<Brief Note>

Rational alteration of the pH profile of sarcosine oxidase by site-directed mutagenesis around the active site

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Summary Sarcosine oxidase (Sox) is widely used in creatinine assays to assess renal function. Additionally, Sox-based reagents and sensors are valuable for measuring sarcosine levels as a potential prostate cancer marker. We conducted structure-based rational alteration of the pH profile through site-directed mutagenesis of *Arthrobacter* Sox (SoxA) around the active site. Amino acid replacements for a positive charge (Thr272-to-Arg) and substrate pocket expansion (Met247-to-Ile) were designed for an optimum pH shift. Consequently, the pH profiles of the mutants shifted from 0.5 to more than 1.5 toward alkaline conditions, although the specific activities significantly decreased. Conversely, the mutant with a decreased positive charge (His271-to-Phe) lost its enzymatic activity owing to the change in interaction with the coenzyme.

Key words: Sarcosine oxidase, *Arthrobacter*, pH Profile, Mutagenesis, Active site

1. Introduction

Monomeric sarcosine oxidase (Sox; EC 1.5.3.1) is a flavoprotein that catalyzes the oxidative demethylation of sarcosine (N-methylglycine) to yield glycine, formaldehyde, and hydrogen peroxide[1]. It contains covalently bound flavin adenine dinucleotide (FAD) as a coenzyme. Sox is also involved in the bacterial metabolisms of compounds such as creatinine, creatine, and sarcosine[2]. Sox is widely used with creatininase and creatinase in enzymatic creatinine assays to evaluate renal function. For example, an enzymatic endpoint spectrophotometric assay using Sox was developed to accurately estimate serum and urine creatinine levels[3]. Recently, sarcosine was identified as a potential urine-based biomarker of prostate cancer progression[4]. Thus, reagents and sensors using Sox are also useful for measuring sarcosine levels as a potential prostate cancer marker[5].

We previously screened a monomeric Sox from *Arthrobacter* sp. TE1826 (SoxA)[6]. Recombinant SoxA can be easily purified from an *Escherichia coli* strain[6]. We also succeeded in altering the substrate affinity of the enzyme and stabilizing it using protein engineering techniques[7-11]. These wild-type and mutant SoxAs are commercially produced and used as diagnostic reagents[12-16]. Since the successful development of SoxA, various diagnostic enzymes have been altered by protein engineering and are now commercially available.

The practical use of enzymes is influenced by their optimum pH. Typically, matching the pH profiles of diagnostic enzymes present in the same

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reagent is desirable. For example, the optimum pH values for practical creatininase, creatinase, and SoxA are reportedly 6.5–7.5, 6.5–7.5, and 7.0–7.5, respectively (https://www.toyobo-global.com/seihin/xr/enzyme/enzyme_list/)[6]. Thus, improvements in the preferred pH profiles of diagnostic enzymes can increase their applicability.

In this study, an *in silico* analysis of the SoxA structure was performed to gain computational insights into how best to rationally alter its pH profile. Amino acid replacements were designed to achieve an optimum pH shift, and the pH profiles of these mutants were altered almost as anticipated. These findings provide information for further improvements in the functionality of enzymes used as diagnostic reagents.

2. Materials and Methods

In silico analysis

The SoxA tertiary structure model was previously constructed by homology modeling based on the structure of *Bacillus* Sox (PDB ID: 2GF3b), with high sequence identity (approximately 85%)[17-19]. No outliers were present in the Ramachandran plot of the models, indicating high-quality three-dimensional structures. Molecular Operating Environment software (Chemical Computing Group, Montreal, Canada) was used to generate mutant structures, docking simulations, interaction predictions, potential energy calculations, FAD atom charge determination, and molecular visualizations[19-21]. Energy minimization was applied for structural refinement. The force field Amber10:EHT was used to add hydrogen atoms and partial charges following the manufacturer's instructions (Chemical Computing Group). The overall SoxA structure was visualized using PyMOL software (https://www.pymol.org/).

