〈Brief Note〉

Altered substrate specificities of mandelate oxidases generated by site-directed mutagenesis of L-lactate oxidase

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Summary We had previously reported the construction of L-mandelate (MAN) oxidase by careful modification of L-lactate (LAC) oxidase from the genus *Enterococcus*. Substrate specificities of five engineered MAN oxidases, including those of the three newly constructed mutants, were investigated. The engineered enzymes exhibited quite different activities with glycerate (GLY) and L-phenyllactate (PHE) compared to those with MAN. Although the five mutants demonstrated an increased MAN oxidase activity, they exhibited decreased GLY and PHE oxidase activities as compared to the wild type LAC oxidase. The current study compared the reactivities of wild type and mutant LAC oxidase using substrate-docking models.

Key words: Protein engineering, Lactate oxidase, Structural model, Substrate docking, Substrate specificity

1. Introduction

Styrene is used in large quantities as an organic solvent in paint industries. Following exposure, it is metabolized in the body of workers to yield mandelate in urine. Therefore, it is possible to evaluate styrene exposure in workers and prevent health damage by measuring mandelate[1]. However, the HPLC method currently used to measure mandelate[2] is complicated and time consuming. Hence, there is a need to develop a highly efficient method for measuring the levels of mandelate in urine. L-mandelate (MAN) oxidase can be exploited for the construction of an enzymatic assay that is capable of simple and high-throughput analysis of mandelate levels in biological samples[3].

L-lactate (LAC) oxidase (EC 1.1.3.2) catalyzes the oxidation of LAC to yield pyruvate and hydrogen peroxide[4-6]. Our previous study has reported the production of MAN oxidase via structure-guided design of engineered LAC oxidase from *Enterococcus* sp. NBRC3427 (LoxL, DDBJ/EMBL/GenBank accession number: LC377909)[7]. LoxL is

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already used in clinical lactate assays and lactate biosensors. The tertiary structure of LoxL was predicted using homology modeling and was used to generate docking simulations between LoxL and LAC, MAN, glycerate (GLY), or L-phenyllactate (PHE)[7]. These models revealed marked steric interference between LoxL and MAN, a bulky and poorly reactive substrate[6]. Therefore, double and triple mutants that can effectively act on MAN were created based on the rational design of engineered LoxL, thereby extending the application of LoxL mutants in healthcare examination[3].

The rational design of the LoxL mutations in the active site and its surroundings resulted in the generation of mutant enzymes with remarkably improved affinities and catalytic efficiencies for MAN. Substrate specificities of these mutant enzymes were significantly improved relative to wild type protein owing to the reduction in steric interference for bulky substrates like MAN. There were obvious correlations between the effect of mutations on the tertiary structure of enzyme and utilization of different substrates[3].

In this study, we constructed MAN oxidases by protein engineering of LoxL. Reactivities of the MAN oxidases towards GLY and PHE were compared to those of the wild type LoxL. These results further enriched our knowledge of the active site structure of LoxL protein.

2. Materials and Methods

In addition to the previously constructed double and triple mutants (MD: A92G+Y121A and MTV: MD+Y211V) that can use MAN, three more triple mutants of LoxL (MD+M207A, MD+M207V, and MD+V210A, respectively) were constructed via site-directed mutagenesis using inverse PCR. Primers used for mutagenesis are listed in Table 1. *E. coli* BL21(DE3) cells were used for recombinant protein expression as previously described[3,7]. The mutant proteins were expressed with a C-terminal His-tag and were purified to homogeneity. The enzymatic properties of the purified proteins were characterized. The enzyme assay was based on the quantification of the amount of hydrogen peroxide produced during substrate oxidation[3,6,7].

MOE (Molecular Operating Environment) software (Chemical Computing Group Inc., Montreal, Canada) was used to model the substrate-enzyme complexes. The docking simulations were performed as described previously[3,7].

