Arg305 of *Streptomyces* L-glutamate oxidase plays a crucial role for substrate recognition

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**Abbreviations:** LGOX, L-glutamate oxidase; LAO, L-amino acid oxidase; KPB, potassium phosphate buffer; MBTH, 3-methyl-2-benzothiazolone hydrazone hydrochloride.

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1. Introduction

L-Glutamate oxidase (LGOX; EC 1.4.3.11) catalyzes the oxidative deamination of L-glutamate to a 2-ketoglutarate via an imino acid intermediate. LGOXs of several kinds have been identified from the genus *Streptomyces* [1–4]. Among them, an enzyme from *Streptomyces* sp. X-119-6 is the sole commercially available enzyme that is useful for biosensors: it has strict substrate specificity and high stability. LGOX is a useful analytical tool for the quantitative assaying of L-glutamate existing in food, in a fermentation process, and in the brain acting as the principal excitatory neurotransmitter. In addition, microfluidic biosensors using LGOX are applicable for the detection of glutamic oxaloacetic transaminase, glutamic pyruvic transaminase and \(\gamma\)-glutamyl transpeptidase activities, which are diagnostic markers of liver function [5,6].

As a similar enzyme, L-amino acid oxidases (LAOs) have been isolated from various sources [7–13]. General LAOs (for example LAO from snake venom) catalyze the oxidation of a variety of \(L\)-amino acids. Meanwhile, several LAOs with strict substrate specificity have been identified: \(L\)-lysine-\(\alpha\)-oxidase [14], \(L\)-phenylalanine oxidase [15,16], and \(L\)-aspartate oxidase [17]. They have an identical catalytic mechanism but different affinities for substrate. Elucidation of the mechanism for substrate recognition create the potential of a molecular design of this class of the enzyme; that is, a development of potent biocatalyst for \(L\)-amino acid sensor through modification of their properties using protein engineering techniques. Particularly, sensor for measurement of \(L\)-histidine and \(L\)-arginine has high demand in the food and medical fields; that is, \(L\)-histidine is one of the essential amino acid for human that has a beneficial effect on anti-obesity [18] and \(L\)-arginine is one of the semiessential amino acid for human that is important for removal of ammonia from human body.

Recently, we have solved the crystal structure of L-glutamate oxidase (LGOX) from *Streptomyces* sp. X-119-6 (PDB code: 2E1M), the substrate specificity of which is strict toward \(L\)-glutamate. By a docking simulation using \(L\)-glutamate and structure of LGOX, we selected three residues, Arg305, His312, and Trp564 as candidates of the residues associating with recognition of \(L\)-glutamate. The activity of LGOX toward \(L\)-glutamate was significantly reduced by substitution of selected residues with Ala. However, the enzyme, Arg305 of which was substituted with Ala, exhibited catalytic activity toward various \(L\)-amino acids. To investigate the role of Arg305 in substrate specificity, we constructed Arg305 variants of LGOX. In all mutants, the substrate specificity of LGOX was markedly changed by the mutation. The results of kinetics and pH dependence on activity indicate that Arg305 of LGOX is associated with the interaction of enzyme and side chain of substrate.

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study, the mechanism for substrate recognition of LGOX is discussed.

2. Materials and methods

2.1. Docking study

The docking study was performed using Internal Coordinate Mechanics (ICM) software (Molsoft L.L.C., La Jolla, USA), a program for comparative protein structure modeling and interactive docking. The structure of l-glutamate was built into the software, and simulation of interactive docking was performed using the rigid form of LGOX and flexible form of l-glutamate. The simulation was repeated 150 times with the calculation of total energy using biased probability Monte-Carlo method as calculated before the conformation was stored [20].

2.2. Enzyme assays

LGOX activity assay involved the determination of 2-ketoglu tarate formed with 3-methyl-2-benzothiazolone hydrazine hydrochloride as described previously (MBTH method) [21]. One unit of LGOX activity was taken as the amount of the enzyme that liberated 1 μmol of 2-ketoglu tarate per minute.

2.3. Construction of an expression vector

The full-sized gene encoding LGOX was amplified using PCR from the chromosomal DNA of Streptomyces sp. X-119-6 using the following primers: 5′-CCACTCAGAGGCGCCATGAGCAGA- GAT-3′ and 5′-AGGTAATCCGCCACCTGACCGTC-3′ (the underlined regions are EcoR I and Pst I). The PCR product was separated by agarose gel electrophoresis and digested by EcoR I and Pst I, then subcloned into the EcoR I and Pst I gap of pMal-c2 (yielding pGox-mal1).

2.4. Site directed mutagenesis

Site-directed mutagenesis was conducted by inverse PCR using a pair of oligonucleotide primers containing a point mutation (see Table S1 in the supplemental material) and the pGox-mal1. The PCR program was as follows: 15 cycles of 10 s at 98 °C, 30 s at 65 °C, and 10 min at 68 °C. The PCR product was treated with DpnI at 37 °C for 1 h. Then the PCR product was transfected into competent cell of Escherichia coli TOP10 according to the manufacturer’s protocol. After the confirmation of the sequence, the extracted plasmid was transformed into E. coli JM109.

