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Kinetics of Thermostable Alanine Racemase of *Bacillus stearothermophilus*

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Unlabeled D- and L-alanine were racemized in deuterium oxide with an alanine racemase of *Bacillus stearothermophilus* at saturated concentration of substrate, and various p^2H and temperature. Samples of the solution were taken at intervals, and all alanine isomers in the samples were transformed into a mixture of diastereomeric derivatives of methyl *N*-(−)-camphanylalaninate. Their ratio was measured on a GC-Mass, and the relative rate was calculated at the initial stage of the reaction. There was little difference in the decrease rate of the optical rotation between the enantiomers. Internal proton-transfer to the antipode was almost zero for either substrate. The α-hydrogen was abstracted 1.2–2.3 times faster from D-alanine than from L-alanine. D-Alanine gave an almost even mixture of deuterium labeled D- and L-alanine, while L-alanine gave a mixture of labeled D- and L-alanine at a ratio of 3:1. These results suggest the racemase builds two different bases in the active site. The base for D-alanine may be closer to the enzyme surface, and that for L-alanine inside.

After its first recognition, alanine (Ala) racemase has been found ubiquitously in the bacterial world.[1-3] The enzyme catalyzes conversion of either L- or D-Ala to the racemic mixture. In microorganisms, D-Ala is used as a component for biosynthesis of cell walls and other metabolites. The racemase used in this article was found and elucidated at first in *Bacillus stearothermophilus* by Soda and his collaborators.[4] The enzyme is a homo-dimeric protein with one molecule of pyridoxalphosphate (PLP) as the cofactor in each subunit. Among several unique qualities, its thermostability is the most characteristic. It keeps the activity to some extent even after partial hydrolysis by a protease.[5] The gene of the enzyme was cloned and expressed in *Escherichia coli*,[4] and the amino acid sequence was compared with other homologous enzymes.[6] The racemase, however, has not been studied kinetically in detail.

Racemization of α-amino acid is accomplished by dissociation and recovery of the chemical bond between the α-hydrogen and α-carbon. The proton once abstracted from the substrate[3] can be transferred within the enzyme-substrate complex to provide the antipode, or diffused into the medium water. The difference can be distinguished by racemization in deuterium oxide using unlabeled D- and L-[α-^{1}H]Ala (D-1 and L-1). The reaction should provide a mixture of four kinds of Ala isomer, D-1, L-1, D-[α-^{2}H]Ala, and L-[α-^{2}H]Ala (D-2 and L-2, respectively). Described here is an analytical method for these isomers, and some discussion of the racemization catalyzed by the thermostable Ala racemase.

Experimental

Chemicals. D-1 (99.98% of optical purity) was purchased from the Peptide Institute Osaka, Japan. Labeled L-2 (99% of optical purity and 98% of deuterium enrichment at α-hydrogen), and D-2 (85% and 98%, respectively) were prepared by repeated racemization of L-1 with our racemase in deuterium oxide, followed by optical resolution of its *N*-acetyl derivative with acylase. (1*S*)-(−)-Camphanic (CPN) acid chloride (98% of chemical purity) was from Aldrich, U.S.A., deuterium oxide from Merck, U.S.A., and other chemicals of reagent grade from Nacalai Tesque, Kyoto, Japan.

Measurements. Optical rotation was taken on a Perking Elmer 241 polarimeter at 436 nm in a micro-cell (optical path, 10 cm) jacketted with a water circuit, which was connected to a water incubator for the racemization. NMR was taken on a JEOL EX-270 (270 MHz to ^{1}H, and 67.8 MHz to ^{13}C). GC-Mass was recorded on a Shimadzu GCMS-6020, with a capillary column of CBP-1 (25 m of length and 0.25 mm inner diameter) at oven, injection, and ionization temperatures of 172°C, 230°C, and 270°C, respectively, with helium as the carrier gas. The p^2H was uncorrected meter-reading of the deuterium oxide solution on a Hitachi-Horiba H-5.

Enzyme. One ml of the homogeneous racemase[2] (6 mg/ml, A_{280} 6.0 in 50 mM Tris–buffer, pH 8.0, 10 mM of PLP and mercaptoethanol) was dissolved in 50 ml of 50 mM phosphate buffer of deuterium oxide (p^2H 7.4). The solution was kept in a refrigerator at 4°C to use for all of the experiment.

