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Structure and function of psychrophilic alanine racemase

Kumio Yokoigawa [a,*], Yoko Okubo [a], Hiroyasu Kawai [a], Nobuyoshi Esaki [b], Kenji Soda [c]

[a] *Department of Food Science and Nutrition, Nara Women's University, Nara 630-8506, Japan*
[b] *Institute for Chemical Research, Kyoto University, Uji, Kyoto 611-0011, Japan*
[c] *Department of Biotechnology, Faculty of Engineering, Kansai University, Osaka 564-0073, Japan*

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Abstract

We describe the structure and function of psychrophilic alanine racemases from *Bacillus psychrosaccharolyticus* and *Pseudomonas fluorescens*. These enzymes showed high catalytic activities even at 0°C and were extremely labile at temperatures over 35°C. The enzymes were also found to be less resistant to organic solvents than alanine racemases from thermophilic and mesophilic bacteria, both in vivo and in vitro. Both enzymes have a dimeric structure and contain 2 mol of pyridoxal 5′-phosphate (PLP) per mol as a coenzyme. The enzyme from *B. psychrosaccharolyticus* was found to have a markedly large K_m value (5.0 μM) for PLP in comparison with other reported alanine racemases, and was stable at temperatures up to 50°C in the presence of excess amounts of PLP. The dissociation of PLP from the *P. fluorescens* enzyme may trigger the unfolding of the secondary structure. The enzyme from *B. psychrosaccharolyticus* has a distinguishing hydrophilic region around residue no. 150 in its deduced amino acid sequence, whereas the corresponding regions of other *Bacillus* alanine racemases are hydrophobic. The position of this region in the three dimensional structure of this enzyme was predicted to be in a surface loop surrounding the active site. This hydrophilic region may interact with solvent, reduce the compactness of the active site, and destabilize the enzyme.

Keywords: Alanine racemase; Psychrophilicity; Thermolability; *Bacillus psychrosaccharolyticus*; *Pseudomonas fluorescens*

1. Introduction

Alanine racemase catalyzes racemization of L- and D-alanine, and provides the latter enantiomer for the construction of the peptidoglycan layer of bacterial cell walls. Accordingly, this type of enzyme is believed to occur widely in bacteria, and is interesting from the standpoint of comparative biochemistry. Alanine racemase has been studied as a target for antibacterial drugs [1–5]. Alanine racemases from the mesophiles *Bacillus subtilis* var. *aterrimus* [6], *Pseudomonas putida* [7], *Streptococcus faecalis* [2], *Staphylococcus aureus* [8], *Escherichia coli* [9], and *Salmonella typhimurium* [10,11], and from a thermophile, *Bacillus stearothermophilus* [12] have been purified and characterized. Several alanine racemase genes from the mesophiles and thermophile including two isozyme genes, *dadB* and *alr*, were cloned, and their DNA and amino acid sequences have been analyzed [13–17]. Some alanine racemase genes from other mesophiles such as *Haemophilus influenzae* [18], *Helicobacter pylori* [19] and *E. coli alr* [20] have also been clarified by whole-genome sequencing. The amino acid sequences of the known alanine racemases

* Corresponding author. Tel.: +81-742-20-3460; fax: +81-742-20-3499.
E-mail address: yokoigawa@cc.nara-wu.ac.jp (K. Yokoigawa).

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show 9–10 conserved regions, including a highly conserved region around the active-site lysyl residue, distributed evenly throughout the enzymes [17]. Thus, alanine racemases from different sources are highly homologous. Therefore, the study of the structural difference between psychrophilic alanine racemase and other enzymes may provide useful information on the nature of psychrophilicity and thermolability.

Several reports have been published on the structural characteristics responsible for the psychrophilicity and thermolability of psychrophilic enzymes. Schlatter et al. [21] and Vckovski et al. [22] reported that psychrophilic lactate dehydrogenase (LDH) was more homologous with thermophilic LDH than mesophilic LDH. They suggested that the psychrophilic enzymes had structural adaptations to low temperature similar to thermophilic enzymes had to high temperature. Feller and Gerday [23], and Davail et al. [24] suggested that the high catalytic activity of a psychrophilic subtilisin at low temperature originated from its highly flexible structure. Preliminary crystal structures of psychrophilic α-amylase [25] and alkaline protease [26] have been reported. However, the enzymological properties of psychrophilic enzymes having pyridoxal 5′-phosphate (PLP) as a coenzyme have not been clarified. In addition, no report has been published on the effect of coenzymes on the thermolability of psychrophilic enzymes.

