〈Brief Note〉

Computational insights for coenzyme interactions in wild-type and mutant sarcosine oxidases

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Summary Sarcosine oxidase from *Arthrobacter* sp. TE1826 (SoxA) is widely used in creatinine and creatine assays. Reagents and sensors with SoxA are useful in measuring sarcosine as a potential prostate cancer marker. In our previous studies, chloride ion (Cl^-) and flavin adenine dinucleotide (FAD) concentrations were individually estimated using different SoxA mutants. In this study, *in silico* analysis of the wild-type and mutant SoxA structures was performed to gain insights into enzyme–FAD interactions. The effect of Cl^- on the stability of SoxA was explained by the formation of a hydrogen bond network, including the coenzyme FAD. The covalent bond with the enzyme was crucial for FAD stability. Mutations in the G-X-G-X-X-G motif, which imparted the Cl^--dependent activity to SoxA, affected the enzyme–FAD interactions rather than the FAD stabilities. Moreover, the inhibitory effects of the mutations on these interactions were structurally recovered in the presence of Cl^-. These computational insights provide an enhanced understanding for applying SoxA to various enzymatic assays.

Key words: Sarcosine oxidase, Flavin, Chloride, Potential energy, Molecular dynamics

1. Introduction

Sarcosine oxidase (EC 1.5.3.1) is a monomeric or heterotetrameric flavoprotein that catalyzes the oxidative demethylation of sarcosine (N-methylglycine) to yield glycine, formaldehyde, and hydrogen peroxide. This enzyme is involved in the bacterial metabolisms of creatinine, creatine, choline, betaine, dimethylglycine, and sarcosine along with the related enzymes. The monomeric sarcosine oxidase is industrially important and is used with creatininase and creatinase for enzymatic creatinine and creatine assays in clinical settings (Fig. 1). Based on its relationship to the glomerular filtration rate, the serum creatinine concentration serves as an important index value of renal function in clinical medicine. An enzymatic endpoint spectrophotometric assay was previously developed for accurately estimating serum creatinine levels. Recently, the metabolite sarcosine was identified as a potential urine-based biomarker for prostate cancer progression.

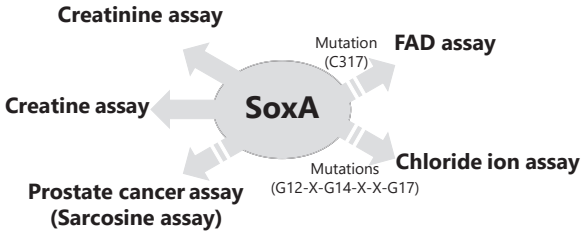

Fig. 1. Various enzymatic assays using the wild-type and mutant SoxAs.

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Sarcosine oxidase might prove useful in the determination of prostate cancer (Fig. 1)[1].

We have previously screened a monomeric sarcosine oxidase from *Arthrobacter* sp. TE1826 (SoxA) and cloned the gene[2]. Recombinant SoxA could be easily purified to homogeneity from an *Escherichia coli* strain. We have also succeeded in altering the substrate specificity and affinity of the enzyme and its stabilization by using protein engineering techniques[3-6]. The wild-type and mutant SoxAs are produced commercially and used in diagnostic reagents[7-9].

SoxA interacts with flavin adenine dinucleotide (FAD) as a coenzyme. The isoalloxazine ring of FAD covalently attaches to the C317 side chain of the catalytic domain through an 8α-S-cysteinyl linkage. The activity of the C317S mutant significantly increases as the concentration of FAD increases, although it exhibits extremely weak activity because of the non-covalent binding to FAD[10]. Therefore, this mutant can be used in the FAD assay (Fig. 1). However, the structural influence of the covalent bond between C317 and the isoalloxazine ring on the enzyme and FAD is not well-known.

