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Stereochemistry of the Transamination Reaction Catalyzed by Aminodeoxychorismate Lyase from *Escherichia coli*: Close Relationship between Fold Type and Stereochemistry

Kwang-Hwan Jhee,*,† Tohru Yoshimura,* Edith Wilson Miles,† Sou Takeda,* Ikuko Miyahara,‡ Ken Hirotsu,‡ Kenji Soda,§ Yasushi Kawata,‖ and Nobuyoshi Esaki*,¶

Institute for Chemical Research, Kyoto University, Gokasho, Uji, Kyoto, 611-0011; †Laboratory of Biochemistry and Genetics, NIDDK, National Institutes of Health, Bethesda, MD 20892-0830, U.S.A.; ‡Department of Chemistry, Graduate School of Science, Osaka City University, Sumiyoshi-ku Osaka 558-8585; §Department of Biotechnology, Faculty of Engineering, Kansai University, Suita, Osaka 564-8680; and ‖Faculty of Engineering, Tottori University, Koyama, Tottori 680-8552

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Aminodeoxychorismate lyase is a pyridoxal 5′-phosphate–dependent enzyme that converts 4-aminodeoxychorismate to pyruvate and *p*-aminobenzoate, a precursor of folic acid in bacteria. The enzyme exhibits significant sequence similarity to two aminotransferases, D-amino acid aminotransferase and branched-chain L-amino acid aminotransferase. In the present study, we have found that aminodeoxychorismate lyase catalyzes the transamination between D-alanine and pyridoxal phosphate to produce pyruvate and pyridoxamine phosphate. L-Alanine and other D- and L-amino acids tested were inert as substrates of transamination. The *pro*-R hydrogen of C4′ of pyridoxamine phosphate was stereospecifically abstracted during the reverse half transamination from pyridoxamine phosphate to pyruvate. Aminodeoxychorismate lyase is identical to D-amino acid aminotransferase and branched-chain L-amino acid aminotransferase in the stereospecificity of the hydrogen abstraction, and differs from all other pyridoxal enzymes that catalyze pro-*S* hydrogen transfer. Aminodeoxychorismate lyase is the first example of a lyase that catalyzes *pro*-R–specific hydrogen abstraction. The result is consistent with recent X-ray crystallographic findings showing that the topological relationships between the cofactor and the catalytic residue for hydrogen abstraction are conserved among aminodeoxychorismate lyase, D-amino acid aminotransferase and branched-chain L-amino acid aminotransferase [Nakai, T., Mizutani, H., Miyahara, I., Hirotsu, K., Takeda, S., Jhee, K.-H., Yoshimura, T., and Esaki, N. (2000) *J. Biochem.* 128, 29–38].

Key words: aminodeoxychorismate lyase, molecular evolution, pyridoxal 5′-phosphate, stereochemistry, transamination.

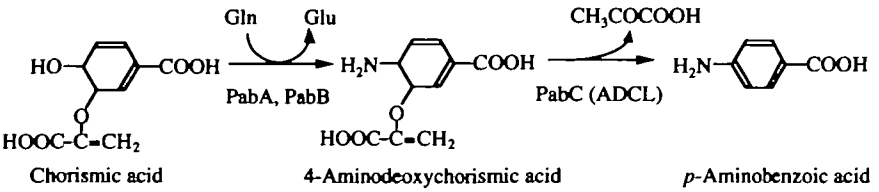
p-Aminobenzoic acid (PABA), a precursor of folic acid, is formed in bacteria from chorismate by PABA synthase (*1*, *2*) and 4-amino-4-deoxychorismate lyase (ADCL, *3–5*). PABA synthase consists of components I and II encoded by *pabB* and *pabA*, respectively, and catalyzes the conversion of chorismate to 4-aminodeoxychorismate (ADC) with L-glutamine as an amino donor (*1*, *2*). ADCL depends on pyridoxal 5′-phosphate (PLP) and converts 4-aminodeoxychorismate to pyruvate and PABA (*3–5*, Scheme 1). ADCL catalyzes the elimination of pyruvate from ADC with concomitant aromatization of the cyclohexadiene ring. The enzyme mechanism can be classed as an α,β-lyase.

PLP enzymes are classified into 5 groups based on similarities in sequence and secondary structure (*6*). ADCL is placed in the fold type IV group together with two aminotransferases, D-amino acid aminotransferase (D-AAT) (*7*) and branched-chain L-amino acid aminotransferase (BCAT) (*8*). *Escherichia coli* ADCL exhibits 23 and 22% sequence identities to *Bacillus* sp. YM-1 D-AAT and *E. coli* BCAT, respectively (*5*). Recently, the crystal structure of *E. coli* ADCL was solved at 2.2 Å resolution with an *R*-factor of 19.9% (*9*). The fold of ADCL is similar to those of D-AAT and BCAT and differs from those of other pyridoxal enzymes studied so far. ADCL is the only example of an α,β-lyase belonging to the fold-type IV family.

