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Role of Tyrosine 265 of Alanine Racemase from *Bacillus stearothermophilus*

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Tyrosine 265 (Y265) of *Bacillus stearothermophilus* is believed to serve as a catalytic base specific to the L-enantiomer of a substrate amino acid by removing (or returning) an α-hydrogen from (or to) the isomer on the basis of the X-ray structure of the enzyme [Stamper, C.G., Morollo, A.A., and Ringe, D. (1998) *Biochemistry* 37, 10438-10443]. We found that the Y265→Ala mutant (Y265A) enzyme is virtually inactive as a catalyst for alanine racemization. We examined the role of Y265 further with β-chloroalanine as a substrate with the expectation that the Y265A mutant only catalyzes the α,β-elimination of the D-enantiomer of β-chloroalanine. However, L-β-chloroalanine also served as a substrate; this enantiomer was rather better as a substrate than its antipode. Moreover, the mutant enzyme was as equally active as the wild-type enzyme in the elimination reaction. These findings indicate that Y265 is essential for alanine racemization but not for β-chloroalanine elimination.

Key words: alanine racemase, β-chloroalanine, chemical rescue.

Alanine racemase [EC 5.1.1.1] is a pyridoxal 5'-phosphate (PLP) enzyme that occurs widely in bacteria and plays a central role in the metabolism of D-alanine, an essential component of peptidoglycans in bacterial cell walls (*1*). The reaction catalyzed by the enzyme is apparently simple, consisting of non-stereospecific rearrangement of the substrate α-hydrogen, but the detailed reaction mechanism including the role of the active-site residues remains unclear. We constructed a mutant of alanine racemase from *Bacillus stearothermophilus* by substituting an alanyl residue for lysine 39 (K39) which binds with PLP (*2*). Through chemical rescue studies with the K39→Ala mutant (K39A) enzyme, we showed that K39 plays two major roles, as follows. It serves as a catalytic base specific to the D-enantiomer of a substrate amino acid: it abstracts an α-hydrogen from D-alanine and transfers a proton from water to the α-position of the deprotonated (achiral) intermediate to form D-alanine (Fig. 1; steps a and b). The other important role of K39 is the mediation of transaldimination: it facilitates the formation of the PLP-aldimine of a substrate amino acid (Fig. 1; steps 1 and 2) (*2*). X-Ray crystallographic studies of the wild-type enzyme suggested that tyrosine 265 (Y265) is the counterpart residue of K39 specifically mediating the α-hydrogen transfer of the L-enantiomer of a substrate amino acid (Fig. 1; steps c and d) (*3-5*).

We examined the roles of Y265 by means of the Y265→Ala mutant (Y265A) enzyme prepared by Kunkel's method (*6*). As shown in Table I, the Y265A mutant showed little activity in both directions of alanine racemization. The results are compatible with the above hypothesis that Y265 is the base specifically abstracting an α-hydrogen from L-alanine and returning a proton to the α-position of the deprotonated intermediate to form L-alanine: the mutation probably abolishes both directions of racemization, because steps c and d in Fig. 1 do not proceed. However, another possible explanation for these results is that the enzyme structure especially at the active site, is changed into an inactive form by the mutation.

Therefore, we examined whether or not the mutant enzyme is also inactive toward β-chloroalanine (β-Cl-Ala). Alanine racemase does not catalyze the racemization of β-Cl-Ala (*7*), but catalyzes the α,β-elimination of both enantiomers to produce chloride, pyruvate and ammonium (*8, 9*). α,β-Elimination of β-Cl-Ala differs markedly from the racemization of alanine in that the step of reprotonation at the α-position of the deprotonated intermediate is not involved. If Y265 acts as a base specifically abstracting an α-hydrogen from the L-enantiomer (shown as Base 2 in Fig. 1), then the Y265A mutant is probably active toward D-β-Cl-Ala but inactive toward L-β-Cl-Ala. However, as shown in Table II, the Y265A mutant acts on both enantiomers of β-Cl-Ala with similar kinetic parameters to those observed for the wild-type enzyme. This is in clear contrast to the roles of the mutant enzyme in the alanine racemization (Table I).