The SoxA–FAD interaction is also recognized in the amino-terminal region. The motif with three glycine residues, G-X-G-X-X-G (where X denotes any residue), interacts with the ADP part of FAD, which consists of adenine nucleoside and diphosphate, and the structural features of the interaction are understood[11]. Some mutant SoxAs in the G-X-G-X-X-G motif sequence showed remarkably decreased activities, which were reactivated by chloride ion (Cl^-) addition[12]. Using this property, we developed a new enzymatic assay for determining serum Cl^- concentrations (Fig. 1)[13]. Moreover, the addition of Cl^- was useful for stabilizing SoxA[14]. The stabilization effect was mechanistically elucidated using differential scanning calorimetry and isothermal titration calorimetry[15]. However, the structural influences of Cl^- on the activities and stabilities of the wild-type and mutant SoxAs are not well-known.

In this study, *in silico* analysis of the wild-type and mutant SoxA structures was performed to gain computational insights into the interactions with the coenzyme FAD. The stabilization effect of Cl^- on SoxA was explained by the formation of a hydrogen bond network, including FAD. The covalent bond with the enzyme was crucial for FAD stability. The mutational effects, which afforded the FAD- and Cl^--dependent activities, affected the SoxA–FAD interactions. These findings provide information for further improvements in the functionality of the enzyme in diagnostic reagents.

2. Materials and Methods

The Molecular Operating Environment software (MOE ver.2022.02, Chemical Computing Group Inc., Montreal, Canada) was used in this study for the generation of three-dimensional protein models; prediction of hydrogen, ionic, and hydrophobic bonds; calculation of potential energies; construction of mutant structures; molecular dynamics simulations; and molecular visualizations[16-19]. Energy minimizations were applied to the models for further structural refinement. Before energy minimizations, the force field Amber10:EHT was used to add hydrogen atoms and partial charges at pH 7 according to the manufacturer's instructions.

In total, 12 models of the wild-type (2 types) and mutant (4 variants) SoxAs in the presence and absence of Cl^- were constructed by homology modeling, based on the structure of *Bacillus* sarcosine oxidase (PDB ID: 2GF3b) with high sequence identity (approximately 85%). The Cl^--containing and non-containing models of SoxA were superposed well on the template structure, with root mean square deviations for atomic Cα positions of 0.44 and 0.45 Å, respectively. There were no outliers on the Ramachandran plot of the models. The modeled three-dimensional structures of the present study were of high quality and could thus enhance understanding of the structure–function relationship of SoxA.

3. Results and Discussion

Stabilization effect of Cl^-

The tertiary structure of Cl^--containing SoxA was constructed as described in the Materials and Methods section. Therefore, a hydrogen bond network was recognized because of some newly formed interactions around

Cl^- (Fig. 2). It consisted of three residues, Y319, T320, and G346, with Cl^- and FAD covalently bound to C317. We hypothesized that the hydrogen bond network contributed to SoxA stabilization. In fact, the calculated potential energy of the SoxA–FAD interaction was decreased by 13 kcal/mol in the presence of Cl^-,

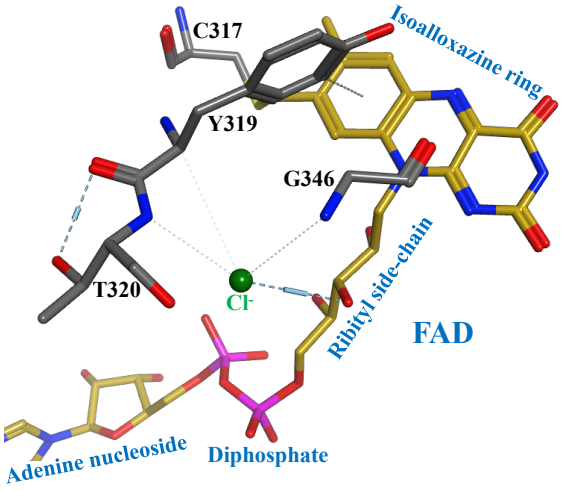

Fig. 2. Hydrogen bond network of SoxA around Cl^-. Nitrogen, oxygen, phosphorus, and sulfur atoms are shown by blue, red, magenta, and light yellow, respectively. Putative hydrogen bonds are represented by dotted lines. Stronger bonds are marked with a cylinder.

