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Implication of a mutation in the flavin binding site on the specific activity and substrate specificity of glycine oxidase from *Bacillus subtilis* produced by directed evolution

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Abstract

Directed evolution was used to expand the substrate specificity and functionality of glycine oxidase by using a high-throughput screening assay based on the 4-aminoantipyrine peroxidase system, with a coefficient of variance below 4%. After screening the library, one mutant with the desired changes was found. The mutant was purified and characterized, showing important changes compared to the wild-type, especially towards cyclic D-amino acids. Amino acid substitution of Ile15 for Val, where the consensus sequence for flavin binding site is placed, seems to be responsible for these changes in specific activity and substrate specificity. The effect of this mutation was explained by using a computer-based three-dimensional model.

Keywords: Glycine oxidase; Directed evolution; High-throughput screening; Pipecolic acid; Homology modelling

1. Introduction

Glycine oxidase (GOX, EC 1.4.3.19,) is a 154 kDa homotetrameric flavoprotein with deaminating activity that was cloned and characterized for the first time by Nishiya and Imanaka (1998). GOX catalyzes the deamination of small size amines (sarcosine, *N*-ethyl-glycine, glycine-ethyl-ester) and some D-amino acids. This deaminating activity is also shown by D-amino acid oxidase (EC 1.4.3.3, D-AAO) but it is only active towards neutral and basic D-amino acids. Although sarcosine (*N*-methyl-glycine) is a substrate of the enzyme, GOX does not catalyse its oxidative demethylation to form glycine and formaldehyde as sarcosine oxidase does (EC 1.5.3.1, SOX) (Job et al., 2002a).

Since GOX and D-AAO catalyze the oxidative deamination of amino acids to yield the corresponding α-keto acids, ammonia (or primary amines) and hydrogen peroxide (Scheme 1), GOX is the object of particular attention as an alternative to D-AAO, since the latter can be used in the industrial production of biosensors (Gemeiner et al., 1993), α-keto acids (Brodelius et al., 1981; Butó et al., 1994; Trost and Fischer, 2002), the resolution of racemic mixtures for the production of pure L-amino acids (Nakajima et al., 1990; Trost and Fischer, 2002), or the industrial bioconversion of cephalosporin C to glutaryl-7-amino cephalosporanic acid and 7-amino cephalosporanic acid (7-ACA) (Szwajcer-Dey et al., 1991; Conlon et al., 1995; Pilone et al., 1995). Due to the low production of recombinant GOX, those industrial applications are still carried out using yeast D-AAO. However, the recombinant production of D-AAO in prokaryotic organisms is difficult and has a low operational stability (Lin et al., 2000). Thus, one biotechnological goal is to find alternatives for yeast D-AAO. Recently, high-level production of GOX has been achieved by fed-batch cultivation (Martínez-Martínez et al., 2007), but a GOX with activity towards classical D-AAO substrates (D-methionine, D-proline) is still needed.

Molecular or directed evolution of proteins has become a powerful tool to achieve improvement in substrate affinity, thermostability or increase in specific activity (Bloom et al., 2005).

Abbreviations: GOX, glycine oxidase; D-AAO, D-amino acid oxidase; SOX, sarcosine oxidase; HT, high-troughput.

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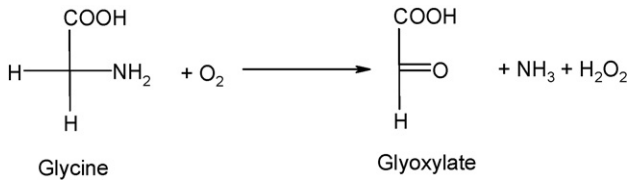

Scheme 1. Reaction catalyzed by glycine oxidase.

Several methods have been developed to introduce changes into the amino acid sequence, including site-directed mutagenesis, random mutagenesis (directed evolution), antibody catalysis, computational redesign, and de novo methods (Shao and Arnold, 1996; Woycechowsky et al., 2007). The aim of this work was to explore the importance of some residues in the activity or substrate specificity of GOX by using directed evolution techniques.

2. Materials and methods

2.1. Reagents, plasmids and cells

Restriction enzymes were obtained from New England BioLabs (Beverly, MA). T4 DNA ligase was from Roche Diagnostics. Taq DNA polymerase and its 10× reaction buffer were from Promega. Genomic DNA was extracted using a DNeasy system. QIAprep spin plasmid miniprep kit and QIAquick PCR purification kit were from QIAgen (USA). Substrates, antibiotics and broths were from Sigma. Ultrafiltration and microfiltration membranes, HiTrap chelating, PD10, Phenyl Sepharose and Superdex 200 prepacked columns were from GE Healthcare (Barcelona, Spain). All other chemicals were of reagent grade and obtained from commercial sources. The pET28a plasmid and *E. coli* Rosetta (DE3) cells were from Novagen (USA).

