〈Original Article〉

Creation of an L-mandelate oxidase via structure-guided design of engineered lactate oxidase

Misaki Hiruta[1] and Yoshiaki Nishiya[1]

Summary Substrate specificity of lactate oxidase from the genus *Enterococcus* was altered by triple mutations designed with the help of the tertiary structure model and L-mandelate docking. Catalytic efficiency of the mutant for L-mandelate was determined to be 9.4 $s^{-1}mmol^{-1}L$. However, catalytic efficiency of the mutant for L-lactate and that of wild-type for L-mandelate could not be precisely estimated due to weak substrate affinity.

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

1. Introduction

Lactate oxidase (EC 1.1.3.2) is a homotetrameric flavoprotein containing one flavin mononucleotide (FMN) per subunit as a coenzyme. The enzyme catalyzes the oxidation of L-lactate to yield pyruvate and hydrogen peroxide[1]. The lactate concentration in the blood rises with fatigue during exercise and acts as a stress indicator as well as an indicator of diseases such as lactic acidosis[2]. Lactate oxidase from *Enterococcus* sp. NBRC3427 (LoxL, DDBJ/EMBL/GenBank accession number: DJ772830 and HV348788), previously developed for use in several applications[3], is utilized in clinical lactate assays and lactate biosensors.

The tertiary structure of LoxL was constructed using homology modeling. This structure model was used to generate docking simulations between LoxL and L-lactate, L-mandelate, L-glycerate, or L-phenyllactate, which revealed marked steric interference between LoxL and L-mandelate[4].

In this study, a triple mutant that acts on L-mandelate, a bulky and poorly reactive substrate, was developed based on the rational design of engineered LoxL, thereby seeking to extend the application of LoxL mutants in health examinations. In paint industries, styrene is handled in large quantities as an organic solvent, and is consequently metabolized in the body of the workers to yield mandelate in urine (Fig. 1). It is possible to evaluate styrene exposure and prevent health damage by measuring mandelate. The current HPLC method used to measure mandelate[5,6] is complicated and time consuming, making the development of a highly efficient method desirable. L-mandelate

[1]Department of Life Science, Setsunan University, 17-8 Ikeda-Nakamachi, Neyagawa, Osaka 572-8508, Japan. To whom correspondence should be addressed.
E-mail: nishiya@lif.setsunan.ac.jp,
Fax: +81-72-838-6599, Tel: +81-72-800-1151
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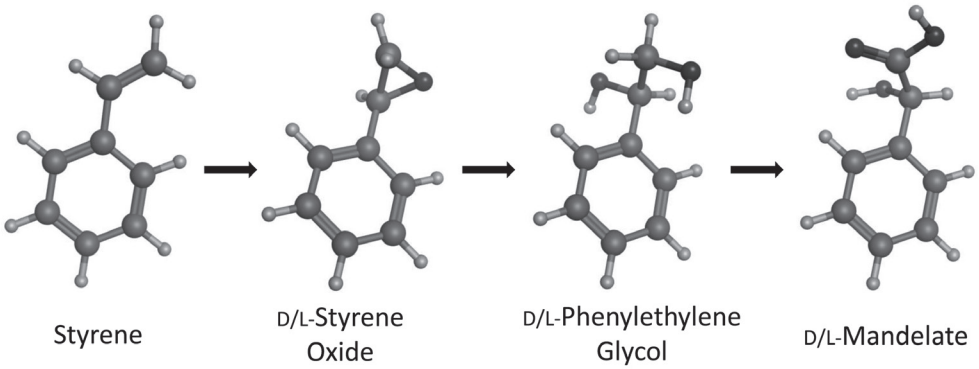

Fig. 1 Styrene metabolism.

oxidase created via structure-guided design of engineered lactate oxidase can contribute to the construction of an enzymatic assay capable of simple and high-through put analysis (Fig. 2).

2. Materials and Methods

MOE (Molecular Operating Environment) software ver. 20151001 (Chemical Computing Group Inc., Montreal, Canada) was used to build the tertiary structures of the LoxL mutants and substrate-enzyme complexes. The docking simulations were performed as described previously[4].

