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# Conversion of the catalytic specificity of alanine racemase to a D-amino acid aminotransferase activity by a double active-site mutation

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Dedicated to Professor Dr. Kenji Soda in honor of his 70th birthday

**Abstract**

Alanine racemase depending on pyridoxal 5′-phosphate catalyzes the interconversion between D- and L-alanine. The enzyme from *Bacillus stearothermophilus* catalyzes the transamination as a side reaction with both substrates once per $3 \times 10^7$ times of the racemization. In this work, we studied the effects of the mutation of Arg219, and that of Arg219 and Tyr265 on the catalysis of *Bacillus* alanine racemase. Arg219 interacting with pyridinium nitrogen of the cofactor is conserved in all alanine racemases. The corresponding residue of aminotransferases is an acidic residue, such as glutamate or aspartate. Mutation of Arg219 to a glutamyl residue resulted in a 5.4-fold increase in the forward half transamination activity with D-alanine and a $10^3$-fold decrease in the racemase activity. The double mutation, Arg219 → Glu and Tyr265 → Ala, completely abolished the racemase activity and increased the forward half transaminase activity 6.6-fold. Arg219 is one of the structural determinants of the catalytic specificity of the alanine racemase.


*Keywords:* Alanine racemase; D-Amino acid aminotransferase; Protein engineering; Pyridoxal 5′-phosphate; Transamination

## 1. Introduction

Alanine racemase (AlaR) [EC 5.1.1.1] belonging to the fold-type III group of pyridoxal 5′-phosphate (PLP)-dependent enzymes [1] is widely distributed in bacteria. The enzyme is indispensable for bacterial cell growth because it provides D-alanine (Scheme 1), a component of the peptidoglycan layer of bacterial cell walls [2]. Thus, the enzyme has been regarded as a target for designing specific inhibitors that serve as an antibacterial agent. AlaR is also important as a biocatalyst: the enzyme of a thermophile, *Bacillus stearothermophilus*, was used for the enzymatic synthesis of various D-amino acids from the corresponding α-keto acids in combination with D-amino acid aminotransferase (D-AAT), alanine dehydrogenase, and formate dehydrogenase [3]. Understanding of the structure–function relationship of AlaR is important for the development of new antibacterial agents and effective catalysts. We have studied the reaction mechanism of *Bacillus* AlaR [4–7] on the basis of its three-dimensional structure [8–10], and postulated a new mechanism of the catalysis [10]. Racemization proceeds through a two-base mechanism with lysine39

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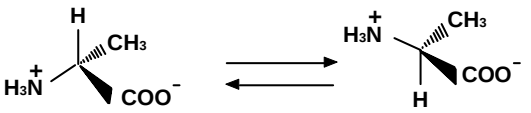


**D-Alanine** **L-Alanine**

Scheme 1. Reaction catalyzed by alanine racemase.

(K39) and tyrosine265 (Y265) that is situated on the opposite to K39 across from PLP [5–12]. Racemization is initiated by a transaldimination. In this step, PLP binds to K39 through an internal Schiff base and forms an external Schiff base with a substrate. When L-alanine is a substrate, the phenolic hydroxyl group of Tyr265' removes α-hydrogen from L-alanine in its external aldimine with PLP and concertedly donates a proton to the carboxylate group of the aldimine. The resultant carboxyl group then donates the proton to the ε-amino group of K39, and, again, in a concerted manner, K39 donates a proton to Cα to form the D-alanyl-PLP aldimine. The product, D-alanine, is released from the resultant D-alanyl-PLP Schiff base by the transaldimination with K39. This mechanism is delineated without formation of a quinonoid intermediate, which has been considered to be an indispensable intermediate of the pyridoxal enzyme reactions. Crystallographic studies have revealed that the N1 atom of the PLP pyridine ring interacts with a basic residue, Arg219 (R219), contrary to aminotransferases, which have an acidic residue (aspartate or glutamate) at the corresponding position [8–10]. R219 probably makes the pyridine nitrogen less protonated, which should destabilize the quinonoid intermediate.