The K39A mutant is identical with the wild-type enzyme in the far-UV (180-200 nm) and near-UV (200-300 nm) CD spectra (*2*). This suggests that the mutant has a secondary structure virtually identical to that of the wild-type enzyme. The mutation probably causes no distortion

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Abbreviations: AspAT, aspartate aminotransferase; bis-tris-propane, 1,3-bis[tris-](hydroxymethyl)aminopropane; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethansulfonic acid; β-Cl-Ala, β-chloroalanine; PLP, pyridoxal 5'-phosphate; Tris, tris(hydroxymethyl)aminomethane.

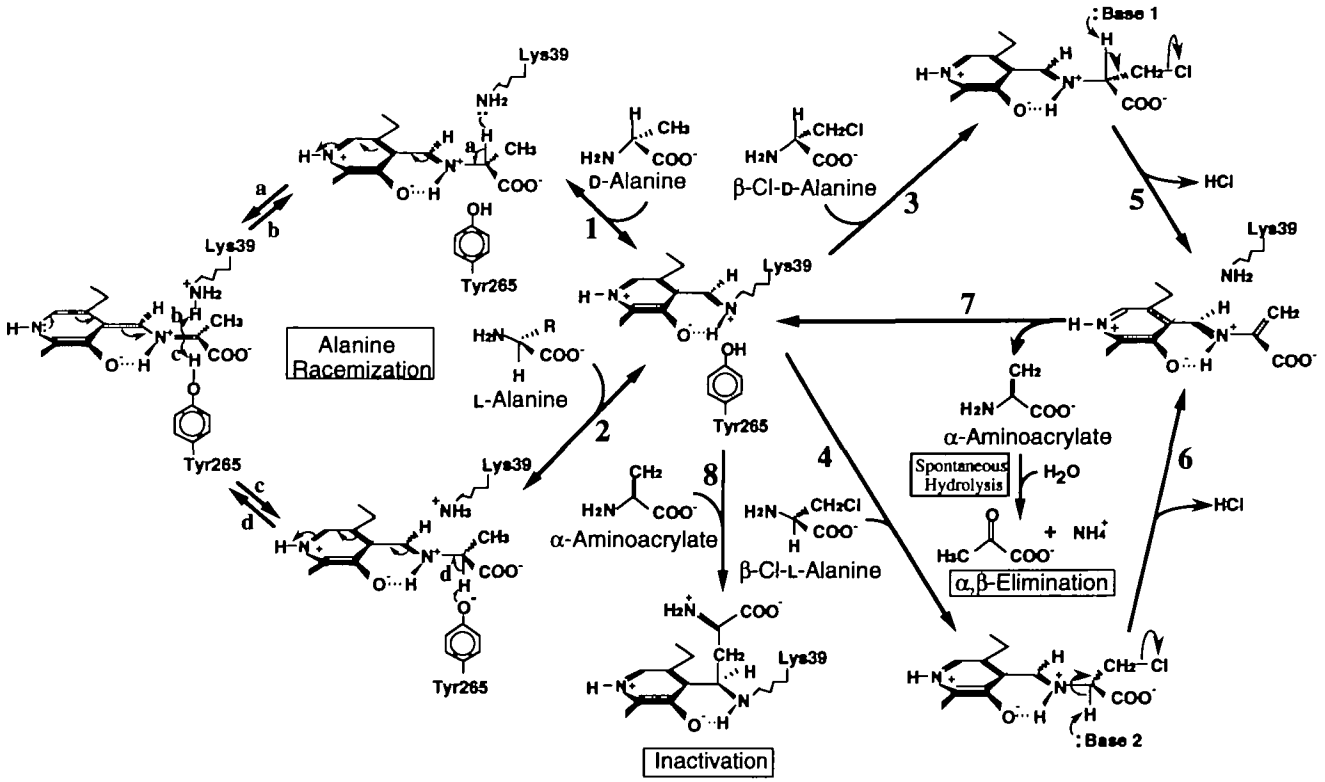

Fig. 1. **Proposed reaction mechanism for the α,β-elimination of β-Cl-Ala catalyzed by alanine racemase.**

TABLE I. **Activities of the wild-type and Y265A mutant alanine racemases.** The Y265A mutant was purified to homogeneity in the same manner as the K39A mutant (*2*). The rates of the reactions from D- to L-alanine and from L- to D-alanine catalyzed by homogeneous preparations of the enzymes were determined as described previously (*2*) except that the reactions were carried out at pH 7.2. The specific activity of an enzyme is expressed as the amount of L- or D-alanine formed per min per mg of enzyme.