Reagents and chemicals

All reagents and chemicals were purchased from either Nacalai Tesque, Inc. (Kyoto, Japan) or Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

SoxA expression and purification

An expression plasmid containing the SoxA-encoding gene (1,167 bp, DDBJ/EMBL/GenBank accession number: D63413) with a C-terminal 6-histidine tag was transformed into *Escherichia coli* JM109 cells and cultured as previously described[22]. The cells were collected and sonicated. The supernatant was subjected to HiTrap Q HP anion-exchange chromatography and His GraviTrap™ chelating columns (Cytiva Life Sciences, Marlborough, MA, USA) as described previously[22]. The protein solution was dialyzed against 20 mmol/L potassium phosphate buffer (pH 7.5) to remove NaCl and imidazole. Wild-type and mutant SoxAs were then purified to homogeneity. Protein concentrations were quantified using the Bradford assay (Takara Bio Inc., Shiga, Japan).

Site-directed mutagenesis

Expression plasmids for the mutants were prepared using the inverse polymerase chain reaction (PCR) with the enzyme solution KOD-plus (Toyobo Co., Ltd., Osaka, Japan) according to the manufacturer's instructions.

Sox activity assay

To assess Sox activity, the enzymatic production of hydrogen peroxide from dissolved oxygen was measured using the 4-aminoantipyrine peroxidase assay[6]. The enzyme solution (0.035 mL) was incubated with a mixture (1.0 mL) containing 100 mmol/L sarcosine, 0.49 mmol/L 4-aminoantipyrine, 2.1 mmol/L phenol, 50 mmol/L Tris-HCl (pH 8.0), and 5.0 U/mL of horseradish peroxidase at 37°C. The time-dependent absorbance increase at 500 nm was monitored against the blank for 5 min. One unit of each assay was defined as the amount of enzyme that catalyzed the oxidation of 1 μmol of substrate per minute at 37°C.

Spectral analyses

Spectrophotometric analyses were performed using a Hitachi U-3900 spectrophotometer (Hitachi Co., Ltd., Tokyo, Japan). The absorption spectra of 20 μmol/L wild-type and mutant SoxAs were measured at 300–600 nm.

3. Results and Discussion

Mutation designs for altering the pH profile

The previously constructed SoxA tertiary structure enhanced our understanding of the structure–function relationship. The first sphere of the enzyme contains residues that directly interact with the active site, whereas the second sphere contains residues that interact with the first sphere and indirectly affect the active site. Both first- and second-sphere residues influence the pH profile, making them targets for structure-based enzyme design (Fig. 1A). Single-point mutations of the first-sphere residues were structurally designed using integrated computational chemistry software to alter the pH profile (see Materials and Methods).

Three different mutations—threonine-272, histidine-271, and methionine-247, replacing arginine, phenylalanine, and isoleucine, respectively—were chosen to shift the pH profile to alkaline side. The Thr272Arg mutation increased the positive charge around the SoxA active site (Fig. 1A). In contrast, the His271Phe mutation decreased its positive charge (Fig. 1A), whereas the Met247Ile mutation expanded the active site (Fig. 1B).

These amino acid substitutions were expected to influence the interaction with the substrate, sarcosine, which changes from a zwitterionic ion to an anionic ion as the pH increases. The positive charge repulsion of the Thr272Arg mutant with sarcosine was predicted to be enhanced at neutral pH and reduced at alkaline pH owing to the decrease in positive charge (Fig. 2). Conversely, the repulsion of the His271Phe mutant with sarcosine was further reduced at neutral pH compared with that of the wild type. Furthermore, the sarcosine in the Met247Ile mutant was predicted to move by positive charge repulsion at neutral pH, resulting in inhibition of the catalytic reaction (Fig. 2).

pH profiles of the mutant SoxA enzymes

Each rationally designed mutant SoxA was overproduced by the recombinant *E. coli* strain and purified to homogeneity (see Materials and Methods). The activities of the Thr272Arg and Met247Ile