The *Bacillus* AlaR catalyzes a half transamination as a side reaction once per $3 \times 10^7$ times of racemization [3]. As the result, the enzyme is converted to the pyridoxamine 5′-phosphate (PMP) form with the production of pyruvate. Transamination occurs if α-hydrogen abstracted from the substrate is transferred to the C-4′ of the cofactor. The *Bacillus* AlaR exhibits a similar structure around the active site to that of D-AAT of *Bacillus* sp. YM-1 catalyzing the transamination of various D-amino acids [8]. When the PLP molecules of AlaR and D-AAT are superimposed, the position of the active-site lysyl residues, K39 of AlaR and lysine145 (K145) of D-AAT, is very similar [8,13].

We are interested in the AlaR structures that determine the catalytic specificity of the enzyme. In this work, we studied the effect of a mutation of R219 interacting with the pyridinium nitrogen of PLP to a glutamyl residue on the transamination catalyzed by *Bacillus* AlaR. Sun and Tony converted R219 to a glutamyl residue and found that the mutation decreased the racemase activity $10^3$-fold [12]. However, they did not show the effect of the R219 mutation on the transamination. We constructed the R219 → Glu (R219E) and R219 → Glu/Y265 → Ala (R219E/Y265A) mutant enzymes. The double mutation completely abolished the racemase activity and increased the rate of transamination with D-alanine 6.6-fold.

## 2. Experimental

### 2.1. Materials

The plasmid pMDalr3 carrying the AlaR gene from *B. stearothermophilus* was prepared as described previously [14]. Phagemid vectors pUC118 and 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, Japan. A mixture of dNTPs was purchased from Boehringer Mannheim, Germany. Alanine dehydrogenase was a gift from Dr. H. Kondo of Unitika, Osaka, Japan. D-AAT of *Bacillus* sp. YM-1 was prepared as described previously [15]. L-Lactate dehydrogenase was purchased from Boehringer Mannheim (Germany). Other chemicals were of analytical grade commercially available.

### 2.2. Site-directed mutagenesis

The plasmid pMDalrY265A encoding the Y265A mutant enzyme was constructed as described previously [7]. Plasmid pMDalrR219E encoding the R219E mutant enzyme was constructed by Kunkel's method [16] with pMDalr3 as a template and the oligonucleotide, 5′-GGCAATGCCGAACTCGACCATATTGAACGT-3′, as a mutagenic primer. Plasmid pMDalrR219E/Y265A encoding the R219E/Y265A mutant enzyme was constructed by a similar method,

except that pMDalrY265A was used as a template. Mutation was confirmed by DNA sequencing by the dye deoxy terminator method with an Applied Biosystems Model 373A automated DNA sequencer.

### 2.3. Enzyme purification and apo-enzyme preparation

The R219E and R219E/Y265A mutant enzymes were purified to homogeneity according to the method for the purification of the Y265A mutant enzyme as described previously [7]. During the purification, the mutant enzymes without activity were detected by sodium dodecyl sulfate gel electrophoresis (SDS-PAGE). Preparation of the apo-enzyme was carried out as described by Kurokawa et al. [3].

### 2.4. Protein assay

Protein concentrations were determined by the measurement of absorbance at 280 nm or by the method of Bradford with bovine serum albumin as a standard [17]. The absorption coefficients at 280 nm were estimated from the amino acid composition of the enzymes [18].

### 2.5. Enzyme assay

AlaR was assayed at 37 °C with L- or D-alanine as a substrate [5,19]. Conversion of D-alanine to L-alanine was determined by following the formation of NADH in a coupled reaction with L-alanine dehydrogenase [19]. The formation of D-alanine from L-alanine was assayed with D-AAT [5]. One unit of the enzyme was defined as the amount of enzyme that catalyzes the racemization of 1 μmol of substrate per minute.

### 2.6. Spectroscopic methods

Absorption spectra were taken with a Shimadzu MPS-2000 or a Beckman DU-640 spectrophotometer. A CD measurement in the far-UV region (200–250 nm) was carried out with a Jasco J-600 recording spectropolarimeter at room temperature with a 1 mm light path length cell at a protein concentration of 0.2 mg/ml at pH 7.2.

