<Research Article>

Chemical modification of glycine oxidase from *Geobacillus kaustophilus* by *N*-methylisothiazolinone

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Summary *N*-Methylisothiazolinone (MIT) is a thiol group modifier and an antimicrobial agent. Glycine oxidase from *Geobacillus kaustophilus* (GoxGk), used for measuring glycine levels, was modified with MIT chemical modification (Biosci Biotechnol Biochem, 88:630-636, 2024), and its enzymatic reaction was analyzed. Substrate inhibition of MIT-modified GoxGk decreased compared to intact GoxGk, leading to a 1.3-fold increase in specific activity. Three cysteine (Cys) residues in GoxGk were replaced with serine. Cys232 and Cys252 of the three cysteines influenced the enzyme reaction. Surprisingly, GoxGk-Cys232Ser had a specific activity approximately three times higher than the wild-type GoxGk. The mutant might be useful for practical applications.

Key words: Glycine oxidase, *Geobacillus*, Chemical modification, Substrate inhibition, Mutagenesis

1. Introduction

Glycine oxidase (Gox, EC 1.4.3.19) was discovered by *in silico* screening in 1998[1,2]. Gox is a flavoprotein that contains flavin adenine dinucleotide (FAD) as a coenzyme[2]. It catalyzes the oxidative deamination of glycine to yield glyoxylate, ammonium, and hydrogen peroxide[2-4] (Fig. 1). Gox plays an important role as the initial enzyme in the bacterial vitamin B1 synthetic pathway[4]. Gox from *Bacillus subtilis* (GoxB) is commercially available and used with the 4-aminoantipyrine-peroxidase system in enzymatic glycine assay[5,6].

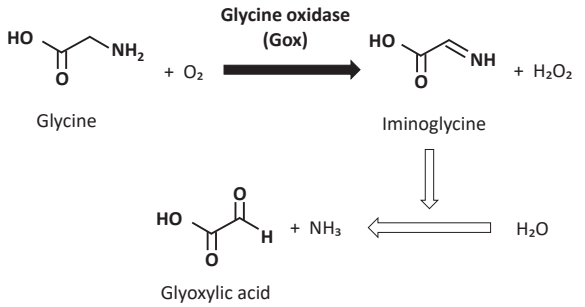

Fig. 1. Glycine oxidase (Gox) reaction. Iminoglycine is non-enzymatically hydrolyzed to glyoxylate and ammonium.

The simple enzymatic measurement of glycine concentrations in various samples is useful for both research and industrial applications. Glycine is a basic molecule with various biological functions. For example, glycine accounts for approximately one-third of the amino acid composition of collagen, a major skin constituent protein[7]. It functions as a building block for various important substances and a neurotransmitters[8]. Glycine is widely used as a safe food additive, such as a shelf life enhancer and seasoning, in a wide variety of foods, including snacks and pickles, because it can be industrially

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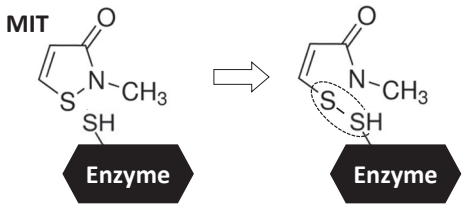

Fig. 2. Chemical modification of thiol groups in enzymes with *N*-methylisothiazolinone (MIT).

synthesized at a low cost. Recently, the sleep-improving effect of glycine has been discovered, and it has been commercialized as a supplement[9].

In our previous study, we investigated the chemical modification of an enzyme from the genus *Arthrobacter*, sarcosine oxidase (SoxA; EC 1.5.3.1; sarcosine:oxygen oxidoreductase [demethylating]), which is one of the related enzymes used in creatinine assay kits, was investigated[10]. The cysteine (Cys) residues in SoxA were modified using *N*-methylisothiazolinone (MIT), a thiol group modifier and antimicrobial agent (Fig. 2). Consequently, only the oxidative half-reaction with oxygen was inhibited. Therefore, SoxA was altered from a sarcosine oxidase to a sarcosine dehydrogenase.

In this study, Gox from *Geobacillus kaustophilus* (GoxGk)[11,12], which is more thermostable than GoxB, was modified by MIT that we used previously[10], and its enzymatic reaction was characterized. Gox has low specific activity and exhibits substrate inhibition, which limits its practical use. We expected that the enzymatic properties of Gox would be improved by MIT chemical modification.