With the exceptions of succinyldiaminopimelate aminotransferase and valine-pyruvate aminotransferase, which have not been classified, the aminotransferases so far studied belong to one of two groups, fold types I and IV (*6*). One of the prominent differences between the reactions catalyzed by these two types of aminotransferases is the stereospecificity of the hydrogen transfer that occurs between C-2 of the substrate and C-4′ of the cofactor during the

¶ To whom correspondence should be addressed. Tel: +81-774-38-3240, Fax: +81-774-38-3248, E-mail: esaki@scl.kyoto-u.ac.jp

Abbreviations: AspAT, aspartate aminotransferase; ADC, 4-aminodeoxychorismate; ADCL, 4-amino-4-deoxychorismate lyase; BCAT, branched-chain L-amino acid aminotransferase; D-AAT, D-amino acid aminotransferase; PABA, *p*-aminobenzoic acid; PMP, pyridoxamine 5′-phosphate; PLP, pyridoxal 5′-phosphate; SDS-PAGE, sodium dodecyl sulfate gel electrophoresis; Tris, tris(hydroxymethyl)-aminomethane.



Scheme 1. **Conversion of chorismate to *p*-aminobenzoate.**

transamination (*10*). D-AAT and BCAT, which belong to the fold type IV family, catalyze hydrogen transfer specifically on the *re*-face of the planar intermediate formed from the substrate and cofactor. In contrast, aminotransferases belonging to the fold-type I family, such as L-asparatate aminotransferase, catalyze *si*-face specific hydrogen transfer (*10, 11*). In addition to aminotransferases, other pyridoxal enzymes such as racemases, lyases, and decarboxylases catalyze the transamination as a side reaction (*12*). Because of their structural similarities, ADCL was expected to catalyze the transamination in a manner similar to D-AAT and BCAT. In this work, we demonstrate that ADCL catalyzes transamination with D-alanine as an amino donor. ADCL shows *re*-face–specific hydrogen transfer during the transamination. In contrast, two lyases, tryptophanase and tryptophan synthase, which belong to the fold type I and II families, respectively, catalyze *si*-face–specific hydrogen transfer.

EXPERIMENTAL PROCEDURES

Materials—E. coli JM109, restriction nucleases, calf intestinal alkaline phosphatase, Pfu DNA polymerase, and T4 DNA polymerase were purchased from Takara Shuzo, Kyoto. AspAT from pig heart and a mixture of dNTPs were purchased from Boehringer Mannheim, Germany. T4 DNA ligase was from New England Biolabs, USA. D-Amino acid oxidase, L-amino acid oxidase, and peroxidase were from Worthington Biochemical, USA. 4-Aminoantipyrine and *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-*m*-toluidine were from Sigma, USA. Tryptophan synthase from *Salmonella typhimurium* (*13*) and D-AAT from *Bacillus* sp. YM-1 (*14*) were prepared as described previously. BCAT of *E. coli* (*15*) was a kind gift from Dr. H. Kagamiyama of Osaka Medical College. Tryptophanase was purified as described previously (*16*). Chorismic acid was prepared from the culture broth of *Aerobacter aerogenes* 62-1 as described previously (*17*).

Construction of pADCL, a Vector for the Overexpression of ADCL—Plasmid pADCL, a vector for the overexpression of ADCL was prepared as follows. The structural gene of *E. coli* ADCL (*pabC*) was amplified by polymerase chain reaction (PCR) with *E. coli* JM109 chromosomal DNA as a template and the following two primers, 5′-CACTAAGAATTCTCTGATAAGG-3′ (forward) and 5′-CGGCATAAAGCTTTTCAC-3′ (reverse). The forward and reverse primers contained *Eco*RI and *Hin*dIII restriction sites, respectively, as indicated by the underlines. The DNA fragment obtained was digested with nucleases, and inserted between the *Eco*RI and *Hin*dIII restriction sites of plasmid pKK223-3. Construction of the plasmid was confirmed by DNA sequencing using the dideoxy terminator method with an Applied Biosystems Model 373A automated sequencer.

