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Structural and catalytic alteration of sarcosine oxidase through reconstruction with coenzyme-like ligands

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ABSTRACT

A sarcosine oxidase (SOX) gene from *Bacillus* sp. (AY626822.2) was expressed in *Escherichia coli* BL21 (DE3) in the form of inclusion bodies. A 3D model of SOX was then built and refined, and molecular docking was used to investigate the interactions between SOX and natural or coenzyme-like ligands, including flavin adenine dinucleotide (FAD); flavin mononucleotide (FMN); riboflavin; isoalloxazine; 7-methyl-8-chloro-10-(1′-D-ribityl) isoalloxazine (7-M-8-C); 7-bromo-8-methyl-10-(1′-D-ribityl) isoalloxazine (7-B-8-M); 7-methyl-8-bromo-10-(1′-D-ribityl) isoalloxazine (7-M-8-B); 7-chloro-8-ethyl-10-(1′-D-ribityl) isoalloxazine (7-C-8-E); 7,8-diethyl-10-(1′-D-ribityl) isoalloxazine (7,8-D); and 3-methyl-7,8-dimethyl-10-(1′-D-ribityl) isoalloxazine (3-M-7,8-D). Unfolded SOX was extracted from inclusion bodies, and reconstructed with these ligands *via* a refolding process. The reconstructed enzymes were then subjected to structural and catalytic analysis. After structural simulation, refinement, and molecular docking, all ligands were able to recognize the coenzyme site of SOX. In addition, when the position 7- or 8-site of the compounds was modified, new pi-cation/sigma interactions were formed in the SOX-ligand complex. Fluorescent detection revealed that all the ligands could be successfully reconstructed with unfolded SOX. Circular dichroism (CD) spectra and nano differential scanning calorimetry (DSC) analysis indicated that the loss of phosphoric acid and adeninein natural coenzymes could significantly reduce the α-helix content, transition temperature (T_m), and calorimetric enthalpy (ΔH). In addition, although reconstruction with the position 7- or 8-site modified compounds led to variations in secondary structure, no significant shifts in T_m and ΔH were observed. Furthermore, in the evaluation of catalytic kinetic parameters, when SOX was reconstructed with ligands containing halogen atoms at the 7- or 8-sites, much higher relative specificities in the presence of organic solvents were noted.

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1. Introduction

Sarcosine oxidase (SOX, EC. 1.5.3.1) is a typical flavoenzyme that catalyzes the oxidative demethylation of sarcosine (N-methylglycine). SOX could be produced by different microorganisms, including *Bacillus* [1], *Corynebacterium* [2], *Streptomyces* [3], *Arthrobacter* [4], and *Pseudomonas* [5]. In our previous research, a mono-sarcosine oxidase (SOX) gene from *Bacillus* sp. (AY626822.2) had been successfully expressed and purified in *Escherichia coli*, and a poly-lysine covalent modification protocol was developed for the promotion of thermo- and pH-stability [6,7]. Recently, SOX has been widely used for the determination of creatinine levels in clinical tests together with creatininase and creatinase [8]. In addition, the SOX composite screen-printed electrode has been developed for the early prediction of cancer development [9]. Furthermore, in the food industry, biosensors and probes based on SOX have been employed in the determination of organic acids and glycerol in wine fermentation [10,11].

According to their subunits, SOX could be arranged into mono- and hetero-tetrameric forms, and their coenzyme domains could be covalently or non-covalently bound with flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN) [12,13]. In our previous studies, mono-SOX from *Bacillus* sp. was confirmed as a FAD non-covalent binding enzyme [6,7]. For practical applications, it is always a challenge to modify the enzyme to enhance its

Abbreviations: CD, circular dichroism; CV, column volume; DSC, differential scanning calorimetry; FAD, flavinadenine dinucleotide; FMN, flavin mononucleotide; RAMD, random acceleration molecular dynamics; SOX, sarcosine oxidase; TCA, trichloroacetic acid; 7-M-8-C, 7-methyl-8-chloro-10-(1′-D-ribityl)isoalloxazine; 7-B-8-M, 7-bromo-8-methyl-10-(1′-D-ribityl)isoalloxazine; 7-M-8-B, 7-methyl-8-bromo-10-(1′-D-ribityl)isoalloxazine; 7-C-8-E, 7-chloro-8-ethyl-10-(1′-D-ribityl)isoalloxazine; 7,8-D, 7,8-diethyl-10-(1′-D-ribityl)isoalloxazine; 3-M-7,8-D, 3-methyl-7,8-dimethyl-10-(1′-D-ribityl)isoalloxazine.

