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# Research Article

# Improving the thermal stability of lactate oxidase by directed evolution

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**Abstract.** Lactate oxidase is used in biosensors to measure the concentration of lactate in the blood and other body fluids. Increasing the thermostability of lactate oxidase can significantly prolong the lifetime of these biosensors. We have previously obtained a variant of lactate oxidase from *Aerococcus viridans* with two mutations (E160G/V198I) that is significantly more thermostable than the wild-type enzyme. Here we have attempted to further improve the thermostability of E160G/V198I lactate oxidase using directed evolution. We made a mutant lactate oxidase gene library by applying error-prone PCR and DNA shuffling, and screened for thermostable mutant lactate oxidase using a plate-based assay. After three rounds of screening we obtained a thermostable mutant lactate oxidase, which has six mutations (E160G/V198I/G36S/T103S/A232S/F277Y). The half-life of this lactate oxidase at 70 °C was about 2 times that of E160G/V198I and about 36 times that of the wild-type enzyme. The amino acid mutation process suggests that the combined neutral mutations are important in protein evolution.

**Keywords.** Lactate oxidase, thermostability, DNA shuffling, directed evolution.

## Introduction

Enzymes isolated from thermophilic bacteria have proved to be of great use in the biotechnology industry because they are able to function at high temperatures without being denatured. However, it is not always possible to obtain the desired enzyme from such bacteria. Improving the thermostability of an enzyme has become a major challenge in protein engineering. Lactate oxidase is widely used in biosensors to measure the concentration of lactate in blood [1]. Recently, it was discovered that lactate oxidase could also be used to monitor the chemoenzymatic D-enantiomerization of DL-lactate [2]. Most lactate biosensors have a limited operational lifetime [3], which should be significantly extended by increasing the enzyme stability. Indeed, we previously demonstrated that increasing the thermostability of lactate oxidase is effective in prolonging the lifetime of the lactate sensor [1]. However, the effect of lactate oxidase thermostability tends to be weaker when immobilized onto a membrane than when in solution [1]. Protein stability can be enhanced through protein engineering using two approaches: rational design or random/combinatorial methods. Rational design approaches for improving enzyme thermostability have been reported, and in some cases were very successful [4]. We have previously isolated a mutant version of lactate oxidase (E160G/V198I) by irrational design that was found to be substantially more thermostable than the wild-type enzyme [5]. However, subsequent attempts to further improve the stability of the mutant lactate oxidase

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using a rational design approach were still unsatisfactory [6]. Directed evolution is based on a random mutagenesis step, such as error-prone PCR [7] or DNA shuffling [8], followed by selection, and is an attempt to mimic natural evolution. Directed evolution of enzymes has proved to be a powerful tool in protein engineering [9], and is currently used in experiments to both improve the properties of an enzyme and analyze the natural evolution process [10]. In this study, we applied directed evolution, based on error-prone PCR, DNA shuffling and plate assay screening, to improve the thermostability of the E160G/V198I mutant lactate oxidase [11].

