Research article

Asn336 is involved in the substrate affinity of glycine oxidase from Bacillus cereus

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A B S T R A C T

Background: Glycine oxidase (GO), a type of L-amino acid oxidase, is of biotechnological interest for its potential in several fields. In our previous study, we have characterized a new glycine oxidase (BceGO) from Bacillus cereus HYC-7. Here, a variant of N336K with increased the affinity against all the tested substrate was obtained by screening a random mutant library of BceGO. It is observed that the residue N336 is invariable between its homogeneous enzymes. This work was aimed to explore the role of the residue N336 in glycine oxidase by site-directed mutagenesis, kinetic assay, structure modeling and substrate docking.

Results: The results showed that the affinity of N336H, N336K and N336R increased gradually toward all the substrates, with increase in positive charge on side chain, while N336A and N336G have not shown a little significant effect on substrate affinity. The structure modeling studies indicated that the residue Asn336 is located in a random coil between β-18 and α-10. Also, far-UV CD spectra-analysis showed that the mutations at Asn336 do not affect the secondary structure of enzyme.

Conclusion: Asn336 site was located in a conserved GHYRNG loop which adjoining to substrate and the isoalloxazine ring of FAD, and involved in the substrate affinity of glycine oxidase. This might provide new insight into the structure–function relationship of GO, and valuable clue to redesign its substrate specificity for some biotechnological application.

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1. Introduction

Glycine oxidase (GO, EC 1.4.3.19), a homotetrameric flavoenzyme, contains non-covalently attached FAD molecule [1,2]. BceGO catalyzes the oxidative deamination of various amines (glycine, sarcosine, N-ethylglycine) and some D-isomer of amino acids (D-alanine, D-proline, etc.) to yield corresponding α-keto acid(s), ammonia/amine, and hydrogen peroxide. GO appears to be stereo-specific in oxidizing the D-amino acids and its substrate specificity partially similar to D-amino acid oxidase (DAAO, EC 1.4.3.3) and sarcosine oxidase (SOX, EC 1.5.3.1). It plays an important role in the biosynthesis of the thiazole ring of thiamine pyrophosphate cofactors in Bacillus subtilis [2]. The broad substrate specificity and stereoselectivity of GO confers it great potential in several biotechnological fields, such as industrial biocatalysis, biosensors and developing glyphosate-resistant crop [3,4,5]. This promotes scientists to search new enzyme, study the structure–function relationship and redesign its application by protein engineering [5,6].

In our previous study, we have reported a new glycine oxidase (BceGO) with glyphosate-oxidative activity from Bacillus cereus and, developed a high through screening method for improving its affinity and activity toward glyphosate [7]. Here, we continued to screen new mutant with higher specificity to glyphosate from a random mutation library of BceGO, and obtained a mutant, N336K, whose $K_m$,app on glyphosate decreased 3.77-fold. Sequence alignment showed that the residue N336 is highly conserved in BceGO and its homogeneous enzymes. Here, we attempted to investigate the role of N336 residue in the catalytic activity of GO by site-directed mutagenesis, three-dimensional structure modeling and ligand docking assay.

2. Materials and methods

2.1. Reagents, strains, and plasmid

Glyphosate, glycine, sarcosine, L-alanine, o-dianisidine dihydrochloride, horseradish peroxidase and FAD were purchased from Sigma (USA). Taq DNA polymerase, T4 DNA ligase and restriction enzymes were purchased from TAKARA (Japan). Fast Pfu polymerase, DNA purification kits, GST Binding Resin and Bradford protein assay kits were purchased from TransGen (Beijing, China), Axygen (USA), Novagen (Germany), and...
Sangon (Shanghai, China), respectively. *Escherichia coli* DH5α and *E. coli* BL21 (DE3) were used as for gene cloning and for protein expression, respectively.

### 2.2. Construction of mutant library and site-directed mutagenesis

The BceGO random mutant library was generated by error-prone PCR used pGEX-GO as the template. The amplification mixture, which contained 20 nM primers, 0.2 mM dATP and dCTP, 0.1 mM dTTP and dGTP, 2 U Taq DNA polymerase and Taq buffer containing 5 mM MgCl2 and 0.5 mM MnCl2 in 100 μL volume, was cycled in a Bio-rad thermal cycler (California, USA) for 30 cycles of 94°C for 30 s, 57°C for 30 s at 54°C, and 160 s at 72°C, and incubation of 10 min at 72°C. Then the PCR products were purified, digested with BamHI and XhoI, cloned into pGEX-6P-1, and transformed into *E. coli* DH5α to obtain the random mutant library.

