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Tyrosine 265 of Alanine Racemase Serves as a Base Abstracting α-Hydrogen from L-Alanine: The Counterpart Residue to Lysine 39 Specific to D-Alanine

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Alanine racemase of *Bacillus stearothermophilus* has been proposed to catalyze alanine racemization by means of two catalytic bases: lysine 39 (K39) abstracting specifically the α-hydrogen of D-alanine and tyrosine 265 (Y265) playing the corresponding role for the antipode L-alanine. The role of K39 as indicated has already been verified [Watanabe, A., Kurokawa, Y., Yoshimura, T., Kurihara, T., Soda, K., and Esaki, N. (1999) *J. Biol. Chem.* 274, 4189-4194]. We here present evidence for the functioning of Y265 as the base catalyst specific to L-alanine. The Y265→Ala mutant enzyme (Y265A), like Y265S and Y265F, was a poor catalyst for alanine racemization. However, Y265A and Y265S catalyzed transamination with D-alanine much more rapidly than the wild-type enzyme, and the bound coenzyme, pyridoxal 5′-phosphate (PLP), was converted to pyridoxamine 5′-phosphate (PMP). The rate of transamination catalyzed by Y265F was about 9% of that by the wild-type enzyme. However, Y265A, Y265S, and Y265F were similar in that L-alanine was inert as a substrate in transamination. The apo-form of the wild-type enzyme catalyzes the abstraction of tritium non-specifically from both (4′*S*)- and (4′*R*)-[4′-^{3}H]PMP in the presence of pyruvate. In contrast, apo-Y265A abstracts tritium virtually from only the *R*-isomer. This indicates that the side-chain of Y265 abstracts the α-hydrogen of L-alanine and transfers it supra-facially to the *pro-S* position at C-4′ of PMP. Y265 is the counterpart residue to K39 that transfers the α-hydrogen of D-alanine to the *pro-R* position of PMP.

Key words: alanine racemase, pyridoxal 5′-phosphate, reaction mechanism, transamination.

Alanine racemase [EC 5.1.1.1] requires pyridoxal 5′-phosphate (PLP) as a coenzyme, and catalyzes the interconversion between L- and D-alanine. The enzyme 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 enzyme gene of *Bacillus stearothermophilus* has been cloned in our laboratory (*2*); the genes from other bacterial sources have likewise been cloned by other groups (*3-8*). The catalytic mechanism of the enzyme has been studied extensively with the aim of developing mechanism-based inactivators specifically targeting the enzyme which can be utilized as antibacterial agents (*9-12*). Figure 1 shows the proposed mechanism for the enzyme. PLP bound with the active-site lysyl residue (A or E) reacts with either alanine enantiomer to form an external Schiff base (B or D). The subsequent α-hydrogen abstraction results in the production of a resonance-stabilized deprotonated intermediate (C). If reprotonation occurs at C-2 of the substrate moiety on the opposite face of the planar intermediate, then an antipodal aldimine (D or B) is formed. The antipode alanine and a PLP form of the enzyme (E or A) are generated through transaldimination with the lysyl residue.

Crystallographic studies of an alanine racemase from *B. stearothermophilus* (*13-15*) have suggested that this dimeric enzyme uses two individual base catalysts specifically abstracting the α-hydrogen from each alanine enantiomer: lysine 39 (K39) and tyrosine 265 (Y265). We have studied the role of K39 by means of site-directed mutagenesis and chemical rescue analyses, and obtained clear evidence that it acts as the base catalyst specific to D-alanine (*16*). We have found that the enzyme uses another residue functioning as the base catalyst specific to L-alanine, which is most probably Y265. Recently, Sun and Toney (*17*) presented evidence supporting our proposal obtained in site-directed mutagenesis studies on arginine 219 (R219), which interacts with Y265 *via* a hydrogen-bonding network.