## Materials and methods

In the first round, we generated the mutant lactate oxidase gene libraries using error-prone PCR and DNA shuffling. Error-prone PCR was conducted using a method proposed by Miyazaki et al. [12] with some minor modifications. The reaction conditions were: 0.2 mM dATP, 0.2 mM dGTP, 1 mM dCTP, 1 mM dTTP, 7 mM $MgCl_2$, 0.15 mM $MnCl_2$, and 5 U Taq in a 100- µl solution. PCR consisted of 30 cycles at 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min followed by one final extension step at 72 °C for 7 min. The amplified fragments were checked and purified by agarose gel electrophoresis. DNA shuffling was performed using a previously reported method [11]. The amplified full-length mutant lactate oxidase genes were cloned back into pKK223-3. The resulting plasmids were transformed into *E. coli* JM109 cells, which were grown on an LL plate (10 mg tryptone/ml, 5 mg yeast extract/ml, 5 mg NaCl/ml, 15 mg agar/ml, 100 µg ampicillin/ml, 1 U horseradish peroxidase/ml, 100 µg 2,2′-azino-di-3-ethylbenzthiazoline sulfonate/ml, and 100 mM L-lactate lithium salt). Purple-bordered colonies of lactate oxidase-positive clones were then picked up with sterile toothpicks and put on an L plate (10 mg tryptone/ml, 5 mg yeast extract/ml, 5 mg NaCl/ml, 15 mg agar/ml, 100 µg ampicillin/ml) and cultured at 37 °C for 12 h. The plates were heated at 70 °C for 3 h and then cooled to room temperature, before being stained with a lactate oxidase activity assay solution (40 mM HEPES pH 7.3, 1.5 mM 4-aminoantipyrine, 3.3 mM phenol, 100 mM L-lactate lithium salt, and 2 U horseradish peroxidase/ml). The red-bordered colonies were selected as thermostable mutants (plate screening). Selected colonies were cultured at 37 °C for 12 h in L-broth before induction of heterologous gene expression by the addition of IPTG (1 mM final). The culture was then continued for 3 h before harvesting the cells by centrifugation. Pellets were suspended in a solution containing 10 mM HEPES (pH 7.3), 1 mM EDTA and 50 µM PMSF and the cells were disrupted by sonication. The cell-free extract was clarified by centrifugation and the protein concentration measured with a BCA Protein Assay Kit (Pierce, Rockford, IL), using bovine serum albumin as the standard. We measured the irreversible thermal inactivation of the crude enzyme using a peroxidase-coupled spectrophotometric method [11]. Residual activity was compared with the parent (E160G/V198I) lactate oxidase using the crude enzyme screening technique. Plasmids derived from clones that gave improved thermostability over the parent lactate oxidase were isolated and used as template for the next round of mutagenesis. Several rounds of mutagenesis and screening were performed in an iterative process until the procedure identified positive clones with the desired properties. Finally, the isolated clones were recombined by DNA shuffling. The wild-type and various mutant lactate oxidases were purified using a previously reported method [5]. The purity of the enzyme was checked by SDS-PAGE using a PhastGel run on a PhastSystem (Amersham Pharmacia, Uppsala, Sweden). Lactate oxidase activity was determined by a peroxidase-coupled spectrophotometric method [11] using $H_2O_2$ as the standard. We measured the irreversible enzyme inactivation at a protein concentration of 50 µg/ml in 40 mM HEPES buffer, pH 7.3. Samples were incubated at 70 °C for various periods of time and then cooled on ice prior to measuring residual enzyme activity.

## Results and discussion

The amino acid changes introduced at each round of screening are given in Table 1. Thermostability of the crude enzyme preparations during each round of mutagenesis are also given in Table 1 and illustrated graphically in Figure 1. In the first round, we screened about

**Table 1.** Amino acid mutation and crude enzyme thermostability in each selection round.

| Round | Clone name | Amino acid sequence | Thermostability[a] (%) |
|---|---|---|---|
| 0 | Parent | E160G/V198I | 40 |
| 1 | 1-17 | E160G/V198I/T103S | 42 |
| 1 | 1-36 | E160G/V198I/L345I | 31 |
| 1 | 1-56 | E160G/V198I/D164V/Q355L | 14 |
| 2 | 2-12 | E160G/V198I/K104R/A232S | 38 |
| 2 | 2-8 | E160G/V198I/T103S | 39 |
| 2 | 2-1 | E160G/V198I/G36S/D75E/V185M | 40 |
| 3 | 3-24 | E160G/V198I/T103S/A232S | 56 |
| 3 | 3-30 | E160G/V198I/G36S/R225K/A232S | 53 |
| 3 | 3-3 | E160G/V198I/G36S/K104R/A232S | 50 |
| 3 | 3-5 | E160G/V198I/T103S | 48 |
| 3 | 3-44 | E160G/V198I/G36S/T103S/A232S/F277Y | 71 |

[a] Remaining activity of crude enzymes at 70 °C and 20 min of heat denaturation.

