Purification and Properties of Glycine Oxidase from *Pseudomonas putida* KT2440

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**Summary**  Glycine oxidase, encoded by the thiO gene, participates in the biosynthesis of thiamin by providing glyoxyl imine to form the thiazole moiety of thiamin. We have purified and characterized ThiO from *Pseudomonas putida* KT2440. It has a monomeric structure that is distinct from the homotetrameric ThiOs from *Bacillus subtilis* and *Geobacillus kaustophilus*. The *P. putida* ThiO is unique in that glycine is its preferred substrate, which differs markedly from the *B. subtilis* and *G. kaustophilus* enzymes that use D-proline as the preferred substrate.

**Key Words**  thiamin biosynthesis, glycine oxidase, thiO, flavin adenine dinucleotide, *Pseudomonas putida*

Thiamin diphosphate is the active form of thiamin (vitamin B₃) and acts as a cofactor for various enzymes that make or break C–C bonds, such as the pyruvate dehydrogenase complex, the 2-oxoglutarate dehydrogenase complex, transketolase, and acetalactate synthase. Most microorganisms and plants can synthesize thiamin de novo, but vertebrates cannot and therefore can only obtain it through their diet. In the de novo biosynthesis pathway, the thiazole and pyrimidine moieties of thiamin are produced separately and combine to form thiamin phosphate, which is then converted to thiamin diphosphate. In *Bacillus subtilis* and most other bacteria, six gene products are involved in the formation of thiazole, whereas the formation of pyrimidine requires only two products. Labeling studies have shown that thiazole is synthesized through complex reactions among deoxy-D-xylulose phosphate, cysteine, and glycine. The glyoxyl imine derived from glycine through the action of glycine oxidase (GO) is incorporated into thiazole, as shown in Fig. 1.

GO is a homolog of sarcosine oxidase, but differs markedly from the latter in that it acts on the C2-carbon atom of the substrate. Thus, glyoxyl imine and hydrogen peroxide are the primary products obtained from the GO reaction, and glyoxyl imine is used for the biosynthesis of the thiazole ring of thiamin. Otherwise, glyoxyl imine is spontaneously hydrolyzed in water to produce glyoxylate and ammonia, which, in addition to hydrogen peroxide, are generally considered as the main products of the GO reaction. Since the discovery of GO in *B. subtilis* by Nishiya and Imanaka, this bacterial GO encoded by the thiO gene has been extensively studied not only structurally but also mechanistically. More than 1,000 orthologs from various bacterial strains are catalogued as ThiO in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.kegg.jp/).

However, there is limited information on the enzymological properties of ThiO from bacterial strains other than *B. subtilis*. The *Geobacillus kaustophilus* ThiO (GOBK) exhibits some unique properties, although it shares the same basic characteristics as the *B. subtilis* ThiO (GOBS): uses flavin adenine dinucleotide (FAD) as a cofactor, is a homotramer, and has broad substrate specificity. *Marinomonas mediterranea* is an interesting bacterium, because it produces an unusual GO that is independent of FAD and probably contains a built-in quinone cofactor. The *M. mediterranea* GO (GOMM) is quite different from GOBS and GOBK with respect to its substrate specificity: that is, it is highly specific toward glycine. Recently, we purified recombinant ThiO of *Pseudomonas putida* KT2440 and found that this ThiO (GOPP) has a monomeric structure that is distinct from the homotetrameric structures of GOBS and GOBK. On the basis of substrate specificity, GOBS and GOBK can be regarded as D-proline oxidases rather than as glycine oxidases, although they definitely participate in the biosynthesis of thiamin described above. Nevertheless, GOPP is distinct from GOBS and GOBK because glycine is the preferred substrate of the enzyme. We here describe the purification and properties of GOPP.

**Methods**  We assayed glycine oxidase by measuring the rate of hydrogen peroxide at 37 °C with a standard reaction mixture (1 mL) containing 100 mM sodium pyrophosphate buffer (pH 8.5), 20 mM glycine, 0.1 mM FAD, 0.5 mM 4-aminopyridine, 2 mM phenol, 5 units of horseradish peroxidase, and an appropriate amount of GOPP. The quinone-imide dye formed from 4-aminopyridine and phenol by the peroxidase reaction was measured at 505 nm.