### 2.7. Assay of the forward half transamination from D- or L-alanine to PLP

Transamination from D- or L-alanine to PLP (forward half transamination) was assayed by following the absorption spectral change of the enzyme. The reaction mixture (0.1 ml) contained a 100 mM potassium phosphate buffer (pH 7.2) or a cyclohexylaminopropanesulfonic acid (CAPS) buffer (pH 10.5), 100 mM D-alanine, and 46 μM enzyme (2 mg/ml). The reaction was started by the addition of D- or L-alanine, and the decrease in the absorption at 420 nm was monitored at 25 °C. Data were computed by direct fits to Eq. (1) using a nonlinear least-squares analysis with KaleidaGraph software (Abelbeck Software, USA),

$$Y = A \exp(-kt) + c, \quad (1)$$

where $Y$ is the percent absorbance change, $A$ the difference value of $Y$ of the exponential phase, $k$ is the rate constant, $t$ the time (min), and $c$ is a constant.

### 2.8. Assay of the reverse half transamination from PMP to pyruvate

Reverse half transamination from PMP to a keto acid was assayed as follows. The reaction mixture (0.1 ml) contained a 100 mM potassium phosphate buffer (pH 7.2) or a CAPS buffer (pH 10.5), 0.25 mM pyruvate, 36 μM PMP, and 23 μM apo-enzyme (1 mg/ml). The reaction was started by the addition of pyruvate, and the increase in the absorption at 420 nm was monitored at 25 °C.

### 2.9. Quantitative analysis of the affinity of the wild-type and mutant enzymes for PLP and PMP

We estimated the affinities of the apo enzymes for the cofactors by measuring the quenching of the emission fluorescence of the tryptophanyl residue caused by the addition of PLP or PMP. The mixture containing a 100 mM bis(2-hydroxyethl)imino-tris(hydroxymethyl)propane (bis–tris-propane) buffer (pH 8.0), various concentrations of PLP or PMP, and 0.9 μM (0.04 mg/ml) apo-form of the wild-type Y265A, R219E, or R219E/Y265A mutant enzyme was incubated at room temperature in the dark for at least 30 min. The emission fluorescence at 340 nm was measured with the excitation wavelength at 280 nm

with a Shimadzu RF-1500 spectrofluorometer. The net intensity of the emission fluorescence of the tryptophanyl residue was obtained by subtraction of the emission fluorescence of free PLP or PMP under the same conditions. The difference between the emission fluorescence at 340 nm of the apo enzyme and that of the holo enzyme (PLP form) was regarded as 100-% quenching. Percentages of the quenching ($Q$%) at various PLP or PMP concentrations were fitted to Eq. (2),

$$Q\% = \frac{[\text{PLP or PMP}]}{K_d^{app}} + [\text{PLP or PMP}], \quad (2)$$

where $K_d^{app}$ and [PLP or PMP] are the apparent dissociation constant and concentration of PLP or PMP, respectively.

## 3. Results

### 3.1. Construction of the mutant enzymes

The purified R219E mutant enzyme exhibited 0.2% specific activity of the wild-type enzyme in both directions of alanine racemization, whereas the R219E/Y265A mutant enzyme showed no detectable racemase activity (Table 1). The CD spectra at 200–250 nm of the R219E/Y265A mutant enzyme was similar to those of the wild-type enzyme (data not shown). As described below, the R219E/Y265A mutant enzyme showed the transamination activity with D-alanine. These results suggest that the double mutation, R219 → E/Y265 → A, gave no gross disorder of the enzyme structure, though the mutant enzyme has no detectable racemase activity.

### 3.2. Forward half transamination catalyzed by the mutant enzymes

When the wild-type enzyme was incubated with D- or L-alanine at pH 7.2, the absorption maximum at 420 nm decreased with a concomitant increase in that at 330 nm [3,7]. This is due to the forward half transamination, a conversion of PLP to PMP. The optimum pH of the alanine racemization is in a range of 9.5–10.0, but the transamination proceeds preferably at neutral pHs [3]. In contrast to the wild-type enzyme, the Y265A mutant enzyme showed spectral change upon incubation with D-alanine but not with L-alanine because Y265 is a catalytic residue abstracting and adding a C-2 hydrogen of L-alanine [7,11,12]. We examined the forward half transamination catalyzed by the R219E and R219E/Y265A mutant enzymes (Fig. 1). The R219E mutant enzyme catalyzed the transamination with both D- and L-alanine at pH 7.2 and 10.5 (Fig. 1, A–D). The shape of the absorption spectrum at 300–360 nm of the mutant enzymes varied according to the pH conditions (Fig. 1). This is due to the pH-dependency of the absorption spectrum of PMP [20]. The R219E/Y265A mutant enzyme catalyzed the transamination with D-alanine but not with L-alanine (Fig. 1, E–H).