PCR-based site-directed mutagenesis was carried out to generate single-mutant [8]. PCR reactions (50 μL) contained 20 ng template (pGEX-GO), 0.2 mM dNTP, 20 nM each primer, 10 μL PCR buffer and 1 unit of *Pfu* DNA polymerase (Transgen, China). The PCR cycling parameters were: 1 cycle of 2 min at 97°C, 20 cycles of 20 s at 95°C, 30 s at 54°C, and 160 s at 72°C, and incubation of 10 min at 72°C. Then the PCR products were treated with *DpnI* to digest the parental DNA at 37°C for 8 h. Finally, *DpnI* digestion mixture was transformed into *E. coli* DH5α competent cells, and the transformant was selected on ampicillin plates. The primers used were listed in Table 1. The desired mutants were validated by DNA sequencing.

### 2.3. Screening for GO mutants

The mutant library was screened by an enzyme-coupled assay using horseradish peroxidase (5 U/mL) and o-dianisidine dihydrochloride as substrates (glycine, 0–300 mM; glyphosate, 0–600 mM; sarcosine, 0–300 mM; o-alanine, 0–600 mM). The absorbance was measured at 450 nm using a microplate reader (Thermo Scientific, Multispec). The data were collected at room temperature from 190 to 260 nm using 1 mm quartz cuvette (400 μL). The conversion to the MoI CD (Δε) in each spectrum was performed with the Jasco Standard Analysis software. Estimation of the secondary structure content from far-UV circular dichroism (CD) spectra was performed by using the CDPro software.

### Table 2

Comparison of the apparent kinetics parameters of the wild-type BceGO and the mutants toward different substrates.

| Substrate | Wild-type | N336H | N336K | N336R | N336A | N336G |
|-----------|-----------|-------|-------|-------|-------|-------|
| Glycine   | k_{cat,app} (s^{-1}) | 0.71 ± 0.03 | 0.37 ± 0.02 | 0.25 ± 0.003 | 0.024 ± 0.0002 | 0.52 ± 0.02 | 0.67 ± 0.03 |
|           | k_{m,app} (mM) | 1.04 ± 0.12 | 0.95 ± 0.11 | 0.79 ± 0.12 | 0.53 ± 0.07 | 1.41 ± 1.23 | 2.25 ± 2.52 |
|           | k_{cat}/K_{m} | 0.68 | 0.39 | 0.32 | 0.045 | 0.37 | 0.3 |
| Glyphosate| k_{cat,app} (s^{-1}) | 0.87 ± 0.02 | 0.23 ± 0.05 | 0.19 ± 0.002 | 0.021 ± 0.0003 | 0.67 ± 0.08 | 0.62 ± 0.02 |
|           | k_{m,app} (mM) | 8.47 ± 2.34 | 42.31 ± 1.84 | 22.45 ± 1.44 | 10.44 ± 0.33 | 68.36 ± 3.15 | 96.73 ± 2.81 |
|           | k_{cat}/K_{m} | 0.01 | 0.0064 | 0.0084 | 0.002 | 0.0098 | 0.0064 |
| Sarcosine | k_{cat,app} (s^{-1}) | 0.98 ± 0.01 | 0.68 ± 0.04 | 0.28 ± 0.003 | 0.035 ± 0.0002 | 0.26 ± 0.03 | 0.59 ± 0.04 |
|           | k_{m,app} (mM) | 1.51 ± 0.18 | 1.39 ± 0.15 | 0.36 ± 0.02 | 0.15 ± 0.006 | 1.56 ± 0.17 | 2.72 ± 1.35 |
|           | k_{cat}/K_{m} | 0.65 | 0.49 | 0.78 | 0.23 | 0.17 | 0.22 |
| D-Alanine | k_{cat,app} (s^{-1}) | 0.81 ± 0.04 | 0.37 ± 0.03 | 0.21 ± 0.001 | 0.028 ± 0.0004 | 0.65 ± 0.01 | 0.46 ± 0.15 |
|           | k_{m,app} (mM) | 34.65 ± 1.22 | 24.63 ± 1.12 | 6.81 ± 0.32 | 1.31 ± 0.04 | 37.6 ± 0.95 | 48.85 ± 1.86 |
|           | k_{cat}/K_{m} | 0.023 | 0.015 | 0.03 | 0.021 | 0.017 | 0.009 |