We here present direct evidence for the functioning of Y265 as the base catalyst specific to L-alanine, obtained by analyzing the transamination [Fig. 1; C→F (or G)→H] catalyzed by the Y265→Ala (Y265A), Y265S, and Y265F mutant enzymes as a side-reaction (*18*).

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Abbreviations: AspAT, aspartate aminotransferase; BCAT, branched-chain L-amino acid aminotransferase; CAPS, 3-(cyclohexylamino)-propanesulfonic acid; PMP, pyridoxamine 5′-phosphate; PLP, pyridoxal 5′-phosphate; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

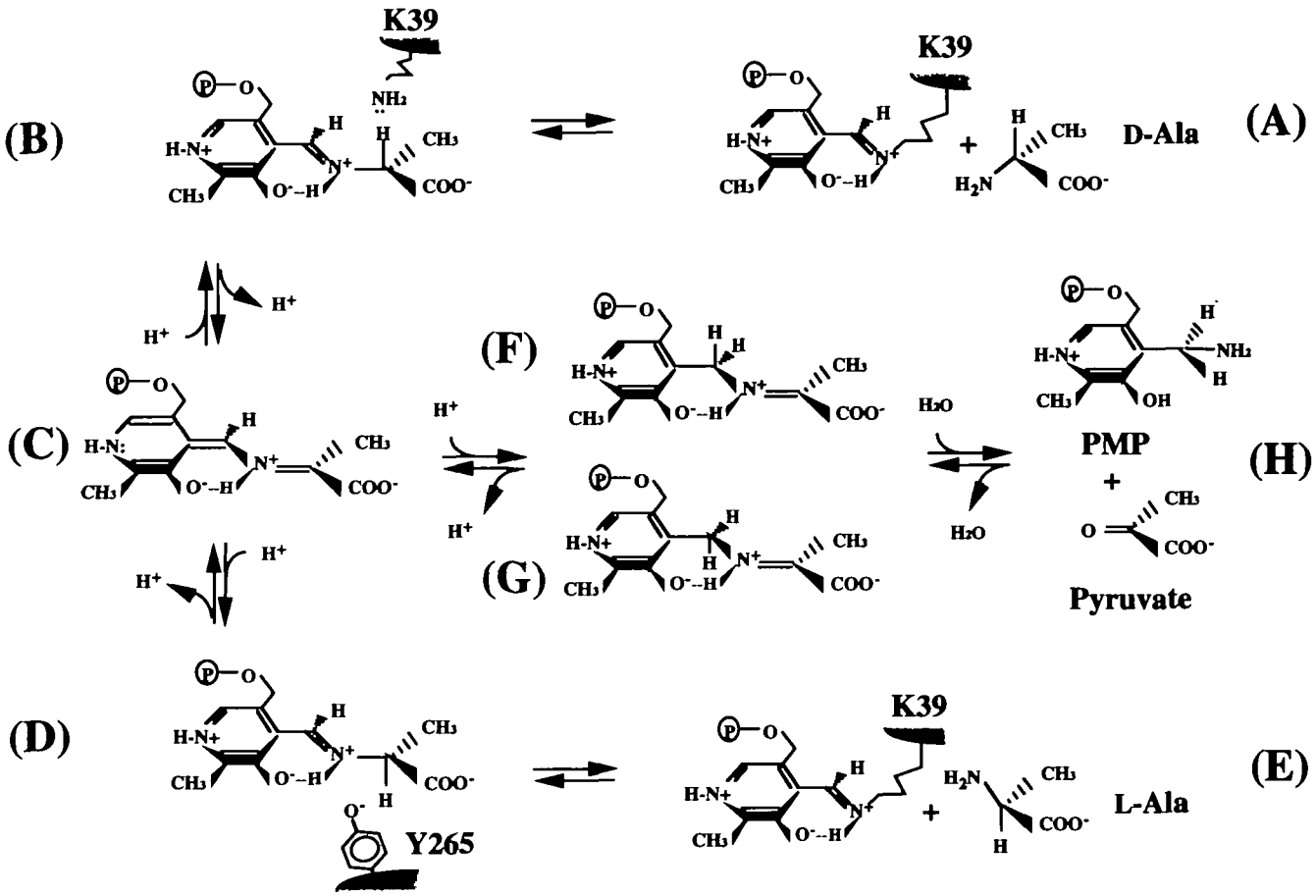

Fig. 1. **Reaction mechanism of alanine racemase.**

EXPERIMENTAL PROCEDURES

Materials—The plasmid, pAR310, carrying the alanine racemase gene from *B. stearothermophilus* was prepared as described previously (*18*). The oligonucleotides used for mutagenesis were synthesized by means of phosphoamidite chemistry. The enzymes for DNA manipulation were purchased from Takara Shuzo, Kyoto. L-Alanine dehydrogenase was a kind gift from Dr. H. Kondo of Unitika (Osaka). D-Amino acid aminotransferase was prepared as described previously (*19*). Branched-chain L-amino acid aminotransferase (BCAT) of *Escherichia coli* K-12 was kindly supplied by Professor H. Kagamiyama and Dr. K. Inoue of Osaka Medical College (*20*). Aspartate aminotransferase (AspAT) from porcine heart was obtained from Sigma (USA). L-Lactate dehydrogenase was purchased from Boehringer Mannheim (Germany). All other reagents and chemicals were of analytical grade.

Site-Directed Mutagenesis—Site-directed mutagenesis was carried out by Kunkel's method (*21*). The four mutagenic primers used were 5′-CGTCGCACCAGCGCTCACCTTTTCGCC-3′ for preparation of Y265A, 5′-CGTCGCACCACTGCTCACCTTTTCGCC-3′ for the Y265→Ser (Y265S) mutant, and 5′-CGTCGCACCAAAGCTCACCTTTTCGCC-3′ for the Y265→Phe (Y265F) mutant. Mutation was confirmed by DNA sequencing by the dye deoxy terminator method with an Applied Biosystems Model 373A automated DNA sequencer.