2. Materials and Methods

Reagents and chemicals

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

GoxGk expression and purification

Expression plasmid of GoxGk was constructed as below. The DNA fragment (1,134 bp, DDBJ/EMBL/GenBank accession number: BA000043-623) containing the GoxGk gene was cloned into *NdeI* and *Bam*HI sites of pET-15b (5,708 bp, Ap^r)[13,14]. This plasmid was kindly provided by Prof. Takaomi Nomura, Faculty of Textile Science and Technology, Shinshu University. It encodes GoxGk with 20 extra amino-terminal residues containing a 6-histidine tag and was transformed into *Escherichia coli* BL21(DE3) pLysS. The transformed cells were cultured in Terrific Broth medium containing 100 μg/mL ampicillin at 37 ℃ and 200 r/m shaker speed for 2 h, and then were induced to synthesize the gene product by adding 100 μmol/L of isopropyl-L-D-thiogalactopyranoside to the culture medium. The cells subsequently cultured for 20–22 h were collected by centrifugation at 4 ℃ and 13,000 xg for 5 min and suspended in buffer A (20 mmol/L potassium phosphate, pH 7.5).

The harvested cells were sonicated on ice, and the cell extracts were centrifuged at 4 ℃ and 13,000 xg for 5 min. The crude extract was heat-treated at 50 ℃ for 20 min and then re-centrifuged. The supernatant was applied to a His GraviTrap Ni-chelating affinity chromatography column (Cytiva Life Sciences, Marlborough, MA, USA), equilibrated with buffer A containing 500 mmol/L NaCl and 20 mmol/L imidazole. The bound proteins were eluted using buffer A containing 500 mmol/L NaCl and 70–500 mmol/L imidazole. The protein solution was dialyzed against buffer A to remove the NaCl and imidazole.

Finally, GoxGK was purified to homogeneity. The molecular weight of the purified GoxGK was estimated to be 42.1 kDa using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentration was quantified using the Bradford assay (Takara Bio Inc., Shiga, Japan).

Chemical modification

The GoxGk sample solution (5.0 μmol/L in buffer A) was mixed with a 1,000-fold molar concentration of MIT. The mixture was allowed to react at room temperature for approximately 20 min and then dialyzed against buffer A to remove the excess MIT.

Peptide-mass fingerprinting

Cys-containing protein fragments were identified using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS)-based peptide mass fingerprinting. The purified protein was digested with 5.0 μg/mL of MS-grade

trypsin (Fujifilm Wako Pure Chemical Corp.) in 50 mmol/L NH_4HCO_3 at 37 ºC overnight. The resulting peptides were mixed with 5.0 mg/mL of α-cyano-4-hydroxycinnamic acid in 50% (v/v) acetonitrile and 0.1% (v/v) trifluoroacetic acid and subjected to MALDI-TOF-MS (AXIMA-Performance, Shimadzu Corp., Kyoto, Japan)[10].

Site-directed mutagenesis

Expression plasmids for the mutants were prepared by inverse PCR using the enzyme solution PrimeSTAR Max DNA Polymerase (Takara Bio Inc.) according to the manufacturer's instructions. The sequences of PCR primers used were listed in Table 1.

Table 1 Specific primers used for Cys-to-Ser mutagenesis

Cys173Ser	Forward	5'- ggcgcttctctgtacgagtatacggaagtg -3'
	Reverse	5'- gtacagagaagcgccggcgga -3'
Cys232Ser	Forward	5'- ggagaatctgtcatggtgcgcgcc -3'
	Reverse	5'- catgacagattctcctttgaccggataaac -3'
Cys252Ser	Forward	5'- aacggctcttacatcgttccgaaatcagga -3'
	Reverse	5'- gatgtaagagccgtttttcgcaaatacagt -3'

Gox activity assay

In the Gox activity assay, the enzymatic production of hydrogen peroxide from dissolved oxygen was measured using a 4-aminoantipyrine peroxidase assay[1]. The enzyme solution (0.035 mL) was incubated with a mixture (1.0 mL) of 10 mmol/L glycine, 0.010% 4-aminoantipyrine, 0.020% phenol, 50 mmol/L Tris-HCl (pH 8.0), and 5.0 U/mL of horseradish peroxidase at 37 ºC, and the time-dependent spectral increase at 500 nm was monitored against the blank for 5 min. One unit of each assay was defined as the amount of the enzyme that catalyzed the oxidation of 1 μmol of substrate per min at 37 ºC.

Computational analysis

Structural influences on chemical modification are discussed based on the GoxGK tertiary structure (PDB ID: 4YSH)[12]. Molecular Operating Environment software (Chemical Computing Group Inc., Montreal, Canada) was used for molecular visualizations[15,16].