Table 1
Racemization and forward half transamination activities of the wild-type and mutant alanine racemases

| Substrate | Racemization activity | | | | Half transamination activity | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | D-Alanine | | L-Alanine | | pH 7.2 | | | | pH 10.5 | | | |
| | $h^{-1}$ | Ratio[a] | $h^{-1}$ | Ratio[a] | D-Alanine | | L-Alanine | | D-Alanine | | L-Alanine | |
| | | | | | $h^{-1}$ | Ratio[b] | $h^{-1}$ | Ratio[b] | $h^{-1}$ | Ratio[c] | $h^{-1}$ | Ratio[c] |
| Wild-type[d] | 1600000 | 100 | 2400000 | 100 | 0.50 | 1.0 | 0.60 | 1.0 | n.d.[e] | – | n.d. | – |
| Y265A[d] | 310 | 0.019 | 620 | 0.026 | 1.9 | 3.8 | n.d. | – | 3.0 | 6.0 | n.d. | – |
| R219E | 3100 | 0.19 | 4800 | 0.20 | 0.60 | 1.2 | 0.10 | 0.17 | 2.7 | 5.4 | 0.80 | 1.3 |
| R219E/Y265A | n.d. | – | n.d. | – | 2.1 | 4.2 | n.d. | – | 3.3 | 6.6 | n.d. | – |

[a] The ratio of activity relative to wild-type activity times 100.
[b] The ratio of activity relative to the wild-type activity.
[c] The ratio of activity relative to the wild-type activity at pH 7.2.
[d] Data were taken from (7).
[e] n.d.: not detected.