Purification of Mutant Enzymes—*E. coli* JM109 cells were transformed with a plasmid encoding one of the above mutant enzymes. The transformant cells were cultivated in 5 liters of Luria-Bertani's medium containing 50 μg/ml ampicillin at 37°C for 10 h. Each mutant enzyme was inducibly produced by the addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside 2 h after inoculation. Cells were harvested by centrifugation at 6,000 rpm for 10 min at 4°C, and then washed with 0.85% NaCl twice. The cells (about 10 g, wet weight) were suspended in 100 ml of 100 mM potassium phosphate buffer (pH 7.2) containing 20 μM PLP, 0.01% 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, and 0.1 mM *p*-toluenesulfonyl-L-phenylalanine chloromethyl ketone.

The following purification procedures were carried out at 4°C unless otherwise specified. After the cell suspension had been sonicated for 20 min, the lysate was centrifuged at 8,000 rpm for 20 min. The supernatant solution was incubated at 60°C for 60 min, cooled on ice, and then centrifuged at 8,000 rpm for 20 min. The supernatant solution was applied to a DEAE-TOYOPEARL 650M column (ϕ3.0×20 cm) equilibrated with buffer A, which comprised 20 mM potassium phosphate buffer (pH 7.2) containing 0.01% 2-mercaptoethanol. The column was washed with 300 ml of buffer A, and then the enzyme was eluted with a linear gradient of 0 to 200 mM KCl in buffer A. The active fractions were brought to 20% saturation with

ammonium sulfate in buffer A and then applied to a Butyl-TOYOPEARL 650M column (ϕ3.0×20 cm) equilibrated with the same buffer. The enzyme was eluted with a linear gradient of 20 to 0% saturation with ammonium sulfate in buffer A. The active fractions were dialyzed against buffer A containing 20 μM PLP and then against buffer A without PLP. The dialysate was concentrated with a MILLIPORE Centriprep-10 concentrator. The purity of the enzyme was determined by SDS-PAGE.