Substrate. L-1 (1.0 g) dissolved in deuterium oxide (10 ml) was lyophilized. The procedure was repeated four times to the product, and the final mass was diluted in 36 ml of 50 mM phosphate buffer (p^2H 7.4). The solution was used for all of the experiments. By the same procedure, the stock solution of D-1 was prepared.

Racemization reaction. The reaction was done in a water incubator, which was connected with water pipes to the polarimeter. The mouth of the reaction test tube was wrapped with plastic film to keep out water moisture. The racemization mixture was composed of 0.2 ml of the enzyme solution and 1.8 ml of L-1 or D-1 stock solution (50 mg, 0.56 mmol). The p^2H of the solution was adjusted with sodium deuteroxide before enzyme addition. After the enzyme addition and a few seconds of mixing, two or

Abbreviations: Ala, alanine; L-1, L-[α-^{1}H]Ala; D-1, D-[α-^{1}H]Ala; L-2, L-[α-^{2}H]Ala; D-2, D-[α-^{2}H]Ala; CPN, (1*S*)-(−)-camphanic; OR, optical rotation; MC, mass chromatogram; PLP, pyridoxalphosphate; SIM, selected ion monitoring.

three drops of the mixture were taken, and quenched immediately as the product of time *zero*. At the same time, about two-thirds of the solution was put into the micro-cell of the polarimeter. By monitoring the decrease of the optical rotation, samples of the solution in the test tube were taken at intervals of several minutes.

Instantaneous quenching of the racemase activity. Each sample was immediately added to a dry ice-cooled mixture of sodium borohydride (0.1 mg), sodium bicarbonate (1 mg), water (0.5 ml), and tetrahydrofuran (THF, 0.5 ml). The mixture was stirred for 3 h at ambient temperature.

Preparation of a mixture of methyl N-(−)-CPN-alaninates **1** *and GC-mass analysis*. Solutions of sodium hydroxide (0.5 N, 1 ml) and CPN acid chloride (2.1 mg, 0.01 mmol) in THF (1 ml) were added successively to the mixture above, and the solution was stirred for 3 h at ambient temperature. After the p^2H of the solution was adjusted to 4–5 with acetic acid, diazomethane in ether was added, and the mixture was soon concentrated *in vacuo* to dryness. Chloroform (1 ml) was added to the residue, and the solution (0.2 μl) was analyzed by GC-mass chromatography.

Standard mixture of **1**. Two kinds of standard mixture of **1** were prepared by the same method above. One of them was a mixture of L-1 and D-1 at a ratio of 100:0, 90:10, 80:20, 70:30, and so on to 0:100, and the other, a 1 : 1 mixture of racemic DL-1 and DL-2 (deuterium enrichment 98%).

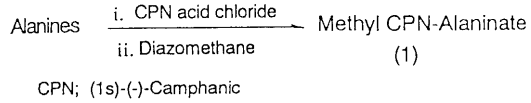
Analytical data of **1**. mp 73.0–74.0°C. $[\alpha]_\lambda^{15}$ (*c* 0.5, ethanol) −44.6′ (λ, 578 nm), −51.8′ (546), −92.0′ (436), and −153′ (365). NMR δ ppm from an internal tetramethylsilane in $CDCl_3$, ^1H: 0.92 (3H, s), 1.11 (3H, s), 1.12 (3H, s), 1.44 (3H, d, J=7.0 Hz), 1.70–1.76 (1H, m), 1.90–1.98 (2H, dt, J=4.6 and 12.5), 2.46–2.54 (1H, m), 3.76 (3H, s), 4.63 (1H, quint, J=7.2), 6.96 (1H, br. d, J=7.0), ^{13}C: 9.63, 16.39, 16.66, 18.42, 28.93, 30.15, 47.71, 52.53, 53.89, 55.18, 92.08, 166.63, 172.41, 178.08. *Anal.* Found: C, 59.33; H, 7.57; N, 4.97%. Calcd. for $C_{14}H_{21}NO_5$: C, 59.35; H, 7.47; N, 4.94%. Mass, unlabeled **1**, *m/z* (M^+) 283. deuterium labeled **1**, 284.

