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Transamination as a Side-Reaction Catalyzed by Alanine Racemase of *Bacillus stearothermophilus*[1]

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The pyridoxal form of alanine racemase of *Bacillus stearothermophilus* was converted to the pyridoxamine form by incubation with its natural substrate, D- or L-alanine, under acidic conditions: the enzyme loses its racemase activity concomitantly. The pyridoxamine form of the enzyme returned to the pyridoxal form by incubation with pyruvate at alkaline pH. Thus, alanine racemase catalyzes transamination as a side function. In fact, the apo-form of the enzyme abstracted tritium from [4′-^{3}H]pyridoxamine in the presence of pyruvate. A mutant enzyme containing alanine substituted for Lys39, whose ε-amino group forms a Schiff base with the C4′ aldehyde of pyridoxal 5′-phosphate in the wild-type enzyme, was inactive as a catalyst for racemization as well as transamination. However, when methylamine was added to the mutant enzyme, it became active in both reactions. These results suggest that the ε-amino group of Lys39 participates in both racemization and transamination when catalyzed by the wild-type enzyme.

Key words: active site lysine, 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 provides D-alanine, which is an indispensable component of the peptidoglycan layer of bacterial cell walls (*1*). We have cloned the gene for thermostable alanine racemase from *Bacillus stearothermophilus*, purified the enzyme, and determined its primary structure (*2, 3*). The three-dimensional structure of the enzyme was also clarified recently (*4*).

Reactions catalyzed by PLP-dependent amino acid racemases probably proceed through the mechanism described below (*4, 5*). PLP bound with the active-site lysyl residue through an internal Schiff base (Fig. 1A) reacts with a substrate to form an external Schiff base (B). The subsequent α-hydrogen abstraction results in the formation of a resonance-stabilized anionic intermediate (C). Reprotonation at C-2 of the substrate moiety on the opposite face of the planar intermediate to that where the proton abstraction occurs produces an antipodal aldimine (D). An isomerized amino acid and a PLP form of the enzyme are generated by the subsequent hydrolysis of the aldimine complex (A). We have proposed that two different bases participate in catalysis: one abstracts the α-hydrogen from the substrate, and the other returns hydrogen to the deprotonated intermediate (*6*). X-ray crystallographic studies have suggested that Tyr265 and Lys39, the PLP-binding lysyl residue, serve as catalytic bases in alanine racemase from *B. stearothermophilus* (*4, 7*).

In addition to aminotransferases, various PLP-enzymes such as amino acid decarboxylases and lyases catalyze the transamination as a side-reaction (*8*). We have found that alanine racemase also catalyzes transamination in the same manner as amino acid racemase with low substrate specificity and arginine racemase (*9*). Transamination is characterized by hydrogen transfer between the C-2 of the substrate and the C-4′ of the cofactor. The transamination catalyzed by amino acid racemases is probably attained through a sequence A→B→C→E (or F)→G (Fig. 1). An equivalent route can be delineated for the antipode: A→D →C→E (or F)→G.

In transamination catalyzed by aminotransferases such as aspartate aminotransferase (*10, 11*) and D-amino acid aminotransferase (*12, 13*), the hydrogen transfer between a substrate and PLP is considered to be mediated by the lysine residue forming the Schiff base with PLP. We have examined the role of Lys39 of alanine racemase in transamination by means of site-directed mutagenesis and chemical rescue experiments. We here show that Lys39 plays an essential role in transamination.

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Abbreviations: Bis-tris, 2,2-bis[tris(hydroxymethyl)-2,2′,2″-nitriloethanol]; Bis-tris-propane, 1,3-bis[tris(hydroxymethyl)methylamino]-propane; CAPS, 3-(cyclohexylamino)propionesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; DAAT, D-amino acid aminotransferase; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)-aminomethane.