Protein Assay—Protein concentrations were determined by measurement of the absorbance at 280 nm or by the method of Bradford (*22*) with bovine serum albumin as a standard. The absorption coefficients at 280 nm were estimated from the amino acid composition of the enzymes (*23*).

Enzyme Assay—Alanine racemase was assayed spectrophotometrically at 37°C with L- or D-alanine as the substrate as follows. Conversion of D-alanine to L-alanine was determined by following the formation of NADH in a coupled reaction with L-alanine dehydrogenase. The assay mixture contained 100 mM CAPS buffer (pH 10.5), 0.15 unit of L-alanine dehydrogenase, 30 mM D-alanine, and 2.5 mM NAD^+, in a final volume of 1.0 ml. The reaction was started by the addition of alanine racemase after pre-incubation of the mixture at 37°C for 15 min. The increase in the absorbance at 340 nm owing to the formation of NADH was monitored.

D-Alanine formed from L-alanine was assayed with D-amino acid aminotransferase. The assay mixture contained 100 mM CAPS buffer (pH 10.5), 30 mM L-alanine, 5 mM α-ketoglutarate, 0.16 mM NADH, 12 units of D-amino acid aminotransferase, and 10 units of L-lactate dehydrogenase. The reaction was started by the addition of alanine racemase after pre-incubation of the mixture at 37°C for 10 min. The decrease in the absorbance at 340 nm was monitored. One unit of the enzyme was defined as the amount of enzyme that catalyzed the racemization of 1 μmol of substrate per min.

Spectrophotometric Measurements—Absorption spectra were obtained with a Shimadzu MPS-2000 or a Beckman DU-640 spectrophotometer.

Assaying of Transamination from D- or L-Alanine to PLP—Transamination from D- or L-alanine to PLP catalyzed by alanine racemase was assayed by following the absorption spectral change of the enzyme in a reaction mixture containing 0.1 M potassium phosphate buffer (pH 7.2), 0.1 M D- or L-alanine and from 1.0 to 1.9 mg of alanine racemase, in a final volume of 1.0 ml. The reaction was started by the addition of D- or L-alanine and carried out at 25°C.

Preparation of (4′S)- and (4′R)-[4′-^{3}H]PMP—(4′*S*)- and (4′*R*)-[4′-^{3}H]PMP were prepared by incubation of randomly labelled [4′-^{3}H]PMP with apo-BCAT and apo-AspAT, respectively (*24*). The reaction mixture for the preparation of (4′*R*)-[4′-^{3}H]PMP (550 μl) contained 0.1 M Tris-HCl buffer (pH 8.0), 0.05% sodium azide, 19 nmol of [4′-^{3}H]PMP, and 40 nmol of apo-AspAT. (4′*S*)-[4′-^{3}H]-PMP (500 μl) was prepared with a mixture containing 45 nmol of [4′-^{3}H]PMP and 60 nmol of apo-BCAT. The reaction was carried out at 32°C for 18 h, and stopped by the addition of HCl to the final concentration of 0.2 M. The reaction mixtures for preparation of the *S*- and *R*-isomers were mixed with 1.9 and 4.5 nmol of unlabelled PMP, respectively, and then further incubated at 32°C for 2 h. After centrifugation, the supernatant solution was dried with a Speed Vac Concentrator (Savant, USA), and the residue was dissolved in 0.2 ml of water. PMPs were purified by reverse phase column chromatography (Waters Ultron S-C_{18} column) with a Shimadzu LC-6A HPLC system as described previously (*24*). Radioactivity was determined with a Beckman LS-6500 scintillation counter and a scintillator Clear-sol II (Nacalai Tesque, Kyoto) (10 ml). The specific radioactivities of the prepared (4′*S*)- and (4′*R*)-[4′-^{3}H]PMP were 0.84×10^6 and 1.32×10^6 dpm/μmol, respectively.

