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# Improvement in thermostability and psychrophilicity of psychrophilic alanine racemase by site-directed mutagenesis

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Dedicated to Professor Dr. Kenji Soda in honor of his 70th birthday

**Abstract**

A psychrophilic alanine racemase from *Bacillus psychrosaccharolyticus* has a higher catalytic activity than a thermophilic alanine racemase from *Bacillus stearothermophilus* even at 60 °C in the presence of pyridoxal 5′-phosphate (PLP), although the thermostability of the former enzyme is lower than that of the latter one [FEMS Microbial. Lett. 192 (2000) 169]. In order to improve the thermostability of the psychrophilic enzyme, two hydrophilic amino acid residues (Glu150 and Arg151) at a surface loop surrounding the active site of the enzyme were substituted with the corresponding residues (Val and Ala) in the *B. stearothermophilus* alanine racemase. The mutant enzyme (ER150,151VA) showed a higher thermostability, and a markedly lower $K_m$ value for PLP, than the wild type one. In addition, the catalytic activities at low temperatures and kinetic parameters of the two enzymes indicated that the mutant enzyme was more psychrophilic than the wild type one. Thus, the psychrophilic alanine racemase was improved in both psychrophilicity and thermostability by the site-directed mutagenesis. The mutant enzyme may be useful for the production of stereospecifically deuterated NADH and various D-amino acids.


*Keywords:* Alanine racemase; Psychrophilicity; Thermostability; Site-directed mutagenesis; *Bacillus psychrosaccharolyticus*

## 1. Introduction

Psychrophilic enzymes are highly active at low temperatures, and appear to have a high potential ability as biocatalysts. Although the enzymes are probably useful for enzymatic processes at low temperatures in various industries such as the food, chemical, and pharmaceutical industries [1], they are thermolabile. In order to improve the thermostability of the enzymes, several reports have been published on the structural characteristics responsible for the psychrophilicity and thermolability of psychrophilic enzymes [2–10]. Feller and Gerday [7], and Davail et al. [6] suggested that the high catalytic activity of a psychrophilic subtilisin at low temperature originated from its highly flexible structure. The hydrophilic and polar surface of a psychrophilic subtilisin was reported to enhance solvent interactions, reduce the compactness of the molecule, and destabilize the psychrophilic enzyme [6]. Similar results were also reported for the psychrophilic enzymes such as phosphoglycerate kinase [4], α-amylase [2,3], alkaline protease [10], and malate

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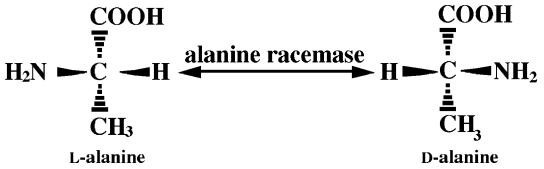


Fig. 1. Racemization of alanine catalyzed by alanine racemase.

dehydrogenase [8]. Therefore, the surface structure of the psychrophilic enzymes may be one of the major factors of the psychrophilicity and thermolability.

Alanine racemase (EC 5.1.1.1) is a pyridoxal 5′-phosphate (PLP)-dependent enzyme catalyzing the racemization of L- and D-alanine (Fig. 1). The enzyme widely occurs in bacteria and in some eukaryotes [11–14], and is useful for the production of stereospecifically deuterated NADH and various D-amino acids [15]. Several alanine racemases from mesophiles, a thermophile and eukaryotes were purified and characterized [16–20]. Several alanine racemase genes were also clarified [18–26] and the three-dimensional structure of a thermostable alanine racemase from *Bacillus stearothermophilus* was determined by X-ray crystallography [25]. We also characterized the mesophilic alanine racemases from four *Shigella* species [27] and the psychrophilic alanine racemases from *Pseudomonas fluorescens* [28–31] and *Bacillus psychrosaccharolyticus* [29,31–33]. The *B. psychrosaccharolyticus* alanine racemase has a high catalytic activity at low temperature, but was labile above 40 °C [32]. In the presence of PLP, however, the psychrophilic enzyme has a higher catalytic activity than a thermophilic alanine racemase from *B. stearothermophilus* even at 60 °C [33]. If the thermostability of the psychrophilic enzyme is improved, the psychrophilic enzyme may be more useful than the corresponding thermophilic enzyme.