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PP_0612 in the KEGG database; http://www.kegg.jp/dbget-bin/www_bget?ppu:PP_0612) consists of 1,098 nucleotides corresponding to 365 amino acid residues with a deduced molecular mass of 38,679.3 Da. The genomic DNA of the bacterium was used as a template to amplify the gene with oligonucleotide primers derived from the nucleotide sequence: forward primer, 5′-CGAGGGATCCGAAATTCATGAGCAAGCAAGTGTTGTTG-3′; reverse primer, 5′-CGACAAGCTTGAATTCTCAGCCCAAAGCCCTTCTGCGCATA-3′. The amplified gene was inserted into the EcoRI site of the pCold I vector (Novagen, Madison, WI) by the In-Fusion HD cloning technique (Takara Bio, Kusatsu, Japan), and *Escherichia coli* DH5α was transformed with the recombinant plasmid. The resulting recombinant plasmid, pCold I-PP_0612, encoded an additional 27-amino acid sequence (MNHKVHHHHHHIEGRHMELGTLEGSEF), including a six-histidine tag at the original amino terminus of PP_0612. Thus, the calculated molecular mass of the recombinant GOPP was 41,767.7 Da.

*E. coli* BL21(DE3) cells harboring pCold I-PP_0612 were grown in Luria-Bertani medium at 37°C until the culture reached an optical density at 600 nm of 0.5. Then, isopropyl-β-d-1-thiogalactopyranoside (1 mM) was added to the culture, which was further incubated at 15°C for 24 h. The harvested cells were suspended in 50 mM Tris-HCl (pH 8.0) containing 150 mM NaCl, 10% glycerol, and 10 mM imidazole, and were disrupted by sonication. The resulting cell extract was mixed with Ni-NTA Superflow beads equilibrated with the same buffer at 4°C for 1 h, and the slurry was loaded onto a HisTrap HP (Bio-Rad, Hercules, CA) column with a volume similar to that of the slurry. The recombinant GOPP was eluted by linearly increasing the imidazole concentration from 20 to 300 mM. The active fractions collected were dialyzed against 50 mM Tris-HCl (pH 8.0), concentrated with an Amicon Ultra Centrifugal Filter 10 MWCO device (Merck Millipore, Tullagreen, Ireland), and the solvent for the final preparation was replaced with 50 mM Tris-HCl (pH 8.0) containing 10% glycerol with an Econo-pac® 10DG-10 desalting column (Bio-Rad). The enzyme was kept frozen at −80°C until use.

**Results and Discussion**

We obtained a homogeneous preparation of the recombinant GOPP as described above. The overall yield in the purification was about 88%, and the specific activity of the final preparation was 0.073 μmol·mg⁻¹·min⁻¹ as determined in the absence of added FAD in the assay mixture. The enzyme produced a single band following sodium dodecyl sulfate-polyacrylamide gel electrophoresis with an estimated molecular mass of 43.5 kDa. Furthermore, a single peak was eluted by gel filtration with an estimated molecular mass of 42.7 kDa under the following conditions: column, TOSOH-TSK gel G3000 SWXL; mobile phase, 50 mM potassium phosphate buffer (pH 7.2) containing 50 mM Tris-Cl (pH 7.2) containing 150 mM NaCl; flow rate, 1 mL/min. This indicates that the recombinant GOPP has a monomeric structure. If this is the case, this structure is quite different from the homotetrameric enzymes GOBS and GOGK (7, 8, 11).

The recombinant GOPP showed an absorption spectrum that is typical of a flavoprotein, with absorption bands at around 380 nm and 450 nm. When the enzyme was treated with 6 M guanidine chloride fol-

### Table 1. Kinetic parameters of *Pseudomonas putida* ThiO (GOPP) for various substrates.

| Substrate       | $V_{\text{max}}$ (μmol·mg⁻¹·min⁻¹) | $K_m$ (mM) | $V_{\text{max}}/K_m$ (μmol·mm⁻¹·mg⁻¹·min⁻¹) |
|-----------------|----------------------------------|------------|---------------------------------------------|
| Glycine         | 0.15                             | 2.43       | 0.064                                       |
| Sarcosine       | 0.17                             | 4.86       | 0.036                                       |
| d-Proline       | 0.13                             | 31.7       | 0.004                                       |
| N-Ethylglycine  | 0.11                             | 7.68       | 0.014                                       |

Fig. 1. Biosynthesis of thiamin.
The rates of hydrogen peroxide production from glycine (panel A), sarcosine (panel B), d-proline (panel C), and N-ethylglycine (panel D) catalyzed by GOPP were determined under standard assay conditions in which glycine was replaced by the other substrates indicated.