Determination of Stereospecificity for Hydrogen Abstraction from PMP—The apo-forms of the wild-type and Y265A enzymes were prepared by dialysis against 100 mM potassium phosphate buffer (pH 7.2) containing 50 mM hydroxylamine and 0.01% 2-mercaptoethanol for 24 h at 4°C, followed by dialysis against the same buffer without hydroxylamine overnight. The reaction mixture (0.1 ml) contained 100 mM CAPS buffer (pH 10.0), 0.5 nmol of (4′*R*)-[4′-^{3}H]PMP [or 1.2 nmol of (4′*S*)-[4′-^{3}H]PMP], 10 nmol of sodium pyruvate, 0.05% sodium azide, and 2.0 nmol of apo-alanine racemase: wild-type or Y265A. The reaction was started by the addition of pyruvate after pre-incubation of the mixture at 32°C for 30 min. The reaction was carried out at 32°C for 18 h, and stopped by the addition of HCl to a final concentration of 0.2 M, followed by drying with a Speed Vac Concentrator. The residue was dissolved in 0.2 ml of water, and then the radioactivity was determined as described above.

RESULTS AND DISCUSSION

Mutation of Y265—We have shown that Y265A is a poor catalyst for alanine racemization (*25*). However, this mutant enzyme is as active as the wild-type enzyme, as judged from its action in α,β-elimination of β-chloroalanine, although this is an anomalous reaction eventually leading to inactivation of the enzyme (*25*). Therefore, Y265A probably has no serious distortion in protein conformation, possibly as a result of the mutation. The low activity of Y265A as to alanine racemization is due to the absence of the phenolyl group of tyrosine, which is essential, particularly for racemization. The essentiality of Y265 in alanine racemization was verified further with other mutant enzymes (Table I). Y265S is slightly more active than Y265A, while Y265F is virtually inactive as to both directions of alanine racemization. The phenolic hydroxyl group of Y265 forms hydrogen bonds with H166 and R219 in the wild-type enzyme (*13*). Removal of this hydroxyl group, and thereby the hydrogen bonds, presumably perturbs the configuration of the bulky phenyl group of Y265F.

Transamination Catalyzed by the Wild-Type and Mutant Alanine Racemases—We have shown that alanine racemase catalyzes transamination as a side-reaction (*18*): both D- and L-alanine serve as substrates in the reaction from PLP to PMP, and both *pro-S* and *pro-R* hydrogen are released from C-4′ of PMP during the reverse reaction from PMP to PLP. This nonstereospecificity is a unique feature of the transamination catalyzed by alanine racemase: aminotransferases catalyze the stereospecific reactions.

In PLP-dependent transamination, the α-hydrogen of

the substrate amino acid is reversibly transferred to C-4′ of the cofactor (*26, 27*). This hydrogen transfer proceeds supra-facially on the planar π-system of the substrate-cofactor complex (Fig. 1C) (*28*). Therefore, the non-stereospecific transamination catalyzed by alanine racemase strongly suggests that the hydrogen transfer occurs on both faces of the planar intermediate (*29, 30*). The hydrogen transfer is most probably mediated by the specific catalytic residue located on each side of the planar intermediate.

Transamination and racemization share the same steps of α-hydrogen abstraction from the substrates (Fig. 1; B$\rightleftarrows$C and C$\rightleftarrows$D). The same catalytic residues are probably used in both reactions. If Y265 is the catalytic residue abstracting the α-hydrogen from L-alanine and returning a proton to C-2 of the deprotonated intermediate to form L-alanine (Fig. 1, C$\rightleftarrows$D), then mutation of Y265 should abolish the transamination activity toward L-alanine. However, the mutant enzyme is expected to retain the transamination activity toward D-alanine, because K39 mediating the α-hydrogen transfer from (and to) D-alanine is intact in the mutant enzyme.