We have purified and characterized a psychrophilic alanine racemase from *Pseudomonas fluorescens* [27–29]. We also cloned and sequenced a psychrophilic alanine racemase gene from *Bacillus psychrosaccharolyticus*, and characterized the encoded enzyme [29–31]. In this paper, we describe the structure and function of these psychrophilic alanine racemases.

2. Purification and properties of psychrophilic alanine racemases

P. fluorescens TM5-2 is a gram negative psychrotroph; we routinely cultivated these cells at 20°C. Alanine racemase from *P. fluorescens* was purified to homogeneity (about 10,000-fold purification) with an overall yield of 17%, and characterized [27]. The purified enzyme was found to have an apparent molecular mass of about 76,000 Da by gel filtration. The

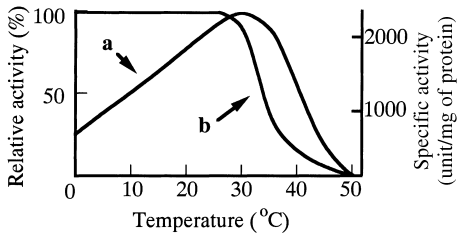

Fig. 1. Effect of temperature on the activity of alanine racemase from *P. fluorescens*. (a) Enzyme activity was measured at various temperatures by the direction from L- to D-alanine; (b) the enzyme solution (5 units/ml in 10 mM potassium phosphate buffer (pH 8.0) containing 10% glycerol, 0.02% sodium azide, 0.01% 2-mercaptoethanol, 0.5 mM EDTA, 10 μM PLP, and 0.1 mM phenylmethanesulfonyl fluoride) was incubated at various temperatures for 1 h. After incubation, any remaining activity was measured at 0°C.

apparent molecular mass of the subunit was estimated to be about 38,000 Da by SDS polyacrylamide gel electrophoresis. These results show that the enzyme is composed of two subunits identical in molecular mass. The temperature dependence of the Vmax was examined by Arrhenius plots, and the activation energy E_a was 6.98 kcal/mol with ΔH, ΔG, and ΔS calculated as 24.2 kcal/mol, 6.4 kcal/mol, and −59 cal/mol deg., respectively. The K_m values at 30°C for D-alanine and L-alanine were 12.8 and 18.9 mM, respectively, and V_{max} values at 30°C for racemization of D-alanine and L-alanine were 1440 and 2400 units/mg, respectively; 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. When these values were used, the calculated K_{eq} for alanine racemization was 1.13, in good agreement with the theoretical value (1.0) for the chemically symmetric reaction. The enzyme showed high activity at low temperature (Fig. 1) [27]. The activity at 0°C was about 25% of the maximum activity observed at 30°C. The alanine racemases from *B. subtilis* and *B. stearothermophilus* were catalytically inert at 0°C. When the psychrophilic enzyme was incubated at greater than 35°C for 1 h, enzyme activity was quickly lost (Fig. 1b). The thermophilic enzyme from *B. stearothermophilus* was quite stable upon heat treatment at 75°C for 1 h, and the mesophilic enzyme from *B. subtilis* was stable up to 55°C under the same conditions.