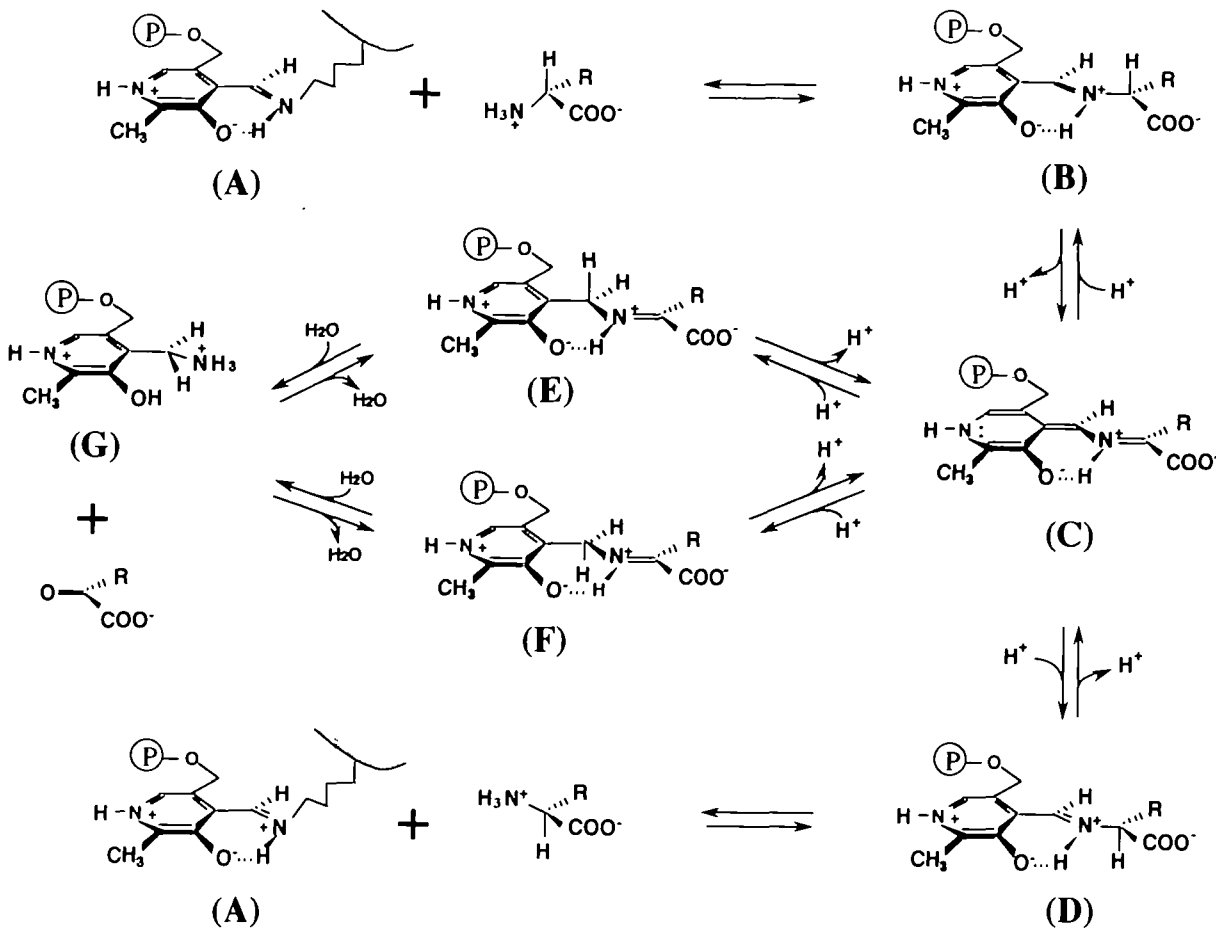

Fig. 1. **Probable reaction mechanism of alanine racemase.** An internal Schiff base (A) is formed between the ε-amino group of Lys39 and the C4′ aldehyde group of PLP. The α-hydrogen of a substrate amino acid is removed from intermediate B or D to form a quinoid intermediate, C. If a proton is returned to the α-position of the substrate moiety of intermediate C, then racemization is accomplished through D or B. However, if the C4′-position of the cofactor moiety of C is protonated, then transamination (*e.g.*, formation of G and α-keto acid) is accomplished through E or F. Tritium is liberated without discrimination from both [4′*R*-^{3}H] and [4′*S*-^{3}H]PMP in the transamination catalyzed by the apo-enzyme with an α-keto acid. Therefore, G is converted to A through E or F as an equivalent intermediate.

MATERIALS AND METHODS

Materials—The plasmid pMDalr3 carrying the alanine racemase gene from *B. stearothermophilus* was prepared as described previously (*14*). Phagemid vectors pUC118, pUC119, helper phage M13KO7, *E. coli* JM109, BW313, BMH71-18 mutS, restriction nucleases, T4 DNA polymerase, T4 DNA ligase, T4 DNA kinase, and calf alkaline phosphatase were purchased from Takara Shuzo, Kyoto. A mixture of dNTPs was purchased from Boehringer Mannheim, Germany. Alanine dehydrogenase was a gift from Dr. H. Kondo of Unitika, Osaka. D-Amino acid aminotransferase was prepared as described previously (*15*). L-Lactate dehydrogenase was purchased from Boehringer Mannheim (Germany). Other chemicals were of analytical grade commercially available.