2.2. Plasmid and library construction

The *B. subtilis yjb*R gene was amplified by standard PCR with the following primers: YJBr-up (5′-G*GAATTC*ATGAAAAGGCATTATGAAGCAGT-3′) derived from the 5′-end and YJBr-down (5′-G*CTCGAG*TCATATCTGAACCGCCTCCTTGC-3′) derived from the 3′-end of the nucleotide sequence of *yjb*R gene (the sequences recognized by *Eco*RI and *Xho*I restriction enzymes are underlined) using the same strategy as that reported (Martínez-Martínez et al., 2006).

Mutagenic PCR was carried out under standard error-prone conditions. Primers YJBr-up and YJBr-down were used to amplify and mutate the template gene, which had been previously subcloned into vector pET28a by ligation and transformation into *E. coli* Rosetta (DE3). 50 nM each of the primers were used and the reaction conditions were: 100 ng template, 10 mM Tris–HCl pH 8.3, 50 mM KCl, 7 mM $MgCl_2$, 0.2 mM dNTP mix, 5 U Taq polymerase (Promega), in a total volume of 100 μl. The PCR reaction was carried out for 30 cycles of 94 °C, 45 s; 55 °C, 45 s; 72 °C, 1 min 10 s, and then 1 cycle of 72 °C for 10 min. The library fragment was purified and digested with *Eco*RI and *Xho*I. The resulting PCR products were ligated to pET28a, which had been previously digested. Plasmids were transferred into *E. coli* Rosetta (DE3) electrocompetent cells but recovery in SOC media was done only for 20 min, instead of 1 h, to avoid duplicated mutants. The error rate of mutants was checked by DNA sequencing.

2.3. DNA sequencing

To locate mutations on the *yjb*R gene and estimate the error rate, mutant plasmids were sequenced at the Molecular Biology Integrated Laboratory of the Centro de Apoyo a la Investigación y Desarrollo (CAID), University of Murcia. The primers used were PETup (5′-ATGCGTCCGGCGTAGA-3′) and PETdown (5′-GCTAGTTATTGCTCAGCGG-3′).

2.4. Library preparation

Transformed *E. coli* Rosetta (DE3) cells were plated on LB agar and incubated at 37 °C overnight. Individual colonies were picked into 96-well plates (QPix2, Genetix, UK) containing 100 μl LB supplemented with 50 μg ml^{-1} and chloramphenicol 34 μg ml^{-1}. On each plate, four wells in column 9 were inoculated with parental transformed cells while four wells in the same column were not inoculated (to check the possibility of cross-contamination). Plates were incubated for 24 h at 30 °C and 150 rpm (Certomat BS1 shaker, B. Braun International, Sartorius group, Germany). Afterwards, cells were replicated with QPix2 system (Genetix, UK) into 96-deep well plates containing 300 μl Terrific broth (TB) media supplemented with 50 μg ml^{-1}, chloramphenicol 34 μg ml^{-1} and 1 mM isopropyl thio-β-D-galactoside (IPTG). Plates were then incubated for an additional 24 h at 30 °C and 175 rpm. After induction, plates were centrifuged for 20 min at 1600 × *g* at 4 °C. Supernatants were removed and pellets were resuspended with a volume of 0.5 mg ml^{-1} lysozyme (Roche Diagnostics) in 75 mM sodium pyrophosphate buffer pH 8.5. After 1 h of incubation at 37 °C, plates were centrifuged with the same parameters as above and supernatants were used for the reactions. All these steps were done with a Starplus robot (Hamilton, Switzerland).

A second screening was done inoculating 10 ml of LB media (supplemented with 50 μg ml^{-1} and chloramphenicol 34 μg ml^{-1}) with most active clones at 37 °C, and parental type was grown in the same conditions. After 12 h, 10 ml of grown cultures were used as precultures for inoculating 100 ml TB media (supplemented with 50 μg ml^{-1} and chloramphenicol 34 μg ml^{-1}) in 1 L flasks. Cultures were grown at 37 °C, 150 rpm, for 5 h, and were then induced with 1 mM IPTG for 24 h at 30 °C.

2.5. Activity screen

To measure the activity of each clone, 50 μl of supernatant was transferred into 96-well plates containing 150 μl substrate solution with the following final concentrations: 10 mM substrate (glycine, D-methionine, D-proline), 0.4 mM 4-aminoantipyrine, 7 mM phenol, 4.4 U ml^{-1} horseradish peroxidase, 0.2 μM FAD and 75 mM sodium pyrophosphate (pH