2.5. Expression and purification of the recombinant LGOX and its variants fused with maltose binding protein E. coli

JM109 cells harboring pGox-mal1or the plasmid for mutant LGOX expression were cultivated at 22 °C for 24 h in 3 l of 2× TY medium. Expression of the LG OX was induced by incubation at 22 °C with 0.5 mM IPTG for 24 h. The harvested cells were suspended in 20 mM potassium phosphate buffer (KPB) (pH 7.4), and then disrupted by ultrasonication on ice. After removal of the cell debris, the supernatant was brought to 40% saturation with ammonium sulfate. The resultant supernatant was brought to 60% saturation with ammonium sulfate. The resultant precipitate was dissolved in 20 mM Tris–HCl buffer (pH7.4) containing 200 mM NaCl and 1 mM EDTA. Next, the solution was loaded onto an Amylose resin affinity column (New England Biolabs, Beverly, MA, USA) equilibrated with the same buffer. The column was washed gently with the equilibrium buffer. Then the enzyme was eluted using the same buffer containing 10 mM maltose.

2.6. Maturation of LGOX and its variants by protease digestion

The purified LGOX and its variants by Amylose resin were single-chain LGOX precursor fused with maltose binding protein. The digestion by metallo-protease from Streptomyces was necessary for maturation of LGOX [22] (the mature form of LGOX has α2β2γ2 structure). To maturation of them, the purified enzymes were treated with the metallo protease from Streptomyces griseus (Sigma–Aldrich Corp., St. Louis, MO, USA) at 30 °C for 24 h. Then it was heated at 50 °C for 30 min to inactivate the protease. After centrifugation, the solution was loaded onto a column (DEAE-Toyopearl 650; Tosoh Corp., Tokyo, Japan) equilibrated with 20 mM KPB (pH 7.4) containing 0.1 M NaCl. After the column was washed with the same buffer, the bound protein was eluted with 20 mM KPB (pH 7.4) containing 0.3 M NaCl. The eluate was pooled and dialyzed against 20 mM KPB (pH 7.4); the dialysate was used as the mature enzyme.

2.7. Biochemical studies

The substrate specificity of wild-type and mutant LGOXs were tested by the MBTH method with 0.5 mM l-tyrosine, 1 mM l-tryptophan and l-aspartate, 5 mM l-leucine, and 10 mM other substrates with the purified enzymes. Effects of pH on activity were examined using Britton-Robinson buffer at a pH range of 4.0–12.0. The activities were measured by the MBTH method at the corresponding pH.

3. Results and discussion

3.1. Conformational simulation of l-glutamate in active site of LGOX

The overall structure and local structure around the active site of LGOX resembles that of LAAO [19]. A previous report by Pawelek et al. of the crystal structure of LAO interacted with o-aminobenzolate (AB) described that three AB molecules are visible within the funnel of the LAO-AB complex [23]. Likewise, the structure of LGOX interacted with ligand provide us with valuable information about the mechanism of the substrate recognition of LGOX. However, the LGOX structure interacting with a mimic of the natural substrate was not analyzed because of the difficulty in obtaining crystals of the LGOX–ligand complex. Therefore, we performed a docking study using the LGOX structure as a receptor and l-glutamate as ligands to investigate the interaction of enzyme with a substrate.

By the simulation, we obtained numerous l-glutamate binding forms around the active site. The l-glutamate molecules were mainly present at three sites: the entrance to the active site, near the active site, and the active center (see Fig. S1 in the Supplemental material). As shown in Fig. 1, the model at the active center indicates that the hydrogen atom on Cα of l-glutamate points toward to isoalloxazine ring of FAD, and the distance between Cα of l-glutamate and N5 of FAD is approximately 5 Å. Although this is not sufficiently close for reaction, l-glutamate, the residues surrounding l-glutamate, and the FAD should be allowed to be flexible. Because it has suitable space and adequate basic residues in the model of l-glutamate binding at the active center, LGOX might have strict specificity for oxidation toward l-glutamate.

3.2. Mutation of Arg305, His312, and Trp564 of LGOX with Ala

In the binding model, the side chains of Arg305 and His312 might produce hydrogen bonding with the side chain of l-glutamate. In
addition, Trp564 also appears to be important for closing up the active site (Fig. 1). To investigate the roles of them in substrate specificity of LGOX, we constructed R305A, H312A, and W564A LGOXs by site-directed mutagenesis. We used a pMal system to produce wild-type and mutant enzymes.