B. psychrosaccharolyticus is an endospore-forming gram positive psychrophile; we routinely cultivated

these cells at 23°C. A psychrophilic alanine racemase gene from *B. psychrosaccharolyticus* was cloned and expressed in *E. coli* SOLR in the plasmid pYOK3 [31]. The psychrophilic alanine racemase was purified to homogeneity (about 50-fold purification) with an overall yield of 15%, and characterized [31]. The purified enzyme was found to have an apparent molecular mass of about 73,000 Da by gel filtration. The molecular mass of the subunit was estimated as about 42,500 Da by SDS polyacrylamide gel electrophoresis, which is in excellent agreement with that predicted from the DNA sequence (42,519 Da). These results show that the enzyme is composed of two subunits identical in molecular mass. The isoelectric point (pI) of the *B. psychrosaccharolyticus* enzyme was found to be pH 5.7 by isoelectric focusing. Since the pI values of other alanine racemases have not been reported, we predicted the values by the method of Patrickios and Yamasaki [32]. The predicted pI values of the enzymes from *B. subtilis*, *B. stearothemophilus*, and *B. psychrosaccharolyticus* were 8.3, 6.5, and 5.9, respectively. The predicted pI value (5.9) of the *B. psychrosaccharolyticus* enzyme showed good agreement with the experimental value (5.7). The pI value of the psychrophilic enzyme is lower than those of other *Bacillus* alanine racemases. When we compared the amino acid composition of the psychrophilic enzyme with those of other *Bacillus* alanine racemases, the psychrophilic enzyme showed lower mol% of such basic amino acids as His and Arg than other *Bacillus* alanine racemases. The activation energy E_a was 5.4 kcal/mol with values of ΔH, ΔG, and ΔS calculated as 5.7 kcal/mol, 20.8 kcal/mol, and -49.1 cal/mol deg., respectively. The K_m values at 30°C for D-alanine and L-alanine were 12.2 and 17.9 mM, respectively, and V_{max} values at 30°C for racemization of D-alanine and L-alanine were 1,010 and 2,000 units/mg, respectively. The enzyme showed its greatest degree of catalysis in the pH range from 8 to 10, and was stable in the pH range from 8 to 10. The enzyme also showed a high catalytic activity at 0°C, and was extremely labile over 35°C. The activity at 0°C (in the direction of L- to D-alanine, 650 unit/mg) was about 30% of the maximum activity observed at 35°C.

Both psychrophilic enzymes contain two mol of PLP per mol as a coenzyme. The enzymes were specific to alanine. 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 did not appear to be racemized when examined by polarimetry.

3. Sensitivity of psychrophilic alanine racemases to organic solvents and denaturants

Thermostable enzymes are known to be resistant to organic solvents and denaturants [33,34]. However, no thermolabile enzyme has been characterized in terms of its resistance to these reagents. Therefore, we examined the effects of denaturants and organic solvents on psychrophilic alanine racemase from *P. fluorescens* [28]. As shown in Fig. 2, the thermolabile enzyme lost about 50% of its initial activity after being incubated at 30°C for 5 min with sodium lauryl sulfate (0.08%), guanidine hydrochloride (1 M), urea (4 M), ethanol (45%), and dimethyl sulfoxide (50%), whereas the thermostable enzyme from *B. stearothermophilus* was resistant to these denaturants at the same concentrations. The resistance of mesophilic alanine racemases to these reagents has not been reported. We observed that the enzymes from *B. subtilis* and *E. coli* in cell-free extracts each lost about 5–25% of their initial activity under the same conditions with 0.08% sodium lauryl sulfate, 1 M guanidine hydrochloride, 4 M urea, 45% ethyl alcohol, and 50% dimethyl sulfoxide. Thus, the thermolabile enzyme was found to be less resistant to organic solvents and denaturants than the thermostable and mesophilic types.

Psychrotrophic bacteria are widely distributed in nature, and often cause deterioration in foods stored at a low temperature [35]. Accordingly, these bacteria have been studied in order to better understand food preservation [36,37]. While psychrotrophs are easily killed by heat treatment, their extracellular hydrolytic enzymes survive, because of their thermostability, and degrade the quality of foods [37]. Therefore, suppressing the growth of psychrotrophs is important for food preservation at low temperature. However, there is no effective method for suppressing the growth of psychrotrophs in foods other than thermal sterilization, which often changes the original taste. Psychrophilic alanine racemase is less resistant to organic solvents