3. Results and Discussion

Our previous findings indicated that Y211 of LoxL limited the reactivity of LoxL enzyme for bulkier substrates[3,7]. Therefore, it can be speculated that other residues lying close to Y211 can alter the substrate specificity of LoxL by moving the side

Table 1 Primers used for construction of mutants

Primer		Sequence
M207A	Forward	5'-GCGGATGCAGTGTATAAAAGCAGCAAACAG
M207A	Reverse	5'- TGTCTGACCAACACCGCTCTGATATGCCTG
M207V	Forward	5'-SYGGATGCAGTGTATAAAAGCAGCAAACAG
M207V	Reverse	5'- TGTCTGACCAACACCGCTCTGATATGCCTG
V210A	Forward	5'-GCGTATAAAAGCAGCAAACAGAAACTGAGC
V210A	Reverse	5'-TGCATCCATGGTCTGACCAACACCG

S; C or G, Y; C or T.

chain of Y211. Thus, M207 and V210 were selected as new targets for mutagenesis, and triple mutants, MD+M207A, MD+M207V, and MD+V210A, were constructed and purified to homogeneity. The ratio of enzyme activities of these mutants for oxidation of MAN to LAC were above 1.2×10^4 times higher than that of wild type protein, while those of the previously constructed MD and MTV mutants were approximately 2.7×10^4 and 1.5×10^5 times higher than the wild type, respectively (Table 2). This suggests that the interaction between LoxL and MAN can be altered by substituting indirectly related residues. M207 and V210, as well as Y211, are located in a long loop in the proximity of active site (Fig. 1). The change in the flexibility of this loop may have contributed to the effects of mutations.

Specific activities of wild type and five mutants for LAC, MAN, GLY, and PHE are summarized in Table 2. The previous results of molecular docking simulations predicted that the reactivity of LoxL for bulky α-hydroxy acids increases in the order MAN<GLY<PHE[7]. The specific activities of wild type protein for MAN, GLY, and PHE reflected those predicted from the simulations (Table 2). However, the mutants having MAN oxidase activity exhibited quite different enzymatic activities. As shown in Table 2, the mutants exhibited improved reactivity for MAN than those observed for GLY and PHE.

The modeled substrate-docking structures were constructed and used in the analyses of structure-function relationships and the effects of mutations (Fig. 1). The MAN-fitting models of the wild type and MTV mutant revealed that Y211 residue in wild type protein produces steric interference with the binding of bulkier substrates and Y211V mutation results in the expansion of active site pocket in mutant proteins. In contrast, the decrease in the reactivity of MTV for GLY is probably caused by the excessively expanded pocket in MTV resulting in weaker interactions with the polar groups of GLY.

No interference was observed between the Y211 residue and binding of PHE, and this can be attributed to the differential orientation of the benzene ring in case of PHE and MAN (Fig. 1). In comparison to the MD and MTV mutants, the wild type enzyme showed higher reactivity for PHE. However, the activity ratio of PHE to LAC was approximately 0.0096 for the wild type enzyme while those for MD and MTV mutants were 0.049 and 0.27, respectively (Table 2). As shown in Fig. 1, expansion of the active site pocket in mutants would have changed the interactions between the peripheral residues lining the active site pocket of enzyme (Y37, Y121, Y211, R179, and H261) and PHE.

To further understand the effect of mutations on substrate specificity of enzyme, the affinities of wild type, MD, and MTV proteins for PHE were investigated (Fig. 2). Although the substrate-MAN oxidase activity curves did not follow the common

Table 2 Substrate specificities of wild type and mutant lactate oxidases for different α-hydroxy acids

Enzyme	Specific Activity (U/mg)			
	L-Lactate (LAC)	DL-Glycerate (GLY)	L-Mandelate (MAN)	L-Phenyllactate (PHE)
Wild type	77	0.51	0.0051	0.74
MD: A92G+Y121A	0.83	0.064	1.5	0.041
MTV: MD+Y211V	0.10	0.025	1.0	0.027
MD+M207A	0.22	0.047	0.24	n.t.
MD+M207V	0.73	0.24	0.57	n.t.
MD+V210A	n.d.	n.d.	0.086	n.t.

n.d.; not detected, n.t.; not tested.