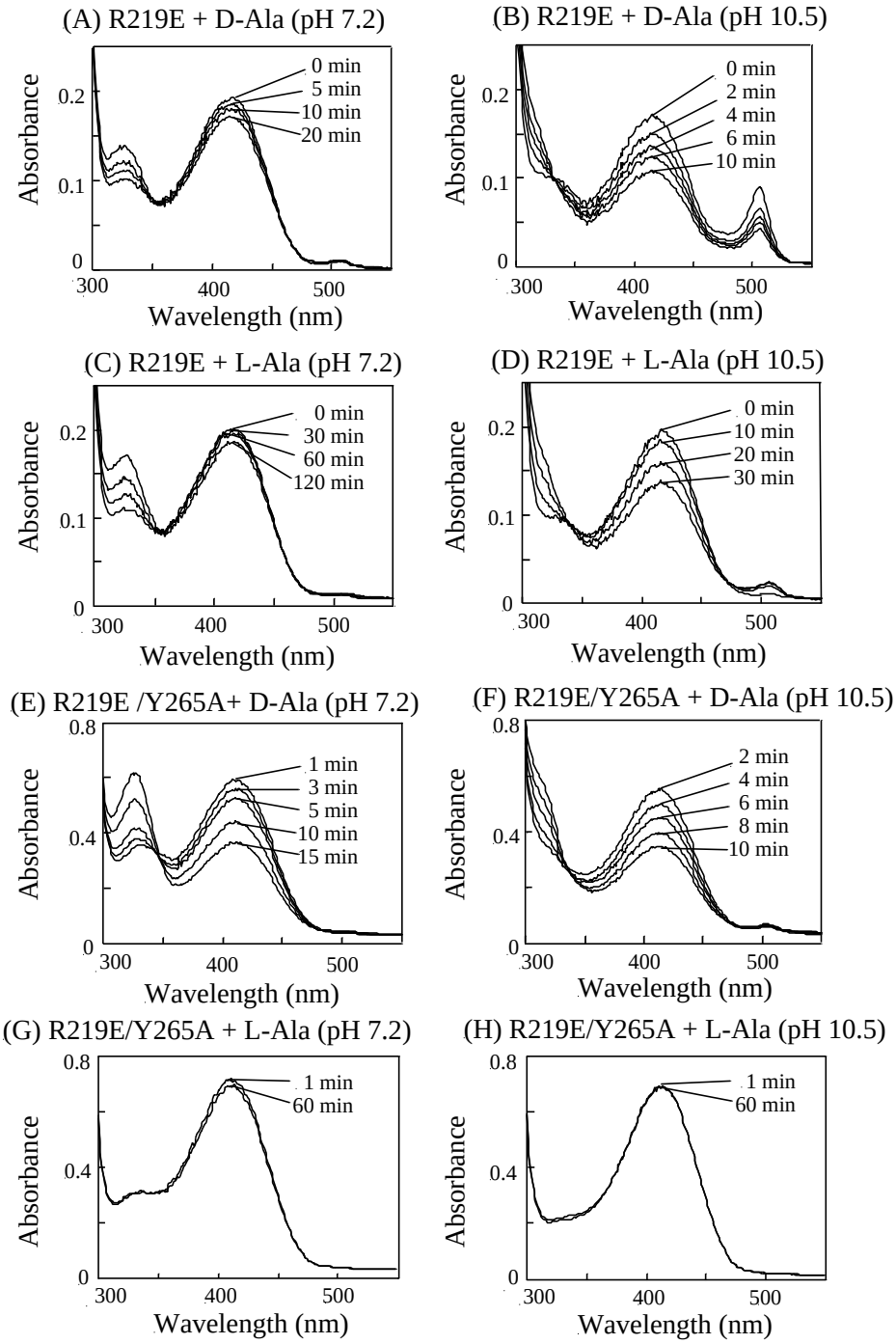


Fig. 1. Forward half transamination catalyzed by the R219E and R219E/Y265A mutant enzymes.

The pseudo-first-order rate constants for the forward half transamination catalyzed by the wild-type and mutant enzymes are summarized in Table 1. The rates of the transamination with D-alanine catalyzed by the Y265A, R219E, and R219E/Y265A mutant enzymes at pH 7.2 were 3.8, 1.2, and 4.2 times higher than that of the wild-type enzymes, and those at pH 10.5 were 6.0, 5.4, and 6.6 times higher, respectively. The rate of the transamination with L-alanine catalyzed by the R219E mutant enzyme at pH 7.2 was lower than that by the wild-type enzyme. This is probably because that the mutation of R219 interacting with Y265 through the hydrogen-bonding network [12] decreased the efficiency of the C-2 hydrogen withdrawal from L-alanine. The partition ratios, which are the turnover number of racemization per one transamination event, were calculated from the data shown in Table 1. The wild-type, Y265A, and R219E mutant enzymes catalyze the transamination of D-alanine once per $3.1 \times 10^7$ (at pH 7.2), $1.0 \times 10^2$ (at pH 10.5), and $1.2 \times 10^3$ (at pH 10.5) times of racemization, respectively. The exact partition ratio for the R219E/Y265A mutant enzyme could not be calculated because the enzyme shows no detectable racemase activity. However, it should be below one, if the racemase activity of the R219E/Y265A mutant enzyme is assumed to be the same as the value of the detection limit of the assay system. The forward half transamination activity of the R219E/Y265A mutant enzyme exceeded its racemase activity.

### 3.3. Overall transamination catalyzed by the R219/Y265A mutant enzyme

The R219E/Y265A mutant enzyme exhibited the highest rate in the forward half transamination among the wild-type and mutant enzymes. We examined the overall transamination catalyzed by the R219E/Y265A mutant enzyme. The R219E/Y265A mutant enzyme was incubated with one of the following pairs of amino acids and keto acids: D-serine and pyruvate; D-alanine and α-ketocaproate; D-alanine and α-ketovalerate; D-alanine and hydroxypyruvate; or D-2-aminobutyrate and pyruvate. However, amino acid analysis showed no amino acid formation from the corresponding keto acids (data not shown).