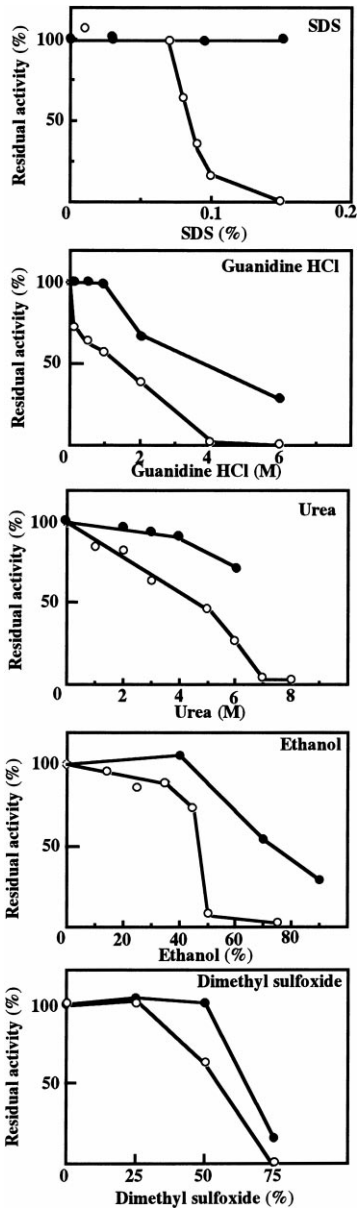

Fig. 2. Effects of denaturants and organic solvents on the activity of alanine racemases from *P. fluorescens* and *B. stearothermophilus*. Each enzyme (final concentration of 0.1 mg/ml) was incubated with various concentrations of denaturants and organic solvents in 10 mM potassium phosphate buffer (pH 7.5) containing 10 μM PLP and 0.01% 2-mercaptoethanol at 30°C for 5 min, before being immediately used for measurement of residual activity. In the measurement, 10 μl of the solution was added to a reaction mixture containing 0.4 mM PLP, 40 mM phosphate buffer (pH 8.3), 250 mM L-alanine in a final volume of 1 ml. (○) The *P. fluorescens* enzyme; (●) the *B. stearothermophilus* enzyme.

and denaturants than the thermophilic and mesophilic types as described above, and we further investigated this difference using ethyl alcohol, which is applicable to the sterilization of foods. Although alcohol is known to have sterilizing power, no report has been published on its effect at a low concentration on the growth and intracellular alanine racemase activity of psychrotrophs in comparison with mesophiles.

To compare the effect of ethyl alcohol on psychrotrophs and mesophiles, both types of cell should be exposed to ethyl alcohol at the same temperature and for the same length of time, because the sterilizing power of ethyl alcohol changes depending on the temperature. However, the optimum growth temperature of psychrotrophs clearly differs from that of mesophiles. In addition, the growth rate of bacteria depends on species. The effect of alcohol on the growth of the bacteria was examined at 22°C for 36 h, since in a preliminary experiment, the three psychrotrophs (*P. fluorescens* TM5-2, *B. psychrosaccharolyticus* ATCC 23296 and *B. psychrophilus* ATCC 23304) and two mesophiles (*B. subtilis* and *E. coli*) grew to early stationary phase at 22°C within 36 h in the absence of ethyl alcohol. As shown in Fig. 3A, the growth of the psychrotrophs was more effectively suppressed by ethyl alcohol than that of the mesophiles [29]. Also, the specific activities of intracellular alanine racemases in the presence of ethyl alcohol were lower in the psychrotrophic cells than the mesophilic ones (Fig. 3B).

The survival ratio and alanine racemase activity of the psychrotrophs and mesophiles after incubation of the cells at 30°C for 1 h with 0–5% ethyl alcohol were also examined [29]. As shown in Fig. 4, all the psychrotrophs tested had lower survival ratios than the mesophiles. Ethyl alcohol is known to extract lipid components from cells and to cause denaturation of membrane-bound proteins. When we measured the absorbance of the supernatant solution at 280 nm after the ethyl alcohol treatment of the cells, no significant difference was observed between the absorbances of the psychrotrophs and mesophiles. However, the alanine racemase specific activities of the psychrotrophs were markedly lower than those of the mesophiles (Fig. 4B). The specific activities of each bacterium before and after the treatment with 0% ethyl alcohol (H_2O) were almost identical. Therefore, ethyl alcohol had probably permeated the bacterial cells and

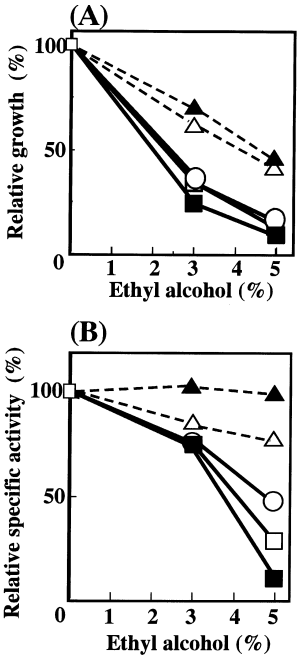

Fig. 3. Growth (A) and intracellular alanine racemase activity (B) of psychrotrophs and mesophiles in the presence of ethyl alcohol. Bacteria were grown at 22°C for 36 h in the growth medium containing ethyl alcohol. Growth was analyzed by measuring the absorbance of each culture broth at 660 nm. The specific activity of alanine racemase was measured with cell extracts in the absence of ethyl alcohol. (○) *P. fluorescens*; (■) *B. psychrophilus*; (□) *B. psychrosaccharolyticus*; (△) *E. coli*; (▲) *B. subtilis*. The results are expressed as mean values of triplicate experiments, for which the standard deviations are below 10%.