The amino acid sequences of the reported alanine racemases are highly homologous, but the psychrophilic alanine racemase from *B. psychrosaccharolyticus* has two distinctive features [32]. First, the psychrophilic enzyme has distinguishing hydrophilic amino acid residues (E150 and R151) at a surface loop surrounding the active site. The corresponding amino acid residues of the *Bacillus subtilis* and *B. stearothermophilus* enzymes are reported to be MA and VA, respectively [26]. The E150 and R151 residues may interact with solvent, reduce the compactness of the active site and destabilize the enzyme. Second, the psychrophilic enzyme has an extremely large $K_m$ value (5.0 μM) for PLP in comparison with other reported alanine racemases (the $K_m$ values, 33–50 nM). Tutino et al. suggested that a more flexible active site in a psychrophilic aspartate aminotransferase resulted in a decrease of the affinity for substrates by loosening the interactions at the active site [34]. The low affinity of the psychrophilic alanine racemase for PLP may be related to the flexible conformation of the active site, and to the high catalytic activity at low temperatures as discussed in our previous report [32].

We now describe the properties of a mutant enzyme (ER150,151VA) of the psychrophilic alanine racemase from *B. psychrosaccharolyticus*. The psychrophilic alanine racemase was improved in both psychrophilicity and thermostability by the site-directed mutagenesis.

## 2. Materials and methods

### 2.1. Enzymes

The psychrophilic alanine racemase from *B. psychrosaccharolyticus* was purified to homogeneity from *Escherechia coli* SOLR carrying a plasmid pYOK3 as previously described [32]. D-Amino acid oxidase (EC 1.4.3.3) from pig kidney (0.13 unit per mg) was obtained from Sigma (St. Louis, MO).

### 2.2. Subcloning of pYOK3 and site-directed mutagenesis

The site-directed mutagenesis on E150 and R151 of the psychrophilic alanine racemase was performed with a plasmid pYOK4 and a Takara LA PCR in vitro Mutagenesis kit (Takara Shuzou, Kyoto, Japan). The plasmid pYOK4 was prepared from a plasmid pYOK3, which contained a 3.3 kb insert DNA including the *B. psychrosaccharolyticus alr* gene [32], as follows. The plasmid pYOK3 was digested with *Kpn*I and *Pst*I to yield a 1.4 kb fragment containing the psychrophilic *alr* gene. The 1.4 kb fragment was recloned into the *Kpn*I and *Pst*I sites of pUC18 DNA and designated pYOK4. The mutagenic oligonucleotide primer was 5′-AATGAAATCGTAGCTTTTCTTCAGAAAACG, whose translation is NEIV$^{150}$A$^{151}$FLQKT. The

nucleotide sequence of the mutant plasmid was determined by the dideoxynucleotide chain termination method with an ABI Prism Dye Deoxy Terminator Cycle Sequencing Core kit and an ABI Prism 310 Genetic Analyzer (Perkin-Elmer, Foster City, CA, USA). In all instances, both strands of the DNA were sequenced in their entirety. The mutant plasmid was fully sequenced to ensure that no mutations occurred except at the desired positions, and designated pYOKVA.

### 2.3. Enzyme purification

*E. coli* JM109 carrying the plasmid pYOKVA was grown at 25 °C for 48 h in TB medium (1.2% tryptone, 2.4% yeast extract, 0.4% glycerol, 17 mM $KH_2PO_4$, 72 mM $K_2HPO_4$) containing 50 μg/ml of ampicillin. The cells were harvested by centrifugation, and washed with 0.85% NaCl. All the purification procedures were done at 0–5 °C. Potassium phosphate buffer (10 mM, pH 8.0) containing 10% glycerol, 0.02% sodium azide, 0.01% 2-mercaptoethanol, 0.5 mM EDTA, 0.05 mM PLP, and 0.1 mM phenylmethanesulfonyl fluoride was used as the standard buffer. The enzyme purification was performed using an Äkta Purifier system (Pharmacia Biotech, Uppsala, Sweden) as previously described [32].