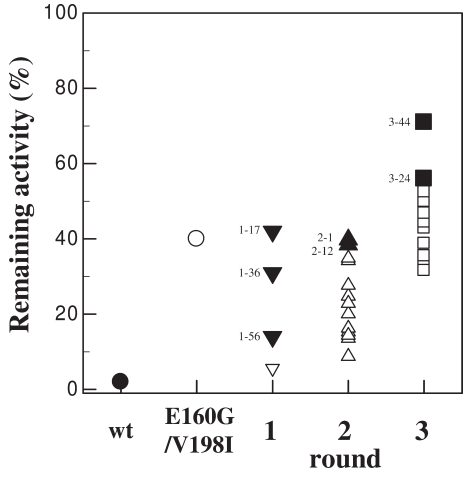


**Figure 1.** Thermostability of crude enzyme preparation at each round of mutagenesis. Lactate oxidase activity of the crude enzyme preparation was measured after heating the solution to 70 °C for 20 min.

7000 colonies and obtained 6 lactate oxidase-positive clones. There were no mutants that were noticeably more thermostable than the parent lactate oxidase (E160G/V198I) from the crude enzyme screening. Two of the 6 isolated clones had the same amino acid sequence as the parent lactate oxidase. However, three of the isolated clones had an additional independent additive mutation (T103S, L345I, D164V/Q355L), and one clone carried an alternative mutation at residue 160 (E160A/R300H). Because the E160A/R300H crude enzyme was considerably less thermostable than the parent lactate oxidase, the three clones with the additive mutations were used as the variants in the next round of mutation and screening. In the second round, we screened about 7400 colonies and obtained 41 lactate oxidase-positive clones. Of these, using plate screening, 19 clones were more thermostable than the wild-type, but none were significantly more thermostable than the E160G/V198I. The three clones that showed almost the same thermostability as the E160G/V198I were used as the variants for the third round of mutation and screening. In the third round, we screened about 7100 colonies and obtained 359 lactate oxidase-positive clones. Of these, 14 were found to be more thermostable than the wild-type using plate screening, and five kinds of mutation were observed. One of 5 clones, designated 3-44, had four additional mutations (G36S/T103S/A232S/F277Y) and showed greater thermostability than E160G/V198I by the plate screening and the crude enzyme screening. Clone 3-44 includes three mutations observed in the first or second round of screening (G36S/T103S/A232S) plus one new mutation (F277Y) (Table 1). We then addressed the question of how the various mutations affect the thermostability of the enzyme. To identify mutations that increase thermostability, we performed DNA shuffling as a molecular backcrossing experiment [8], using a mixture of genes corresponding to the parent lactate oxidase E160G/V198I and clone 3-44. We isolated 7 clones that were more thermostable than E160G/V198I from the 240 lactate oxidase-positive clones obtained by plate screening. Using the crude enzyme assay, all 7 clones exhibited almost the same thermostability as the variant 3-44. Six of the 7 clones had the same amino acid sequence as clone 3-44, including some silent mutations, and 1 clone had an additive mutation (V305I). The crude enzyme thermostability assays from each round of mutation (Table 1) show that the G36S, T103S, A232S mutations have small effect on the lactate oxidase thermostability. DNA shuffling experiments between the parent lactate oxidase E160G/V198I and clone 3-44 suggests that the F277Y mutation alone does not have obvious effect on lactate oxidase thermostability, but was effective in combination with other mutations. Similar phenomena were observed in our previous study [5], as well as the enzyme study done by Akanuma et al. [13].

The thermostability and enzymatic properties of the purified wild-type and mutant lactate oxidases are shown in Figure 2 and Table 2. The thermal inactivation curve of the 3-44 at 70 °C was obviously less steep than that of E160G/V198I (Fig. 2), and the half-life of 3-44 at 70 °C was about two times that of E160G/V198I, and about 36 times that of the wild-type (Table 2). The $K_m$ and $V_{max}$ values of 3-44 are almost identical to those of E160G/V198I, indicating that the G36S, T103S, A232S and F277Y mutations had very little effect on the catalytic activity of lactate oxidase. Position 160 is in $\alpha_3$ helix constituting part of $(\beta/\alpha)_8$ barrel, and the amino