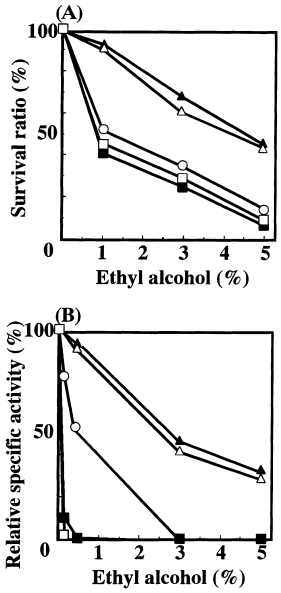

Fig. 4. Survival ratio (A) and intracellular alanine racemase activity (B) of bacterial cells exposed to 0–5% ethyl alcohol. Bacterial cells grown to the early stationary phase at 22°C for 36 h in the absence of ethyl alcohol were harvested by centrifugation, and washed twice with 0.85% NaCl. The cells were suspended in 0–5% ethyl alcohol, and incubated at 30°C for 1 h. The survival ratio was determined by surface plating onto Plate Count Agar (Difco) at 22°C after washing twice with 0.85% NaCl. The specific activity of alanine racemase was measured with the extracts of the bacterial cells treated with ethyl alcohol at 30°C for 1 h. (○) *P. fluorescens*; (■) *B. psychrophilus*; (□) *B. psychrosaccharolyticus*; (△) *E. coli*; (▲) *B. subtilis*. The results are expressed as mean values of triplicate experiments, for which the standard deviations are below 7%.

inactivated the enzyme. Although the enzymes of the psychrotrophs were easily inactivated by 0.1% ethyl alcohol, the corresponding survival ratio was markedly high. In these cells, cell injury apart from the inactivation of the enzyme may have been at a low level, and therefore, the cells may have been able to synthesize enough alanine racemase to form colonies during culture on Plate Count Agar (Difco Laboratories, Detroit, MI). The enzyme may also have been synthesized to some extent during the culture on plate count agar in those cells that did not show detectable enzyme activity after the treatment with more than 3% ethyl alcohol. When cell extract (0.1 ml) of each psychrotroph, unexposed to the alcohol treatment, was suspended in 1 ml of 3% ethyl alcohol and incubated at 30°C for 1 h, the enzyme was completely inactivated. Thus, the alanine racemases of psychrotrophs appear to be easily inactivated by ethyl alcohol both in vivo and in vitro. This information may be useful to the food industry in efforts to suppress the growth of psychrotrophs and psychrophiles.

4. Role of PLP on thermolability and psychrophilicity of alanine racemase

To clarify the reason for the susceptibility of psychrophilic alanine racemases to denaturants and organic solvents, the denaturation of the *P. fluorescens* enzyme was examined with respect to the dissociation of PLP and the unfolding of the enzyme protein [28].

We used urea as a denaturant for this study, because the enzyme needed to be inactivated to various extents for a detailed analysis of the denaturation process, and only urea inactivated the thermolabile enzyme with pseudo-first-order kinetics. To avoid renaturation of the denatured enzymes, the enzyme solutions containing urea were directly used for spectrophotometric measurements. As well, the K_m values of the denatured enzymes for L-alanine were determined in the reaction mixtures containing the appropriate concentration of urea.

The absorption spectra of the *B. stearothermophilus* and *P. fluorescens* enzymes show maxima at 420 nm [12,27] in the visible region due to the aldimine linkage formed between the formyl group of PLP and the ε-amino group of the active site. Using this fact as a basis, we examined spectrophotometrically the dissociation of PLP from the enzyme proteins during their denaturation with urea. As shown in Fig. 5, the absorbance at 420 nm decreased with increasing urea concentration [28]. This indicates that, with the addition of urea, the aldimine linkage was hydrolyzed to produce the aldehyde form of PLP. No further appreciable change in the absorbance of the thermolabile and thermostable enzymes was observed above 3.5 and 4.0 M urea, respectively. The K_m values of the thermolabile and thermostable enzymes for L-alanine increased markedly at 3.5 and 4 M urea, respectively (Fig. 6). Therefore, the increase in K_m values and hydrolysis of the aldimine linkage occurred at the same urea concentration. Although the two enzymes were active after being treated with 4 M urea (Fig. 2), the PLP-dissociated enzymes (apoenzymes) may have been partially reconstituted with PLP by 100-fold dilution of the enzyme solutions containing urea with the standard reaction mixture; PLP is essential for the activities of these enzymes.