### 2.4. Enzyme and protein assay

Enzyme assays were routinely done at 0 °C for 10 min. The standard reaction mixture contained 40 mM potassium phosphate buffer (pH 8.3), 100 mM L-alanine, and enzyme in a final volume of 0.04 ml. The reaction was started by addition of the enzyme, and stopped by addition of 0.04 ml of 2N HCl. After neutralization with 2N NaOH, the D-alanine formed was measured with D-amino acid oxidase [35]. To assay the L-alanine formed from D-alanine, L-alanine was replaced with D-alanine in the standard reaction mixture. The L-alanine formed was determined using L-alanine dehydrogenase [17]. Substrate specificity of alanine racemase was examined using a P-1030 polarimeter (Jasco Co., Tokyo, Japan). A unit of the enzyme was defined as the amount of enzyme that catalyzed the formation of 1 μmol of D- (or L-) alanine per min. The specific activity was expressed in units per mg of protein. Protein was measured using a Bio-Rad protein assay kit (Nippon Bio-Rad Laboratories, Osaka, Japan) and bovine serum albumin as the standard.

### 2.5. Circular dichroic analysis

The circular dichroic (CD) measurements were carried out using a Jasco J-600 recording spectropolarimeter at 25 °C with a 1 mm light path-length cell under a nitrogen atmosphere. The instrument was calibrated with (+)-10-camphorsulfonic acid, $\Delta\varepsilon = +2.37\,M^{-1}\,cm^{-1}$ at 290.5 nm. To calculate the mean residue ellipticity ($\theta$), the mean residue weight was taken as 112.0 for the enzyme protein.

## 3. Results and discussion

### 3.1. Purification of the ER150,151VA mutant enzyme

The mutant enzyme was purified about 60-fold with an overall yield of 30%. The purified enzyme was homogeneous based on SDS-polyacrylamide gel electrophoresis (data not shown). The mutant enzyme was identical to the wild type one with respect to the apparent molecular mass, subunit structure, and N-terminal amino acid sequence. The specific activity of the mutant enzyme at 0 °C in the direction from L- to D-alanine was 980 units/mg. This value was higher than that of the wild type enzyme (650 units/mg). When the PLP contents of the two enzymes were determined by a fluorometric method [36] after dialysis at 4 °C for 24 h against 10 mM potassium phosphate buffer (pH 8.0), both enzymes had 1.0 mol of PLP per mol of subunit. Therefore, the difference in the activity at 0 °C between the two enzymes was not due to the amount of bound PLP. The mutant enzyme was stable at 4 °C for 3 weeks and at −80 °C for over 60 days in the standard buffer.

### 3.2. Effect of temperature on the activity

We examined the thermostability of the mutant and wild type enzymes. To avoid the renaturation of the denatured enzymes, the remaining activity was immediately measured after incubation at various temperatures for 1 h. As shown in Fig. 2, the mutant enzyme showed a higher thermostability than the wild

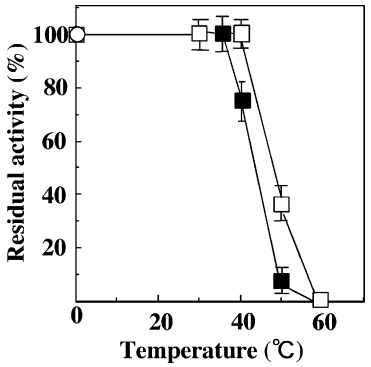


Fig. 2. Thermostability of the mutant and wild type enzymes. The enzyme solutions (3 units/ml of 10 mM potassium phosphate buffer, pH 8.3) were incubated at various temperatures. After the incubation for 1 h, the enzyme solutions were cooled at 0 °C for 5 min and the remaining activity was immediately measured at 0 °C. The results are expressed as the mean values of triplicate experiments, for which the standard deviations are shown. Open symbols, the mutant enzyme; solid symbols, the wild type enzyme.

