (1.0 ml) of 95 mmol/L sarcosine or L-proline, 0.47 mmol/L 4-aminoantipyrine, 2.0 mmol/L phenol, 50 mmol/L Tris-HCl (pH 8.0), and 5U of horseradish peroxidase per ml at 37℃ for 10 minutes.

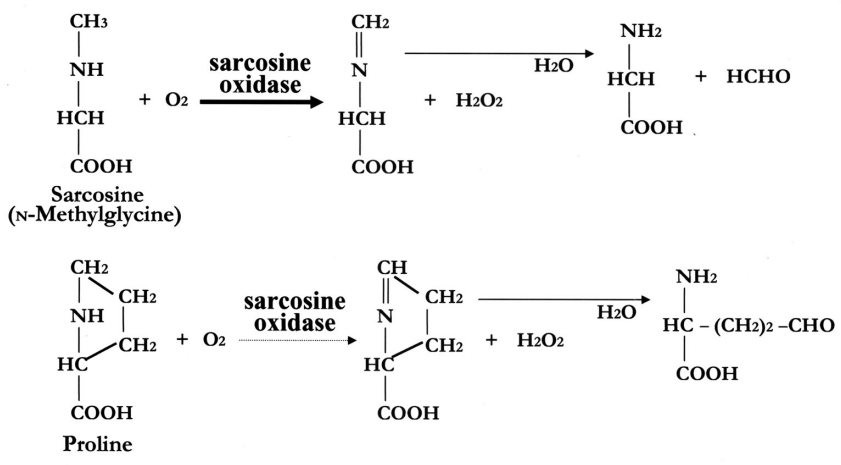

Fig. 1 Reactions of sarcosine oxidase to sarcosine and L-proline.

The reaction was stopped by adding 2.0 ml of 0.25% SDS solution, and the A_{500} was measured against the blank. One unit of activity was defined as the formation of 1 micromole of hydrogen peroxide per minute at 37℃ and pH 8.0. Reaction mixtures containing several concentrations of substrate solution were used to determine the K_m and k_{cat} values.

3. Results

1. Random mutagenesis and isolation of mutant SoxAs

Alteration of the L-proline oxidase activity of SoxA was achieved by means of phenotypic selection from recombinant cells carrying a mutagenized plasmid[8]. As a result, each two clones showing increased and decreased L-proline oxidase activities, respectively, were found among about 10,000 colonies. Recombinant plasmids were extracted from these clones, and the DNA sequences were determined. The L-proline oxidase activity-decreasing clones had Val94-Gly (codon: GTT→GGT) and Lys322-Arg (codon: AAA→AGA) mutations, respectively, and the mutant enzymes were designated as V94G and K322R. On the other hand, the L-proline oxidase activity-increasing clones had Asp234-Gly (codon: GAT→GGT) and Ile297-Thr (codon: ATT→ACT) in addition to Ala308-Val (codon: GCA→GTA), respectively, and the mutant enzymes were designated as D234G and I297T+A308V.

We also tried to re-mutagenize the V94G- and K322R-encoding genes, and obtained two clones showing further decreased L-proline oxidase activity. They had Glu250-Gln (codon: GAA→CAA) as well as Val94-Gly and Tyr224-Thr (codon: TAC→ACC) as well as Lys322-Arg, respectively, and the mutant enzymes were designated as V94G+E250Q and Y224T+K322R.

2. Purification and characterization of mutant SoxAs

Each recombinant strain producing the wild-type or mutant SoxA was cultured, and the sarcosine oxidase was purified to homogeneity. Upon SDS-PAGE analysis, each purified enzyme was seen to migrate as a single protein band (data not shown); the molecular mass of each enzyme was determined to be ~43 kDa.

The K_m and k_{cat} values of the purified wild-type and mutant SoxAs for sarcosine and L-proline were estimated from Lineweaver-Burk plots (Table 1). The K_m value of V94G for L-proline is approximately two times higher than that of the wild type. In contrast, the k_{cat} value of K322R for L-proline is approximately half that of the wild type. As a result, the catalytic efficiency (k_{cat}/K_m) of each mutant for L-proline is lower than that of the wild type due to a decrease in the binding affinity ($1/K_m$) or kcat. The K_m and kcat of both mutants for sarcosine were close to those of the wild type. Thus, the substrate specificities of V94G

Table 1 Kinetic parameters of wild-type and mutant SoxAs for sarcosine and L-proline

