〈Original Article〉

L-Thioproline: a new substrate for monomeric sarcosine oxidase selected by *in silico* analysis

Yoshiaki Nishiya[1], Chisa Yoshida[1] and Tasuku Okajima[1]

Summary Monomeric sarcosine oxidase (Sox) is used in clinical creatinine assays and serum creatinine concentration is an index of renal function. The assay couples creatininase, creatinase, and peroxidase. Wild-type Sox reacts slightly with L-proline and thus L-proline might interfere with the assay. Consequently, mutant enzymes with weakened oxidase activities towards L-proline have been developed using protein engineering techniques. Here, the affinities of Sox towards L-proline analogues and derivatives were investigated by tertiary structure comparisons and molecular docking simulations. L-Thioproline was predicted as a new substrate based on the results of *in silico* selection. As expected, Sox exhibited weak oxidase activity towards L-thioproline, with much lower K_m and k_{cat} values than those observed using sarcosine as the substrate. Typical substrate inhibition was observed at relatively high concentrations of L-thioproline. Spectral analyses under aerobic and anaerobic conditions showed that the first reductive half-reaction for L-thioproline occurs very slowly. A charge transfer Michaelis complex based on L-thioproline-enzyme interaction was clearly observed as a change in long-wavelength absorption. These results broaden our understanding of the reactivity and substrate specificity of Sox.

Key words: sarcosine oxidase, *in silico*, molecular docking, substrate affinity, substrate inhibition

1. Introduction

The concentration of creatinine in serum is an important index of renal function in clinical medicine due to its relationship to the glomerular filtration rate[1-3]. The spectrophotometric enzymatic endpoint assay is an accurate method for estimating serum creatinine concentration. Sarcosine oxidase (Sox, EC 1.5.3.1; sarcosine:oxygen oxidoreductase) is a monomeric or heterotetrameric flavoprotein that catalyzes the oxidative demethylation of sarcosine (N-methylglycine) to yield glycine, formaldehyde, and hydrogen peroxide[4-6]. The catalytic reaction of monomeric Sox, which contains flavin adenine dinucleotide (FAD) as the coenzyme, is a well-known

[1]Department of Life Science, Setsunan University, 17-8 Ikeda-Nakamachi, Neyagawa, Osaka 572-8508, Japan.

To whom correspondence should be addressed.
Dr. Yoshiaki Nishiya
Department of Life Science, Setsunan University, 17-8 Ikeda-Nakamachi, Neyagawa, Osaka 572-8508, Japan.
Fax: +81-72-838-6599, E-mail: nishiya@lif.setsunan.ac.jp

Received for Publication September 22, 2016
Accepted for Publication October 26, 2016

example of a typical ping-pong Bi-Bi mechanism consisting of the following anaerobic reductive half-reaction (1) and aerobic oxidative half-reaction (2):

(1) CH_3NHCH_2COOH + Sox-FAD
→ CH_2NCH_2COOH + Sox-$FADH_2$

(2) O_2 + Sox-$FADH_2$ → H_2O_2 + Sox-FAD

Sox is involved in the bacterial metabolism of creatinine, together with the related enzymes creatininase (EC 3.5.2.10) and creatinase (EC 3.5.3.3)[1-3]. Monomeric Sox is industrially important and is used with creatininase, creatinase, and horseradish peroxidase for the enzymatic assay of creatinine in clinical settings, and SoxA and SoxB from the genera *Arthrobacter* and *Bacillus*, respectively, are produced commercially as diagnostic reagents[4,5,7,8].

Enzymes used as diagnostic reagents must exhibit extremely low reactivity towards substrate analogues and derivatives that may be present in clinical samples and that could interfere with the assay. For example, L-proline reacts slightly with Sox[9]. It would therefore be advantageous to minimize interference in clinical assays by improving the substrate specificity of Sox, and especially to weaken its L-proline oxidase activity. We have used random and site-directed mutagenesis techniques to decrease the L-proline oxidase activity of Sox without decreasing its sarcosine oxidase activity[10], and one of these mutant enzymes is now produced commercially for use as a diagnostic reagent.

The X-ray crystallographic structures of SoxB and several mutants have been solved[11-15] and models of the enzyme-substrate complex can be constructed by computer-aided docking. We previously constructed SoxB-substrate docking models to understand how Sox reacts with both L- and D-substrates[16]. The resulting insights will help improve the functionality of the enzyme.