We examined the effect of pH on the enzyme with the following buffers: 100 mM potassium phosphate (pH 5.5–7.5), 100 mM sodium pyrophosphate (pH 8.0–8.5), and 50 mM sodium pyrophosphate mixed with 50 mM sodium carbonate (pH 9.0–10). The enzyme showed the highest activity at pH 8.5, and was most stable in the pH range from 8.0 to 10 upon incubation at 25˚C for 1 h. We also found that the enzyme showed maximum activity at 40˚C when assayed at pH 8.5.

We examined the substrate specificity of the recombinant GOPP with various amino acids and derivatives under standard conditions. The enzyme showed similar activity toward glycine and sarcosine as the best substrates, as opposed to N-ethylglycine (relative activity, 40%), d-proline (25%), d-alanine (10%), glycine ethyl ester (5%), and glycyglycine (3%). We determined the kinetic parameters $K_m$ and $V_{max}$ for glycine, sarcosine, d-proline, and N-ethylglycine, as shown in Table 1. Martinez-Martinez et al. (11) showed that GOGK is inhibited at high concentrations of substrates such as glycine, sarcosine, and glycine ethyl ester. However, no such inhibition was observed with GOPP (Fig. 2). Another point to be noted is that GOGK (11) and GOBS (6) show the highest activity towards d-proline. However, d-proline was less preferred as a substrate for GOPP with a high $K_m$ value (Table 1). Moreover, GOGK accepts various d-amino acids as substrates, such as d-norvaline, d-valine, d-arginine, etc. The substrate specificity of GOPP is shown in Table 2.
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Glycine oxidase from \textit{Pseudomonas putida}.

\begin{itemize}
\item \textbf{GOBS}: NKRHEAYAVIGGVSGLSAKAYLIIAGNQYAFTSGMRTGSSSAAAMAGHSGKEDG
\item \textbf{GOGK}: NTHYKYNKGGGSLGAAINIIKHHAIRHYKTVKNQGGSADSEFSTSS
\item \textbf{GOPP}: \texttt{--WKNQVIVYVGQGQLTAFNLKQAVYDVGQYEVGSLAVGGSDVLPSVVFYRMPSP}
\end{itemize}

GOPP is located in a different clade. This suggests that the unique substrate specificity of GOPP may represent a common feature of GO homologs belonging to the betaproteobacteria/gammaproteobacteria clade.

The homotetrameric structure of GOBS has been clearly demonstrated by X-ray crystallography (7, 8). The main interactions between monomers A and B involve hydrogen bonds between the following pairs of amino acid residues: Lys155 and Asp232, and Lys162 and Asp233. Similarly, hydrogen bonds also occur to maintain the interaction between monomers A and C with the Lys283 and Glu276 residue pair. Pairwise hydrophobic interactions also play important roles for the subunit interaction as follows: between monomers A and B, Phe152 and Trp230; between monomers A and C, Phe297 and Val294. It is interesting to note that only 3 of the 10 amino acid residues are conserved in GOPP relative to GOBS: Lys155 for Lys155; Glu233 for Asp233; and Val294 for Val294 (Fig. 4). However, the counterparts to be paired are not conserved in GOPP relative to GOBS: Ala232 for Asp232; Leu160 for Lys162; or His297 for Phe297. Conversely, 11 important amino acid residues forming cofactor- and substrate-binding sites are highly conserved between GOBS and GOPP, respectively: Asp32 for Glu34; Ser41 for Thr43; Gly46 for Gly48; Ile47 for Met49; Ile173 for Val174; Tyr246 for Tyr246; Arg302 for Arg302; His327 for His327; Gly331 for Gly331; Leu332 for Ile332; and Val333 for Leu333. Therefore, one may assume that GOPP has a monomeric structure due to the absence of appropriate amino acid residues that interact pairwise at the subunit interfaces. GOGK is a homotetramer in the same manner as GOBS, but the 13 amino acid residues at the subunit interfaces in GOBS listed above are not conserved well in GOGK (11). This indicates that factors other than the 13 amino acid residue interactions described above may contribute to the organization of the quaternary structure. Therefore, elucidating the reason for the unique monomeric structure of GOPP awaits determination of its X-ray structure. In addition, GOPP shows much narrower substrate specificity than GOBS and GOGK. The X-ray structure of GOPP will also likely provide clues toward understanding this unique substrate specificity.

\textbf{Conflict of interest statement}

There are no conflicts of interest associated with this work.

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