Site-Directed Mutagenesis—Plasmid pAR310 was constructed by ligation of the 1.4-kb *Eco*RI-*Hin*dIII fragment from pMDalr3 into the *Eco*RI-*Hin*dIII sites of vector pUC118. Plasmid pAR420 was constructed by ligation of the 0.5-kb *Eco*RI-*Acc*I fragment from pMDalr3 together with the *Acc*I-*Acc*I linker into the *Eco*RI-*Acc*I sites of vector pUC119. ssDNA was prepared from the *E. coli* BW313 transformed with pAR420 and infected with M13KO7 phage. It was used as a template for the site-directed mutagenesis. Two oligonucleotides, 5′-ATTATG-GCGGTCGTGGCAGCGAACGCCTATGGA-3′ and 5′-CT-GGTGAGTATTCAACCAAGTC-3′, were synthesized by the phosphoamidite method and used for the replacement of Lys39 by an alanyl residue and the elimination of *Sca*I site of the pUC119 vector, respectively. Site-directed mutagenesis was carried out by Kunkel's method (*16*) in combination with the USE (Unique-Site Elimination) method (*17*). Mutation was confirmed by DNA sequencing by the dye deoxy terminator method with an Applied Biosystems Model 373A automated DNA sequencer. The 0.5-kb *Eco*RI-*Acc*I fragment of the obtained plasmid was ligated into the *Eco*RI-*Acc*I sites of the plasmid pAR310.

Purification of the Enzymes—The cloned wild-type alanine racemase of *B. stearothermophilus* was purified as described previously (*2, 14*). The mutant enzyme was purified as follows. *E. coli* JM109 cells were transformed with the plasmid pAR310 bearing the mutant alanine

racemase gene. Transformant cells were cultivated in 3 liters of Luria-Bertani's medium containing ampicillin (60 μg/ml) at 37°C for 10 h. The mutant enzyme was produced by induction by the addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside at 2 h after inoculation. Cells were harvested by centrifugation at 6,000 rpm for 10 min at 4°C and washed with 0.85% NaCl twice. 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 phenylmethyl-sulfonyl fluoride, and 0.1 mM *p*-toluensulfonyl-L-phenylalanine chloromethyl ketone. The purification procedures described below were carried out at 4°C unless otherwise specified. After the cell suspension was sonicated for 20 min, the lysate was centrifuged at 8,000 rpm for 20 min. The precipitate was resuspended in the same buffer, sonicated and centrifuged again. The supernatant solution was combined and incubated at 60°C for 20 min, cooled on ice, and then centrifuged at 8,000 rpm for 20 min. The supernatant solution was brought to 60% saturation with ammonium sulfate, and the precipitate obtained was dissolved in 10 ml of 20 mM potassium phosphate buffer (pH 7.2) containing 0.01% 2-mercaptoethanol (buffer A). After dialysis against buffer A, the enzyme solution was applied to a DEAE-Toyopearl 650M-column (ϕ3.0×42 cm) equilibrated with buffer A. The column was washed with 300 ml of buffer A, then the enzyme was eluted with a linear gradient from 0 to 200 mM KCl in buffer A. The active fractions were concentrated with a Millipore Centriprep-10 concentrator, dialyzed against 5 mM potassium phosphate buffer (pH 7.2) containing 0.01% 2-mercaptoethanol (buffer B), and loaded onto a Gigapite column (ϕ2.0×32 cm) equilibrated with buffer B. The enzyme was eluted with buffer B. Purity of the enzyme was determined by SDS-polyacrylamide gel electrophoresis.

Protein Assay—Protein was assayed by the method of Bradford (*18*) with bovine serum albumin as a standard.

Enzyme Assay—Conversion of D-alanine to L-alanine catalyzed by alanine racemase was assayed by measurement of L-alanine formed with L-alanine dehydrogenase. The reaction mixture contained 100 μmol of CHES buffer (pH 9.0), 30 μmol of D-alanine, and alanine racemase 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 10 min. After incubation of the reaction mixture at 37°C for an appropriate period, the reaction was stopped by heating at 100°C for 10 min. After centrifugation, a 100-μl aliquot was withdrawn and incubated with 250 μmol of CHES buffer (pH 9.0), 2.5 μmol of NAD^+, and 0.15 unit of alanine dehydrogenase in a final volume of 1.0 ml at 37°C for 30 min. The amount of L-alanine was assayed as the amount of NADH formed by measuring the increase in absorbance at 340 nm.