	D- to L-Ala		L- to D-Ala	
	Specific activity (μmol/min/mg)	Ratio (%)	Specific activity (μmol/min/mg)	Ratio (%)
Wild-type	58.8	100	215	100
Y265A	0.037	0.06	0.073	0.03

of the enzyme gross structure. However, the K39A mutant showed no activity toward either enantiomer of β-Cl-Ala at pH 7.2 and 9.0. This is probably due to the inability of the enzyme to catalyze transaldimination (Fig. 1; steps 3 and 4). However, exogenous methylamine facilitated α,β-elimination of β-Cl-Ala in the same manner as observed in alanine racemization (*2*); methylamine mimics the side chain of K39 of the wild-type enzyme and mediates transaldimination to form the PLP aldimine of β-Cl-Ala. The aminoacrylate formed is liberated from the Schiff base through another transaldimination with methylamine *via* a step similar to step 7 in Fig. 1. The PLP-binding lysyl residues of aspartate aminotransferase (*10*), tryptophan synthase (*11*), D-amino acid aminotransferase (*12, 13*), and aromatic L-amino acid decarboxylase (*14*) are known to mediate transaldimination to release reaction products. The methylamine-assisted α,β-elimination was accelerated by increases in pH (Fig. 2) and the methylamine concentration (Fig. 3). These findings suggest that deprotonated methylamine is the active species catalyzing the transaldimination reaction. The absorption spectrum of the wild-type enzyme suggests that the imine nitrogen of the internal Schiff base is protonated between pH 5.5–12.0 (*5*). This suggests that the transaldimination steps (Fig. 1; steps 1, 2, 3, 4, and 7) proceed through an attack on the imine bond by a deprotonated amino group of the substrate or K39 in the reactions catalyzed by the wild-type enzyme. The amino groups of a substrate amino acid and K39 are most probably depronated in the alkaline pH region (around 10), corresponding to the optimum pH for the alanine racemization. K39A showed no activity toward β-Cl-Ala in the absence of methylamine (Fig. 3). This suggests that the amino group of β-Cl-Ala does not act as a base substituting for methylamine. This is probably due to steric hindrance by other groups attached to the α-carbon of β-Cl-Ala.

D-Amino acid aminotransferase catalyzes the α,β-elimination of β-Cl-Ala as a secondary function in the same manner as in the cases of alanine racemase and other pyridoxal enzymes (*15–21*). Soper and Manning (*21*) showed that the step of α-proton abstraction from the substrate is not rate-limiting in the α,β-elimination of β-bromo-D-alanine catalyzed by D-amino acid aminotransferase in contrast to the transamination of ordinary D-amino acids catalyzed by the same enzyme. It may be reasonable to assume a mechanism in which the α-hydrogen of β-Cl-Ala is readily liberated without the participation of particular basic residues: Bases 1 and 2 in Fig. 1 are water molecules. The α-hydrogen removal possibly proceeds concomitantly with the release of chloride (Fig. 1;

TABLE II. **Kinetic parameters of the wild-type and mutant alanine racemases in the α,β-elimination of β-Cl-Ala.** The α,β-elimination of β-Cl-Ala was determined as follows. The reaction mixtures (1 ml) contained 100 mM HEPES buffer (pH 7.2), 0.16 mM NADH, 5.5 units of lactate dehydrogenase, and various concentrations of β-Cl-D- or L-Ala. The reactions were started by the addition of homogeneous preparations of wild-type and mutant alanine racemases, and performed at 37°C. The decrease in absorbance at 340 nm was monitored with a Shimadzu MPS-2000 spectrophotometer.

	β-Cl-D-Ala			β-Cl-L-Ala		
	k_{cat} (s^{-1})	K_m (mM)	Partition ratio	k_{cat} (s^{-1})	K_m (mM)	Partition ratio
Wild-type	4.6	0.15	72	1.2	2.8	61
Y265A	3.8	0.12	83	1.2	0.54	79
K39A	n.d.[a]	–	–	n.d.[a]	–	–
K39A+MA[b]	0.07	0.030	18	0.07	0.54	19

[a]Activity was detectable at neither pH 7.2 nor 9.0. [b]The reaction was carried out in the presence of 100 mM methylamine in 100 mM CHES buffer (pH 9.0).