3. Results and Discussion

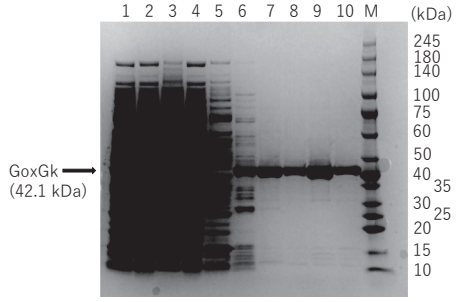

Fig. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of crude and purified GoxGk. Lane 1, crude extract; lane 2, cell debris precipitate; lane 3, supernatant after heat treatment; lanes 4-10, Ni-chelating affinity chromatography fractions 1-7. Fraction 1 contained the void volume. Fractions 2, 3, 4, and 5-7 were eluted with 20, 70, 100, and 500 mmol/L imidazole, respectively.

Comparison of the reactions between intact and MIT-modified GoxGks

Recombinant GoxGk was purified to homogeneity, as described in the Materials and Methods section. Finally, approximately 1.5 mg of GoxGk with an amino-terminal 6-histidine tag was obtained. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns of crude and purified GoxGk, including the Ni-chelating affinity chromatography fractions, are shown in Fig. 3. Highly purified GoxGk was obtained from fraction numbers 5-7 and used in the subsequent experiments.

The thiol groups of the Cys residues in GoxGk were modified by MIT as described in the Materials and Methods section. The Gox activity of MIT-modified GoxGk was measured and compared with that of intact GoxGk, resulting in an approximately 1.3-fold increase in the specific activity at a final glycine concentration of 10 mmol/L. Therefore, the relationships between the reaction rates and the concentration of each substrate

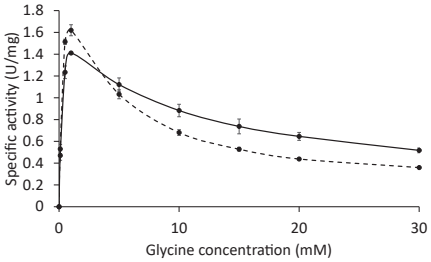

Fig. 4. Specific activities of intact and MIT-modified GoxGk at various glycine concentrations Dotted and solid lines indicate the activities of intact and modified GoxGk, respectively. Error bars: standard deviation; n = 3.

Table 2 Kinetic parameters of intact and MIT-modified GoxGk for glycine

Enzyme	K_m (mmol/L)	K_i (mmol/L)	k_{cat} (s^{-1})	k_{cat} / K_m ($s^{-1}mmol/L^{-1}$)	Spec. Act. (U/mg)
Intact GoxGk	0.35 ± 0.056	3.4 ± 0.60	7.3 ± 0.62	21 ± 1.8	0.68
Modified GoxGk	0.25 ± 0.028	9.5 ± 1.8	5.0 ± 0.26	20 ± 1.2	0.88

Gox activity assay is described in the Materials and Methods section. Kinetic parameter values are presented as mean ± SD.
K_m: Michaelis constant, K_i: inhibition constant, k_{cat}: turnover number, Spec. Act.: specific activity.

Table 3 Glycine oxidase activities of wild-type and mutant GoxGk

Enzyme	Modifiable residues	Spec. Act. (U/mg)		Activity ratio (Modified/Intact)
		Intact	MIT-Modified	
Wild type	Cys173,Cys232,Cys252	0.68	0.88	1.3
Cys173Ser	Cys232,Cys252	0.49	0.65	1.3
Cys232Ser	Cys173,Cys252	1.7	1.5	0.87
Cys252Ser	Cys173,Cys232	0.83	0.86	1.0

Gox activity assay is described in the Materials and Methods section.
Spec. Act.: specific activity.

(glycine) for both enzymes were investigated, and the results are shown in Fig. 4. Intact GoxGk exhibits a typical substrate inhibition mode. In contrast, MIT-modified GoxGk exhibited relatively weak substrate inhibition.

The kinetic parameters for the activity of intact and MIT-modified GoxGk were estimated (Table 2). The K_i value of the MIT-modified GoxGK was 2.8-fold higher than that of intact GoxGk, whereas there was no significant difference between the K_m values of the two enzymes. The MIT-modifying effect of GoxGk was mainly derived from the reduction of substrate inhibition. To the best of our knowledge, this is the first report of the improvement of substrate inhibition by chemical modification of the enzyme.