2.4. Enzyme expression and purification

The recombinant BceGO and its mutant were purified by affinity chromatography using the methods described previously [7]. Briefly, the recombinant plasmids were transformed into the host *E. coli* BL21 (DE3). Recombinant cells grew at 37°C in LB medium containing 100 μg/mL ampicillin. Protein expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.2 mM, when the OD600 reached 0.6. After an overnight induction at 22°C, 1.5 L culture was collected and disrupted by the high pressure homogenizer (NiroSoavi, Italy). Then, the supernatant of the lysate was mixed with 1.5 L GST-Bind Resin that had been equilibrated with 50 mM dithiothreitol. The resin was washed with dithiothreitol buffer (50 mM, pH 7.5) to elute the unspecific-binding protein. Finally, the GST-free recombinant protein was prepared by on-column cleavage with PreScission protease [10]. The concentration of the wild-type BceGO and mutants was measured by the method of Bradford assay [11]. The purity of the protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

### 2.5. Determination of kinetic parameters

The kinetic parameters of wild-type BceGO and mutants were assayed using a fixed amount of enzyme and various concentrations of substrates (glycine, 0–300 mM; glyphosate, 0–600 mM; sarcosine, 0–300 mM; o-alanine, 0–600 mM). The absorbance was measured at 450 nm using a microplate reader (Thermo Scientific, Multispec). The initial reaction velocities under various concentrations of each substrate were fitted to the Lineeweaver-Burk transformation of the Michaelis– Menten equation to figure out apparent kinetic parameters (*i.e.*, k_{m,app} and V_{max}). Further, the k_{cat,app} was calculated by the equation: k_{cat,app} = V_{max} / [E], in which [E] is the total amount of enzyme in the reaction mixture.

### 2.6. Circular dichroism and secondary structure prediction

Secondary structure of BceGO was predicted by using the program PSIPRED [12]. Circular dichroism (CD) spectra of BceGO and variants were recorded with a Jasco-810 CD spectrometer (Jasco Corp., Japan). The data were collected at room temperature from 190 to 260 nm using 1 mm quartz cuvette (400 μL). The conversion to the MoI CD (Δε) in each spectrum was performed with the Jasco Standard Analysis software. Estimation of the secondary structure content from far-UV circular dichroism (CD) spectra was performed by using the CDPro software.
software package (available at [http://lamar.colostate.edu/~sreeram/CDPro/main.html](http://lamar.colostate.edu/~sreeram/CDPro/main.html)), including three executable programs (SELCON3, CDSSTR, and CONTIN/LL) [13]. In this study, the percentages of α-helix and β-sheet for each protein sample were averaged by the calculations from the CDPro software package. The circular dichroism data were expressed in terms of the mean residue ellipticity ($\theta_{\text{mrw}}$), which were calculated using Equation 1 [14]:

$$\theta_{\text{mrw}} = \frac{M_w \cdot \theta_{\text{obs}} \cdot 100}{N \cdot d \cdot c}$$  \hspace{1cm} \text{[Equation 1]}$$

where $\theta_{\text{obs}}$ is the observed ellipticity in degrees, $M_w$ is the molecular weight of wild-type and variants proteins, and $N$ is the number of residues in BceGO (369), $d$ is the path length of quartz cuvette (0.1 cm), $c$ is the protein concentration (mg/mL), and the constant number 100 stems from the conversion of the molecular weight to mg/dmol.

2.7. Molecular modeling analysis

To obtain a reasonable model, the structure of BceGO was built with homology modeling in InsightII program (version 2005). The crystal structure of glycine oxidase from B. subtilis (Protein Data Bank code: 1RYI) was used as the template. The binding conformation of the ligands in the BceGO active site was obtained with the docking module in MOE 2009.10, and the result description was prepared using software PyMol 0.99.

3. Results and discussion

3.1. Mutagenesis of BceGO

A random mutant library of BceGO was constructed by error-prone PCR to screen new mutants with low affinity and increased activity toward glyphosate by the method of high throughput colorimetric assay. Asn336Lys mutant was selected from 7000 clones, which showed improved specificity toward glyphosate than the wild type. Its apparent $K_m$ value decreased 3.77, 1.32, 4.19 and 5.09-fold glyphosate, glycine, sarcosine and D-alanine, respectively (Table 2). However, the turnover numbers (the $k_{\text{cat, app}}$) were lower than the wild-type BceGO. Protein sequence alignment showed that Asn336 is highly conserved in the GO family, and locates in the loop connecting β-strands 18 and α-helices 10 (Fig. 1). To elucidate the role of this invariable Asn336, it was substituted with other positively charged amino acids (i.e., His and Arg) and small amino acids (i.e., Ala and Gly) by site-directed mutagenesis.