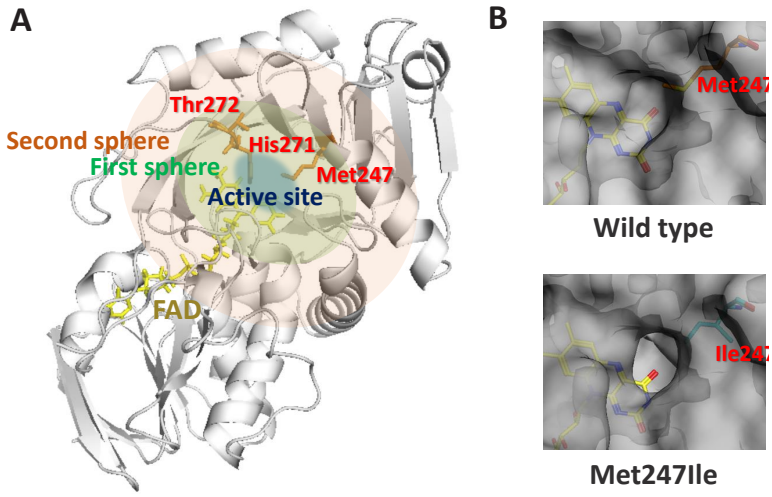

Fig. 1. *Arthrobacter* sarcosine oxidase (SoxA) structure and designed mutations. A. Overall SoxA structure and three mutated target residues (Met247, His271, and Thr272). The active site, first sphere, and second sphere are indicated using blue, green, and orange shaded areas, respectively. The yellow sticks represent FAD. B. Close-up views of the active sites using surface models of wild-type SoxA and the Met247Ile mutant. Carbon atoms of FAD, Met247, and Ile247 are shown by yellow, orange, and light blue, respectively. Nitrogen, oxygen, and sulfur atoms are shown by blue, red, and dark yellow, respectively.

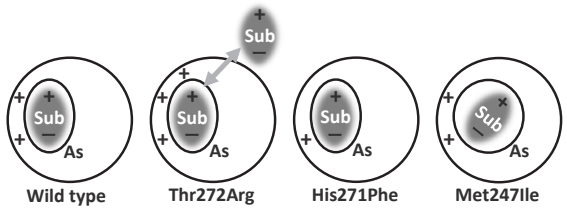

Fig. 2. Prediction of substrate binding for wild-type and mutant *Arthrobacter* sarcosine oxidase (SoxA) at neutral pH. As: active site of SoxA; Sub: substrate (sarcosine); +: positive charge; –: negative charge. Double-sided arrow indicates that the substrate is likely to leave the active site due to electrostatic repulsion.

mutants at pH 8.0 were estimated to be 0.011 and 0.88 U/mg, respectively. These values were much lower than those of wild-type SoxA (17 U/mg). No activity was detected with the His271Phe mutation.

Concordantly, the pH profiles of the Thr272Arg and Met247Ile mutants shifted by approximately 1.0 and above 1.5, respectively, toward the alkaline side (Fig. 3). In addition to the structurally predicted mutational effects, the calculated atomic charges of FAD were altered by these amino acid substitutions. The C2 and C4 atoms of the isoalloxazine rings of FAD in Thr272Arg and Met247Ile indicated a decrease in positive charge at pH 7.0, and the N3 atoms showed an increased negative charge (data not shown).

The reaction mechanism of Sox was previously elucidated using fragment molecular orbitals and mixed quantum mechanics/molecular mechanics methods, labeled the hydrogen-atom-coupled electron-transfer (HACET) mechanism[23]. The charge on the isoalloxazine ring plays a vital role in this mechanism. Specifically, the C2, N3, and C4 atoms in the isoalloxazine rings are close to the carboxyl group of the binding substrate in the active site; thus, the low activities of the Thr272Arg and Met247Ile mutants may be caused by charge fluctuations in the negative direction.

Effects of the His271Phe mutation

In our previous study, the importance of the covalent attachment between the Cys317 residue of SoxA and the isoalloxazine ring of FAD for catalytic function was elucidated[15,19]. We also demonstrated from molecular dynamics simulations that mutations in the Gly12-to-Gly17 motif, located in the amino-terminal region of SoxA, increased the potential energy of the SoxA–FAD interaction, resulting in markedly reduced activity[9,19].