Cys-to-serine (Ser) mutants of GoxGk

Three Cys residues (Cys173, Cys232, and Cys252) are present in GoxGk, and their thiol groups do not covalently bind. There is no information on which Cys residues affect Gox activity through chemical modification. Cys-containing fragments of MIT-modified GoxGk were identified by peptide mass fingerprinting. However, the MIT-modified fragment masses were not detected after digestion (data not shown). Although this could not be clearly explained, the MIT modification might affect the ionization efficiency. In our previous study, the chemically modified fragments of MIT-modified SoxA were not detected[10].

To clarify the effects of MIT modification on enzymatic activity, GoxGk mutants with Cys residues replaced to Ser were constructed, purified to homogeneity, and characterized (Table 3). The specific activities of intact and MIT-modified GoxGk-Cys173Ser were similar to those of the wild-type GoxGk. Thus, the MIT modification of Cys173 was considered ineffective in the enzymatic reaction. However, Cys232Ser and Cys252Ser did not affect the specific activity induced by MIT modification. Accordingly, the substrate inhibition of GoxGk appeared to be reduced by chemical modifications at Cys232 and Cys252, that is, the modification effect was cooperatively mediated by the binding of MIT to both Cys residues.

Unexpectedly, the specific activities of intact Cys232Ser and Cys252Ser (1.7 and 0.83 U/mg) were approximately 2.5- and 1.2-fold higher than that of the wild-type (0.68 U/mg), respectively (Table 3).

Structural discussion

Reduced substrate inhibition and the accompanying increased specific activity of the Gox reaction with glycine were caused by the MIT modification of the Cys residues Cys232 and Cys252. This was confirmed based on the tertiary structure of GoxGk (Fig. 5)[12].

Cys232 is located near the active site and close to

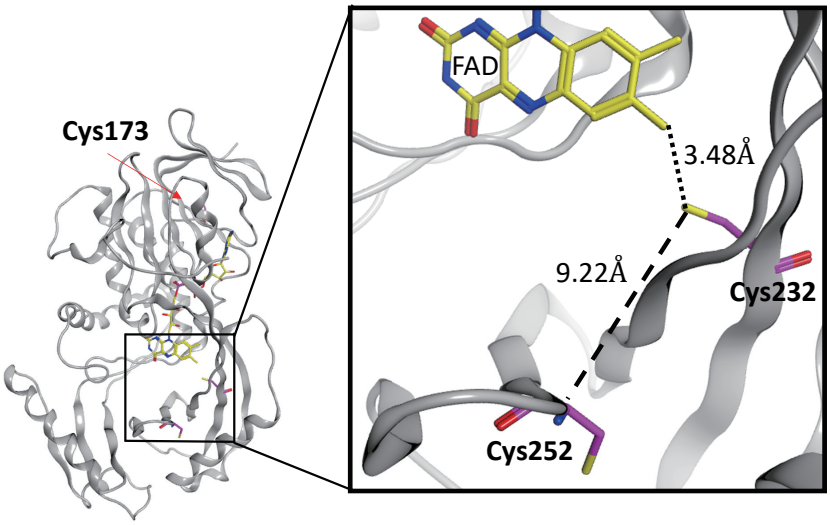

Fig. 5. The tertiary structure of GoxGk including a noncovalent FAD (PDB ID: 4YSHa). A: Overall structure. B: Close-up view of the region around the active site. The nitrogen, oxygen, phosphorus, and sulfur atoms are shown in blue, red, magenta, and light yellow, respectively. The distances between the isoalloxazine ring and the side chain of Cys232 and between Cys232 and Cys252 are represented in Å.

the isoalloxazine ring of FAD (shortest distance: approximately 3.5 Å). In contrast, Cys252 was located in a loop structure near the substrate-binding pocket. In contrast, Cys173 is located far from the active site. The thiol groups of Cys232 and Cys252 were approximately 9.2 Å apart; thus, they could not be expected to directly affect each other. The altered enzymatic reaction was likely related to the interaction between the MIT-modified thiol groups and their associated structural changes, such as reduced flexibility of the loop structure and narrowing of the active site.

Cys-to-Ser mutations at positions 232 and 252 resulted in increased activity. In particular, the specific activity of GoxGk-Cys232Ser was approximately three times higher than that of the wild-type GoxGk. This cannot be explained structurally at present, although the mutant might be useful for practical applications. Further mutational and structural analyses are currently underway.

Conflicts of interest

The authors declare no conflict of interest.

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