Enzyme	Substrate						
	Sarcosine			L-Proline			Ratio
	Km (mmol/L)	kcat (1/s)	kcat/Km [X]	Km (mmol/L)	kcat (1/s)	kcat/Km [Y]	[Y/X]
Wild type	3.6	14	3.9	110	0.28	2.5×10^{-3}	6.4×10^{-4}
V94G	4.4	14	3.2	210	0.22	1.0×10^{-3}	3.1×10^{-4}
K322R	4.6	14	3.0	140	0.15	1.1×10^{-3}	3.7×10^{-4}
V94G+E250Q	3.4	14	4.1	220	0.25	1.1×10^{-3}	2.7×10^{-4}
Y224T+K322R	3.8	15	3.9	130	0.14	1.1×10^{-3}	2.8×10^{-4}
2M (V94G+K322R)	5.7	14	2.5	180	0.11	6.2×10^{-4}	2.4×10^{-4}
4M (V94G+K322R +Y224T+E250Q)	3.4	15	4.4	200	0.073	3.7×10^{-4}	8.4×10^{-5}
D234G	4.9	2.7	0.55	70	0.17	2.5×10^{-3}	4.5×10^{-3}
I297T+A308V	9.1	9.6	1.1	130	0.48	3.7×10^{-3}	3.4×10^{-3}

and K322R were improved over that of the wild-type enzyme.

Regarding the catalytic efficiency for L-proline, two double mutants, V94G+E250Q and Y224T+K322R, were almost the same as V94G and K322R, respectively (Table 1). However, the catalytic efficiencies for sarcosine grew slightly by the mutational effects of E250Q and Y224T. Thus, the substrate specificities of V94G+E250Q and Y224T+K322R were improved over those of V94G and K322R.

The catalytic efficiency of D234G for L-proline was the same as that of the wild-type enzyme, though the catalytic efficiencies for sarcosine were quite different from each other (Table 1). Since the k_{cat} of D234G for sarcosine was much lower than that of the wild type, the L-proline oxidase activity of D234G seemed to be increased. In the case of I297T+A308V, the K_m for sarcosine was higher than that of the wild type. Moreover, the k_{cat} for L-proline was elevated, thus increasing the L-proline oxidase activity of I297T+A308V.

3. Construction, purification, and characterization of multiple mutants

As mentioned above, we were able to obtain four mutants, the substrate specificities of which were improved. They seemed to be suitable for practical use, given that their L-proline oxidase activities were obviously decreased and yet their kinetic parameters for sarcosine were close to those of the wild-type enzyme. To determine whether or not the mutational effects of these four mutants were additive, multiple mutants were constructed by site-directed mutagenesis. Two- and four-point mutants were designated as M2 (V94G+K322R) and M4 (V94G+K322R+Y224T +E250Q), respectively.

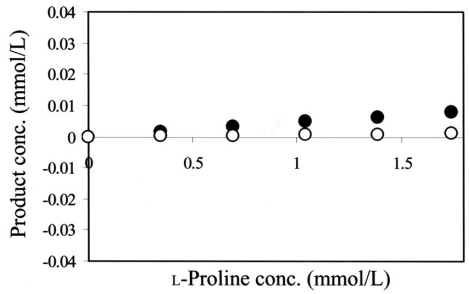

Fig. 2 Simulation of interferences of L-proline with enzyme reaction. It was assumed that the assay mixture contained 5.0 U/ml of enzyme and was incubated at 37℃ and pH 8.0 for 5 minutes. ●: Wild-type SoxA, ○: M4.

Each recombinant strain producing the multiple mutant SoxA was cultured, and sarcosine oxidase was purified to homogeneity. The molecular mass of each enzyme was the same as that of the wild type as determined by SDS-PAGE analysis.

The kinetic parameters of the purified multiple mutants for sarcosine and L-proline were estimated and compared with that of the wild-type SoxA (Table 1). As a result, the mutational effects were additive, since the reactivities of M2 and M4 for L-proline were decreased stepwise. In particular, the k_{cat} value of M4 for L-proline is approximately 1/4th that of the wild type, and the K_m of M4 is approximately two times higher than that of the wild type. The K_m and k_{cat} of M4 for sarcosine are almost the same as those of the wild type. Accordingly, the ratio of the catalytic efficiency for L-proline to that for sarcosine declined to about 1/8th that of the wild type. As a result, the substrate specificity of M4 was recognized to be highly desirable.

We simulated interference of L-proline to enzymatic creatinine assay using the wild-type SoxA or M4 (Fig. 2). The kinetic parameters of each enzyme for sarcosine and L-proline were used in the simulation. Calculated interferences reflected the reactivities of both enzymes for L-proline, i.e., the positive interference of M4 was markedly reduced compared with that of the wild-type enzyme.