### 3.4. Reverse half transamination catalyzed by the Y265A, R219E, and R219/Y265A mutant enzymes

Kurokawa et al. [3] have spectroscopically demonstrated that the wild-type enzyme catalyzes the reverse half transamination. Addition of pyruvate and PMP to the apo wild-type enzyme resulted in the decrease in the absorption maximum at 330 nm with a concomitant increase in that at 420 nm [3]. We attempted to detect the reverse half transamination catalyzed by the Y265A, R219E, and R219/Y265A mutant enzymes by monitoring the absorption spectral change. When the 23 μM apo Y265A mutant enzyme was incubated with 36 μM PMP and 250 μM pyruvate at pH 7.2 or 10.5, absorption spectral changes were observed (Fig. 2, A, B). In contrast, no spectral changes were observed with the R219E (Fig. 2, C, D) or R219E/Y265A mutant enzymes (Fig. 2, E, F) at either pH. However, when the R219E/Y265A mutant enzyme was incubated with 230 μM PMP, a time-dependent increase in the absorption maximum at 420 nm was observed (inset figure of Fig. 2, F). These results suggest that the mutation of R219 lowers the affinity for PMP.

When the apo R219E/Y65A mutant enzyme was incubated with 1.5, 4.0, and 10 times excess amount of PLP in the presence of 100 mM D-alanine, 73, 67, and 83% PLP, respectively, were converted to PMP within 30 min (data not shown). These results indicate the multiple turnovers of the forward half transamination under the conditions. The PMP formed during the transamination was probably exchanged with the free PLP in the reaction mixture. The results also indicate the low affinity of the R219E/Y265A mutant enzyme for PMP.

### 3.5. Affinity of the wild-type and mutant enzymes for the cofactors

We quantitatively estimated the affinities of the wild-type, Y265A, R219E, and R219E/Y265A mutant enzymes for the cofactors, PLP and PMP. Previously, the apparent dissociation constant ($K_d^{app}$) for PLP of the wild-type enzyme was obtained by measuring the racemization activity of the apo enzyme incubated with various concentrations of PLP [21]. This method is not suitable for the mutant enzymes or the PMP