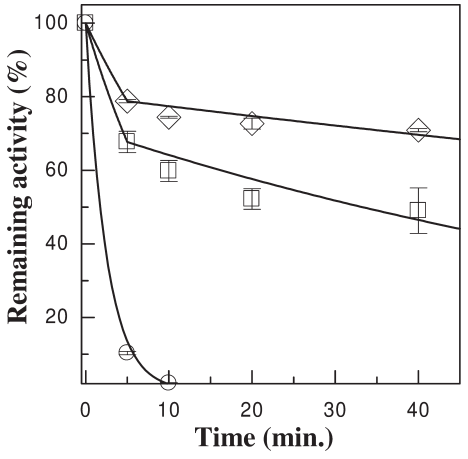


**Figure 2.** Thermal inactivation curves of wild-type, variant E160G/V198I and variant 3-44 (E160G/V198I/G36S/T103S/A232S/F277Y) lactate oxidases. Purified enzyme in 40 mM HEPES buffer (pH 7.3) was heated to 70 °C for various periods of time. The remaining enzyme activity of wild-type (○), E160G/V198I (□) and 3-44 (◊) lactate oxidases are expressed as a percentage of original activity. Results are the mean of triplicate experiments ± SD.

**Table 2.** Enzymatic properties and stability of purified lactate oxidases.

| Type | Enzymatic properties | | | | Stability |
|---|---|---|---|---|---|
| | 25 °C | | 35 °C | | $t_{1/2}$ at 70 °C (min)[a] |
| | $K_m$ (mM) | $V_{max}$ (μmol $H_2O_2$/s/mg protein) | $K_m$ (mM) | $V_{max}$ (μmol $H_2O_2$/s/mg protein) | |
| wt | 0.529 | 0.811 | 0.863 | 1.08 | 1.7 |
| E160G/V198I | 24.3 | 0.507 | 50.7 | 0.793 | 30.3 |
| 3-44 | 25.5 | 0.871 | 47.6 | 0.935 | 61.4 |

[a] The half-life was calculated from Figure 2.

acid substitution at position 160 might have affected the enzyme activity through the $\alpha_3$ helix flexibility containing the interaction with the other residues, such as Glu 130 or Arg 203. Although the structure of the lactate oxidase has not been elucidated, the 3-D structure of a spinach-derived glycolate oxidase, which belongs to the same flavin mononucleotide (FMN)-dependent $\alpha$-hydroxy acid oxidase family, is known [14]. We previously predicted the tertiary and quaternary structures (tetramer structures) of the lactate oxidase, based on homology modeling using the glycolate oxidase structure as a template [15].

The single subunit of lactate oxidase probably possesses a common $(\beta/\alpha)_8$ barrel structure, which is common to the FMN-dependent $\alpha$-hydroxy acid oxidase family. However, the only glycolate oxidase main chain loop from 1GOX (Protein Databank ID) in which the coordinates of residues 189-197 were lacking, is deleted in the lactate oxidase model. The side chain conformations between lactate oxidase and glycolate oxidase residues involved in the FMN-binding or substrate-binding sites are well fitted. The side chain conformations between the paired hydrophobic core residues, which are in the $\alpha$-helices and $\beta$-strands comprising $(\beta/\alpha)_8$ barrel, are also almost superimposed. All the bond lengths and bond angles were found to be standard values. No abnormal van der Waals contacts were found between main chain atoms, between side chain atoms, or between main chain atoms and side chain atoms. The geometry of the main chain dihedral angles ($\phi$, $\psi$) was presented as a Ramachandran plot (data not shown). All residues had desirable ($\phi$, $\psi$) values that lie inside the expected regions of the plot. Moreover, no abnormal van der Waals contacts were found between lactate oxidase and FMN or between lactate oxidase and substrate analog, pyruvate.

Our model shows that all the mutations (E160G, V198I, G36S, T103S, A232S and F277Y) are present on the surface of the enzyme (Fig. 3) and are located far from the subunit interaction region of the lactate oxidase tetramer structure (Fig. 4). Thus, it appears that these mutations do not strengthen the various intermolecular forces acting between the individual subunits. Based on our lactate oxidase model, residue 36 is at the N1 helix position [16] in an $\alpha_B$ helix, residue 103 is at the N' helix position in an $\alpha_1$ helix, residue 232 is at the C2 helix position in an $\alpha_4$ helix, and residue 277 is at the N2 helix position in an $\alpha_6$ helix. These four residues are commonly found at the end of the helix. Intriguingly, all six mutations were lo-