To produce mutants of interest, the gene encoding a double mutant (MD: A92G + Y121A) was first artificially synthesized by the manufacturer (Life Technologies, Foster City, CA, USA). The codon usage was adapted to the codon bias of the *Escherichia coli* genes (DDBJ/EMBL/GenBank accession number: LC377909). The genes encoding two triple mutants (MTV: A92G + Y121A + Y211V and MTA: A92G + Y121A + Y211A, respectively) were constructed via site-directed mutagenesis using inverse PCR (Fig. 3). Plasmid construction with pET29a, recombinant strain preparation of *E. coli* BL21(DE3), and bacterial cultivation were carried out as previously described[4]. The genes with a C-terminal His-tag were expressed, and each enzyme was purified to homogeneity. The enzymatic properties of the purified product were then characterized.

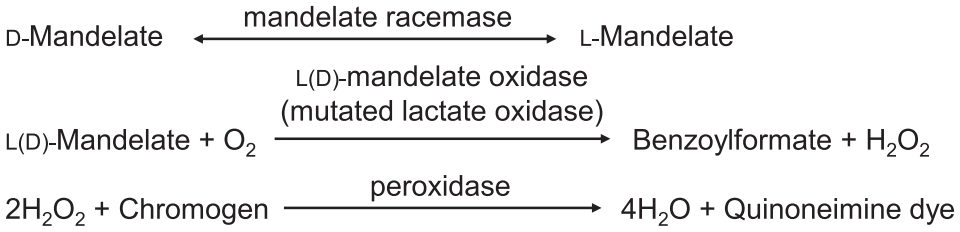

Fig. 2 Enzymatic assay method for mandelate proposed in this report.

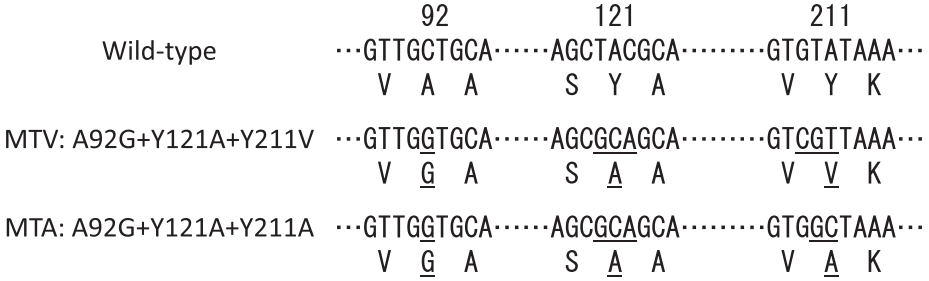

Fig. 3 Mutational points of site-directed mutagenesis.

The enzyme assay was based on the amount of hydrogen peroxide produced during substrate oxidation[4].

3. Results and Discussion

The substrate-fitting model revealed a steric interference between the wild-type and L-mandelate (Fig. 4). The reactivity of the wild-type LoxL for L-mandelate was remarkably low, and the activity ratio of L-mandelate to L-lactate (final concentrations of L-lactate and L-mandelate in the assay mixtures are 24 and 12 mmol/L, respectively) was estimated to be approximately 6.6×10^{-5}.