Overexpression and Purification of ADCL—A 0.5 liter culture of recombinant *E. coli* JM109 cells transformed with pADCL was grown aerobically at 37°C in Super Broth (KD medical) containing 200 mg/liter ampicillin. When the OD_{650} reached 2.5, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture to a final concentration of 0.1 mM, and the cells were cultivated for another 16 h at 30°C. Cultivation in triple-indented Tunair flasks with loose fitting plastic caps (Shelton Scientific Manufacturing INC, USA) (*18*) gave a high yield of cells. After washing with 0.85% NaCl containing 1 mM dithiothreitol (DTT), the cells (15 g, wet weight) were resuspended in Buffer BP consisting of 50 mM Na/Bicine (pH 7.8), 1 mM EDTA, 1 mM DTT, and 0.1 mM PLP and disrupted twice with a French press at 8,000 p.s.i. The suspension was centrifuged at 12,000 ×*g* for 30 min. To 50 ml of supernatant solution, 8 ml of a 2% solution of protamine sulfate in Buffer BP was added and the solution was stirred for 20 min at room temperature. After centrifugation at 12,000 r.p.m., the supernatant solution was fractionated with ammonium sulfate at pH 7.5. The 30–75%-saturated ammonium sulfate fraction was dialyzed against 3 changes of Buffer BP at 4°C for 6 h. The dialyzed enzyme solution (50 ml) was loaded onto a 2.5 × 20 cm column of DEAE-Sephacel, which was then washed with 300 ml of Buffer BP. The enzyme was eluted with 1-liter of a linear gradient from 0 to 0.5 M NaCl in Buffer BP. Fractions containing the 30-kDa protein corresponding to ADCL were identified by SDS–polyacrylamide gel electrophoresis (SDS-PAGE). The fractions eluted with 0.13–0.17 M NaCl were pooled and treated with ammonium sulfate at 30% saturation. The enzyme solution was loaded onto a 2.5 × 20 cm Butyl-Sepharose 4B column equilibrated with Buffer BP containing ammonium sulfate at 30% saturation. The enzyme was eluted with a 600 ml linear gradient of 30 to 0% saturated ammonium sulfate in Buffer BP. The fractions eluted with 10–15% ammonium sulfate were concentrated to 20 mg/ml protein and dialyzed against Buffer KP, consisting of 5 mM potassium phosphate (pH 7.8), 1 mM EDTA, 1 mM DTT, and 0.02 mM PLP. The dialyzed enzyme solution (20 ml) was applied to a Gigapite (Seikagaku-Kogyo, Tokyo) column (2.5 × 20 cm) equilibrated with Buffer KP. After the column was washed with 400 ml of Buffer KP, the enzyme was eluted with a 1-liter linear gradient from 5 to 500 mM potassium phosphate in Buffer KP. The fractions eluted with buffer containing 100–150 mM potassium phosphate were concentrated to 30 mg/ml, dialyzed against Buffer K (50 mM potassium phosphate, pH 7.8, containing 1 mM EDTA, 1 mM DTT, and 0.02 mM PLP), and stored at –85°C. The purity of the protein was estimated to be >95% by SDS-PAGE.

N-Terminal Amino Acid Sequence Determination—The N-terminal amino acid sequence of the purified ADCL was determined with a Shimadzu PPSQ-10 Protein Sequencer.

Measurement of the Activity of the Purified ADCL—Because of the difficulty in obtaining 4-aminodeoxychoris-

mate, a substrate of ADCL, we confirmed the ADCL activity of the purified enzyme with a coupling system with component I of PABA synthase (PabB). Construction of plasmid pPABA, a PabB over-producer, was carried out according to a method similar to that for the preparation of pNPAB (*3*) except that pKK223-3 was used as a vector instead of pHN1$^+$. The crude PabB preparation was obtained by ammonium sulfate fractionation (25–60%) of the cell-free extract of *E. coli* JM109 cells transformed with pPABA as described by Ye *et al.* (*3*), except that *E. coli* JM109 was used as the host cells instead of *E. coli* XA90.

The reaction mixture (100 μl) containing 50 mM Tris-HCl buffer (pH 7.5), 5 mM $MgCl_2$, 50 mM ammonium sulfate, 2 mM ammonium chorismate, 200 μg of the crude PabB protein, and 7.5 μg of the purified ADCL was incubated at 25°C for 4 h. The reaction was stopped by the addition of 10 μl of 2 M HCl. The formation of PABA was analyzed by HPLC under the conditions described by Green and Nichols (*4*).

Spectroscopic Methods—Absorption spectra of ADCL were obtained with a Hewlett-Packard 8452 diode array spectrophotometer thermostated at 25°C with a Peltier junction temperature controlled cuvette holder. CD measurements were carried out at 25°C in a Jasco J-715 spectropolarimeter interfaced with a personal computer (Japan Spectroscopic, Easton, MD). All spectroscopic data were obtained at 25°C with the enzyme in Buffer B consisting of 50 mM Na/Bicine (pH 7.8), 1 mM EDTA, and 1 mM DTT.

Protein Assays—Protein concentrations were determined with a Bio-Rad protein assay reagent using bovine serum albumin as a standard. The concentration of apo ADCL was also determined from the absorbance at 278 nm using the absorption coefficient $A^{1\%}_{1cm}$ = 7.9, which was determined from the amino acid sequence and the absorbance of the enzyme in 6 M guanidine hydrochloride by the Edelhoch method (*19*, *20*). The absorption coefficient of the holo-ADCL ($A^{1\%}_{1cm}$ = 9.0) was determined from the absorbance of the holo enzyme reconstituted by incubation of apo-ADCL (1 mg/ml) with 50 μM PLP for 2 h at 25°C, followed by 6 h dialysis with a Mini dialysis kit (Pierce, USA).