The unfolding of the *P. fluorescens* enzyme with urea was followed by a CD analysis: the mean residual ellipticity at 222 nm was measured at 20°C and plotted against the concentration of urea (Fig. 7) [28]. The

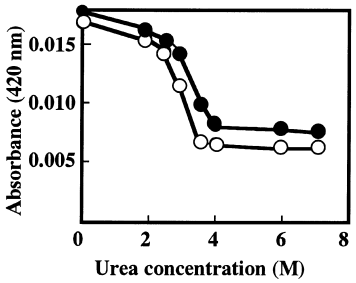

Fig. 5. Effect of urea on the absorbance of the *P. fluorescens* alanine racemase at 420 nm. Each enzyme (final concentration of 0.1 mg/ml) was incubated with various concentrations of urea in a 10 mM potassium phosphate buffer (pH 7.5) containing 0.01% 2-mercaptoethanol at 30°C for 5 min. The absorbance at 420 nm of the enzyme solutions was then immediately measured. (○) The *P. fluorescens* enzyme; (●) the *B. stearothermophilus* enzyme.

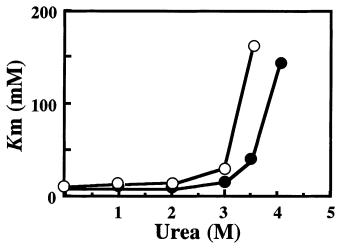

Fig. 6. Effect of urea on the K_m values of the thermolabile and thermostable enzymes. Each enzyme (final concentration of 0.1 mg/ml) was incubated with various concentrations of urea in 10 mM potassium phosphate buffer (pH 7.5) containing 10 μM PLP and 0.01% 2-mercaptoethanol at 30°C for 5 min, before being immediately used for measurement of the K_m value. The K_m values were measured with the standard reaction mixture containing urea at the respective concentration to avoid refolding of the enzyme protein during assay. (○) The *P. fluorescens* enzyme; (●) the *B. stearothermophilus* enzyme.

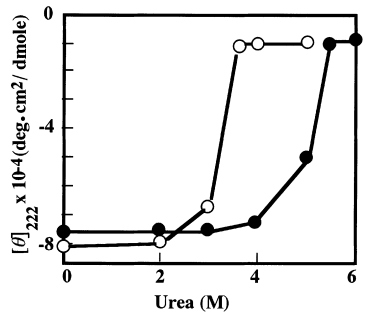

Fig. 7. Effect of urea concentration on the mean residual ellipticity at 222 nm of the thermolabile and thermostable enzymes. Each enzyme (final concentration of 0.1 mg/ml) was incubated with various concentrations of urea in 10 mM potassium phosphate buffer (pH 7.5) containing 10 μM PLP and 0.01% 2-mercaptoethanol at 30°C for 5 min. The CD spectrum of the enzyme solution was then measured. (○) The *P. fluorescens* enzyme; (●) the *B. stearothermophilus* enzyme.

ellipticity decreased with increasing concentration of urea. The transition midpoints of the thermolabile and thermostable enzymes, defined by the horizontal base lines and plateau, were at around 3.5 and 5.5 M, respectively. No further appreciable change in the ellipticity of the thermolabile and thermostable enzymes was observed above 3.5 and 5.5 M urea, respectively. These results suggest that the *P. fluorescens* and *B. stearothermophilus* enzymes are completely denatured by incubation with >3.5 and 5.5 M urea, respectively. Although Fig. 2 shows that both enzymes were active even after being treated with 6 M urea, the denatured enzymes may have been partially refolded and reconstituted with PLP during the measurement of residual activity.

Thus, enzyme unfolding and hydrolysis of the aldimine linkage of the thermolabile enzyme occurred at the same urea concentration (3–3.5 M), whereas each of those changes occurred at different urea concentrations in the *B. stearothermophilus* enzyme. Although this denaturation process is not parallel to the result with urea in Fig. 2, partial renaturation may have occurred under the assay conditions. This renaturation was unexpected because the thermostable enzyme was reported to be unable to renature below pH 8.5 after denaturation with guanidine hydrochloride [12]. Both reversible and irreversible denaturation states may be present in alanine racemases.