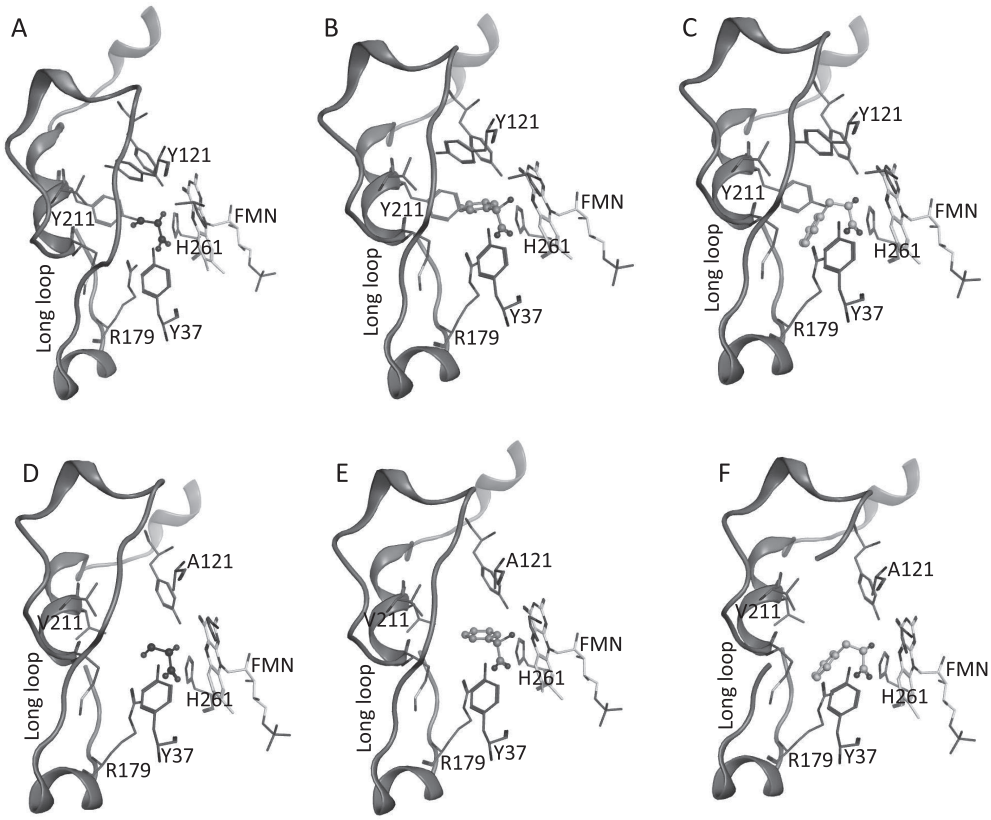

Fig. 1 GLY-, MAN-, and PHE-fitting models of wild type LoxL and MTV mutant. Close-up views of the active site in wild type and mutant protein in complex with glycerate, mandelate, and phenyllactate are shown. Side chains of the amino acid residues and flavin mononucleotide (FMN) are shown as stick drawings. Each compound is shown as ball-and-stick drawing. (A) wild type and GLY, (B) wild type and MAN, (C) wild type and PHE, (D) MTV and GLY, (C) MTV and MAN, (F) MTV and PHE.

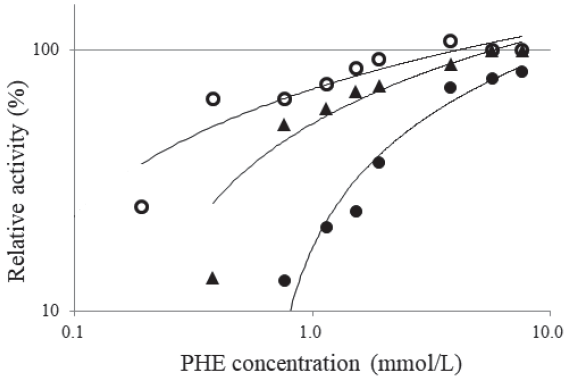

Fig. 2 Substrate concentration-dependent PHE oxidase activity. PHE concentrations and the relative activities of wild type and mutant enzymes are indicated on log scale. Open circle: wild type, filled circle: MD, filled triangle: MTV.

Michaelis-Menten kinetics, the affinities of mutants for PHE were lower than that of the wild type protein. This experiment demonstrates that the mutations inhibited the binding of PHE in the active site.

As expected, the altered substrate specificities of the LoxL mutants could be interpreted using the substrate-docking models. This study will extend the application of LoxL mutants in medical and food industries. For example, bulky α-hydroxy acids are used as raw materials for pharmaceuticals and for the treatment of liver diseases and also have potential applications in food preservation.

Conflicts of interest

The authors have no conflicts of interest.

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