Preparation of Apoenzymes—ADCL (5 mg/ml) was incubated at 25°C for 30 min in Buffer B containing 0.7 M NaCl and 0.2 M hydroxylamine adjusted in advance to pH 8.0. The bound PLP-oximes were removed from the enzyme by gel filtration on a PD-10 column (Amersham Pharmacia Biotech) eluted with Buffer B. The resultant enzyme (3 mg/ml), which still exhibited an absorbance band centered at 330 nm, was incubated at 30°C for 25 min with 1 M KSCN. The precipitate formed was removed by centrifugation, and excess KSCN was removed from the supernatant by gel filtration on a PD-10 column. The final preparation of apo-ADCL still had an absorbance band centered at 330 nm (Fig. 2A). Similar absorbance bands centered at 330–340 nm are also observed for several apo enzymes including D-AAT (*14*), L-lysine α-ketoglutarate ε-aminotransferase (*21*), and the β-form of cytosolic aspartate aminotransferase (*22*). Two of the components that contribute to the 340 nm band of the apo aspartate aminotransferase have been identified as PMP and the *N*-phosphopyridoxyl derivative of 5-oxoproline (*22*). Apo-tryptophanase was prepared by incubating the holo-enzyme with 50 mM phenylhydrazine in 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA and 1 mM DTT for 3 h at 30°C, followed by dialysis against the same buffer. Apo-AspAT, apo-D-AAT, apo-BCAT (*10*), and apo-tryptophan synthase (*23*) were prepared as reported previously.

Determination of the Stereospecificities of C4′ Hydrogen Transfer from PMP in the Half-transamination Reactions Catalyzed by ADCL, Tryptophanase, and Tryptophan Synthase—Stereospecifically labeled pyridoxamine 5′-phosphate (PMP), (4′*S*)-[4′-^{3}H]PMP and (4′*R*)-[4′-^{3}H]PMP were prepared as described previously (*10*). The specific radioactivities of the pro-*S* and pro-*R* labeled PMPs were 1.20 × 10^6 and 1.08 × 10^6 dpm/mmol, respectively. The reaction mixtures contained 10 μmol of Tris-HCl buffer (pH 8.0), 0.02% sodium azide, 0.5 μmol of pyruvate, 3.01 nmol of (4′*S*)-[4′-^{3}H]PMP or 3.34 nmol of (4′*R*)-[4′-^{3}H]PMP, and 7 nmol of each apo-enzyme in a final volume of 200 μl. The reactions were carried out at 25°C for 8 h and stopped by the addition of 100 μl of 2 M HCl. The mixtures were frozen in liquid nitrogen and dried with a Speed Vac Concentrator. The residues were dissolved in 200 μl of distilled water and used for the radioactivity assay. Radioactivity was determined with a Packard Tri-Carb scintillation spectrometer with Clear-sol II (Nacalai Tesque, Kyoto) as the scintillator. The tritium abstracted from the C4′ of PMP was exchanged with the solvent hydrogen during the course of the half transamination reaction (*10*). Tritium released from the labeled PMP is expressed as a volatile radioactivity in the solvent, and can be estimated by subtraction of the final radioactivity from that initially added to the reaction mixture. Control experiments showing non-enzymatic tritium abstraction were carried out without enzyme. The reactions with apo-AspAT and apo-D-AAT were carried out under the same conditions except that α-ketoglutarate was used as the amino acceptor.

Assay of Pyruvate Formed from D-Alanine during the Forward Half Transamination Catalyzed by ADCL—Half transamination from PLP to PMP was carried out with 50 mM D-alanine and 1 mg (33.7 μM) of ADCL in 1 ml of Buffer B. The reaction was carried out at 25°C and terminated by the addition of HCl to a final concentration of 1 M at the indicated times. After centrifugation of the reaction mixture, the supernatant solution was adjusted to pH 8.0 with NaOH, and the amount of pyruvate was determined by coupled assay with NADH and lactate dehydrogenase (*24*).

Calculation of the Rate Constants for the Forward Half Transamination—Rate constants for the forward half transamination were obtained by following the breakdown of the internal Schiff base (decrease in absorbance at 414 nm), formation of the PMP enzyme (increase in absorbance at 328 nm), and pyruvate formation. Data were computated 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 % absorbance change or stoichiometry of pyruvate, A is the difference value of Y in the exponential phase, k is the rate constant, t is time (min), and c is a constant.

Assay of the D-Alanine formed from Pyruvate during the Reverse Half Transamination Catalyzed by ADCL—Half transamination from PMP to PLP was carried out in a reaction mixture containing 3 mg/ml (0.1 mM) of apo-ADCL, 0.1 mM PMP, and 0.1 mM pyruvate in 1 ml of Buffer B. The reaction was carried out at 25°C and terminated by the

addition of HCl to a final concentration of 1 M at the indicated times. After centrifugation of the reaction mixture, the supernatant solution was adjusted to pH 8.0 with NaOH, and the amount of D-alanine was assayed in the coupling system with D-amino acid oxidase and peroxidase (*25*).

RESULTS

Overexpression and Purification of ADCL—pADCL gave a very high level of *E. coli* ADCL gene expression. We obtained 250 mg of pure ADCL from a 0.5 liter culture by three column chromatography steps. The N-terminal 20 amino acid sequence was identical to that deduced from the *pab*C gene (data not shown). ADCL activity was confirmed with an assay system containing chorismate, ammonium sulfate, partially purified PabB and purified ADCL. HPLC analysis of the reaction mixture indicated the complete loss of chorismate with the formation of PABA and an unknown compound with a retention time (*Rt*) of 3.6 min, which corresponded to that of 4-aminodeoxychorismate (*4*). The extent of the conversion of chorismate to PABA was about 20%. Because no PABA was formed in the absence of ADCL, we concluded that the purified enzyme exhibited ADCL activity.