Conversion of L-alanine to D-alanine was assayed with D-amino acid aminotransferase and lactate dehydrogenase as follows: the reaction mixture contained 100 μmol of CHES buffer (pH 9.0), 30 μmol of L-alanine, and alanine racemase in a final volume of 1.0 ml. The reaction was started by addition of alanine racemase after pre-incubation of the mixture at 37°C for 10 min. After proceeding at 37°C for an appropriate period, the reaction was stopped by heating at 100°C for 10 min, and the mixture was centrifugated. D-Alanine in a 100-μl aliquot of the supernatant solution was determined as the amount of NADH consumed in the assay mixture containing 250 μmol of Bis-Tris propane buffer (pH 8.5), 0.16 μmol of NADH, 5 μmol of α-ketoglutarate, 2.2 units of D-amino acid aminotransferase, and 5.5 units of lactate dehydrogenase in a final volume of 1.0 ml. After incubating the assay mixture at 37°C for 60 min, the decrease in absorbance at 340 nm was measured.

Preparation of Apoenzymes—The wild-type and mutant alanine racemases were converted to apo-forms by dialysis against 100 mM potassium phosphate buffer (pH 7.2) containing 30 mM hydroxylamine and 0.01% 2-mercaptoethanol at 4°C for 24 h. The enzyme solutions were then dialyzed against 20 mM potassium phosphate buffer (pH 7.2) containing 0.01% 2-mercaptoethanol at 4°C for 8 h. Formation of the apo-enzyme was confirmed by its absorption spectrum and by determination of its racemase activity in the presence or absence of 10 mM PLP.

Release of Tritium from [4′-^{3}H]PMP Catalyzed by the Wild-Type and K39A Mutant Enzymes—[4′-^{3}H]PMP (specific radioactivity, 2.44×10^6 dpm/μmol) randomly labeled was prepared as described previously (*19*). The reaction mixture (100 μl) for the determination of tritium release containing 10 μmol of CHES buffer (pH 9.0), 8.7 nmol of each enzyme, 8.0 nmol of [4′-^{3}H]PMP, and 8.5 μmol of pyruvate was incubated at 30°C with or without 50 μmol of methylamine hydrochloride. At the indicated time, a 15-μl aliquot of the mixture was withdrawn and mixed with 15 μl of 1 M HCl. The mixture was immediately frozen in liquid nitrogen and dried with a Speed Vac Concentrator. The residue was dissolved in 200 μl of H_2O, and the radioactivity of the solution was determined with a Packard Tri-Carb scintillation spectrometer with Clear-sol I (Nacalai Tesque, Kyoto) as a scintillator. Tritium released from PMP was determined as volatile radioactivity, which was estimated by subtraction of the radioactivity finally remaining from the radioactivity initially added to the reaction mixture.

RESULTS

Transamination Catalyzed by Alanine Racemase—When alanine racemase of *B. stearothermophilus* was incubated with D-alanine at pH 7.2, absorbance at 420 nm decreased

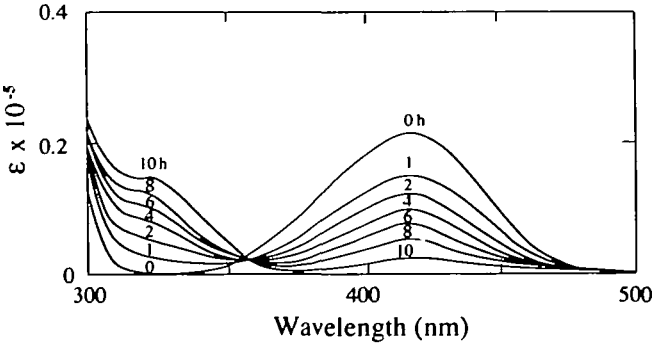

Fig. 2. Spectral change of the alanine racemase during incubation with D-alanine. Alanine racemase (0.1 mg/ml) was incubated with 100 μmol of potassium phosphate buffer (pH 7.2), 30 μmol of D-alanine, and 0.01% 2-mercaptoethanol at 25°C in a final volume of 1 ml. The UV-visible spectra (500-300 nm) were taken at the indicated times.

with increase in absorbance at 330 nm. Concomitantly, the enzyme was inactivated (Fig. 2). The enzyme showed an absorption spectrum characteristic of PMP-form after 12 h (Fig. 4A, b). PMP is inert as a cofactor for the racemase reaction, this inactivation being due to conversion of PLP to PMP by transamination. When the PMP-form enzyme was incubated with pyruvate, it was converted to PLP-form with concomitant recovery of the racemase activity (see below). Therefore, alanine racemase catalyzes both directions of transamination: from PLP to PMP, and from PMP to PLP.

Effect of pH on the Transamination Catalyzed by Alanine Racemase—The optimum pH values for alanine racemase reaction are in the range 9.5 to 10 for both directions of enantiomeric conversion: from D to L, and from L to D (Fig. 3A). However, both inactivation and conversion into the PMP-form due to transamination occurred only slowly in this pH range (Fig. 3B). Rather, the optimum pH for the transamination seemed to be in an acidic region (Fig. 3B). On the other hand, the other direction of transamination, *i.e.*, from PMP to PLP with α-keto acid, proceeded with concomitant recovery of racemase activity in a basic pH range 9 to 10 (Fig. 3B).