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properties, owing to factors such as, elevations in temperature, pH fluctuations, or the presence of organic solvents [14,15]. In previous reports, to investigate the structure and function of SOX, most studies have been focused on the amino acid residues at the oxygen activation site, *e.g.*, mutations on Lys 265 and Arg 49 [12,13,16]. In addition, random acceleration molecular dynamics (RAMD) simulations were used to investigate the catalytic mechanism of SOX [17].

As previously reported, coenzymes could play vital roles in the native structure and activity of flavoenzymes, and the loss of natural coenzymes usually leads to misfolding and inactivation. In previous studies, deflavination and reconstitution protocols have been used for other flavoenzymes [18]. For example, a 5-deazaFAD–reconstituted dihydroxyacetone phosphatesynthase exhibited only 3–5% native activity [19]; when FMN was replaced by FAD, an azoreductase (*Bacillus* sp.) revealed approximately 60% native activity [20]; and following reconstruction with 8 Cl-FAD, the activity of acholesterol oxidase (*Brevibacterium sterolicum*) increased up to 3.5-fold [21]. However, alteration of SOX, based on coenzyme-like ligands has been seldom explored and reported.

In the present study, a homologous model of SOX from *Bacillus* sp. was simulated. A group of compounds was used as coenzyme-ligands for this model, and was further tested using structural and catalytic assays.

2. Experimental

2.1. Materials

The mono-SOX gene from *Bacillus* sp. (AY626822.2) was maintained in our laboratory. Reduced L-glutathione (GSH), oxidized L-glutathione (GSSG), FAD, FMN, riboflavin, isoalloxazine, 7-methyl-8-chloro-10-(1′-D-ribityl) isoalloxazine (7-M-8-C), 7-bromo-8-methyl-10-(1′-D-ribityl) isoalloxazine (7-B-8-M), 7-methyl-8-bromo-10-(1′-D-ribityl) isoalloxazine (7-M-8-B), 7-chloro-8-ethyl-10-(1′-D-ribityl) isoalloxazine (7-C-8-E), 7,8-diethyl-10-(1′-D-ribityl) isoalloxazine (7,8-D), and 3-methyl-7,8-dimethyl-10-(1′-D-ribityl) isoalloxazine (3-M-7,8-D) were purchased from Sigma-Aldrich (USA).

All other chemicals were of analytical reagent grade and were obtained from local companies.

2.2. Simulation of SOX-ligand interaction

The amino acid sequence of the *Bacillus* sp. SOX gene (AY626822.2) was submitted to the SWISS-MODEL service (http://swissmodel.expasy.org/) [21–23]. After homologous alignment, several mono-sarcosine oxidase models were employed as templates (Protein Data Bank [PDB] ID: 1zov, 2a89, 3bhf, 3m13, 3m0o, and 1l9c). FAD was depleted after the model was built, and further energy minimization was performed using the Discovery Studio (DS) CHARMm program to remove geometric restraints. FMN, riboflavin, isoalloxazine, 7-M-8-C, 7-B-8-M, 7-M-8-B, 7-C-8-E, 7,8-D, and 3-M-7,8-D were used as ligands (Fig. 1), and the 2D planar structures were rapidly translated into sensible 3D structures by the 3D optimization algorithm in the ChemBio3D program. The 3D structure of SOX and ligands were submitted to the SwissDock service (http://www.swissdock.ch/).

The homologous model of SOX and the ligands under investigation were refined and minimized to a low energy state using the CHARMm force field implemented in the DS 2.5 program. The convergence gradient was set to 0.01 kcal/mol, and 10,000 steps of the steepest descent algorithm were performed following 50,000 steps of the conjugate gradient algorithm. A spherical cut-off of 14 Å was used for non-bonding interactions, and the other parameters were set to their default values. To validate the docking protocol, FAD was docked in the coenzyme-binding site, and the results were compared with those of other ligands. The LigandFit module in the DS 2.5 program was used for the docking process [24]. The successful poses were evaluated using a set of scoring functions, such as LigScore1, LigScore2, PLP1, and PLP2 as implemented in the DS 2.5 program.