Spectroscopic Properties of the Purified ADCL—The absorption spectrum of the purified ADCL exhibited major peaks at 278, 414, and 334 nm with absorbance ratios of 1:0.18:0.05 (Fig. 1A). The absorption maximum at 414 nm is attributed to the ketoenamine tautomer of the bound PLP. The maximum at 334 nm is more difficult to assign and may be attributable to the enolimine tautomer of the bound PLP or to a substituted aldamine, carbinolamine, or PMP. The ratio of the peak at 414 nm to that at 334 nm (3.6:1) is much greater than that (1.6:1) reported previously (*5*). One possible explanation for this difference is that our preparation was freshly prepared and the previous preparation had undergone aging. Aging of either L-lysine α-ketoglutarate ε-aminotransferase (*21*) and tryptophan synthase (unpublished observation) results in increased absorption near 340 nm and decreased absorption near 410 nm. The absorption spectrum of ADCL was pH-independent between pH 6 and 9 and was not affected by temperature between 20 and 40°C (data not shown). The CD spectra of the enzyme exhibited a negative CD band at 414 nm (Fig. 1C) as found for D-AAT (*14*), BCAT (*26*), and alanine racemase (*27*).

Half-Transamination Reaction from PLP to PMP—The addition of D-alanine to holo-ADCL resulted in a time-dependent decrease in the absorption peak at 414 nm, with a concomitant increase in the absorbance at 328 nm (Fig. 1A). The rates of the decrease in the absorbance at 414 nm (k = 0.138 ± 0.001 min^{-1}) and increase in absorbance at 328 nm (k = 0.141 ± 0.001 min^{-1}) were essentially the same (Fig. 1B). The clear isobestic point at 353 nm provides evidence for two interconverting species. When we prolonged the incubation of the reaction mixture, we observed a very slow increase in the absorption maximum at 328 nm without accompanying pyruvate formation. The absorption maximum at 328 nm, which is the absorption maximum of free PMP at the same pH, disappeared upon dialysis or ultrafiltration of the reaction mixture. These results suggest that the PLP form of the enzyme is converted to the PMP form by a half-transamination reaction with D-alanine. The PMP produced is gradually released from the enzyme because of the low affinity with ADCL. The further increase in the absorbance at 328 nm after the completion