When Y265A was incubated with D-alanine, the absorp-

TABLE I. **Racemization activities of the wild-type, Y265A, Y265S, and Y265F alanine racemases.**

	D- to L-alanine			L- to D-alanine		
	Specific activity (units/mg)	Turnover number (h^{-1})[a]	Ratio (%)	Specific activity (units/mg)	Turnover number (h^{-1})[a]	Ratio (%)
Wild-type	630	1,600,000	100	940	2,400,000	100
Y265A	0.12	310	0.019	0.24	620	0.026
Y265S	0.28	720	0.045	0.36	930	0.039
Y265F	0.010	26	0.0016	0.010	26	0.0011

[a]Turnover numbers were calculated from the specific activities and molecular mass of alanine racemase (about 43,000).

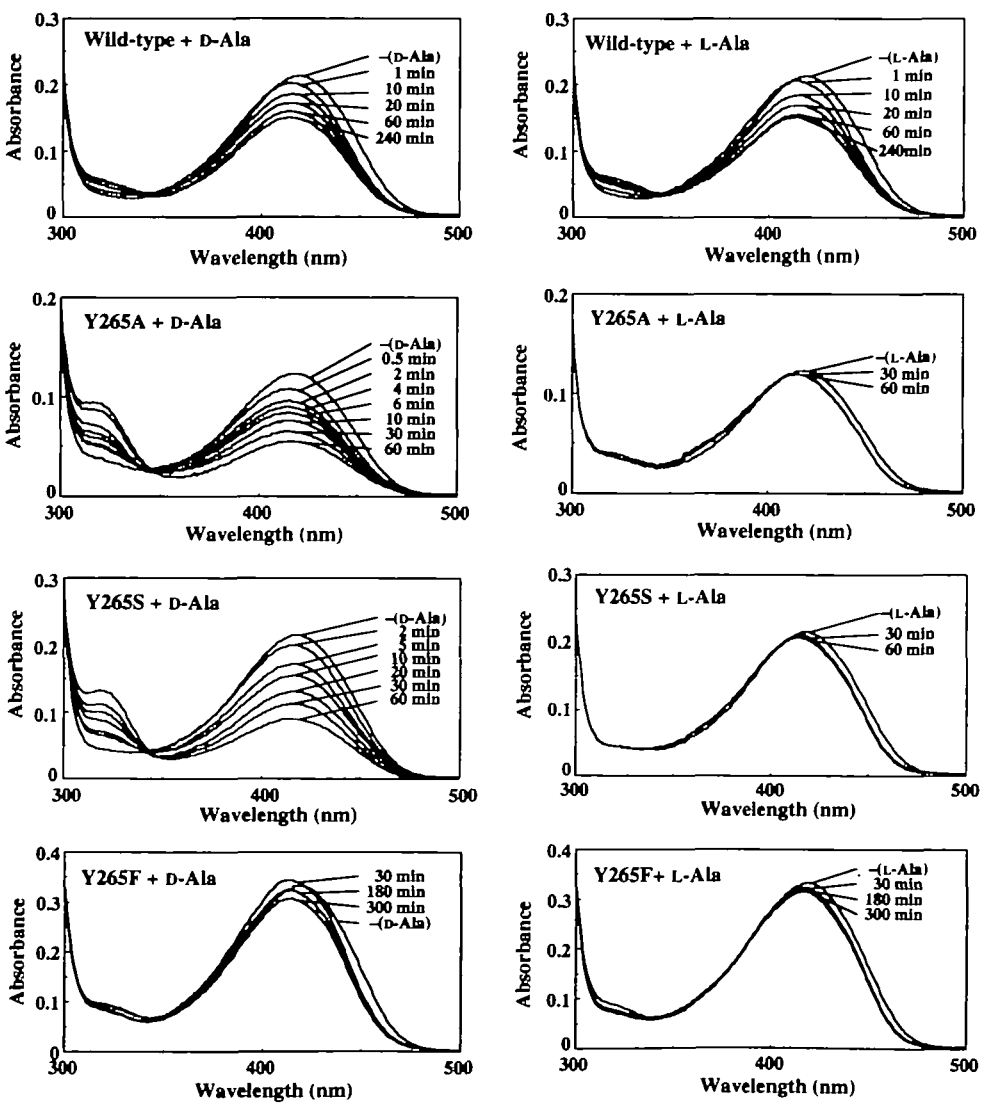

Fig. 2. **Spectral change of the wild-type, Y265A, Y265S, and Y265F alanine racemases caused by incubation with L- or D-alanine.** The UV-visible spectra (300-500 nm) were obtained at the indicated times.

tion maximum around 420 nm decreased with a concomitant increase in the absorbance at 330 nm (Fig. 2). This is due to the conversion of PLP to PMP by Y265A through transamination with D-alanine. The pseudo-first-order rate for the decrease in absorbance at 420 nm was about 3.8 times higher than the corresponding rate for the wild-type enzyme (Table II). In contrast, when L-alanine was used as the substrate, only an insignificant absorption change was observed for Y265A (Fig. 2). Similar results were obtained with Y265S, which also showed high stereoselectivity toward D-alanine (Fig. 2). However, the transamination by Y265S proceeded at a slightly lower rate than that by Y265A (Table II). Y265F is a very poor catalyst not only for alanine racemization (Table I) but also for transamination (Table II). However, Y265F also acts selectively on D-alanine in transamination (Fig. 2). Thus, all three mutant enzymes are similar to each other in the selective action toward D-alanine in transamination, although transamination of L-alanine, if it occurs at a rate lower than 0.006 h^{-1}, could not be assayed accurately with our present method (Table II).