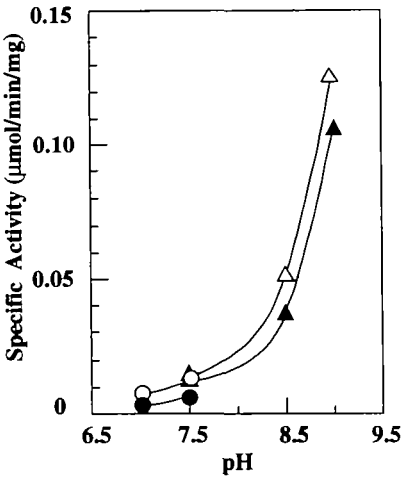

Fig. 2. **Effects of pH on the rates of α,β-elimination of β-Cl-D- and L-Ala catalyzed by the K39A mutant enzyme in the presence of methylamine.** The reaction was carried out with 10 mM β-Cl-D-Ala (open symbols) or β-Cl-L-Ala (closed symbols), 100 mM HEPES buffer (circles, pH 7.0 and 7.5) or bis-tris-propane buffer (triangles, pH 7.5, 8.5, and 9.0), and 75 μg of a homogeneous preparation of K39A in the presence of 100 mM methylamine. Other conditions were the same as in the footnote to Table II.

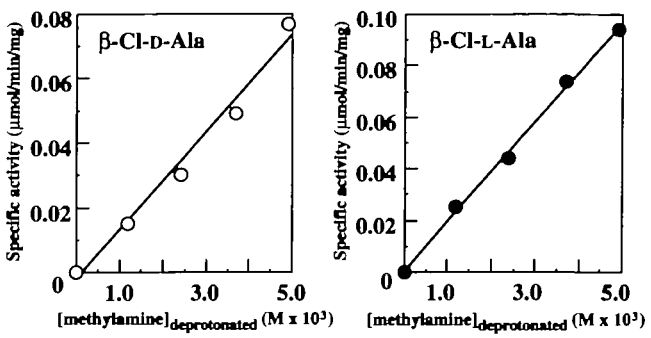

Fig. 3. **Effects of the concentration of methylamine on the rates of α,β-elimination of β-Cl-D- and L-Ala catalyzed by the K39A mutant enzyme.** The reaction mixture (1 ml) contained 100 mM CHES buffer, whose pH was adjusted to pH 9.0 with tetramethylammonium hydroxide, 0.16 mM NADH, 5.5 units of lactate dehydrogenase, 10 mM β-Cl-D- or L-Ala, and various concentrations of methylamine. The ionic strength was maintained at 0.5 with tetramethylammonium chloride. The reaction was started by the addition of 65 μg of K39A and performed at 37°C. The specific activities obtained were plotted against the concentrations of deprotonated methylamine, which were calculated from the K_a value of methylamine (10.6) (*22*), pH 9.0, and the concentration of methylamine added ($[methylamine]_{total}$), according to the equation: $[methylamine]_{deprotonated} = [methylamine]_{total}/1+[H^+]/K_a$.

steps 5 and 6). This mechanism is consistent with our finding that Y265 is not required for α,β-elimination of β-Cl-Ala.

Various PLP-enzymes are inactivated by β-Cl-Ala at a rate specific for each enzyme while catalyzing multiple turnover numbers of α,β-elimination. This rate is referred to as the partition ratio and is expressed as the average number of α,β-elimination events catalyzed by an enzyme before its complete inactivation. The wild-type and Y265A mutant enzymes showed similar partition ratios. This is compatible with the inactivation mechanism for alanine racemases proposed by Walsh and his coworkers (*8, 19*): the PLP-aldimine of the bound lysyl residue is modified with the common achiral product, aminoacrylate, formed in the α,β-elimination (Fig. 1; step 8). The K39A mutant enzyme rescued by methylamine showed much a lower partition ratio than the wild-type and Y265A mutant enzymes. Methylamine probably serves as a base that accelerates the chemical modification with aminoacrylate, although further studies are required for clarification of the mechanism.

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