Substrate Specificity for the Transamination Catalyzed by Alanine Racemase—Various amino acids and α-keto acids effectively inactivated and reactivated alanine racemase, respectively, by transamination (Table I). However, both enantiomers of alanine as well as pyruvate served as the best inactivator and reactivator, respectively. This reflects the substrate specificity of the enzyme, because the second best inactivator was serine: its L-enantiomer is racemized at a rate of about 0.6% of that of L-alanine. However, α-aminobutyrate, another substrate for racemization, was inert as an inactivator, whereas non-substrate branched-chain amino acids inactivated the enzyme, though they did so slowly. A similar disagreement between an amino acid and its α-keto counterpart was found for α-aminobutyrate/α-ketobutyrate and valine/α-amino-*iso*-valerate. It is well known that α-keto analogs of good amino donors are not necessarily good amino acceptors for aminotransferases. Alanine racemase is similar to aminotransferases in this respect.

Role of the Active-Site Lysyl Residue in the Transamination Catalyzed by Alanine Racemase—Both abstraction of α-hydrogen from the substrate and its transfer to the C-4′ position of the cofactor are mediated by the lysine residue bound to PLP in aminotransferase reactions (*10-13*). We replaced the corresponding lysine residue, Lys39, of alanine racemase by alanine by site-directed mutagenesis. The resultant K39A mutant enzyme showed no racemase activity. However, it exhibited a similar absorption spectrum to that of the wild-type enzyme. The absorption spectrum of the mutant enzyme was not changed by incubation with 30 mM D-alanine at pH 7.2, 8.0, 9.0, and 10.5 at 25°C for 12 h (data not shown). When the PMP-form of the wild-type enzyme was incubated with pyruvate at pH 10, the absorption maximum around 330 nm decreased with a concomitant increase in that around 420 nm (Fig. 4A). In contrast, no spectral change was observed for the PMP-form of the mutant enzyme under the same conditions (Fig. 4B, c): the mutant enzyme did not catalyze transamination. Therefore, Lys39 is essential for catalysis of not only racemization but also transamination. This is compatible with the conclusion of the X-ray crystallographic studies of

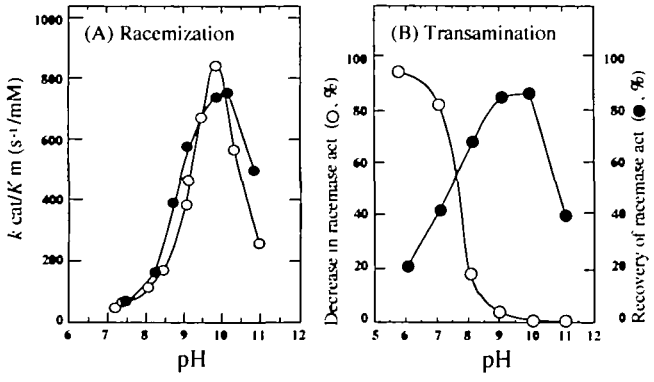

Fig. 3. **pH-profiles of the racemase reaction.** A, pH-profiles for the k_{cat}/K_m of the alanine racemase reaction. Conversions of D-alanine to L-alanine (○) and L-alanine to D-alanine (●) were assayed as described within the text, except for Bis-tris-propane (pH 7-9), CHES (pH 9-10), or CAPS (10-11) buffers, which were used as buffers for the racemase reaction. B, pH-profiles for the inactivation and reactivation of the enzyme due to transamination. The reaction mixture (100 μl) for the conversion of the PLP form of enzyme to the PMP form (○) contained 10 μg of holo-enzyme, 1 μmol of D-alanine, 0.01% 2-mercaptoethanol, and 10 μmol of Bis-tris (pH 6), Bis-tris propane (pH 7-8), CHES (pH 9-10), or CAPS (pH 11) buffer. After incubation of the reaction mixture at 25°C for 12 h, a 10-μl aliquot was withdrawn and subjected to the racemase assay with D-alanine as a substrate. The ratio of the decrease in the enzyme's specific activity to that of the holo-enzyme was plotted against the reaction pH. The reaction mixture (100 μl) for the conversion of the PMP form of enzyme to the PLP form (●) contained 35 μg of holo-enzyme, 2 μmol of PMP, 1 μmol of pyruvate, and buffers as described above. Other conditions were the same as for the conversion of the PLP form of enzyme to the PMP form. The ratio of the specific activity of the enzyme to that of the holo-enzyme used was plotted against reaction pH.