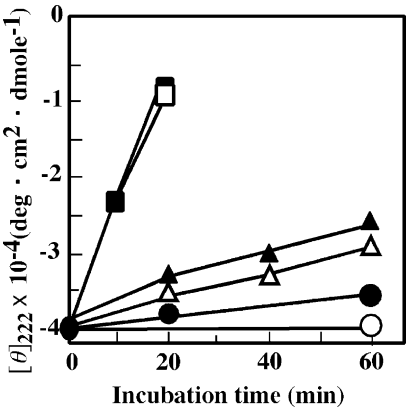


Fig. 3. The mean residue ellipticity at 222 nm of the mutant and wild type enzymes. The mutant and wild type enzymes (0.05 mg ml$^{-1}$) were incubated at 40 (circles), 50 (triangles), and 60 °C (squares) in 10 mM potassium phosphate buffer, pH 8.0. Sample solutions taken at the indicated times were cooled at 0 °C for 5 min, and then the mean residue ellipticities at 222 nm of the enzyme solutions were immediately measured. The results are expressed as the mean values of triplicate experiments, for which the standard deviations are below 10%. Open symbols, the mutant enzyme; solid symbols, the wild type enzyme.

type one. The ER150,151VA mutation may enhance the local rigidity of the enzyme or reduce the solvent interaction leading to the increased thermostability. When we examined the $K_m$ values for PLP of the two enzymes using the apoenzymes prepared as previously described [32], the mutant enzyme showed a markedly lower $K_m$ value (0.23 μM) for PLP than that of the wild type one (5.0 μM). Although the residues E150 and R151 locate at the surface loop surrounding the active site and cannot directly participate in binding the cofactor as reported previously [32], the ER150,151VA mutation was considered to influence the active site structure.

The thermophilic alanine racemase from *B. stearothermophilus* has a number of residues involved in maintaining the PLP cofactor in the active site; Val37, Lys39, Tyr43, Ala63, Ala65, Leu83, Leu85, His127, Arg136, Glu161, Tyr164, His166, His200, Asn203, Ser204, Arg219, Tyr354, Tyr265′, Met312′, Asp313′, and Gln314′ (residues labeled with a prime are from the other monomer) [25]. Except for Tyr164, these amino acid residues are also conserved in the wild type [32] and mutant enzymes. Thus, both active sites of the wild type and mutant enzymes appear to be stabilized through binding of PLP with many amino acid residues. This is supported by the previous result that the dissociation of PLP from the wild type enzyme destabilized the enzyme protein [32,33]. However, the mutant enzyme having a high affinity for PLP may have a more stable active site than the wild type one having a low affinity for PLP by a high ability of maintaining the cofactor in the active site.

The V151 and A152 residues in the *B. stearothermophilus* alanine racemase were reported to locate in an α-helix by X-ray crystallography [25], and the E150 and R151 in the *B. psychrosaccharolyticus* enzyme was considered to locate in the corresponding α-helix by prediction of the three dimensional structure [32]. To better assess the difference in the thermostability between the mutant and wild type enzymes, we measured the ellipticity at 220 nm, corresponding to α-helix structure, of the two enzymes incubated at high temperatures. To avoid refolding of the unfolded enzymes, the ellipticity was immediately measured at 25 °C after the incubation. Fig. 3 shows the mean residue ellipticity at 222 nm of the two enzymes incubated at 40, 50 and 60 °C. The negative value of the mean residue ellipticity of the wild type enzyme decreased more rapidly during the incubation at 40 and 50 °C than that of the mutant enzyme. These results are quite consistent with those in Fig. 2. Therefore, the