In this report, we investigated the affinities of SoxB toward L-proline analogues and derivatives using *in silico* techniques and selected L-thioproline as a new substrate based on our findings. It was previously experimentally demonstrated that Sox has weak L-thioproline oxidase activity. To our knowledge, this is the first example of using *in silico* analysis to select a new substrate for a diagnostic enzyme. By further developing this method for diagnostic reagents, several compounds that interfere with the assay can be easily predicted.

2. Materials and Methods

Materials

SoxB and SoxA used were from Asahi Kasei Pharma (Tokyo) and Toyobo (Osaka), respectively, as reported previously[16-19]. Other compounds were purchased from Nacalai Tesque (Kyoto) or Yashima Pure Chemicals (Osaka).

Enzyme assay and characterization

The enzyme assay is based on the measurement of hydrogen peroxide produced during substrate oxidation. A 4-aminoantipyrine peroxidase system was used for the enzyme assay, as described previously[4]. The final assay mixture contained 100 mmol/L sarcosine or an appropriate concentration of another substrate, 0.49 mmol/L 4-aminoantipyrine, 2.1 mmol/L phenol, 50 mmol/L Tris-HCl (pH 8.0), and 5 U/mL of horseradish peroxidase. Enzyme solution (35 μL) was incubated with the assay mixture (1000 μL) at 37°C, and the amount of quinoneimine dye formed by the coupling of 4-aminoantipyrine, phenol, and horseradish peroxidase was measured spectrophotometrically at 500 nm against a sample blank. One unit of activity was defined as the formation of 1 μmol of hydrogen peroxide (0.5 μmol of quinoneimine dye) per min at 37°C and pH 8.0. Reaction mixtures consisting of various concentrations of substrate solution were used to determine the K_m and k_{cat} values.

Spectral analysis

Spectrophotometric analyses were conducted using a Hitachi U-3900 spectrophotometer (Hitachi Co., Ltd., Tokyo). Anaerobic experiments were conducted in a total volume of 1000 μL as described by Wagner and Jorns[20]. The reaction mixtures incorporated an oxygen-scavenging system consisting of glucose oxidase (8.4 U/mL), glucose (8 mmol/L),

and catalase (220 U/mL).

Molecular docking

Molecular docking studies were performed using the software suite Autodock ver. 4.2[21] and a grid-based docking procedure was used. The ligand structures were obtained from the Protein Data Bank (sarcosine; PDB ID: 3qse, L-proline; PDB ID: 2eiw) and the PubChem database. Gasteiger charges for the ligands were calculated using Autodock Tools. The enzyme model obtained from the X-ray crystal structure (PDB ID: 1el5, resolution: 1.80 Å) was prepared with Autodock Tools by deleting all water molecules, adding polar hydrogens, and loading charges. The hydrogen atoms of the histidine residues were predicted using the software package Reduce[22]. AutoGrid settings with a 30 × 30 × 30 grid size and a grid spacing of 0.375 Å were used to prepare each grid, and the grid was localized at the active site of the respective enzyme-substrate complex. Five billion conformations were evaluated using the Lamarckian genetic algorithm. The program Pymol[23] was also used for molecular visualization and simulation of substrate docking by utilizing the pair-fitting function. The coordinates for sarcosine, L-proline, and L-thioproline were generated by superposing the positions of the Cα, methylamino, and carboxyl groups (total of 6 pairs) onto those of the substrate analogue dimethylglycine in the SoxB structure, and the root mean square deviations of the three substrates were 0.154 , 0.630, and 0.218 Å, respectively.

3. Results and Discussion

Molecular docking

Molecular docking studies enhance our understanding of enzyme-substrate interactions and thus are useful for better understanding enzymatic assays. Predictions made using Autodock previously indicated that L-proline can bind efficiently to Sox[16]. The calculated binding energy of L-proline (-6.0 kcal/mol) was similar to that of sarcosine (-4.9 kcal/mol), whereas the affinity of Sox for L-proline ($1/K_m$ value) was markedly lower than that of sarcosine. This suggests that the enzyme-L-proline complex might cycle between the binding form to the reactive form at an extremely low frequency; indeed, modeling showed that the predicted configuration of L-proline was clearly unreactive towards the flavin ring in Sox.