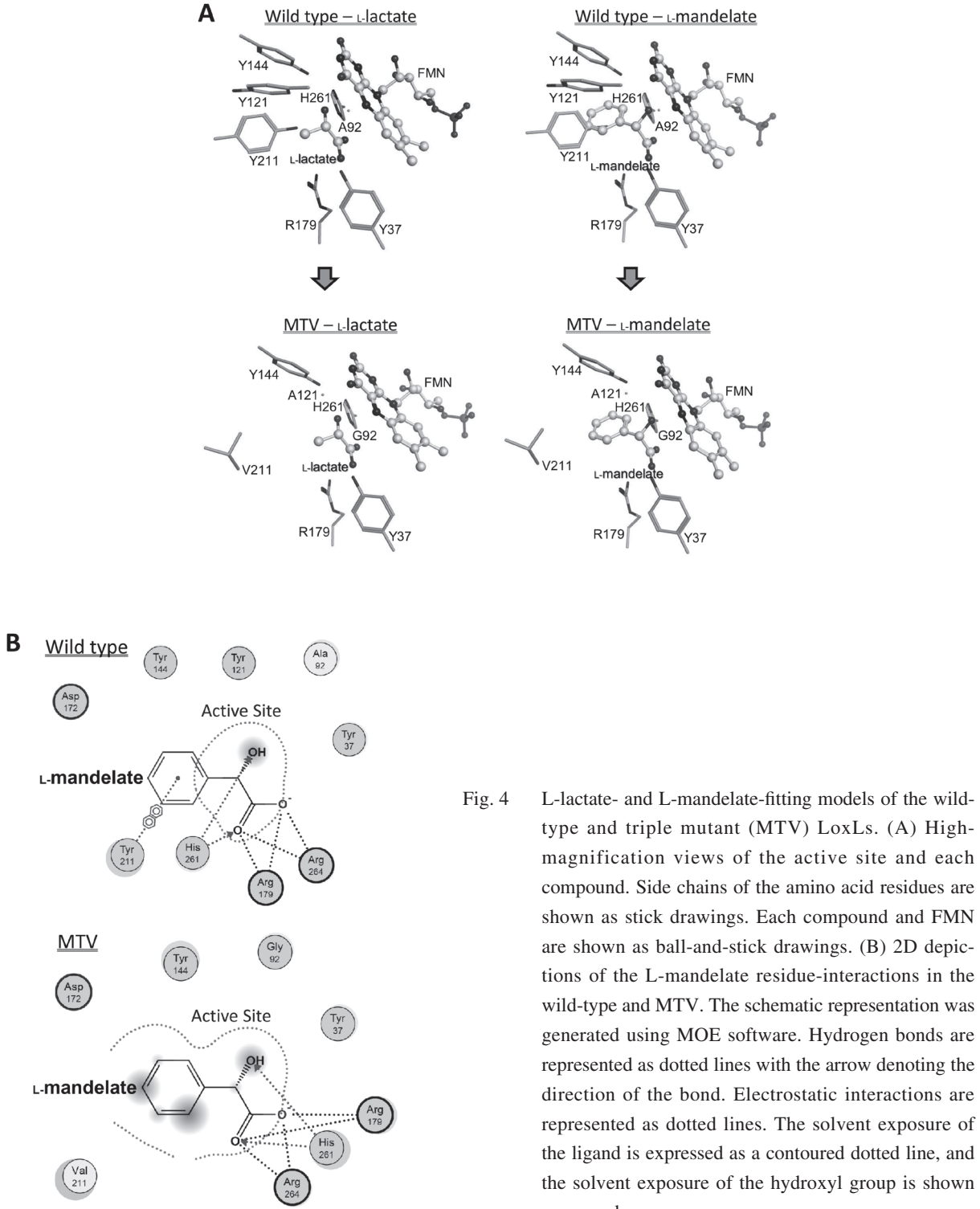

Fig. 4 L-lactate- and L-mandelate-fitting models of the wild-type and triple mutant (MTV) LoxLs. (A) High-magnification views of the active site and each compound. Side chains of the amino acid residues are shown as stick drawings. Each compound and FMN are shown as ball-and-stick drawings. (B) 2D depictions of the L-mandelate residue-interactions in the wild-type and MTV. The schematic representation was generated using MOE software. Hydrogen bonds are represented as dotted lines with the arrow denoting the direction of the bond. Electrostatic interactions are represented as dotted lines. The solvent exposure of the ligand is expressed as a contoured dotted line, and the solvent exposure of the hydroxyl group is shown as a smudge.

Previous studies on the lactate oxidase from *Aerococcus viridans* (LoxA) demonstrated that the A95G mutant altered substrate specificity toward several α-hydroxy acids. The turnover number of A95G for L-mandelate was approximately 6.3% of that for L-lactate, while that of the wild-type LoxA for L-mandelate was approximately 0.0012% of that for L-lactate[7,8]. On the other hand, The K_m value of A95G for L-mandelate was approximately 3 times higher than that for L-lactate, while K_m of the wild-type for L-mandelate was approximately 1/3rd that for L-lactate[8]. The LoxL A92G mutation of LoxL, corresponding to the A95G of LoxA, was also expected to exhibit similar effects, and thus it was selected to enhance the L-mandelate oxidase activity.

As shown in Fig. 4, the bulky side chains of Y121 and Y211 are structurally considered to be responsible for the steric interference caused by L-mandelate binding. Notable steric interference was particularly observed between the side chain of Y211 and the benzene ring of L-mandelate. Therefore, a triple mutant (MTV: A92G + Y121A + Y211V), which was expected to reduce the steric interference by expanding the hydrophobic pocket in the proximity of the active site, was designed (Fig. 4). All three mutations did not cause any conflict with the catalytic residue of LoxL (H261) and the coenzyme FMN, and were predicted to give almost no influence.