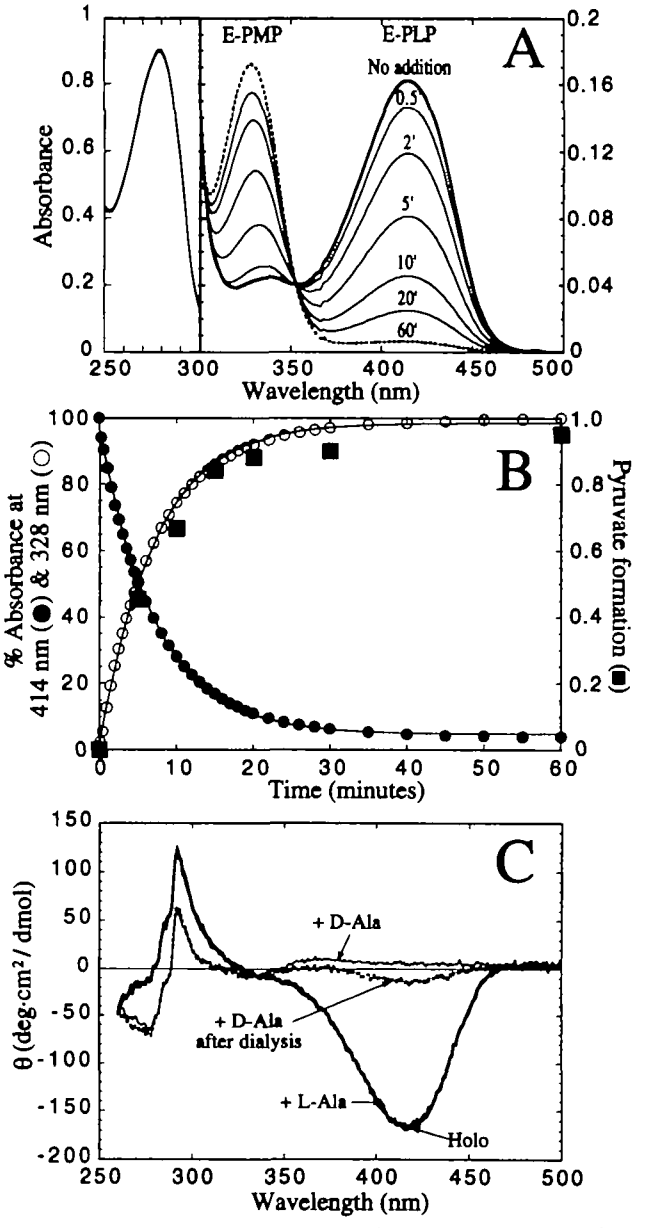


Fig. 1. Half-transamination reaction from PLP to PMP with D-alanine catalyzed by ADCL. A: Effect of D-alanine on the absorption spectrum of holo-ADCL. The reaction mixture contained 1 mg of ADCL in Buffer B in a final volume of 1 ml. The reaction was initiated by the addition of D-alanine to a final concentration of 50 mM and carried out at 25°C. The spectra were recorded at the indicated times. B: Time courses of the decrease in absorbance at 414 nm (E-PLP), increase in absorbance at 328 nm (E-PMP), and pyruvate formation. The absorbance data are expressed as the percentage of the maximum absorbance change. Pyruvate formation is expressed as the molar ratio of the amount of pyruvate formed to the amount of enzyme monomer used. C: CD spectra of holo-ADCL before and after incubation with 50 mM D- or L-alanine for 1 h at 25°C. The spectrum of ADCL after incubation with D-alanine followed by dialysis is also shown. The protein concentrations were 1 mg/ml.

of pyruvate formation is probably due to the difference in the absorption coefficients between free PMP and ADCL-bound PMP. If we assume that the ADCL-bound PLP is completely converted to the bound-PMP after 1-h incubation (Fig. 1A), the absorption coefficient of the free PMP at 328 nm (9,400) is calculated to be 1.66 times that of the bound PMP (5,670).

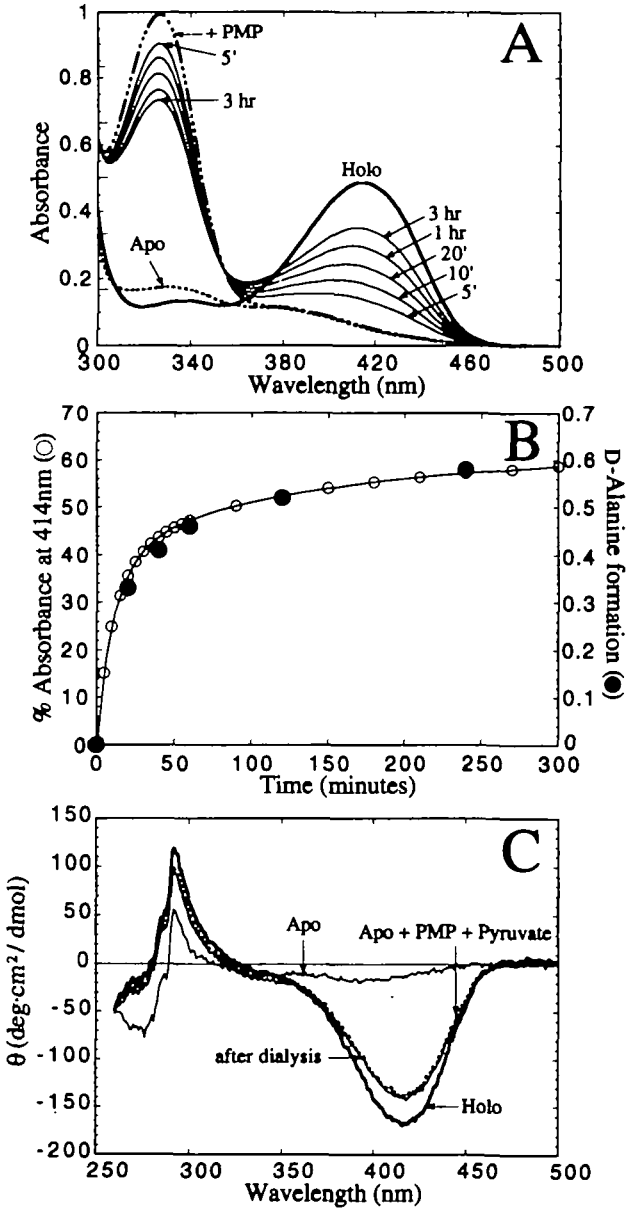


Fig. 2. **Half-transamination reaction from PMP to PLP with pyruvate catalyzed by ADCL.** A: Effect of pyruvate on the absorption spectrum of the PMP-form of ADCL. The reaction mixture contained 0.1 mM (3 mg) apo-ADCL and 0.1 mM of PMP in Buffer B in a final volume of 1 ml. The reaction was initiated by the addition of pyruvate to a final concentration of 0.1 mM and carried out at 25°C. The spectra were recorded at the indicated times. B: Time courses of the increase in absorbance at 414 nm and of D-alanine formation. The absorbance data are expressed as the percentage of the maximum absorbance change. D-Alanine formation is expressed as the molar ratio of the amount of D-alanine formed to the amount of enzyme monomer used. C: CD spectra of apo-ADCL before (Apo) and after incubation with PMP and pyruvate for 5 h at 25°C (Apo + PMP + pyruvate). Also shown is the spectrum after incubation with PMP and pyruvate followed by dialysis. The reaction was carried out under the conditions used in A. CD spectra were obtained for samples diluted 3-fold to a final protein concentration of 1 mg/ml.