Results

D-1 and L-1 were treated preliminarily with deuterium oxide to substitute exchangeable hydrogens for feuterium. The racemization was done in phosphate buffer of deuterium oxide at 40°C and 50°C, and at p^2H 7.0, 8.0, and 9.0, respectively. Samples of the solution were taken at intervals, and the enzyme in each was quenched completely and instantaneously.

Ala isomers in the sample were thoroughly converted to the corresponding mixture of diastereomeric derivatives **1** (Scheme 1). CPN acid chloride was the best among those tested, although a large excess was required for the completion. Figure 1 shows a gas chromatogram (GC) of the specimen prepared from a sample of 55 min racemization of D-1 at 40°C, p^2H 8.0. Among five peaks therein, the fourth is the diastereomer **1** of D-Ala, and the fifth **1** of L-Ala.

Those two peaks were completely separated from each other, and the GC of **1** prepared from pure L-1 showed no peak to be assigned as the derivative **1** from pure D-1 (*vice versa*). The diastereomer mixture **1** prepared from a mixture of D-1 and L-1 of known ratio, showed a fine correlation to the area ratio of peaks 4 and 5. This means that CPN acid chloride is sufficient in its optical purity and stability as a chiral reagent for the preparation of the derivative, and that no epimerization of **1** occurs through the experiment.



Scheme 1.

The derivative **1** gave a stable mass chromatogram (MC, Fig. 2). Spectrum A is the derivative **1** of unlabeled L-1, and B from a mixture of L-2 and L-1 (88 : 12). Each spectrum shows fragment ions of *m/z* 224 and 225 as the base peak, and *m/z* 283 and 284 as the molecular ion peak (M^+), respectively. The M^+ was too small and useless for quantitative analysis. The base peak is reasonably assigned to labeled or unlabeled *N*-camphanylethylidenammonium

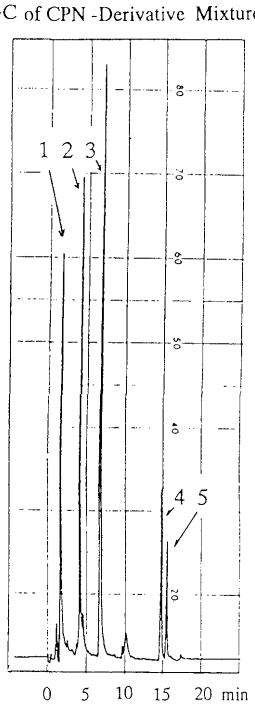


Fig. 1. The Specimen is from a Mixture Containing CPN-derivatives **1** Prepared from a Sample of 55-min Racemization of D-1 at 40°C, and p^2H 8.0.

Peak 4 is methyl *N*-CPN-D-alaninate, and Peak 5 methyl *N*-CPN-L-alaninate. Other three peaks are solvent, methyl ester of CPN acid, and dimethyl ester of CPN acid hydrolyzate, respectively.

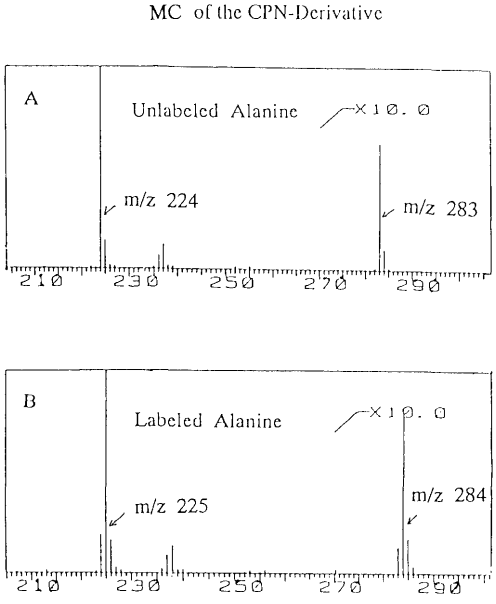
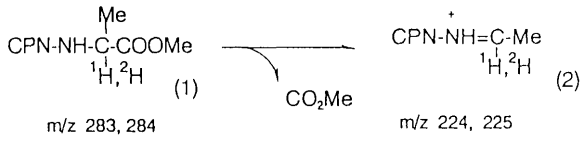


Fig. 2. Spectrum A is a MC of the CPN-derivative of L-1, and B of the Derivative of Racemic [α-^{2}H]Alanine.