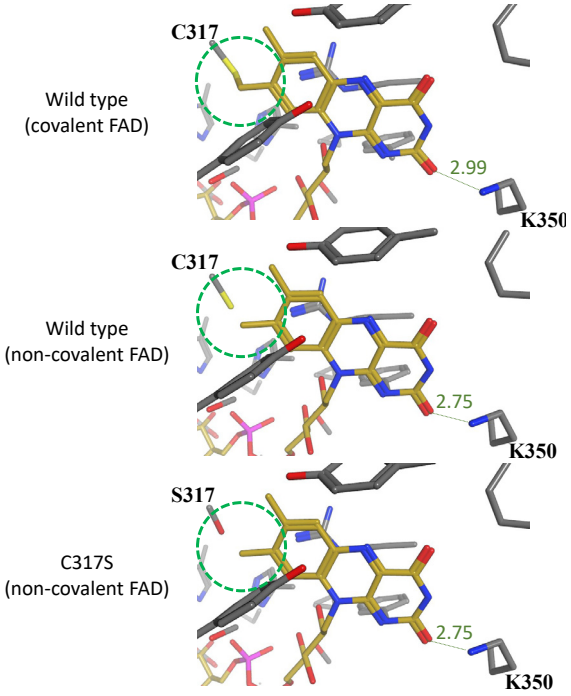

Fig. 3. Close-up views of the three SoxA structures. Two types of wild-type, including covalent and non-covalent FAD, and a mutant C317S were modeled, and the regions around the isoalloxazine ring of FAD are shown. Nitrogen, oxygen, phosphorus, and sulfur atoms are shown by blue, red, magenta, and light yellow, respectively. The distance between the isoalloxazine ring and the side chain of K350 in each enzyme is represented in Å.

Table 1 Catalytic efficiencies and energy calculations of wild-type and mutant SoxAs

Enzyme	Cl^-	Catalytic efficiency	Potential energy		δAffinity (kcal/mol)	δStability (kcal/mol)
			FAD (kcal/mol)	Interaction (kcal/mol)		
Wild type (covalent FAD)	-	1.0	-17.4	-372		
	+	1.0	-16.4	-385		
Wild type (non-covalent FAD)	-	nt	4.5	-438		
	+	nt	11.9	-473		
C317S (non-covalent FAD)	-	nd	2.8	-447		
	+	nd	8.9	-480		
S15P	-	0.0038	-30.4	-304	0.22	1.20
	+	0.33	-22.9	-339	-3.55	1.27
M16G	-	0.000017	-7.1	-357	2.08	3.11
	+	0.079	-12.9	-387	-7.97	3.24
S15P+M16G	-	nd	-10.6	-292	-0.74	4.04
	+	nd	-19.7	-315	-3.13	3.71

δAffinity and δstability scores of each mutant represent the differences from those of the wild type.
Catalytic efficiencies (k_{cat}/K_m) were calculated using the kinetic parameters reported in ref. 10 and 12, and shown as relative values to that of the wild-type.
nt, Not tested; nd, not detected.

although that of FAD was barely influenced by Cl^- (Table 1), resulting in the thermostability of SoxA increasing by approximately 14°C[14,15].

Role of the covalent attachment with FAD

To elucidate the importance of the covalent attachment between the C317 residue of SoxA and the isoalloxazine ring of FAD, the tertiary structures of non-covalent FAD-containing SoxA and C317S mutant were constructed (Fig. 3). The non-covalent FAD-containing SoxA was a virtual protein; that is, this could not be prepared because the isoalloxazine ring of FAD and the C317 side chain were always covalently bonded through an 8α-S-cysteinyl linkage. In contrast, the C317S mutant could not covalently attach FAD. When the potential energies of both structures were estimated, those of the SoxA–FAD interactions were decreased compared with that of the wild-type. In contrast to the potential energies of the SoxA–FAD interactions, those of FAD itself in both structures were quite different. These potential energies were remarkably increased as compared to that of the wild-type, and the addition of Cl^- was ineffective in decreasing the potential energies (Table 1).