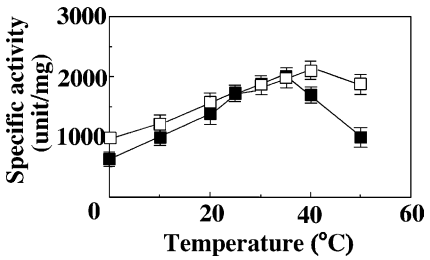


Fig. 4. Effect of temperature on the catalytic activities of the mutant and wild type enzymes. The enzyme assay was done at various temperatures for 10 min. The results are expressed as the mean values of triplicate experiments, for which the standard deviations are shown. Open symbols, the mutant enzyme; solid symbols, the wild type enzyme.

mutant enzyme protein may have a more rigid conformation than the wild type one.

We also examined the catalytic activity of the mutant and wild type enzymes at various temperatures (Fig. 4). At high temperatures (40–50 °C), the specific activities of the mutant enzyme were higher than those of the wild type enzyme. This showed a good agreement with the thermostability results in Fig. 2. However, the mutant enzyme showed higher activities at low temperatures (0–20 °C) than those of the wild type one. Although the difference in the cold-activity between the wild type and mutant enzymes was small, the ER150,151VA mutation that enhanced the thermostability of the wild type enzyme did not sacrifice the original cold-activity.

### 3.3. Kinetic characterization

We examined the kinetic parameters of the two enzymes at pH 8.3 (Table 1). The $K_m$ values for L-alanine of the mutant enzyme measured at 0 and 30 °C were similar to the respective one of the wild type enzyme. The $k_{cat}$ value for L-alanine of the mutant enzyme measured at 30 °C was identical to that of the wild type one, whereas the mutant enzyme had a higher $k_{cat}$ value than that of the wild type one when measured at 0 °C. When we examined the temperature dependence of the rate constant by Arrhenius plots (Fig. 5), both enzymes showed a linear Arrhenius plot but had different slopes. The activation energy $E_a$ of the mutant enzyme was found to be lower than that of the wild type one (Table 1). A change in the rate constant of

Table 1
Kinetic parameters of the mutant and wild type alanine racemases

| Kinetic parameters | Mutant enzyme | Wild type enzyme |
|---|---|---|
| $K_m$ for L-Ala (mM) | | |
| At 0 °C | 14.8 ± 1.2 | 15.0 ± 0.8 |
| At 30 °C | 15.0 ± 1.0 | 17.9 ± 1.1 |
| $k_{cat}$ ($s^{-1}$) | | |
| At 0 °C | 700 ± 60 | 460 ± 30 |
| At 30 °C | 1280 ± 80 | 1310 ± 90 |
| $k_{cat}/K_m$ ($s^{-1}\,M^{-1}$) | | |
| at 0 °C | 47000 ± 4000 | 31000 ± 2000 |
| at 30 °C | 85000 ± 6000 | 73000 ± 5000 |
| $E_a$ (kcal/mol) | 3.1 ± 0.3 | 5.4 ± 0.4 |
| $\Delta H^*$ (kcal/mol) | 2.5 ± 0.2 | 4.8 ± 0.4 |
| $\Delta G^*$ (kcal/mol) | 24.1 ± 1.5 | 24.1 ± 1.4 |
| $\Delta S^*$ (cal/mol degree) | −71.3 ± 5.6 | −63.7 ± 5.0 |

chemical reaction is known to obey the equation:

$$k = Ae^{-E_a/RT}$$

in which $E_a$ is the activation energy, $R$ gas constant and $T$ the temperature in Kelvin. Therefore, the decrease in the $E_a$ value leads to a decrease in the temperature dependence of the reaction rate. Gerday et al. also reported that reactions displaying small $E_a$ values had reaction rates that decrease only slightly with a