Both spectra show the main part. The mass conditions are described in Experimental section.



Scheme 2.

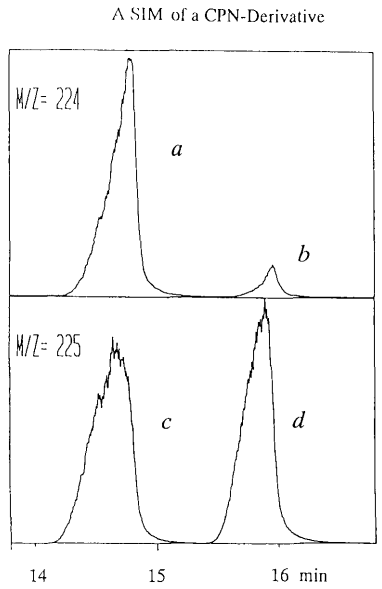


Fig. 3. A Set of SIM Focused on *m/z* 224 and 225 of the Same Sample as in Fig. 1.

Peaks *a* to *d* correspond to D-1, L-1, D-2, and L-2, respectively. The SIM conditions are described in Experimental section.

ion **2** which can be generated by demethoxycarbonylation of **1** (Scheme 2). The ion *m/z* 224 keeps an α-hydrogen in unlabeled Ala, and the ion *m/z* 225 a deuterium in labeled Ala. These ions were suitable for ratio analysis of unlabeled and labeled Ala isomers.

The ratio was taken by selected ion monitoring (SIM) focused to these ions on the GC-Mass. Figure 3 shows a SIM spectrum scanned synchronously with peaks of 4 and 5 on the GC of Fig. 1. The peaks from *a* to *d* correspond to the isomer of D-1, L-1, D-2, and L-2, respectively. Model analyses of diastereomer derivative **1** prepared from a 1 : 1 mixture of DL-1 and DL-2, gave fine a one-to-one match to the SIM ratio of these four Ala isomers, after a reasonable correction below. The result showed no migration of α-hydrogen or deuterium among the diatereomers through the experiment. It enabled us to calculate the ratio of four Ala isomers by this method.

As can be seen in Fig. 2A, although the derivative **1** has no deuterium atom, the MC shows a small satellite peak of *m/z* 225 by an intensity of 15.2% of the base peak. The fragment appeared constantly at the same intensity in every derivative of unlabeled Ala. The same was observed in Fig. 2B of labeled Ala, where the satellite peaks was *m/z* 226. This demanded correction of SIM areas to get the real ratio of Ala isomers. The ratio was calculated by proportional allotment of their ratios, since the total amount of Ala was constant through the reaction.

$$[\text{L-1}]/[\text{D-1}] = b/a$$

$$[\text{D-2}]/[\text{D-1}] = (c/a) - 0.152$$

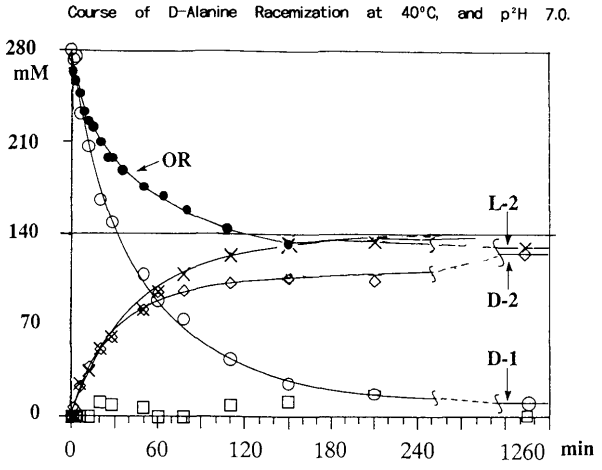


Fig. 4.