We screened various tertiary structures of L-proline analogues and derivatives by *in silico* analysis and several are shown in Figure 1(A). The Cα-N-C bond angles of L-proline, 3,4-dehydro-L-proline, trans-4-hydroxy-L-proline, and 3,4-epoxy-L-proline were quite different from that of sarcosine, whereas those of cis-3-hydroxy-L-proline and L-thioproline were almost the same as that of sarcosine. Therefore, of the candidate new substrates screened, L-thioproline was selected due to its availability and probable low steric interference with the catalytic site.

Molecular docking simulations of compounds with the SoxB structure were performed and compared, and the interactions of sarcosine, L-proline, and L-thioproline with the coenzyme FAD are shown in Figure 1(B). The docking study indicated that L-proline interacted quite differently with the Sox binding site compared with sarcosine. In particular, the distance between the C5 atom of L-proline and the N5 atom of the FAD was too large to allow effective electron transfer for the reductive half-reaction of Sox, suggesting that transformation to the reactive form and the reduction of FAD by L-proline are rate-limiting. Proline dehydrogenase (EC 1.5.99.8) is a flavoprotein that binds the substrate L-proline to the si-face of the flavin ring[24] whereas Sox binds the substrate to the opposite side (re-face) [Fig. 2 (3E2S)]. Another proline dehydrogenase bends the L-proline structure[25] for rapid reaction [Fig. 2 (3AXB)]. In contrast, the distances between L-thioproline (positions N and C5) and the FAD (positions C4a and N5) were similar to those of sarcosine, suggesting that Sox should have high affinity for the L-thioproline structure and thus should efficiently adopt the reactive form.

Reactivity toward L-thioproline

The activities of SoxB and SoxA were assayed

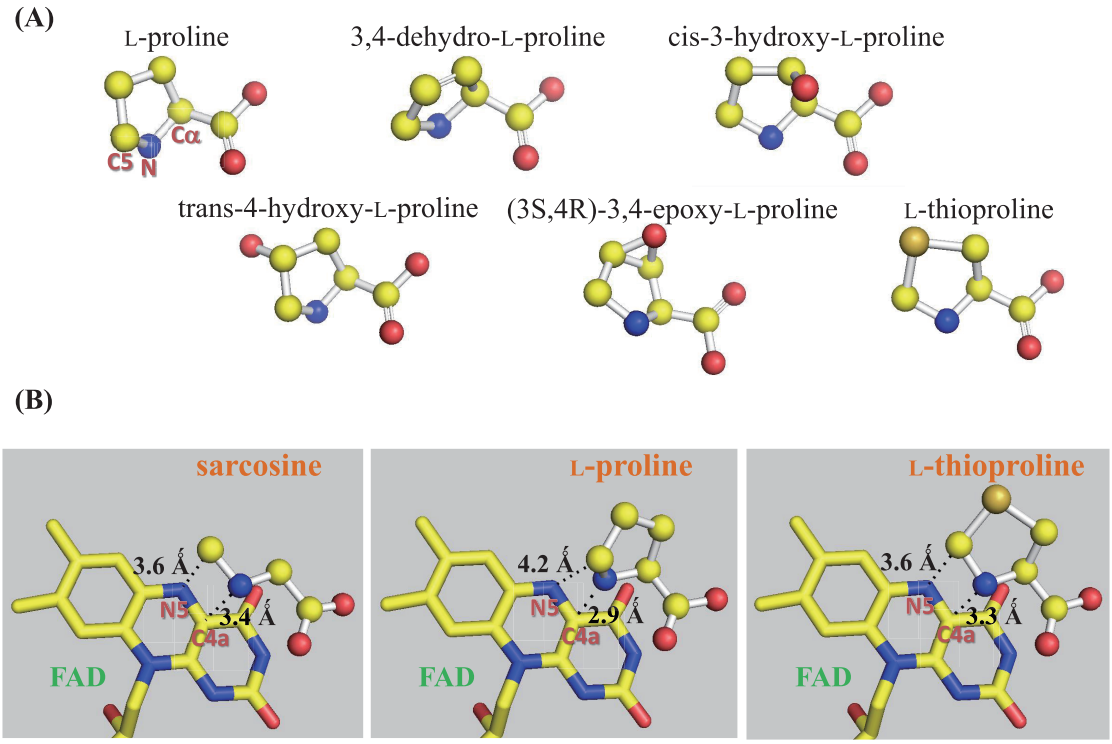

Fig. 1 Comparison of tertiary structures. (A) Structural comparison of L-proline analogues and derivatives. Each compound is represented by a ball-and-stick model. Carbons, oxygens, nitrogens, and sulfurs are in yellow, red, blue, and gold, respectively. (B) Molecular dockings of compounds with SoxB. Close-up views of the active site show the interactions with FAD. Compounds and FAD are shown by ball-and-stick models and stick drawings, respectively.