The product from D-alanine in the half-transamination reaction is pyruvate as evidenced by its reduction with lactate dehydrogenase in the presence of NADH. The rate of pyruvate formation (k = 0.131 ± 0.008 mol pyruvate/mol enzyme/min) was nearly the same as the rate of increase in the absorption at 328 nm (PMP formation) and the rate of decrease at 414 nm (PLP reduction) (Fig. 1B). The amount of the pyruvate formed suggests that about 95% of the PLP form of the enzyme was converted to the PMP form after 1 h incubation (Fig. 1B). The characteristic negative CD band centered at 414 nm disappeared after incubation with D-alanine (Fig. 1C).

The addition of L-alanine caused no changes in the absorption (data not shown) or CD spectra (Fig. 1C). Glycine, L-serine, D-serine, D-isoleucine, D-methionine, D-lysine, D-glutamic acid, and D-aspartic acid were also inert as substrates for the half transamination. The addition of D- or L-cysteine resulted in a rapid increase in the absorbance at 330 nm due to the formation of a thiazolidine adduct, as reported previously (5).

Half-Transamination Reaction from PMP to PLP—Incubation of apo-ADCL with stoichiometric amounts of PMP and pyruvate resulted in a time-dependent increase in the absorbance at 414 nm with a concomitant decrease in absorbance at 328 nm (Fig. 2A).[2] This indicates the reverse transamination from PMP to PLP. About 75% of the negative 414 nm CD band of the holoenzyme was restored by the half transamination reaction (Fig. 2C). The product from pyruvate by half-transamination is D-alanine, because it reacted with D-amino acid oxidase. The rate of D-alanine formation was nearly the same as the rate of increase in the absorbance at 414 nm, reflecting PLP formation (Fig. 2B). The amount of D-alanine formed suggests that 60% of the PMP is converted to PLP after 6 h incubation (Fig. 2B).

Stereospecificity of Hydrogen Transfer during the Reverse Half-Transamination—We determined the stereospecificities of the hydrogen transfer occurring in the reverse half transamination reactions catalyzed by ADCL, tryptophan synthase ($\alpha_2\beta_2$), and tryptophanase. When (4′*S*)-[4′-^{3}H]PMP or (4′*R*)-[4′-^{3}H]PMP was incubated with the apo-form of

TABLE I. **Stereospecificity of the hydrogen transfer from PMP in the half-transamination reaction.**

Reaction system	(4′*S*)-[4′-^{3}H]PMP[a]		(4′*R*)-[4′-^{3}H]PMP[a]	
	^{3}H-released[b]		^{3}H-released[b]	
	(dpm)	(%)[c]	(dpm)	(%)[c]
apo-AspAT + α-KG[d]	1,800	50	0	0
apo-Tryptophanase + pyruvate	2,850	79	0	0
apo-Tryptophan synthase + pyruvate	2,700	75	0	0
apo-ADCL + pyruvate	0	0	2,380	66
apo-D-AAT + α-KG[d]	54	1.5	2,800	78
Without enzyme	0	0	0	0

[a]The initial radioactivity in the reaction mixture was 3,608 dpm. [b]Volatile radioactivity. [c]Ratio of the released radioactivity to that initially added to the reaction mixture. [d]α-Ketoglutarate.

[2] We used a three-times higher concentration of ADCL for this reaction than for the forward half transamination reaction (Fig. 1) to facilitate the detection of PLP formation.

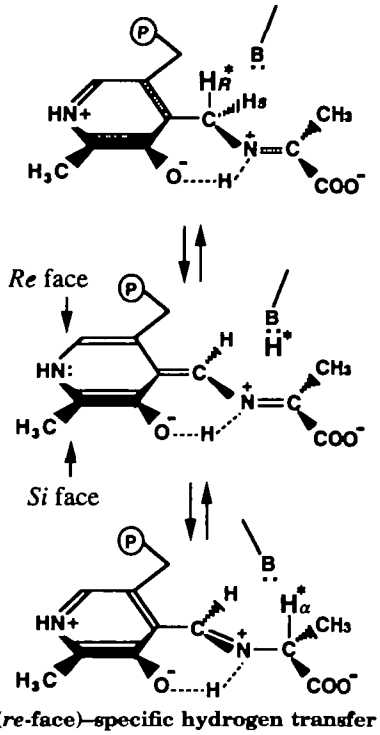


Fig. 3. **pro-*R* (*re*-face)–specific hydrogen transfer between C-2 of pyruvate and C4′ of PMP catalyzed by ADCL in the reverse half-transamination reaction.** Lys159 was proposed as a catalytic base (–B:) by crystallographic studies on ADCL (*9*).