By using the cell free extract of them treated with metallo-protease from *Streptomyces*, the activities of mutants toward glycine and 19 kinds of L-amino acids were investigated. By the investigation, we found that all mutants exhibited the activity for L-glutamate oxidation significantly lower than wild type; the mutation of Arg305 with Ala resulted in about 20-fold decrease in L-glutamate oxidation, and the activities of other mutants were even lower than that of R305A LGOX (data not shown). The mutations of His312 and Trp564 with Ala exerted little influence on substrate specificity. In contrast, R305A LGOX catalyzed the oxidation of some L-amino acids, whereas wild-type LGOX exhibits no oxidation activity toward L-amino acids except L-glutamate.

3.3. Effect of mutation of Arg305 on substrate specificity of LGOX

Because the side chain of Arg behaves strong base, it seems to interact strongly with the side chain of the substrate L-glutamate. Therefore, we focused the residue Arg305 and investigated its roles in substrate specificity of LGOX. We constructed R305X LGOX by site-directed mutagenesis, then matured and purified it by three purification steps as described in Section 2. As judged by SDS-PAGE, the wild-type LGOX and the mutants (R305A, R305L, R305D, R305K LGOXs) could be produced and purified as described in materials and methods (see Fig. S2 in the Supplemental material).

By the investigation of specific activities of R305X mutants toward glycine and 19 kinds of L-amino acids, we found that all mutants catalyzed the oxidation of L-leucine, L-phenylalanine, and L-histidine (Fig. 2). Moreover, the specificity of mutant enzymes toward respective L-amino acids was different from each other. That is, R305A, R305L, R305K LGOXs showed the highest activity toward L-histidine, however, the activity of R305D LGOX toward L-arginine was higher than that toward L-histidine.

3.4. Effect of pH on oxidative activity

To obtain information on the ionizable groups involved in the formation of an ion bridge, the activities toward L-glutamate, L-histidine, and L-arginine were determined at pH between 5 and 10. Fig. 3 shows the effect of pH on the initial velocities of the parental and mutant enzymes using respective substrates. The curves for L-histidine oxidation by mutant enzymes were narrower than that for L-glutamate oxidation by wild type. In addition, the pH range of L-histidine oxidation by R305K LGOX was shifted to the alkaline side. The similar curve was observed in L-arginine oxidation by R305D LGOX. From these results, we speculate that the formation of the ion bridge between the residue 305 of LGOX and the substrate affects the pH dependence of oxidative activity. That is, in such pH ranges, the side chain of the substrate tends to form an ion bridge with the residue, and the formation of an ion bridge leads to an increase in oxidative activity.

3.5. Kinetic study

We also characterized the kinetics of the wild-type and R305X LGOXs; the results are summarized in Table 1. Although all mutation resulted in the loss of activity toward L-glutamate, the catalytic efficiency toward L-histidine, L-phenylalanine, L-leucine, or L-arginine of all mutants could be measured because of the enhancement of activity toward them by the mutation. In terms of catalytic efficiency toward L-histidine, R305L LGOX displayed the highest value among the LGOX variants. The $k_{cat}$ value of R305L LGOX for L-histidine oxidation showed only 1.3- or 2-fold higher than those of other mutants. However, the $K_m$ value of R305L for L-histidine oxidation was over 5.5-fold lower than those of other mutant enzymes. The result indicates that the change of
the environment around the substrate binding site by mutation affects l-histidine binding more strongly than catalytic activity.

In contrast to the l-histidine oxidation, the $k_{cat}$ and $K_m$ values for l-phenylalanine oxidation were different remarkably among the R305 variants of LGOX. The mutants that have hydrophobic residue at the position of 305 displayed the higher $k_{cat}$ value for l-phenylalanine oxidation. With a focus on l-phenylalanine, the substrate specificity of LGOX was markedly changed by the substitution of Arg305 with other amino acid residue. The results of kinetics and pH dependence on activity indicate that Arg305 of LGOX is associated with the formation of an ion bridge.

This shift leads to a change in the distance between the substrate and the catalytic center (i.e. N5 atom of isoaahxazine ring of FAD), resulting in a change in catalytic activity. Furthermore, in R305D LGOX, it is considered that an ion bridge was formed between the side chain of the substrate l-arginine and that of the substituted residue. In fact, only R305D LGOX had the catalytic activity toward l-arginine among the mutant enzymes (Fig. 2 and Table 1). It is thought that activity with l-arginine is enhanced by a suitable distance because of the formation of an ion bridge, that is, this formation of an ion bridge is a factor in the enhancement in catalytic activity.

In conclusion, we have found that Arg305 of LGOX plays a crucial role for substrate recognition. The substrate specificity of LGOX was markedly changed by the substitution of Arg305 with other amino acid residue. The results of kinetics and pH dependence on activity indicate that Arg305 of LGOX is associated with the interaction of enzyme and side chain of substrate. Through the investigations, we obtained the enzymes that have oxidative activity towards l-histidine and l-arginine that have functions important from a primarily medical viewpoint.

Appendix A. Supplementary data


References