We can calculate apparent partition ratios between transamination and racemization from the data shown in Tables I and II: the wild-type enzyme catalyzes the transamination of D-alanine at a ratio of about once per 3×10^6 times of racemization, while the mutant enzymes do so at ratios of about once per 200-600 times. Transamination proceeds preferably at neutral pHs, but little at alkaline pHs such as 10.5 (*18*), which is the optimum pH for alanine racemization (*17, 18*). This is the reason why we examined transamination at pH 7.2 in this study. We previously reported that at pH 7.2 both the wild-type and Y265A enzymes catalyze alanine racemization at rates of about 10-30% those at pH 10.5 (*25*). Therefore, the above ratios determined at different pHs are probably not much different from the partition ratios that could be obtained under the same conditions at pH 7.2. The partition ratio between transamination and racemization catalyzed by the wild-type enzyme should be a few orders of magnitude higher than those in the cases of the mutant enzymes.

The results presented here are consistent with the hypothesis that Y265 is the catalytic residue specifically abstracting the α-hydrogen from L-alanine. The mutant enzymes as to Y265 are poor catalysts for alanine racemization. This is quite reasonable because racemization is not accomplished without the step of conversion between C and D (Fig. 1). Presumably, in the cases of the mutant enzymes, the hydroxyl anion in solvent water is functionally substituted for the hydroxyl group of Y265 of the wild-type enzyme, although with only low efficiency. The apparent rate constant for transamination with D-alanine increased more than threefold with the mutation of Y265 to alanine or serine. The anionic intermediate, C, is probably accumulated in the reaction catalyzed by the mutants, and the intermediate, F, is produced much more readily as compared with in the reaction catalyzed by the wild-type enzyme. Thus, the partition ratios between transamination and racemization catalyzed by the mutant enzymes are much lower than that in the case of the wild-type enzyme (Tables I and II).