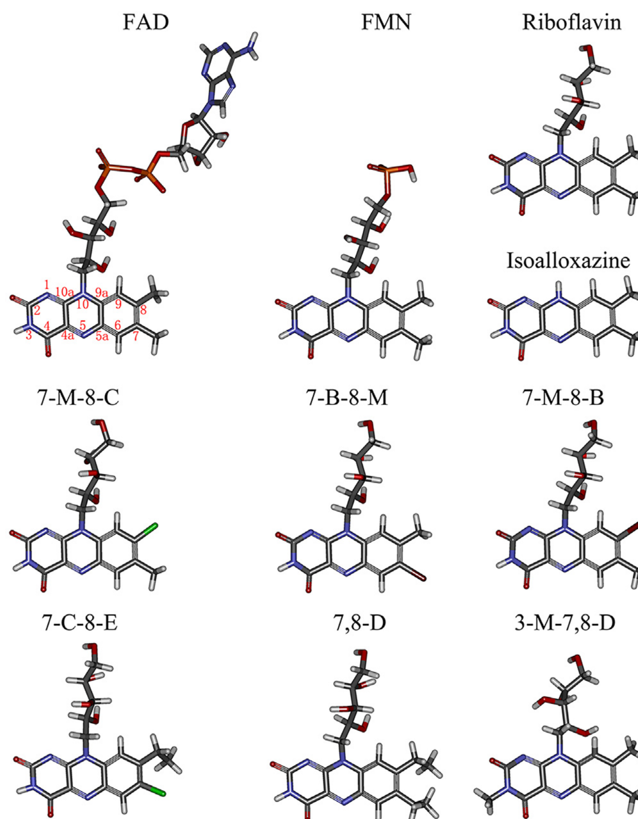

Fig. 1. Structure of coenzyme-like ligands.
The natural coenzyme and coenzyme-like ligands used in the present study: flavin adenine dinucleotide (FAD); flavin mononucleotide (FMN); riboflavin; isoalloxazine; 7-methyl-8-chloro-10-(1′-D-ribityl) isoalloxazine (7-M-8-C); 7-bromo-8-methyl-10-(1′-D-ribityl) isoalloxazine (7-B-8-M); 7-methyl-8-bromo-10-(1′-D-ribityl) isoalloxazine (7-M-8-B); 7-chloro-8-ethyl-10-(1′-D-ribityl) isoalloxazine (7-C-8-E); 7,8-diethyl-10-(1′-D-ribityl) isoalloxazine (7,8-D); and 3-methyl-7,8-dimethyl-10-(1′-D-ribityl) isoalloxazine (3-M-7,8-D).

2.3. Preparation of unfolded SOX

A pET28a (+)-sox-His-tag plasmid was built and transformed into *E. coli* strain BL21 (DE3), according to the methods outlined in our previous study [6]. *E. coli* cells were cultivated at 37 °C overnight in LB broth containing 100 μg/mL of kanamycin. The culture was then transferred into 100 mL of LB medium, with the same antibiotic concentration and cultivated at 37 °C until the OD_{600nm} was up to 0.6. The expression of SOX was induced with 50 g/L lactose for 8 h at 37 °C. The cells were harvested by centrifugation at 8000*g* for 30 min at 4 °C, further resuspended in lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Triton X-100, 5 mM EDTA, and 5 mM dithiothreitol (DTT), and then disrupted by sonication at 0 °C for100 cycles of 3 s with 6 s intervals. The mixture was incubated with 10 mM $MgSO_4$ at 25 °C for 20 min. The lysate was collected by centrifugation at 8000*g* for 30 min at 4 °C. In the supernatant, the soluble SOX was collected and further purified as native control. The inclusion body pellet was washed five times with lysis buffer and collected.

The inclusion body pellet was dissolved in a buffer solution (10 mM Tris with 8 M urea, pH 8.0) for 1 h at 4 °C while stirring.

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