TABLE I. **Substrate specificity for the transamination catalyzed by alanine racemase.**

PLP form of enzyme[a]		PMP form of enzyme[b]	
Amino acids	Decrease in activity (%)	Keto acids	Recovery of activity (%)
D-Alanine	100	Pyruvate	100
L-Alanine	94		
L-α-Aminobutyrate	0	α-Ketobutyrate	31
DL-β-Aminobutyrate	0		
DL-α-Amino-*iso*-butyrate	0		
DL-Valine	4.2	α-Keto-*iso*-valerate	0
DL-Leucine	2.8		
DL-Isoleucine	2.8		
DL-Serine	64	β-Hydroxypyruvate	21
β-Alanine	0		
DL-Lysine	0		
		α-Ketovalerate	19
		α-Ketocaproate	0
		α-Ketoglutarate	0

[a]The PLP form of enzyme (10 μg) was incubated with 0.5 μmol of each amino acid, 10 μmol of potassium phosphate buffer (pH 7.0), and 0.01% 2-mercaptoethanol in a final volume of 100 μl at 25°C for 12 h. The remaining racemase activity was assayed with D-alanine as a substrate. [b]The apo-enzyme (50 μg) was incubated with 2.5 μmol of each α-keto acid, 1 μmol of PMP, 10 μmol of K_2HPO_4-KOH (pH 10.0), and 0.01% 2-mercaptoethanol in a final volume of 100 μl at 25°C for 12 h. The recovered racemase activity was assayed with D-alanine as a substrate.

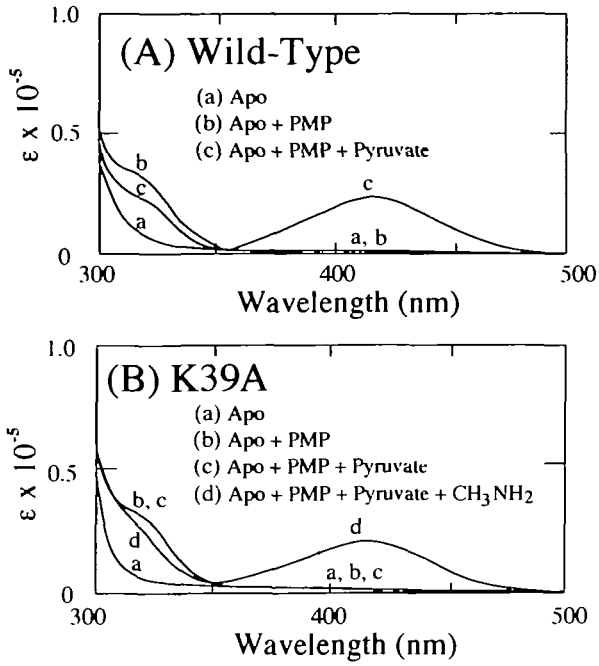

Fig. 4. **Spectral changes of the apo-alanine racemase by incubation with PMP and pyruvate.** The wild-type (A) or K39A mutant enzyme (B) (each 0.18 mg) was incubated with PMP, pyruvate, and/or methylamine in 500 μl of 100 mM K_2HPO_4-KOH buffer (pH 10.0) at 25°C for 12 h. The UV-visible spectra (500-300 nm) were taken: a, the apo-enzyme; b, the apo-enzyme in the presence of 5 nmol of PMP; c, the apo-enzyme in the presence of 5 nmol of PMP and 10 nmol of pyruvate; d, the apo-enzyme in the presence of 5 nmol of PMP, 10 nmol of pyruvate, and 50 μmol of methylamine hydrochloride.

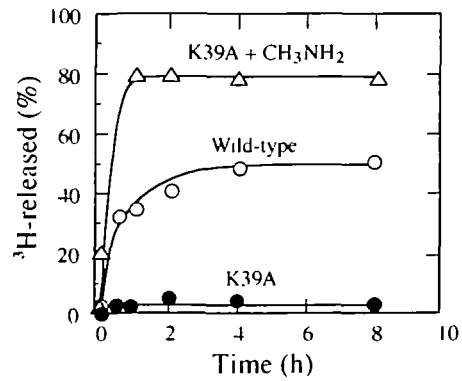

Fig. 5. **Release of tritium from a racemic mixture of 4′*R* and 4′*S*-[4′-³H]PMP by incubation with pyruvate and apo-alanine racemase.** The conditions are shown in the text. The enzymes used are: ○, wild-type; ●, K39A; △, K39A with methylamine.