Our results suggest that the thermolabile enzyme is denatured by treatment with urea through one detectable phase, and that the thermostable enzyme is denatured through two phases: phase 1 involves hydrolysis of the aldimine linkage; phase 2 involves protein unfolding. Toyama et al. have reported that the thermostable enzyme is unfolded by treatment with guanidine hydrochloride through two phases: phase 1 involves dissociation of the dimer into monomers and hydrolysis of the aldimine linkage; phase 2 involves unfolding of the monomer [34]. Subunit dissociation of the thermolabile enzyme may occur simultaneously with destruction of the secondary structure. This denaturation process of the thermolabile enzyme may be related to its susceptibility to denaturants; the dissociation of PLP from the enzyme may trigger the unfolding of the secondary structure.

We also examined the role of PLP in *B. psychrosaccharolyticus* alanine racemase [31]. Although the enzyme was resolved to the apoenzyme by dialysis at 4°C for 24 h against a PLP-free standard buffer (10 mM potassium phosphate buffer, pH 8.0, 0.02% sodium azide, 0.1 mM phenylmethanesulfonyl fluoride, 10% glycerol, 0.01% mercaptoethanol, and 0.5 mM EDTA) supplemented with 10 mM NH_2OH, the apoenzyme was not activated by the addition of PLP. The enzyme may be irreversibly denatured by the NH_2OH treatment. When the holoenzyme was dialyzed at 4°C against the PLP-free standard buffer supplemented with ammonium sulfate at 30% saturation, the enzyme was gradually resolved to the apoenzyme over 16 days. The activity was restored to 60% of the original activity by dialysis against the standard buffer at 4°C for 48 h. The K_m value for PLP at 30°C was estimated to be 5.0 μM. However, the value for the thermophilic enzyme from *B. stearothermophilus* was found to be 50 nM under the same assay conditions. Although the K_m value for PLP of the *B. subtilis* alanine racemase has not been reported, the value of the mesophilic enzyme from *S. typhimurium alr* is reported to be 33 nM [10]. Thus, the psychrophilic enzyme showed low affinity for PLP. The low affinity for PLP may reflect a flexible conformation of the active site, and may be related to the high catalytic activity of the psychrophilic PLP enzyme, initiated by a transaldimination reaction, at low temperature. In addition, the dissociation of PLP from the enzyme may be related to the thermolability of the psychrophilic alanine racemase. In fact, the presence of an excess amount of PLP (0.4 mM) stabilized the psychrophilic enzyme up to 50°C (Fig. 8) [31].

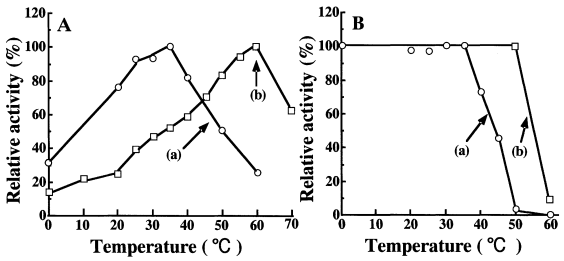

Fig. 8. Effects of PLP on the *B. psychrosaccharolyticus* alanine racemase: (A) enzyme activity was measured at various temperatures in the absence (a) and presence (b) of 0.4 mM PLP; (B) the enzyme solution (3 units/ml in 10 mM potassium phosphate buffer, pH 8.3) was incubated at various temperatures for 1 h in the absence (a) and presence (b) of 50 μM PLP. Any remaining activity was measured at 0°C.

5. Structure of psychrophilic alanine racemase

Alanine racemases from *P. fluorescens* and *B. psychrosaccgarolyticus* are more thermolabile and psychrophilic than other reported alanine racemases. The distinctive thermolability and psychrophilicity presumably reflect the structural differences. The *B. psychrosaccharolyticus* alanine racemase gene encodes a protein of 383 amino acids [31]. The *N*-terminal amino acid sequence from Met1 to Val50 of the enzyme purified from a clone was identical to the deduced amino acid sequence. When the amino acid sequence of the psychrophilic enzyme was analyzed by the FASTA program using GenBank/EMBL databases, the psychrophilic enzyme showed 32–57% homology with reported alanine racemases. The thermophilic enzyme from *B. stearothermophilus* showed the highest sequence-homology (57%) with the psychrophilic enzyme. When we compared the hydropathy profile of the psychrophilic enzyme with those of alanine racemases from *B. subtilis* and *B. stearothermophilus* by the method of Kyte and Doolittle [38], the profiles were similar as a whole but different in a region around residue no. 150 (Fig. 9) [31]. To analyze distinguishing amino acid residues in the region of the psychrophilic enzyme, the amino acid sequences of alanine racemases from three *Bacillus* species (*B. psychrosaccharolyticus* [31], *B. subtilis* [13], and *B. stearothermophilus* [17,39]) were linearly aligned according to a mutation data scoring matrix [40]. Although various amino acid residues in the psychrophilic enzyme were different from the corresponding amino acid residues in the other two, the psychrophilic enzyme was found to contain two distinguishing hydrophilic residues, glutamate and arginine, in position 150 and 151, respectively.