4. Discussion

Sarcosine oxidase acts slightly on L-proline, which may interfere with a creatinine assay. By random and site-directed mutagenesis, we succeeded in creating an enzyme possessing improved substrate specificity. Several mutant SoxAs showing decreased L-proline oxidase activity were isolated. In particular, the L-proline oxidase activity of the multiple mutant M4 was

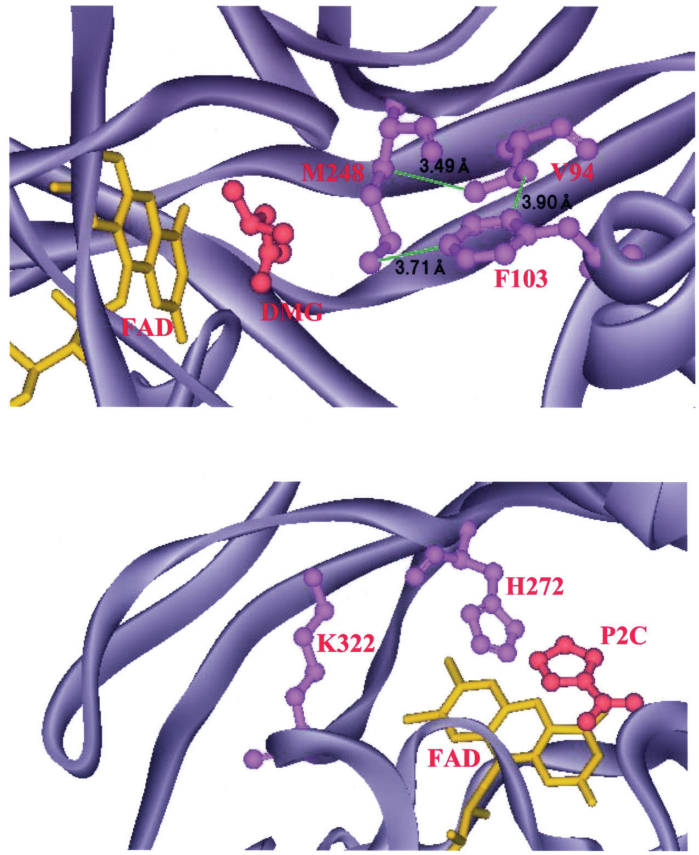

Fig. 3 Localized SoxA structure around each mutation. FAD is the coenzyme, flavin adenine dinucleotide. (A) View of residues around V94 with F103 and M248. DMG is the substrate analog, dimethylglycine. (B) View of residues around K322 and H272. P2C is the substrate analog, pyrrole-2-carboxylate.

considerably altered from that of the wild-type enzyme. The catalytic efficiency of M4 for L-proline was approximately 1/8th that of the wild type, whereas that for sarcosine was the same level as that of the wild type.

The influences of L-proline concentrations on the enzymatic assay were simulated as shown in Fig. 2. M4 was scarcely interfered with by L-proline, though the wild type was positively interfered with. M4 was successfully applied to the creatinine assay with creatininase and creatinase (data not shown).

The structural model helps to enhance our understanding of the enzyme-substrate interaction. The three-dimensional structure of sarcosine oxidase provided a reasonable starting point for analysing the mutational effects. We discussed several mutational effects in terms of the three-dimensional structure model of SoxA (Fig. 3).

The V94 residue did not directly interact with the substrate, but it lay on the scaffold region of M248, which was close to the side chain of the substrate (Fig. 3A). There has been a recent report about sarcosine oxidase and N-methyltryptophan oxidase to the effect that the side chain of M248 or its corresponding residue played an important role in the enzyme-substrate interaction. The V94G substitution extended the area around M248 and altered the hydrophobicity of the scaffold region. This would affect the location of M248 and change the substrate affinity of SoxA. We previously described the alter-

ation of the substrate specificity of SoxA[5]. Mutant enzymes, in which the Phe103 residue (F103) was replaced with Gly, Ala, Val, Leu, and Thr, respectively, exhibited a rather striking change in substrate specificity. For example, the catalytic efficiency of F103A for N-methylvaline is approximately 40 times higher than that of the wild type, whereas that for sarcosine is approximately 1/100th that of the wild type. The F103 residue was also close to both V94 and M248 (Fig. 3A). The region containing these three residues is most likely to be extremely important to the substrate specificity of sarcosine oxidase.

Although the K322 residue was at a distance from the substrate binding site, it was close to the H272 residue, which is located in the active center (Fig. 3B). H272 affects the reaction turnovers to sarcosine and L-proline, as previously described[13]. The side chain of R322 in K322R might indirectly weaken the L-proline oxidase activity of SoxA via its influence on H272.

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