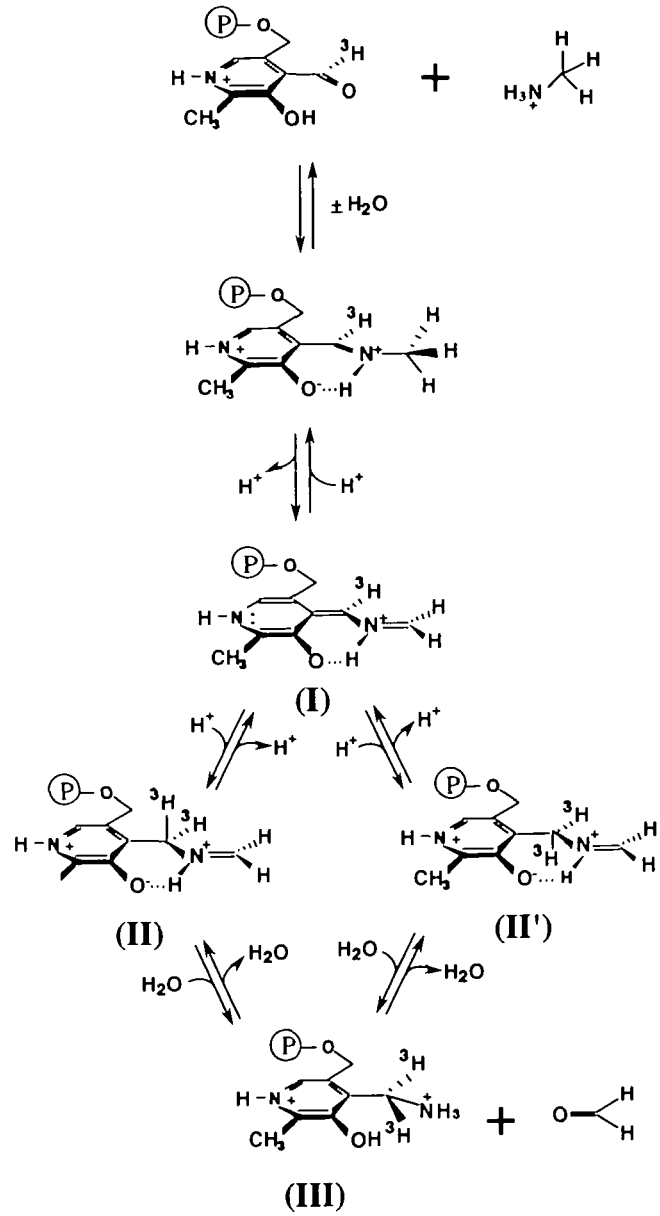

Fig. 6. **Schiff base formation between exogenous methylamine and PLP catalyzed by K39A mutant enzyme.** PMP (III) forms a Schiff base with α-keto acid in the same manner as shown in Fig. 1, and then is converted to PLP. Tritium is released from [4-³H]PMP to solvent water in the step of interconversion between (I) and (II) [or II′)].

the enzyme (*4, 7*): Lys39 and Tyr265 serves as catalytic bases abstracting α-hydrogen of substrates.

The inactive mutant enzymes [*e.g.*, aspartate aminotransferase (*20*) and D-amino acid aminotransferase (*13*)] containing alanine substituted for the PLP-binding lysine are activated by addition of alkylamines, which serve as a base to fulfil the function of the ε-amino group of the lost lysine. The K39A mutant alanine racemase catalyzed racemization only in the presence of high concentrations of alkylamines (*21*). When the PMP-form of the K39A mutant enzyme was incubated with pyruvate at pH 10 in the presence of 0.5 M methylamine, a similar spectral change to that found for the PMP-form of the wild-type enzyme was observed (Fig. 4A, c; 4B, d). Thus, the mutant enzyme is also activated by methylamine, which allows the enzyme to catalyze the transamination between PMP and pyruvate. On the other hand, the PLP-form of the mutant enzyme showed no spectral change upon incubation with 30 mM D-alanine even in the presence of 0.5 M methylamine at various pH values: 7.2, 8.0, 9.0, and 10 (data not shown). Therefore, the other half-reaction from PLP to PMP could not be facilitated by methylamine under the conditions.