3.2. Purification of BceGO and its variants

In order to characterize the enzyme, the wild-type BceGO and variants with GST tag were produced in E. coli BL21 (DE3) and purified by GSH-agarose affinity chromatography. GST-free recombinant fusion proteins were prepared via on-column cleavage by using PreScission protease. As a result, target proteins with high homogeneity and apparent molecular masses of 41 kDa were obtained (Fig. 2).

3.3. Kinetic parameters of BceGO variants

As shown in Table 2, it was observed that $K_m$ values against all substrates (i.e., glycine, glyphosate, sarcosine and D-Alanine) declined alone with the increase of positive charge on the side chain of residue 336. Especially, the $k_{\text{cat, app}}$ values of N336R toward substrates decreased 28-41-fold as compared to wild-type BceGO. It means that substitution at N366 with positively charged residues is able to improve the affinity toward glyphosate.
for the substrates. The both substitutions N336A and N336G did not significantly affect the substrate affinity (the $K_{\text{m,app}}$ value) for all the substrates. Additionally, the turnover number ($k_{\text{cat}}$) of the five mutants toward all tested substrate decreased to some different degrees (Table 2), suggesting that Asn336 is also involved in the catalytic efficiency of BceGO.

### 3.4 Analysis of protein secondary structure

The program PSIPRED predicted that the residue Asn336 was located in a conservative random coil region. A quantitative analysis of the protein secondary structure for wild-type BceGO and variants has been carried out using SELCON3 program. The data showed that the CD spectra of wild-type GO and mutants (N336H, N336K, N336R, N336A, and N336G) were similar (Fig. 3, Table 3). This result suggested that the mutation at Asn336 did not affect the content in secondary structure.

### 3.5 Structure modeling and substrate docking analysis

Protein homology modeling and ligand docking assay revealed the substrates matching the BceGO active site and oriented to the isoxaloxazine ring of the flavin cofactor (Fig. 4). The three dimensional structure of GO from *B. subtilis* showed the active site of GO containing FAD-binding domain and substrate-binding domain including a conserved Rossmann fold [αβ] motif. Both theoretical and experimental studies have indicated that the positive charge in the vicinity of the active site might cause alteration in the loop connecting β-strands 18 and α-helices 10, thereby impacting the charge distribution in vicinity of the flavin. In this work, introduction of basic amino acid to site 336 didn’t impair BceGO secondary structure and increased the affinity (the $K_{\text{m,app}}$ value) to all substrates, indicating that the positive charge near the flavin contributed to the binding of substrates to BceGO, which was accordant with previous findings [16].

### 4. Conclusion

In this work, we investigated the role of Asn336 in the active cavity of BceGO. Together with experimental data and model analysis, it was concluded that the high conserved residue Asn336 played a crucial role in the substrate affinity of BceGO, and positively charged residue could improve its substrate affinity, significantly. This study provides new insights into the structure–function relationship of glycine oxidase and valuable clue to redesign the substrate specificity by protein engineering.

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### Conflict of interest statement

There are no conflicts of interest.

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### Table 3

Secondary structure of GO and variants were calculated by SELCON3 program (CDPro software package).

| Components (%) | Wild-type | N336A | N336H | N336K | N336R | N336G |
|----------------|-----------|-------|-------|-------|-------|-------|
| α-Helix        | 20        | 20    | 21    | 20    | 19    | 19    |
| β-Sheet        | 29        | 29    | 30    | 28    | 29    | 29    |
| Turn           | 20        | 20    | 21    | 22    | 22    | 19    |
| Random         | 31        | 30    | 30    | 30    | 30    | 33    |

### Figure 4

Protein homology modeling and ligand docking assay. The structure of BceGO was generated by homology modeling in InsightII 2005 with GO from *B. subtilis* (PDB code: 1RYI) as the template. Ligand docking assay was carried out with the docking module in MOE 2009.10. The cofactor FAD was colored in yellow, and the substrates were rendered as ball-and-stick representations (Cyan, glyphosate. Blue, glycine. Green, sarcosine. Tan, D-alanine). This figure was prepared with Pymol 0.99.
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