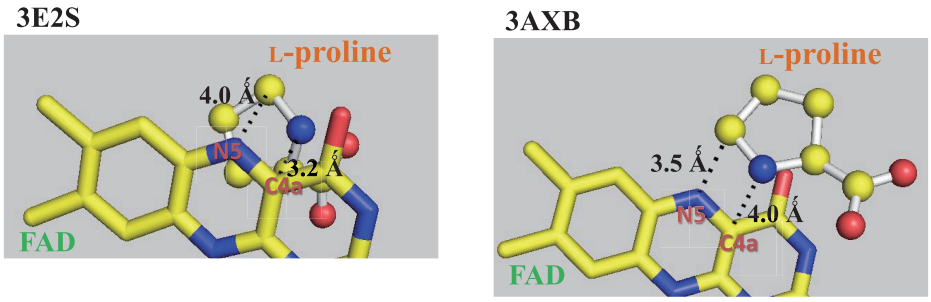

Fig. 2 Close-up views of the active sites of two L-proline dehydrogenases. L-Proline and FAD are shown by ball-and-stick models and stick drawings, respectively. Carbons, oxygens, and nitrogens are in yellow, red, and blue, respectively.

using various concentrations of L-thioproline; both Sox enzymes reacted with L-thioproline (Fig. 3), albeit extremely weakly compared to sarcosine. L-Thioproline is therefore a new substrate for monomeric Sox selected by *in silico* analysis. The rapid decrease in activity observed at relatively high concentrations of L-thioproline [Fig. 3(A)] indicates remarkable substrate inhibition of monomeric Sox by L-thioproline, unlike sarcosine and other conventional substrates. The dependence of the reaction rates on L-thioproline concentration was fitted to the substrate inhibition equation[26]. The K_m and K_i (inhibitory constant) values of SoxB for L-thioproline was estimated to be higher than those of SoxA, as is the case for the K_m values of the enzymes for sarcosine[4,5]. As shown in Figure 3(B), the profiles of the oxidase activities towards L-thioproline were dependent on the enzyme