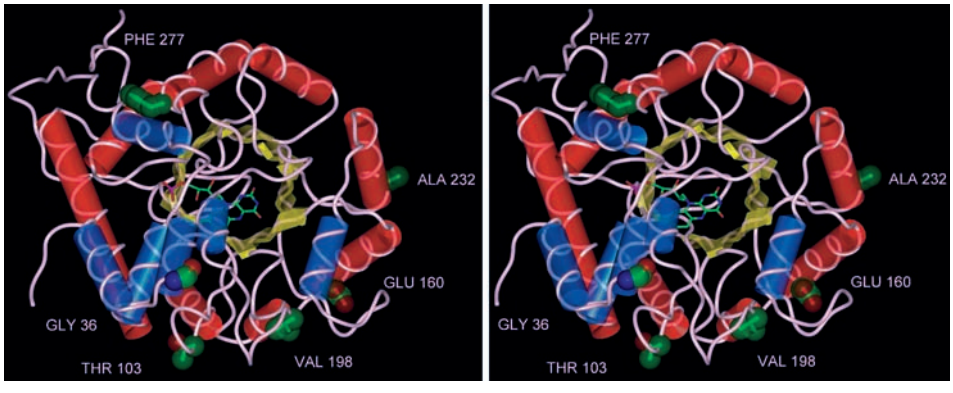


**Figure 3.** Stereoview of the model structure of lactate oxidase. The main-chains and side-chains of Thr 103, Glu 160, Val 198, Ala 232 and Phe 277 are displayed using van der Waals' surface (CPK) model, and the main chain of Gly 36 is displayed using the CPK model. The FMN molecule is shown as a stick model. The eight helices constituting the $(\beta/\alpha)_8$ barrel structure are illustrated using a red cylinder, and the other helices are illustrated in blue. The sheets are shown using yellow arrows. The mutation site labels are white. The atom-type colors are as follows: carbon (green), oxygen (red) and nitrogen (blue). The picture was made using Insight II software (Version 4.3, Accelrys Inc.) on an ONYX2 workstation (Silicon Graphics, Inc.).

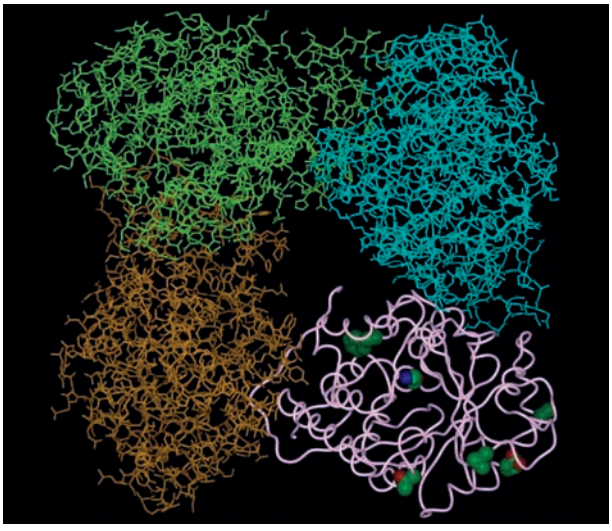

**Figure 4.** Quaternary model structure of lactate oxidase. Only one subunit is displayed using the white ribbon model. The side chains of Thr 103, Glu 160, Val 198, Ala 232 and Phe 277 are displayed using van der Waals' surface (CPK) model, and the main chain of Gly 36 is displayed using the CPK model. The other three subunits (yellow, green and cyan), both major chains and side chains, are represented as stick models.

cated in, or near, a helix that constitutes part of the $(\beta/\alpha)_8$ barrel (Fig. 3). Thus, we infer that these mutations contribute to stabilizing the $(\beta/\alpha)_8$ barrel structure of the lactate oxidase. However, it is more difficult to explain the combinational effect of these mutations for improving the thermal stability of the lactate oxidase from our model structure. More details of the side chain conformation in the 3-D structure are needed to analyze the effect of multiple mutations, and saturation mutagenesis of position in 160, 198, 36, 103, 232 and 277 also might be effective [17]. We are currently engaged in determining the X-ray crystal structure of wild-type lactate oxidase, which might reveal how the amino acid substitutions identified in this study contribute to increasing the thermal stability of the enzyme.

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