ADCL in the presence of pyruvate, tritium was released exclusively from the *R*-enantiomer into the solvent (Table I). These results suggest that the pro-*R* hydrogen at C4′ of PMP is transferred to C-2 of pyruvate on the *re*-face of the planar π-intermediate formed from PMP and pyruvate (Fig. 3). About 66% of the tritium in (4′*R*)-[4′-^{3}H]PMP was released during the reaction. This value is consistent with the extent of conversion of PMP to PLP as determined from the absorption spectral change. In the reactions with tryptophan synthase and tryptophanase, the pro-*S* hydrogen at C4′ of PMP was specifically released into the solvent (Table I). These two enzymes catalyze hydrogen transfer on the *si*-face of the intermediate (Fig. 3). The result with tryptophan synthase is consistent with the findings that tryptophan synthase transfers hydrogen to the pro-*S* position of the C4′ of PMP in the transamination catalyzed as a side reaction in the presence of 2-mercaptoethanol (*28*), and that the pro-*S* hydrogen is abstracted from C4′ of PMP by apo-tryptophan synthase in the presence of indole-3-pyruvic acid (*29*). D-AAT and AspAT catalyze the pro-*R*–and pro-*S*–specific hydrogen abstractions, respectively, as reported previously (Table I, *10*). These results verify the stereospecificity of the labelling. Tritium was not released into the solvent non-enzymatically (Table I).

DISCUSSION

Here we report that ADCL catalyzes forward and reverse half-transamination reactions between PLP and D-alanine, and PMP and pyruvate, respectively. In addition to the aminotransferases, most pyridoxal enzymes catalyze transamination as a side reaction (*12*). In most such cases, the transamination results from a side reaction in which a quinonoid intermediate in the reaction with a normal substrate partitions between two pathways. In one pathway (the normal reaction), the quinonoid intermediate is protonated or reacts with an electrophile at the α-carbon. In the other pathway (transamination reaction), the quinonoid intermediate is protonated at the C4′ of the pyridoxyl moiety to yield a ketimine intermediate. In contrast, ADCL does not appear to carry out a side-transamination reaction with its normal substrate, ADC, but does carry out a transamination reaction with D-alanine. Because ADC is aromatized with a concomitant release of pyruvate, ADC cannot undergo a transamination reaction. The ability of ADCL to catalyze the transamination of D-alanine to form pyruvate may reflect the similarity of the binding sites of ADCL and D-AAT. Thus, the new transamination reaction results from the ability of ADCL to bind a different substrate that can undergo an alternative reaction.

The X-ray crystallography of *E. coli* ADCL demonstrated the conservation of several amino acid residues among ADCL, BCAT, and D-AAT, including the PLP binding lysyl residue (Lys159 of ADCL), the glutamyl residue interacting with pyridinium nitrogen of PLP (Glu193), and the arginyl residue interacting with the phosphate group of PLP (Arg59) (*9*). However, the residues corresponding to Tyr30, Arg98, and His100 of D-AAT, which are the major determinants of the substrate binding of D-AAT (*30*), are not conserved in the active-site of ADCL (*9*). The amino acid residues of ADCL possibly involved in binding D-alanine and pyruvate are not deduced from the comparison of the structures of ADCL and D-AAT. In contrast to D-AAT, BCAT acts on L-amino acids. The carboxylate of an L-amino acid is situated on the phosphate side of the cofactor, and is recognized by the OH of Tyr95 and the two main chain NH groups of Thr257 and Ala258 of BCAT (*8*). In ADCL, the β-turn corresponding to the α-carboxylate recognition site of BCAT is blocked from the active site by the side-chain of Leu258. This may be the reason L-alanine is inert as a substrate for ADCL. The active-site model of the ADCL bound to ADC suggests that Arg107 forms salt bridges with the two carboxylates of ADC (*9*). If the carboxylate of D-alanine also binds to Arg107, the α-hydrogen is likely to be located near the PLP binding Lys159, the probable catalytic residue for the hydrogen abstraction.

In ADCL, PLP has the same orientation toward the protein as that found in D-AAT (*7*) and BCAT (*8*). Lys159 is located on the *re*-face of the bound cofactor. The observed stereospecificity of the hydrogen transfer catalyzed by ADCL is consistent with the active site structure of the enzyme. In this experiment, we also demonstrated that tryptophanase and tryptophan synthase, which belong to the fold type I and II groups, respectively, catalyze the pro-*S*–specific hydrogen abstraction from C4′ of PMP. These results are consistent with the active-site structures of both enzymes: the catalytic residues, Lys87 of tryptophan synthase (*31*) and Lys266 of tryptophanase (*32*), are situated on the *si*-face of the cofactor. To our knowledge, pyridoxal enzymes belonging to the same folding group show the same stereospecificity for the hydrogen transfer during transamination. L-Aspartate aminotransferase (*10*), L-ornithine aminotransferase (*33*), L-glutamate decarboxylase (*34*), and L-aspartate β-decarboxylase (*35*), members of the fold-type I family, and L-tryptophan synthase and L-serine

hydroxymethyltransferase (*36*), members of the fold-type II family, catalyze *si*-face–specific hydrogen transfer. BCAT, D-AAT (*10*), and ADCL, members of the fold-type IV family, show *re*-face–specific hydrogen transfer. The active site structure with the catalytic lysyl residue situated on a specific face of the cofactor has been conserved during evolution among pyridoxal enzymes belonging to the same folding groups. This is consistent with the fact that the residues involved in cofactor binding, Lys159, Glu193, and Arg59 of ADCL, are conserved among BCAT and D-AAT (*9*).

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