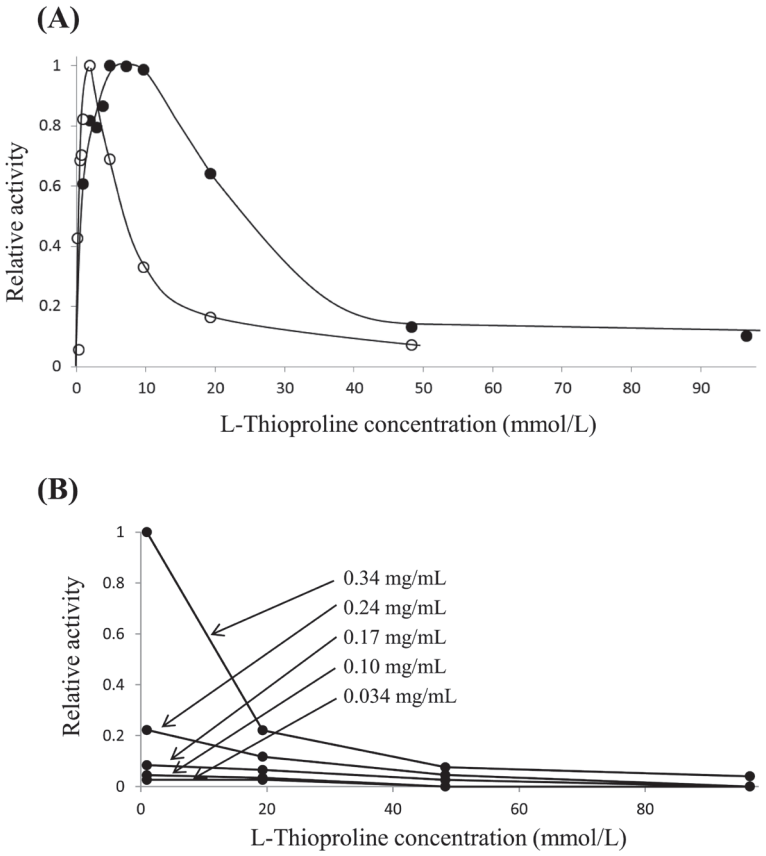

Fig. 3 Effects of L-thioproline concentrations on the Sox activities. (A) Activities of SoxA and SoxB with L-thioproline. Each enzyme solution of approximately 10 mg/mL was prepared by dilution with 20mmol/L potassium phosphate buffer (pH 7.5). Final concentrations of enzymes in assay mixtures were 0.34 mg/mL. The results of SoxA and SoxB were represented by open and closed circles, respectively. (B) The enzyme concentration-activity relationship. SoxB solutions of approximately 1.0-10 mg/mL, of which final concentrations in assay mixtures were 0.034-0.34 mg/mL, were used in measuring L-thioproline oxidase activities.

Table 1. Kinetic parameters of SoxB

Substrate	K_m (mmol/L)	K_i (mmol/L)	k_{cat} (s^{-1})	k_{cat}/K_m (%)
Sarcosine	17	-	21	100
L-Proline	240	-	0.15	5.1×10^{-2}
L-Thioproline	5.3	4.3	1.3×10^{-2}	0.25

concentration and the ratio of L-thioproline to enzyme, consistent with substrate inhibition.

The kinetic parameters of SoxB for L-thioproline were estimated from the substrate inhibition equation by nonlinear curve fitting using the Microsoft Excel Solver tool and compared with those for sarcosine and L-proline (Table 1). The K_m value for L-thioproline was approximately 3 and 45 times lower than that for sarcosine and L-proline, respectively. The catalytic efficiency (k_{cat}/K_m) for L-thioproline was remarkably smaller than that for sarcosine due to the extremely low value of k_{cat}.

Spectral analysis under aerobic conditions

Spectral analysis is a well-known technique to observe FAD and thus the spectral properties of SoxB were examined in the presence and absence of substrate to obtain information regarding enzyme-bound FAD.

SoxB exhibited an absorption spectrum characteristic of flavoprotein, with a peak at 374 nm and 454 nm, identical to those obtained using free FAD (Fig. 4). These peaks are considered to represent enzyme-bound oxidative FAD. Under aerobic conditions, the absorption spectrum of SoxB with and without L-proline was identical, whereas with sarcosine, the FAD-dependent peaks disappeared due to FAD being converted to the reductive form. Unlike L-proline and L-thioproline, sarcosine is rapidly oxidized by the anaerobic reductive half-reaction of SoxB. Dissolved oxygen required as a substrate for the second oxidative half-reaction would be limited due to the rapid first reductive half-reaction and thus the enzyme-bound FAD would be almost entirely in the reductive form. Accordingly, the concentration of dissolved oxygen should gradually increase to normal after the sarcosine is exhausted and the concentration of oxidative FAD would subsequently increase. Indeed, the spectrum with sarcosine measured 6 h after initiation of the reaction was essentially the same as that without substrate or with L-proline, as shown in Figure 4(B).