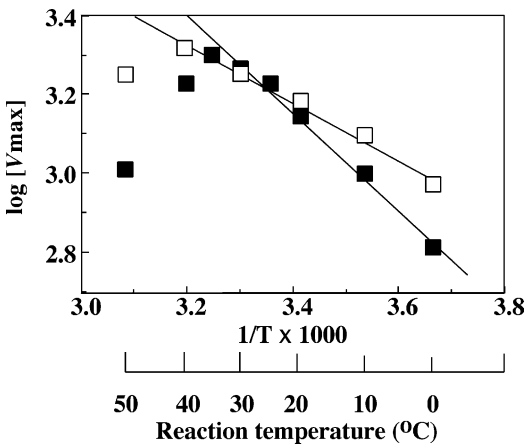


Fig. 5. Arrhenius plots for $V_{max}$ values of the mutant and wild type enzymes. The $V_{max}$ values were measured at various temperatures using L-alanine as the substrate. The results are expressed as the mean values of triplicate experiments, for which the standard deviations are below 8%. Open symbols, the mutant enzyme; solid symbols, the wild type enzyme.

decrease in the reaction temperature [37]. Therefore, the low $E_a$ value of the mutant enzyme may be related to the high catalytic activity at low temperatures.

The mutant enzyme had a lower $\Delta H^*$ value and higher negative $\Delta S^*$ value than the respective one of the wild type enzyme (Table 1). Lonhienne et al. reported that the main adaptation of psychrophilic enzymes lay in the significant decrease in $\Delta H^*$, and that $\Delta S^*$ exerted an opposite and negative effect on the gain in $k_{cat}$ [38]. Therefore, these kinetic parameters of the mutant and wild type enzymes also indicated that the mutant enzyme was more psychrophilic than the wild type one. These are the opposite results to our prediction that the psychrophilicity and thermostability are inversely related. The reason for the discrepancy is obscure at present. However, there is a difference between the psychrophilicity and thermostability experiments in their assay conditions. The thermostability of the wild type and mutant enzymes were examined in the absence of L-alanine, whereas the enzyme activities at various temperatures were inevitably in the presence of the substrate. PLP-dependent enzymes are well known to resolve an internal Schiff base formed between PLP and the active site Lys residue to form an external Schiff base between PLP and their substrate during the initial step of the enzyme reaction. The psychrophilic alanine racemase has only the internal Schiff base in the absence of L-alanine, but has both external and internal Schiff bases during the enzyme reaction in the presence of the substrate. A conformational change upon binding of the substrate is frequently observed among PLP-containing enzymes [39]. Tai and Cook recently reported that an active site conformation changed from open to closed as one went from the internal to external Schiff base in O-acetylserine sulfhydrylase [40]. The psychrophilic alanine racemase probably has similar open and closed structures of the active site. On the basis of the two types of active site structures, one possible explanation for the discrepancy is that the mutant enzyme having the closed active site may be more psychrophilic than the corresponding wild type one, but the mutant one having the open active site may be more thermostable than the corresponding wild type one because of the high affinity of the mutant one for PLP.

Alanine was the exclusive substrate of the mutant enzyme; L-lysine, L-arginine, L-glutamine, L-methionine, L-leucine, L-homoserine, L-asparagine, L-serine, L-cysteine, L-threonine, L-valine, L-glutamic acid, L-aspartic acid, L-proline, L-tyrosine, L-tryptophan, L-phenylalanine, L-histidine, L-isoleucine, and L-α-aminobutyrate were not racemized when examined by polarimetry. The mutant enzyme had its maximum reactivity in the pH range from 8 to 10 when examined in 40 mM phosphate, Tris–HCl, or carbonate buffer at several pHs, and was stable in the pH range from 8 to 10. The $K_m$ and $V_{max}$ values of the mutant enzyme at 30 °C for D-alanine were 13.2 mM and 1110 units/mg, respectively. These results were similar to those of the wild type one as previously described [32].

Recently, psychrophilic aldehyde dehydrogenase with high thermostability was isolated from a psychrophile [41]. Enzymes having psychrophilicity and high thermostability are probably useful for various enzymatic processes in industries. We have described the properties of the ER150, 151VA mutant of a psychrophilic alanine racemase. The mutant enzyme was more thermostable and psychrophilic than the wild type one. The mutant enzyme may be useful for the production of stereospecifically deuterated NADH and various D-amino acids.

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