Stereospecificity for the Hydrogen Abstraction from the C-4′ Carbon of PMP Catalyzed by the Wild-Type and Mutant Alanine Racemases—We have established a simple method for determining the stereospecificity for the hydrogen abstraction from C-4′ of PMP catalyzed by PLP enzymes by means of stereospecifically labelled [4′-^{3}H]PMP (*24, 29, 31*). This is based on the finding that tritium abstracted from [4′-^{3}H]PMP is eventually released into the solvent water through exchange with solvent hydrogen (*24*). Therefore, we can readily determine the stereospecificity by measuring the radioactivity in the solvent water. We have found that apo-forms of amino acid racemase with low substrate specificities and alanine racemase catalyze nonstereospecific hydrogen abstraction from C-4′ of PMP in the presence of pyruvate (*30*).

When the apo-form of the wild-type alanine racemase was incubated with pyruvate and [4′-^{3}H]PMP, tritium was released from both the (4′*S*)- and (4′*R*)-[4′-^{3}H]PMP enantiomers (Table III). However, the ratio of tritium released from (4′*S*)-[4′-^{3}H]PMP by Y265A was much lower than that from (4′*R*)-[4′-^{3}H]PMP (Table III). Thus, Y265A is virtually 4′*R*-specific, indicating that Y265 serves as a catalytic residue abstracting the *pro-S* hydrogen from C-4′ of PMP (Fig. 1, G→C).

Our observation is consistent with the model that the α-hydrogen of L-alanine is transferred supra-facially by Y265 to the *pro-S* position at C-4′ of PMP on the planar π-system of the substrate-cofactor complex C (Fig. 1) through steps D⇄C⇄G. K39 plays the counterpart role to Y265, being specific to D-alanine and the *pro-R* hydrogen of PMP (Fig. 1, B⇄C⇄F) (*16*). This situation for the PLP-binding lysine (K39) of alanine racemase is identical to that of D-amino acid aminotransferase (K145) (*24, 29*), which suggests that the topological situation of K39 of alanine racemase is similar to that of K145 of D-amino acid aminotransferase. Crystallographic studies on both enzymes have verified that this is the case (*13, 32*).

Sun and Toney (*17*) suggested that the pK_a value of the phenolic hydroxyl group of Y265 is lowered through hydrogen-bonding with R219 and H166. Thus, a tyrosine residue, whose hydroxyl group usually has a high pK_a value, can serve as a catalytic base abstracting the α-hy-

TABLE II. **Rate constants for the transamination from D- or L-alanine to PLP catalyzed by the wild-type, Y265A, Y265S, and Y265F alanine racemases.**

	D-Alanine		L-Alanine	
	Rate constant (h^{-1})	Ratio	Rate constant (h^{-1})	Ratio
Wild-type	0.50	1	0.60	1
Y265A	1.9	3.8	n.d[a]	0.0
Y265S	1.7	3.4	n.d[a]	0.0
Y265F	0.044	0.088	n.d[a]	0.0

[a]Not detectable.

TABLE III. **Release of tritium from stereospecifically labelled [4′-^{3}H]PMPs catalyzed by the wild-type and Y265A alanine racemases.**

	(4′*R*)-[4′-^{3}H]PMP[a]		(4′*S*)-[4′-^{3}H]PMP[b]	
	^{3}H released		^{3}H released	
	(dpm)	(%)	(dpm)	(%)
Without enzyme	0	0	0	0
apo-wild-type	335	52	299	33
apo-Y265A	357	56	50	5

[a]The initial radioactivity in the reaction mixture was 639 dpm. [b]The initial radioactivity in the reaction mixture was 917 dpm.

drogen of L-alanine. We have shown that H166A is only 15% as active as the wild-type enzyme in both directions of alanine racemization (data not shown). This is consistent with the proposed function of H166. Further studies, such as the characterization of the H166A and R219A mutant enzymes, and their X-ray crystallographic analyses, are underway.

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