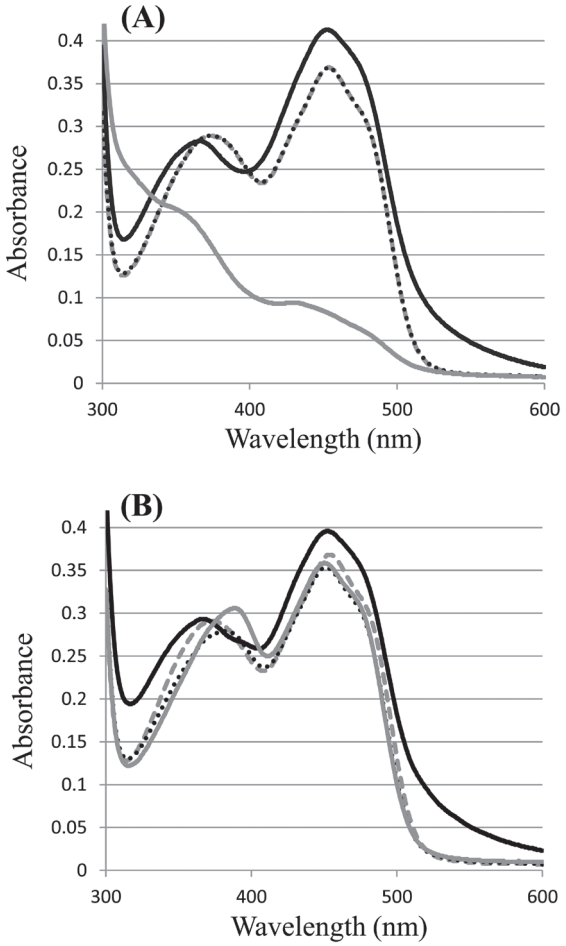

Fig. 4 Spectral profiles of Sox with substrates. Approximately 2.0 mg/mL (47 μmol/L) of SoxB enzyme and 8.0 mmol/L of substrate were used for each analysis. Reactions were conducted under aerobic conditions in 50 mmol/L Tris-HCl buffer (pH 8.0). Solid lines, dotted lines, and grey solid lines indicate the results obtained using L-thioproline, L-proline, and sarcosine, respectively. Grey dotted lines are the spectra of the uncomplexed enzyme. Curves were recorded immediately (A) and 6 h (B) after adding each substrate.

The spectrum obtained with L-thioproline differed considerably from the others: although two peaks were obtained, one was slightly shifted toward shorter wavelength, as shown in Figure 4(A). This difference in profile suggests that FAD binds with the enzyme under different conditions compared to in the presence of other substrates and may reflect changes in the SoxB-FAD interaction. Moreover, a dramatic increase in long-wavelength absorption (λ > 500 nm) was apparent in spectra obtained immediately and 6 h after mixing enzyme and substrate (Fig. 4). This is attributable to a charge transfer interaction between the oxidative FAD and L-thioproline, as previously described for enzyme-inhibitor complexes and rapid reaction kinetic studies under anaerobic conditions[20,27,28]. The turnover of L-thioproline is extremely slow: less than 4.5×10^{-4} and 6.3×10^{-2}% of the rates observed with sarcosine and L-proline, respectively (Table 1). Thus, the charge transfer Michaelis complex generated by L-thioproline could be monitored in manual mixing experiments (Fig. 4).

Spectral analysis under anaerobic conditions

To investigate the reductive half-reaction of Sox toward L-thioproline, spectral analysis under

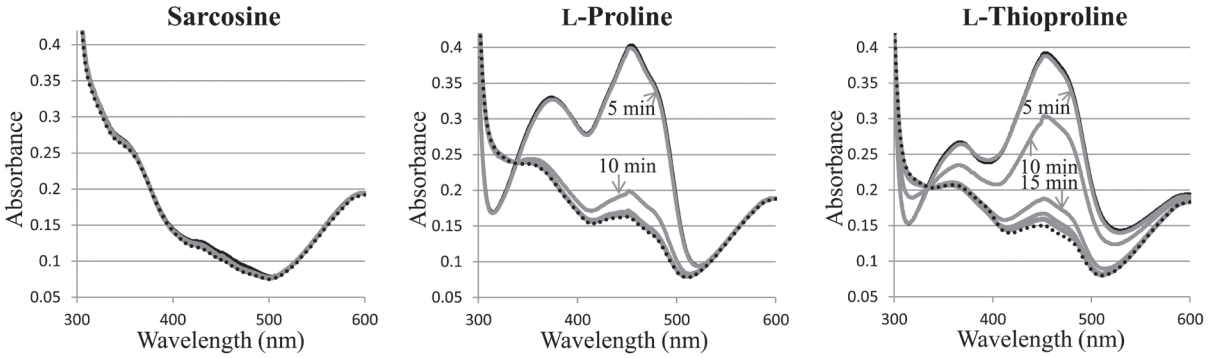

Fig. 5 Anaerobic reductions of Sox with various substrates. Approximately 47 μmol of enzyme and 8.0 mmol of substrate per L were used for the analyses. Reactions were conducted under anaerobic conditions in 50 mmol/L Tris-HCl buffer (pH 8.0). Curves were recorded immediately (solid lines), 5-30 min (grey lines), and 60 min (dotted lines) after adding each substrate.

anaerobic conditions (single turnover) was performed using the oxygen-scavenging system described in the Materials and Methods section, and the results were compared with those obtained using sarcosine and L-proline (Fig. 5). As expected, two peaks due to the oxidative FAD immediately disappeared from the spectrum of SoxB with sarcosine whereas these peaks disappeared 15 and 20 min after initiating the reaction with L-proline and L-thioproline, respectively, because of the slow reductive half-reaction rates. Long-wavelength absorption resulting from the charge transfer Michaelis complex was apparent only in spectra with L-thioproline and decreased as the reaction proceeded.