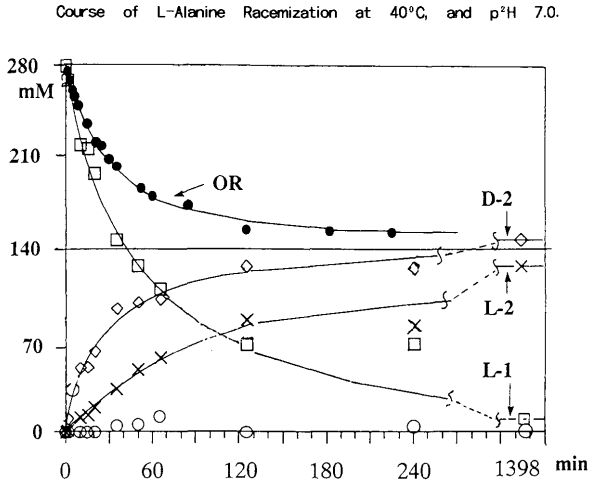


Fig. 5.

$$[\text{L-2}]/[\text{L-1}] = (d/b) - 0.152$$

$$[\text{L-2}]/[\text{D-2}] = (d - 0.152b)/(c - 0.152a)$$

$$[\text{D-1}] + [\text{L-1}] + [\text{D-2}] + [\text{L-2}] = 280 \text{ (mM)}$$

Figures 4 and 5 show results of the racemization of D-1 and L-1 at 40°C and p^2H 7.0, respectively. These are the courses of decrease of starting Ala and optical rotation (OR) of the solution, and increases of labeled L-2 and D-2. The value of OR was converted to the unit of mM for the benefit of comparison with the others. Formation of unlabeled enantiomer was at the analytical limit. Since all of the other experiments showed similar courses, and these curves never followed any simple-ordered kinetics, the results were summarized on a unit of v_0 mM/10 minutes by taking the difference in mM of these components at the first ten minutes, where 3–4 samples were plotted (Table).

The starting substrate, D-1 decreased faster than L-1 by ratios of $v_{0,\text{D-1}}/v_{0,\text{L-1}} = 1.2$–$2.3$. The decrease rate of OR was close between each D- or L-Ala, and quite compatible with the increase rate of the corresponding enantiomer formation. For the products, the substrate D-1 gave almost evenly labeled L-2 and D-2, while L-1 provided predominantly D-2 by 3 times to L-2 isomer. As to the pH and temperature shuffling, no large difference was observed in the rates of racemization and product formation.

Table Relative Rate of Alanine Components in the Racemization in the First Ten Minutes

Substrate	Temperature °C	p²H	v_0 mM/10 minutes[a]				
			D-[α-¹H]Alanine	L-[α-¹H]Alanine	D-[α-²H]Alanine	L-[α-²H]Alanine	OR[b]
D-[α-¹H]Alanine	40	7.0	−59	ND[c]	28	28	−30
		8.0	−78	ND	39	37	−38
		9.0	−100	ND	49	48	−50
	50	7.0	−78	ND	34	34	−35
		8.0	−70	ND	33	33	−35
		9.0	−63	ND	32	32	−37
L-[α-¹H]Alanine	40	7.0	ND	−50	39	12	−36
		8.0	ND	−47	34	12	−34
		9.0	ND	−42	33	11	−30
	50	7.0	ND	−53	42	12	−42
		8.0	ND	−50	37	11	−36
		9.0	ND	−47	36	11	−35

[a] Decrease (−) and increase amount (mM) of alanine components in the racemization in the first ten minutes.
[b] Value of the optical rotation, which is converted to the same unit of mM.
[c] ND, not detected for the analytical limit.

Discussion

Since being concerned with α-proton abstraction from the substrate at its rate-determining step, the enzyme-catalyzed racemization have always been discussed in relation to its *active base* at the reaction site.[1-3] One-base mechanism proposes one active base working on either substrate enantiomer for the proton abstraction and its transfer. Then on this theory, some of the intact α-proton from the substrate has been expected in the provided antipode.

Another mechanism supposes two active bases working cooperatively for proton abstraction and for its addition to the reaction intermediate to give the antipode. The proton once removed from the substrate to one of these bases can be diffused into medium water without its migration to the antipode. The other base, already conjugated with a proton from the medium, donates it to the intermediate from the opposite side to provide the antipode of the substrate.