In our previous study, the catalytic efficiency of C317S could not be estimated due to the extremely weak activity[10]. Further, its absorption spectrum was not characteristic of flavoproteins. In this study, the relationship between energetic tendencies and enzymatic properties demonstrated that the covalent bond of FAD with SoxA was important for the catalytic function.

Effects of mutations on the SoxA–FAD interaction

In our previous study, we had demonstrated that the mutations in the G12-X13-G14-X15-X16-G17 motif, located at the amino-terminal region of SoxA, were responsible for the Cl^--dependent activity. However, the reasons behind the decreased mutant activities and recovery of activity following the addition of Cl^- were not explained by tertiary structure models. In this study, *in silico* structure analysis of three mutant SoxAs with S15P, M16G, and S15P+M16G mutations in the motif was performed.

The motif mutations, especially S15P and S15P+M16G, increased the potential energy of the SoxA–FAD interaction rather than that of FAD itself, as compared with those of the wild-type (Table 1). Therefore, these mutations should affect the stabilities of the enzyme–FAD interactions rather than those of FAD itself. In contrast, the potential energies of the SoxA–FAD interactions were decreased by Cl^- addition to a

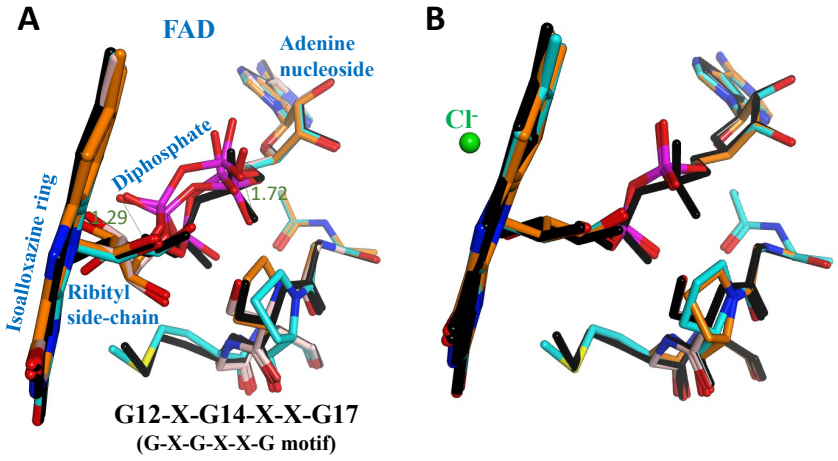

Fig. 4. Close-up views of superposed G-X-G-X-X-G motifs and FAD. The wild-type (black), S15P (cyan), M16G (beige), and S15P+M16G (orange) tertiary structures are superposed. Nitrogen, oxygen, phosphorus, and sulfur atoms are shown by blue, red, magenta, and light yellow, respectively. The distances between the corresponding diphosphate oxygen atoms in the wild-type and S15P+M16G are represented in . A, absence of Cl-; B, presence of Cl-.

greater extent in the motif mutants than that in the wild-type. For example, the potential energy of S15P was decreased from -304 to -339 kcal/mol, whereas that of the wild-type was decreased by only 13 kcal/mol (Table 1). Thus, SoxA–FAD interactions were clearly recovered in the presence of Cl^-.

To reveal the affinity and stability of FAD with the enzyme in the SoxA–FAD interaction in greater detail, molecular dynamics simulations of the mutation residues were performed. As shown in Table 1, the motif mutants resulted in increased δstability score (the difference from that of the wild-type) rather than increased δaffinity score; that is, SoxA became energetically unstable by the mutations. Their δaffinities were, however, remarkably decreased in the presence of Cl^-, and the energetic alterations of S15P and M16G led to the recovery of the enzymatic activity.

Structural comparison of the G-X-G-X-X-G motif and FAD interaction area further revealed the markedly reduced activities of the mutants and the recovery following Cl^- addition (Fig. 4). P15 and G16 had little effect on the locations of the isoalloxazine ring and adenine nucleoside in FAD. However, they altered the conformations of the diphosphate and ribityl side-chain (Fig. 4A). The Cl^- atom, which was on the opposite side of the G-X-G-X-X-G motif across from FAD, clearly restored the structural changes caused by the mutations (Fig. 4B). Thus, the Cl^--dependent activities of the SoxA G-X-G-X-X-G motif mutants were thought to have been acquired by the inhibitory effect of the Cl^- atom on the partial conformational change of FAD.