Comparison of reductive half-reactions

The above results supported the tentative reductive half-reactions of Sox toward sarcosine, L-proline, and L-thioproline shown in Figure 6. In contrast to the complexes formed with sarcosine and L-thioproline, the enzyme-substrate complex with L-proline suggested from docking simulations (Fig. 1) is transferred from the substrate-binding form ([ES]) to the reactive complex ([ES*]) at an extremely low frequency. Transfer from the [ES] to the enzyme-product complex ([EP]) with L-proline and L-thioproline is considerably slower than with sarcosine, as shown in the spectral analysis (Figs. 4 and 5). In particular, the [ES*] to [EP] reaction rate (k_2') of L-thioproline was predicted to be exceedingly low because, like sarcosine, the structural conversion of [ES] to [ES*] L-thioproline was straightfoward (Fig. 1). A delay in the formation of [EP] is likely related to the substrate inhibition of Sox by L-thioproline (Fig. 3). Final formation of the reductive enzyme and product is assumed to be a rate-limiting step dependent on the turnover number, which decreased markedly in the order sarcosine, L-proline, and L-thioproline (Table 1). Differences in the spectral profile between sarcosine and L-proline/L-thioproline around 450 nm (Fig. 5) might reflect the release of the final product from the enzyme.

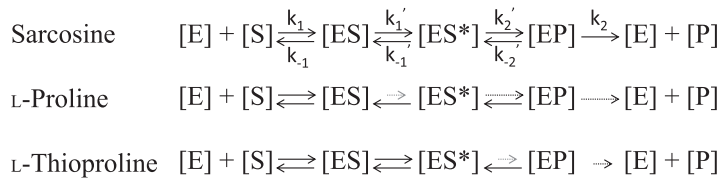

Fig. 6 Schemes of the anaerobic reductive half-reactions of Sox with sarcosine, L-proline, and L-thioproline.

The research described in this paper first used *in silico* analysis followed by experiments to understand the substrate affinity and reactivity of an enzyme used in diagnostic assays. The application of enzymes to diagnostics requires investigation of whether or not various compounds in specimens and reagents influence the enzyme reaction. In future, effective *in silico* analysis of enzyme-compound interactions should be useful in both practical and fundamental studies.