PLP-mediated Ala racemization can be described by the general scheme of aldimine and ketimine intermediates.[1-3] The α-carbon of the Ala moiety keeps a chiral sp^3 hybrid in the aldimine intermediate. Supposing it is trapped by at least two common binding sites, the α-hydrogen of one enantiomer is opposite to the others. Subsequent α-hydrogen abstraction provides a ketimine intermediate, which is planar and achiral, and has an α-carbon of the enantiotopic sp^2 hybrid.

On their study of Ala racemase, Henderson and Johnston[7] analyzed inhibitor effects on D-1 and L-1, and proposed a swinging door model catalyzed by a single base. Floss and his colleagues[8] measured differences of racemization activity among three Ala racemases to L-1 in deuterium oxide and to L-2 in light water at a single turnover condition. They concluded two of them obey a single base mechanism. In our previous study[9] of a low substrate-specific amino acid racemase using NMR and optical rotation, several common amino acids were racemized in deuterium oxide and some [α-²H]-amino acids in light water. We observed a typical isotope effect showing the rate-determining step is the step of α-hydrogen abstraction and enzyme specificity favoring to D-isomer formation. These studies, however, have been mainly discussed on the basis of the results of the substrate, one of the related compounds, or on analyses of single product.

The method described here provides an analysis of all isomeric alanines in the racemization. The initial concentration (280 mM) of D-1 and L-1 was 105 and 66 times the Km[2] of the enzyme (2.65 mM and 4.25 mM, at pH 9.0 and 50°C), respectively. By saturating the enzyme with substrate, the reaction rate closely resembled to the V_{max}, so that the results can be interpreted under steady condition.

The results show several marked enzymatic specificities. To explain them, one possible model could be an equilibrium of two dissymmetric enzyme conformations, as suggested in their study of proline racemase[10] by Knowles and his collaborators.[11] Our Ala racemase, however, shows almost unchanged activity through wide ranges of pH and temperature. This suggests a hard enzyme which does not allow any conformational flexibility.

Another is consideration of the steric configuration and specificity of the reaction site, as deiscussed in some papers.[9,12] No α-hydrogen transfer from starting Ala to the antipode suggests two active bases at the reaction site. The result that D-1 gives an almost even mixture of D-2 and L-2, shows that the proton once abstracted from the aldimine intermediate of D-1 to *the base specific for* D-*Ala* is not transferred to *the other base*, but diffused easily from the base into the medium, deuterium oxide, and a deuteron in the medium is exchanged on the base, followed by its return to the ketimine intermediate from the same face of the proton abstraction to give D-2. On *the other base*, which is specific for L-Ala at the opposite side to the base for D-Ala, and already conjugated with medium deuterons, one of deuterons is transferred to the intermediate from the back face of the ketimine intermediate to provide L-2.

Since the rate of decrease of OR is close to either Ala enantiomer, the proton abstraction activity of these two bases should resemble each other. Then the racemic mixture

from L-1 as the starting substrate can be a mixture of D-2, L-1, and L-2. This means that some of the α-proton once abstracted from L-1 to the L-Ala specific base is kept on the base, and returned to the ketimine intermediate to reproduce L-1 without any proton-deuteron exchange. The other portion of the proton can be exchanged with deuteron by a rather slow equilibrium between the conjugated acid and medium deuterium oxide, followed by the deuteron transfer to give L-2. Through the step, the base for D-Ala works compatibly as a deuteron donor to the same intermediate to give D-2.

The base for D-Ala can be close to the enzyme surface at the reaction site, while the base for L-Ala inside. The location of these bases, and thereby efficiency of proton-deuteron exchange may result in the dissymmetry of the product distribution depending on starting substrates. The base to D-Ala can be ε-amino group of a lysine residue in the protein. Since the amino group holds at most three hydrogen or deuterium atoms to be a conjugated acid as ammonium ion, the possibility of deuteron transfer to the ketimine intermediate increases. The base for L-Ala can be a histidine residue, which holds two protons or deuterons to be the corresponding conjugated acid, and enhances the possibility of the proton return to reproduce L-1.

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