These computational insights provide an improved understanding for applying SoxA to various enzymatic assays.

Conflicts of interest

The authors have no conflicts of interest.

Acknowledgements

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References

1. Zhao L, Yang J, Gong M, Li K, and Gu J: Specific screening of prostate cancer individuals using an enzyme-assisted substrate sensing platform based on hierarchical MOFs with tunable mesopore size. J Am Chem Soc, 143:15145-15151, 2018.
2. Nishiya, Y and Imanaka, T: Cloning and sequencing of the sarcosine oxidase gene from *Arthrobacter* sp. TE1826. J Ferment Bioeng, 75:239-244, 1993.
3. Nishiya, Y and Imanaka, T: Alteration of substrate specificity and optimum pH of sarcosine oxidase by random and site-directed mutagenesis. Appl Environ Microbiol, 60:4213-4215, 1994.
4. Nishiya, Y and Imanaka, T: Highly conservative sequence in carboxyl terminus of sarcosine oxidase is important for substrate binding. J Ferment Bioeng, 84:591-593, 1997.
5. 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.
6. 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.
7. Nishiya Y and Kawamura Y: Some properties of FAD (flavin adenine dinucleotide)-containing oxidases improved by protein engineering and application to the enzymatic assays [Jpn]. J Anal Bio-Sci, 20:149-156, 1997.
8. Nishiya Y, Yamamoto K, Kawamura Y, and Emi S: Development and Improvement of creatinine-degrading enzymes [Jpn]. Nippon Nogeikagaku Kaishi, 75:403-405, 2001.
9. 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.
10. Nishiya, Y: A mutant sarcosine oxidase in which activity depends on flavin adenine dinucleotide. Protein Expr Purif, 20:95-97, 2000.
11. Nishiya, Y: Similarities and individualities of flavin adenine dinucleotide linked oxidases for diagnostic use [Jpn]. J Anal Bio-Sci, 39:189-194, 2016.
12. Nishiya, Y and Imanaka, T: Analysis of interaction between the *Arthrobacter* sarcosine oxidase and the coenzyme flavin adenine dinucleotide by site-directed mutagenesis. Appl Environ Microbiol, 62:2405-2410, 1996.

13. Nishiya, Y, Kawamura, Y, and Imanaka, T: Enzymatic assay for chloride ion with chloride-dependent sarcosine oxidase created by site-directed mutagenesis. Anal Biochem, 245:127-132, 1997.
14. Nishiya, Y and Kawamura, Y: Effects of chloride ion on activity and stability of sarcosine oxidase. J Anal Bio-Sci, 20:375-377, 1997.
15. Sugimoto, H, Nishiya, Y, Miyake, H, and Tanaka, A: Stabilization mechanism of chloride ion on thermal denaturation of *Arthrobacter* sarcosine oxidase. Netsu Sokutei, 35:76-80, 2008.
16. Nishiya, Y and Hirayama, N: Molecular modeling of the *Arthrobacter* sarcosine oxidase. J Anal Bio-Sci, 25:343-346, 2002.
17. Nishiya Y and Hirayama N: Structure-function relationship of the diagnostic enzyme sarcosine oxidase [Jpn]. J Anal Bio-Sci, 26:191-195, 2003.
18. Shimozawa, Y, Himiyama, T, Nakamura T, and Nishiya, Y: Increasing loop flexibility affords low-temperature adaptation of a moderate thermophilic malate dehydrogenase from *Geobacillus stearothermophilus*. Protein Eng Des Sel, 34:gzab026, 2021.
19. Shimozawa, Y, Himiyama, T, Nakamura T, and Nishiya, Y: Structural analysis of diagnostic enzymes: Differences in substrate specificity between malate dehydrogenase and lactate dehydrogenase [Jpn]. J Anal Bio-Sci, 45:151-159, 2021.