References

1. Nishiya Y: Application of protein–engineered oxidases to clinical enzymatic assays. Recent Res Devel Anal Biochem, Transworld Research Network, 135-151, 2002
2. Nishiya Y, Yamamoto K, Kawamura Y, and Emi S: Development of creatinine-degrading enzymes for application to clinical assays [Jpn]. Nippon Nogeikagaku Kaishi, 75: 857-862, 2001
3. Fossati P, Prencipe L, and Berti G: Enzymic creatinine assay: a new colorimetric method based on hydrogen peroxide measurement. Clin Chem, 29: 1494-1496, 1983
4. Nishiya Y and Imanaka T: Cloning and sequencing of the sarcosine oxidase gene from Arthrobacter sp. TE1826. J Ferment Bioeng, 75: 239-244, 1993
5. Suzuki K, Sagai H, Imamura S, and Sugiyama M: Cloning, sequencing, and overexpression in Escherichia coli of a sarcosine oxidase-encoding gene linked to the Bacillus creatinase gene. J Ferment Bioeng, 77: 231-234, 1994
6. Chlumsky LJ, Zhang L, Ramsey AJ, and Jorns MS: Preparation and properties of recombinant corynebacterial sarcosine oxidase: Evidence for posttranslational modification during turnover with sarcosine. Biochemisty, 32: 11132-11142, 1993
7. Nishiya Y, Zuihara S, and Imanaka T: Active site analysis and stabilization of sarcosine oxidase by the substitution of cysteine residues. Appl Environ Microbiol, 61: 367-370, 1995
8. Nishiya Y, Toda A, and Imanaka T: Gene cluster for creatinine degradation in Arthrobacter sp. TE1826. Mol Gen Genet, 257: 581-586, 1998
9. Ohsawa S, Muto T, Mamada K, Yoshida T, Iida S, and Yonemitsu H: Evaluation of an enzymatic reagent for the determination of creatinine [Jpn]. J Anal Bio-Sci, 17: 332-337, 1994
10. Nishiya Y and Kishimoto T: Alteration of L-proline oxidase activity of sarcosine oxidase and a structural interpretation. J Anal Bio-Sci, 33: 161-166, 2010
11. Trickey P, Wagner MA, Jorns MS, and Mathews FS: Monomeric sarcosine oxidase: Structure of a covalently flavinylated amine oxidizing enzyme. Structure, 7: 331-345, 1999
12. Zhao, G, Song H, Chen ZW, Mathews FS, and Jorns MS: Monomeric sarcosine oxidase: Role of histidine 269 in catalysis. Biochemistry, 41: 9751-9764, 2002
13. Chen Z, Zhao G, Martinovic S, Jorns MS, and Mathews FS: Structure of the sodium borohydride-reduced N-(cyclopropyl) glycine adduct of the flavoenzyme monomeric sarcosine oxidase. Biochemistry, 44: 15444–15450, 2005
14. Zhao G, Bruckner RC, and Jorns MS: Identification of the Oxygen Activation Site in Monomeric Sarcosine Oxidase: Role of Lys265 in Catalysis. Biochemistry, 47: 9124-9135, 2008
15. Jorns MS, Chen Z, and Mathews FS: Structural characterization of mutations at the oxygen activation site in monomeric sarcosine oxidase. Biochemistry, 49: 3631–3639, 2010
16. Nishiya Y, Nakano S, Kawamura K, and Abe Y: Monomeric sarcosine oxidase acts on both L- and D-substrates. J Anal Bio-Sci, 35: 426-430, 2012
17. Nishiya Y and Nakano S: Screening of enzyme stabilizers using thermal shift assays on the basis of structural informations. Int J Anal Bio-Sci, 2: 58-63, 2014
18. Nishiya Y: Altered substrate affinity of monomeric sarcosine oxidase by the mutation of phenylalanine-103 or histidine-348. Int J Anal Bio-Sci, 1: 21-26, 2013
19. Nishiya Y and Abe Y: Comparison of the substrate specificity of L-pipecolate oxidase and bacterial monomeric sarcosine oxidase, and structural interpretation of the enzymes. Int J Anal Bio-Sci, 3: 140-145, 2015
20. Wagner, MA and Jorns MS: Monomeric sarcosine oxidase: 2. kinetic studies with sarcosine, alternate substrates, and a substrate analogue. Biochemistry, 39: 8825–8829, 2000
21. Morris GM, Huey R, Lindstrom W, Sanner MF, Belew RK, Goodsell DS, and Olson AJ: AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. J Comput Chem, 30: 2785-2791, 2009
22. Word JM, Lovell SC, Richardson JS, and Richardson DC: Asparagine and glutamine: Using hydrogen atom contacts in the choice of side-chain amide orientation.

J Mol Biol 285: 1735-1747, 1999

23. Nishiya Y.: Structural comparison of creatinases for investigating substrate binding. Int J Anal Bio-Sci, 2: 143-147, 2014
24. Ostrander EL, Larson JD, Schuermann JP, and Tanner JJ: A conserved active site tyrosine residue of proline dehydrogenase helps enforce the preference for proline over hydroxyproline as the substrate. Biochemistry, 48: 951-959, 2009
25. Sakuraba H, Satomura T, Kawakami R, Kim K, Hara Y, Yoneda K, and Ohshima T: Crystal structure of novel dye-linked L-proline dehydrogenase from hyperthermophilic archaeon Aeropyrum pernix. J Biol Chem, 287: 20070-20080, 2012
26. Szegletes T, Mallender WD, Thomas PJ, and Rosenberry TL: Substrate Binding to the Peripheral Site of Acetylcholinesterase Initiates Enzymatic Catalysis. Substrate Inhibition Arises as a Secondary Effect. Biochemistry, 38: 122-133, 1999
27. Wagner MA, Trickey P, Chen Z, Mathews FS, and Jorns MS: Monomeric sarcosine oxidase: 1. flavin reactivity and active site binding determinants. Biochemistry, 39: 8813-8824, 2000
28. Zhao G and Jorns MS: Spectral and kinetic characterization of the Michaelis charge transfer complex in monomeric sarcosine oxidase. Biochemistry, 45: 5985–5992, 2006