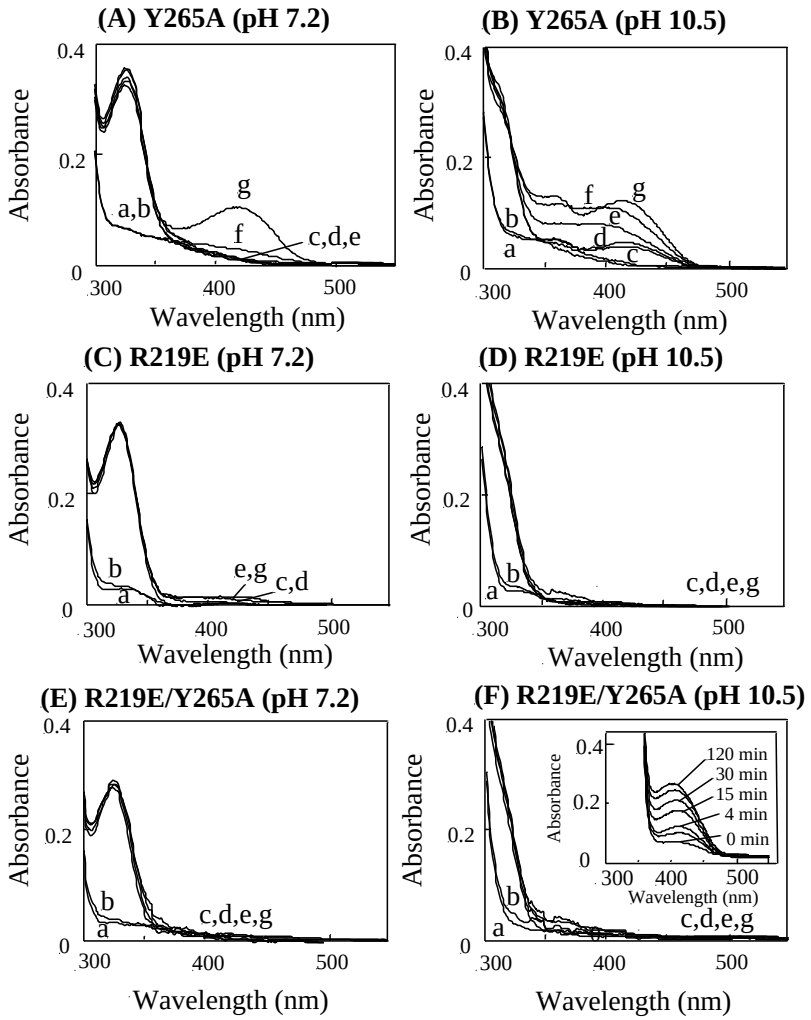


Fig. 2. Reverse half transamination catalyzed by the Y265A, R219E, and R219E/Y265A mutant enzymes. The absorption spectra of apo-enzyme after 0 h (a) and 24 h incubation (b), those of apo-enzyme + PMP after 0 h (c) and 24 h incubation (d), and those of apo-enzyme + PMP + pyruvate after 0 h (e), 5 h (f) and 24 h (g) incubation are shown. The reaction shown in the inset figure of (F) was conducted with 230 μM PMP and a 100 mM bis-tris-propane buffer (pH 8.0). Other conditions are described in the text.

enzymes, which lack the racemase activity. During the course of this study, we found that the emission fluorescence of the tryptophan residue of the apo enzyme was higher than those of the holo and PMP enzymes. Binding of the cofactors probably causes the quenching of the fluorescence. We obtained the $K_d^{app}$ values for PLP and PMP by measuring the extent of the decrease in the fluorescence of the tryptophanyl residues in the presence of various concentrations of cofactors. The plots of the percent of quenching ($Q$%) against the concentration of PLP or PMP ([PLP or PMP]) gave a hyperbolic curve which could be fitted to the equation, $Q\% = [\text{PLP or PMP}]/K_d^{app} + [\text{PLP or PMP}]$ (Fig. 3). The $K_d^{app}$ of the wild-type AlaR obtained by this method ($1.9 \pm 0.2$ μM) is in good agreement with that obtained by the previous method (1.5–2.0 μM, [21]). The $K_d^{app}$(s) of the wild-type and mutant enzymes for PLP and PMP are shown in Table 2. The

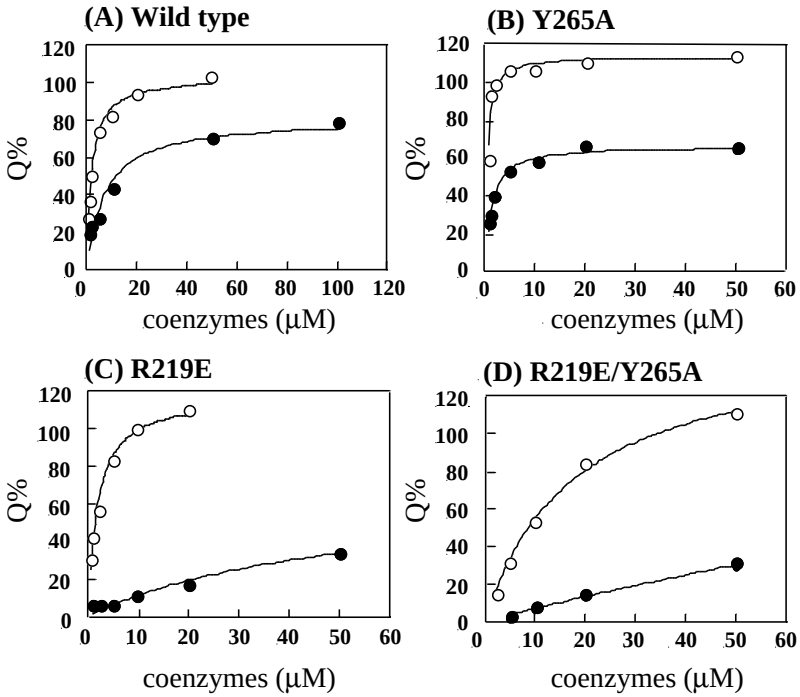


Fig. 3. Determination of $K_d^{app}$ for PLP and PMP of the wild-type, Y265A, R219E and R219E/Y265A mutant enzymes. The percent of quenching (*Q*%) is plotted against the concentration of PLP (open circle) and PMP (closed circle). Details are given in the text.