On the basis of the structure-guided design of engineered lactate oxidase, MTV and other two mutants (MD: A92G + Y121A, MTA: A92G + Y121A + Y211A) were constructed as described in the Materials and Methods section. L-mandelate to L-lactate activity ratios of all three mutants were improved. MD, MTA, and MTV mutants showed higher reactivity for L-mandelate (approximately 290, 160, and 200 times, respectively) when compared to the wild-type, and their L-mandelate to L-lactate activity ratios were approximately 1.8, 1.1, and 10, respectively.

The K_m and k_{cat} values of the wild-type and mutant LoxLs were calculated using the Lineweaver-Burk plots. Details of the kinetic parameters of the purified enzymes are shown in Table 1. Among all the mutants, MTV showed the highest affinity and catalytic efficiency (k_{cat}/K_m) for L-mandelate. The catalytic efficiencies of MD, MTA, and MTV for L-mandelate were 0.57, 1.7, and 9.4 $s^{-1}mmol^{-1}L$, respectively. However, the catalytic efficiency of the wild-type could not be estimated since its activity was barely detected. Moreover, the K_m values of MTV and MTA for L-lactate could not be precisely estimated due to their weak substrate affinities. These results correlated with the design of the engineered enzyme based on the structural prediction.

As expected, the altered substrate affinities of the LoxL mutants, as predicted by the structure-guided design described in this report, were demonstrated experimentally. Expansion of the hydrophobic pocket would have changed the interaction between the peripheral residues and L-mandelate, as observed with the L-mandelate-fitting model. However, the turnover number for L-mandelate could not be sufficiently improved (Table 1).

Previous studies of other enzymes have shown

Table 1 Kinetic parameters of wild type and mutants

Enzyme	L-Lactate			L-Mandelate		
	K_m (mmol/L)	k_{cat} (1/s)	k_{cat}/K_m	K_m (mmol/L)	k_{cat} (1/s)	k_{cat}/K_m
Wild type	1.0	320	320		n.e.	
MD: A92G+Y121A	38	2.7	0.071	7.9	4.5	0.57
MTA: A92G+Y121A+Y211A		n.e.		1.5	2.5	1.7
MTV: A92G+Y121A+Y211V		n.e.		0.17	1.6	9.4

n.e.; not estimated

that imidazole could enter the active site and inhibit enzyme activity[9]. Investigation of the wild-type, MD, and MTV activities at high concentrations of imidazole are presented in Fig. 5. Although the activity of the wild-type did not decrease, the residual activity of MD showed a tendency to decrease with increasing imidazole concentration. Moreover, the residual activity of MTV was even lower than that of MD. The inhibitory effect of imidazole tended to be higher as the space widened due to mutagenesis. Expansion of the substrate binding pocket was thought to be directly related to the inhibitory effect of imidazole.

The rational design of the LoxL mutations in the active site and its surroundings resulted in mutant enzymes with remarkably improved affinities and catalytic efficiencies for L-mandelate. Substrate specificities were significantly improved owing to the reduction in steric interference for the bulky substrates. There were obvious correlations between the tertiary structures of the substrates and mutational effects. The MTV mutant was most effective because Y211 was located near the long loop that resided over the active site. Aside from the direct interaction between the enzyme and the substrate as well as the expansion of the hydrophobic pocket, the change in the flexibility of the long loop in the proximity of the active site may have contributed to the mutational effects of MTV.

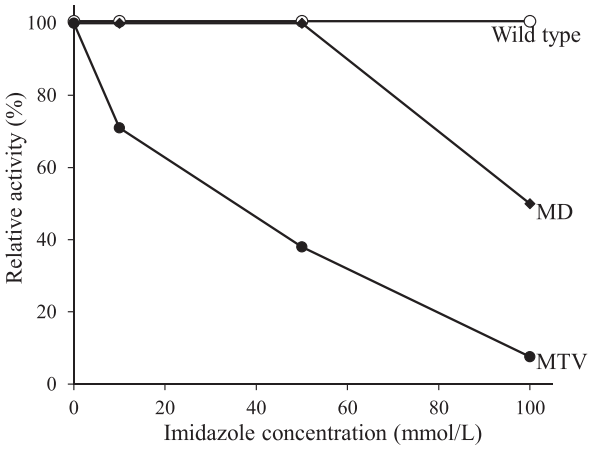

Fig. 5 Effects of imidazole on the enzyme activities. Approximately 0.3~40 μg/mL of each enzyme in a reaction mixture was assayed. Open circle, closed rhombus, and closed circle indicate wild-type, MD, and MTV, respectively.

Conflicts of interest

The authors have no conflicts of interest.

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