In the current study, an *in silico* structure analysis of the His271Phe mutant was performed. The apo-forms of the wild-type and His271Phe enzymes were covalently attached to FAD at Cys317, and both structures were optimized by docking simulation. The

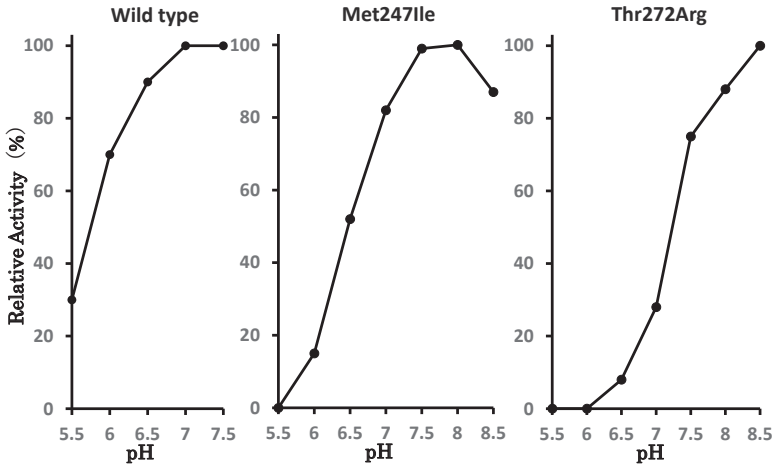

Fig. 3. pH profiles of wild-type and mutant strains of *Arthrobacter* sarcosine oxidase (SoxA). The SoxA activity of each strain was assayed using 50 mmol/L potassium phosphate buffer at pHs 5.5-8.5, instead of 50 mmol/L Tris-HCl buffer, pH 8.0.

FAD conformation of the simulated wild-type structure was similar to that of the SoxA tertiary structure. In contrast, the FAD of the simulated His271Phe structure indicated altered conformations of the diphosphate and ribityl side chains (Fig. 4). This suggests that the His271Phe enzyme is incapable of maintaining normal interactions with FAD, consistent with previous studies[9,19]. Notably, the absorption spectra of the wild-type and His271Phe enzymes were quite different (Fig. 5). The two absorption peaks derived from FAD were not observed in the spectrum of the His271Phe enzyme. The lack of activity with the His271Phe enzyme may be because it could not maintain FAD binding owing to the mutational effect.

Collectively, these computational insights provide an improved understanding of applying SoxA to various enzymatic assays.

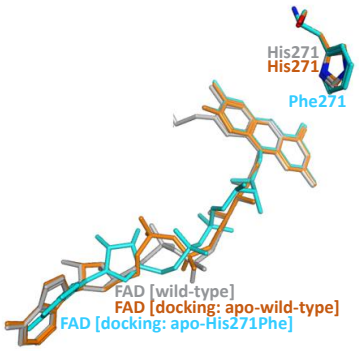

Fig. 4. Wild-type and His271Phe FAD conformations of *Arthrobacter* sarcosine oxidase (SoxA). His271 and FAD of the SoxA structure are shown as gray sticks. Structures constructed via the apo-SoxA-FAD docking simulation are represented using orange sticks. Phe271 and FAD of the structure, constructed using the apo-His271Phe docking simulation, are shown as cyan sticks.

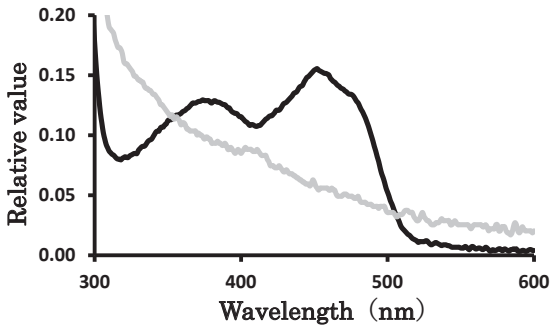

Fig. 5. Absorption spectra of wild-type and His271Phe *Arthrobacter* sarcosine oxidase (SoxA). Black and gray represent the absorption spectra of wild-type and His271Phe SoxA, respectively.

Conflicts of interest

The authors have no conflicts of interest.

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