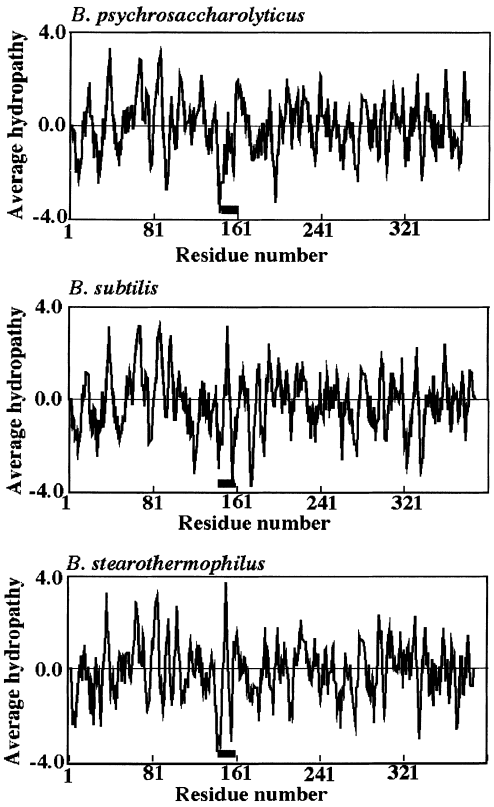

Fig. 9. Comparison of the hydropathy profiles of the three alanine racemases. Consecutive hydropathy averages are plotted for a five-residue window advancing from the N- to the C-terminus. Relative hydrophobicity and hydrophilicity were recorded in the range +4.0 to −4.0 for each of the three sequences, which had been aligned by introducing gaps to maximize identities. The region distinguishing *B. psychrosaccharolyticus* enzyme from the other two enzymes is indicated by a heavy line.

Fig. 10 shows the three dimensional structure of the C^{α}-atoms of the psychrophilic enzyme predicted with the thermophilic enzyme from *B. stearothermophilus* as a reference [31]. The region containing Glu150 and Arg151 is located at a surface loop surrounding the substrate binding site. Davail et al. [24] reported that the hydrophilic and polar surface of a psychrophilic

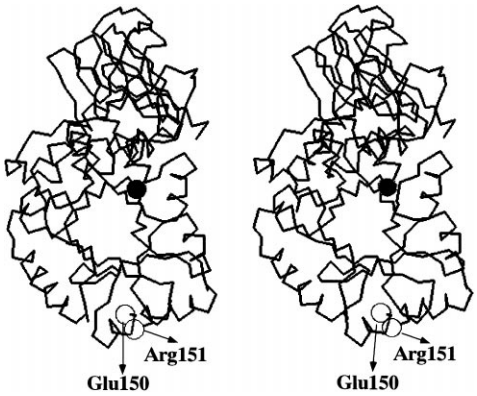

Fig. 10. Stereo diagram of the C^{α}-atoms in the psychrophilic alanine racemase from *B. psychrosaccharolyticus*. The black ball shows the C^{α}-atom of the active site Lys residue. The white balls show the Glu150 and Arg151 residues.

subtilisin gives rise to improved solvent interactions, reduces the compactness of the molecule, and destabilizes the psychrophilic enzyme. Recently, Kim et al. [41] reported that the flexibility of active site residues, the surface charge, and intersubunit ion pair interactions may be the major factors for efficient catalytic activity of psychrophilic malate dehydrogenase at low temperature. Therefore, the existence of the hydrophilic region of the psychrophilic alanine racemase may be one of the reasons for its thermolability. The psychrophilic alanine racemase also differs from other alanine racemases in that it has an acidic pI. This low pI value may be a result of the surface polarity and hydrophilic nature of enzyme molecule. The low affinity of the psychrophilic enzyme for PLP may be related to the flexible conformation of the active site, and to the high catalytic activity at low temperatures.

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