Various aminotransferases catalyze a stereospecific

abstraction of tritium from [4′-^{3}H]PMP and transfer to the α-position of an amino acceptor, for example, D-amino acid aminotransferase and branched-chain L-amino acid aminotransferase, from the 4′-*R* isomer; the other aminotransferases, from 4′-*S* isomer. The lysyl residue bound to PLP mediates the abstraction and transfer in the aspartate aminotransferase (*10, 11*) and D-amino acid aminotransferase reactions (*12, 13*). However, only a small portion of the abstracted tritium is transferred to the amino acceptor, and the rest is released into solvent due to an exchange with solvent hydrogen (*19*). Therefore, we can readily determine the stereospecificity by measuring the radioactivity released into solvent. We have found that the apo-form of alanine racemase catalyzes the release of tritium from both 4′-*S* and 4′-*R* enantiomers of [4′-^{3}H]PMP into solvent in the presence of pyruvate (*22*). In contrast to aminotransferases, alanine racemase is characterized by a non-stereospecific abstraction from both enantiomers. When we incubated the apo-form of the wild-type alanine racemase with a random-labeled preparation of [4′-^{3}H]PMP (*i.e.*, a mixture of 4′-*S* and 4′-*R* enantiomers) and pyruvate, radioactivity was released into solvent water with time and reached a plateau; about 50% of the initial radioactivity was released into solvent water (Fig. 5). We found in a separate experiment that the original activity was essentially recovered under the same conditions (data not shown). These results indicate that PMP was totally converted to PLP, which is still labeled with ^{3}H at the C-4′ position, by the transamination catalyzed by the racemase. In contrast, no tritium was released by incubation of the apo-form of the K39A mutant enzyme with [4′-^{3}H]PMP and pyruvate. However, tritium was released when the reaction was performed in the presence of 0.5 M methylamine. Nearly 80% of the initial radioactivity in the [4′-^{3}H]PMP was released into solvent water (Fig. 5). The release of more than 50% of the initial tritium is probably caused by the mechanism shown in Fig. 6. PMP is probably reproduced by a transamination between [4′-^{3}H]PLP and methylamine, although formaldehyde presumably produced from methylamine in this transamination remains to be identified. These results, however, showed that Lys39 is also essential for the abstraction of C-4′ hydrogen of PMP.

DISCUSSION

In addition to aminotransferases, various PLP-enzymes catalyze transamination as a side reaction. Meister *et al.* reported the first example catalyzed by aspartate β-decarboxylase (*23, 24*). Kynureninase (*25, 26*) methionine α-decarboxylase (*27, 28*) arginine racemase (*9*), and α-amino-ε-caprolactam racemase (*29*) were also demonstrated to catalyze transamination. Their activities can be controlled through transamination (*8, 9, 23-26*). In this work, we have shown that alanine racemase also catalyzes transamination as a side reaction. Alanine racemase activity may be also controlled through transamination by means of alanine (and other amino acids such as serine) as a negative effector, and by pyruvate (and other α-keto acids) as a positive effector. It is as yet unclear whether such a control is of physiological significance, but transamination provides strong evidence of the structure-function relationship of PLP-enzymes, particularly their stereochemical characteristics by means of stereospecifically-labeled [4′-^{3}H]PMP.

We can calculate the partition ratio between the normal reaction (*i.e.*, racemization) and the side reaction (*i.e.*, transamination) on the basis of the rate of racemization and that of the transamination obtained from the absorption spectral change (Fig. 2): the enzyme catalyzes transamination of D-alanine at a ratio of once per 2×10^7 times of normal reaction. This value is much higher than the partition ratio of arginine racemase, 4×10^5, which was the highest value reported before this study (*8*).

The pH-profiles for both half-reactions of transamination differ from each other (Fig. 3B). This is the characteristic feature of the transamination catalyzed by alanine racemase. The results of deuterium isotope effect at Cα of substrate alanine indicated that the step of α-hydrogen abstraction [*i.e.*, formation of C from B (or D) in Fig. 1] is not rate-determining. It may be interesting to note that the optimum pH range for the conversion of PMP to PLP, which is shown in terms of recovery of racemase activity in Fig. 3B, is similar to that of racemization (Fig. 3A). Therefore, racemization and transamination from PMP to PLP possibly share the same step which is kinetically crucial, such as the step of protonation at the α-position of the substrate moiety of the intermediate C to produce either B or D (Fig. 1). The racemization and the other direction of transamination (*e.g.*, from PLP to PMP) proceed through the usual step of either A→B→C or A→D→C. The two reactions diverge at the intermediate C: transamination is accomplished through intermediate E or F, whereas racemization is accomplished through D or B. According to the calculated partition ratio between racemization and transamination, one can assume that the conversion from C to D (or B) is much faster than that from C to E (or F), by about 10^7-fold. This indicates that conditions that are unfavorable to the formation of D (or B) are of great advantage to the formation of E (or F). In fact, the pH-profile for transamination from PLP to PMP, which is shown as the decrease in racemase activity in Fig. 3B, looks like an inverted form of the profile for racemization in the region from pH 6 to 10 (Fig. 3A). This is probably a reason for the characteristic pH profile in the transamination from PLP to PMP. The failure of methylamine to facilitate K39A-mediated transamination from PLP to PMP probably stems from the same reason: racemization is much faster than transamination, and one could not observe the transamination. During the time of our experiments, transamination could not be observed. Further studies are required for a full explanation of these unique features of alanine racemase. Detailed studies with various site-specific mutants are currently in progress.

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