Y265A mutant enzyme showed higher affinity towards PLP and PMP than the wild-type enzyme. The R219E mutant enzyme showed similar $K_d^{app}$ for PLP to that of the wild-type enzyme, but the $K_d^{app}$ for PMP was more than five times higher than that of the wild-type enzyme. The $K_d^{app}$(s) of the R219E/Y265A mutant enzyme for PLP and PMP were 10 and 50 times higher than those of the wild-type enzyme, respectively. The increased $K_d^{app}$ for PMP is consistent with the results that the R219E/Y265A mutant enzyme showed no reverse half transamination with the catalytic amount of PMP (Fig. 2).

Table 2
$K_d^{app}$ values of the wild-type and mutant enzymes for PLP and PMP

| | $K_d^{app}$ for PLP (μM) | $K_d^{app}$ for PMP (μM) |
|---|---|---|
| Wild-type | 1.9 ± 0.20 | 7.9 ± 2.6 |
| Y265A | 0.40 ± 0.070 | 1.2 ± 0.20 |
| R219E | 1.8 ± 0.20 | 50 ± 33 |
| R219E/Y265A | 18 ± 2.0 | >410 |

## 4. Discussion

Conversion of the substrate and catalytic specificities by protein engineering or directed evolution has been carried out with various pyridoxal enzymes for understanding their structure–function relationships. Onuffer and Kirsch converted aspartate aminotransferase (AspAT) to tyrosine aminotransferase by multiple active-site mutation [22]. Yano et al. converted AspAT to branched-chain L-amino acid aminotransferase by directed-evolution [23]. Tyrosine-phenol lyase was endowed with a new function, dicarboxylic acid β-lyase activity, by a double mutation [24]. Graber et al. reported the conversion of the catalytic specificity of AspAT to that of L-aspartate β-decarboxylase by a triple active-site mutation [25]. In this work, we succeeded in converting the catalytic specificity of the *Bacillus* AlaR to D-AAT activity by a double mutation, R219 → E and Y265 → A.

The R219 of the *Bacillus* AlaR interacts with the N1 of the cofactor [8]. The corresponding position

of aminotransferases is occupied by an aspartyl or a glutamyl residue. Single mutation of R219 to a glutamyl residue resulted in 5.4-fold increase in the half transamination activity with D-alanine and $10^3$-fold decrease in the racemase activity. The double mutation, R219 → E and Y265 → A, completely abolished the racemase activity and increased the forward half transaminase activity 6.6-fold (pH 10.5). The double mutation increased the ratio of transaminase activity to racemase activity more than $7.3 \times 10^7$-fold. The glutamyl residue interacting with N1 of the cofactor is favorable for the transamination and unfavorable for the racemization. In the *E. coli* AspAT, Asp222 interacts with N1 of PLP and forms a hydrogen bond with the nitrogen [26]. The hydrogen bonding increases the p$K_a$ of the N1 proton and keeps the N1 as a protonated form. This makes the cofactor serve as a more efficient electron sink, which is advantageous for stabilizing the quinonoid intermediate, a resonance-stabilized anionic intermediate [26]. The arginyl residue at the position 219 of AlaR is unfavorable for the protonation of N1. The quinonoid intermediate formed upon the AlaR reaction, if formed, is probably unstable. This is consistent with the results that the R219E (10 and Fig. 2, B, D) and R219E/Y265A (Fig. 2, F) mutant AlaRs exhibited the absorption maximum at around 510 nm, corresponding to that of the quinonoid intermediate during the incubation with D-alanine. In contrast, the wild-type AlaR did not show a similar absorption spectrum under the conditions [7,11] or in the presence of concentrated D- and L-alanine, a preferable condition for the quinonoid intermediate formation (data not shown). These results sustain our proposal that the AlaR reaction proceeds without the formation of the quinonoid intermediate [11].

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