Unique Stereospecificity of D-Amino Acid Aminotransferase and Branched-Chain L-Amino Acid Aminotransferase for C-4′ Hydrogen Transfer of the Coenzyme

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Abstract: D-Amino acid aminotransferase and branched-chain L-amino acid aminotransferase, which show a significant sequence homology,[6] are unique in their stereospecific catalysis of *pro-R* C-4′ hydrogen transfer through the coenzyme–substrate Schiff base intermediates in contrast to other various aminotransferases catalyzing the *pro-S* hydrogen transfer. D-Amino acid aminotransferase abstracted (*R*)-^{1}H from (4′*S*)-[4′-^{2}H]pyridoxamine in a half reaction of transamination with an amino acceptor. Branched-chain L-amino acid aminotransferase catalyzed the *pro-R* specific hydrogen exchange of pyridoxamine 5′-phosphate with the solvent hydrogen. When D-amino acid aminotransferase and branched chain L-amino acid aminotransferase were incubated with (4′*R*)-[4′-^{3}H]pyridoxamine 5′-phosphate and (4′*S*)-[4′-^{3}H]pyridoxamine 5′-phosphate in a half reaction of transamination with an amino acceptor, and also an overall transamination, ^{3}H was released into the solvent exclusively from (4′*R*)-[4′-^{3}H]pyridoxamine. Thus, these two enzymes are categorized into the same group on the basis of stereospecificity of the hydrogen transfer but different from all other aminotransferases showing the *pro-S* stereospecificity.

Introduction

In all the reactions of pyridoxal 5′-phosphate (PLP) dependent aminotransferases studied so far,[1] a proton is added or removed on the *si* face at C-4′ of the plane of the conjugated π-system of the cofactor–substrate imine (external Schiff base intermediate). This suggests the similar orientation of the catalytic base with the bound cofactor in the active sites of enzymes and also that the enzymes may have evolved from a common ancestral protein.[2] Comparison of their amino acid sequences shows that all the aminotransferases are considerably homologous with one another.[3] However, D-amino acid aminotransferase (D-AAT) of *Bacillus* sp. YM-1[4] and branched-chain L-amino acid aminotransferase (BCAT) of *Escherichia coli*[5] show a significant homology with each other in their primary structures[6] but are different from all other aminotransferases. The circular dichroism (CD) spectra of various aminotransferases show positive bands at the wavelengths where their internal Schiff bases absorb, but, in contrast, those of D-AAT and BCAT show negative ones.[6] Thus, we were interested in stereospecificity of D-AAT and BCAT for the C-4′ hydrogen transfer. We here provide the first evidence for the C-4′ hydrogen transfer of the coenzyme–substrate imine intermediate on the *re* face in the transamination reactions catalyzed by both enzymes.

Results

Hydrogen Transfer at C-4′ of Pyridoxal by D-AAT. Both PLP and pyridoxamine 5′-phosphate (PMP) are too tightly bound with the D-AAT protein to dissociate. Accordingly, the stereospecificy of D-AAT for transfer of the C-4′ hydrogen of cofactor was determined with the stereospecifically C4′-deuterated pyridoxamine (PM), which serves as a coenzyme as poor as pyridoxal (PL), but readily dissociates from the enzyme protein, in the first place. PL (6 mM, **1a**), L-aspartate (12 mM), and 50 mM *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid (EPPS) buffer (p^{2}H 8.5) were incubated with apoaspartate aminotransferase (AspAT) in 12 mL of ^2H_2O at 37 °C for 5 h. AspAT catalyzes protonation of PL at the *pro-S* position of the C-4′ to produce (4′*S*)-[4′-^{2}H]PM during transamination. The (4′*S*)-[4′-^{2}H]PM **2a** thus formed was isolated by ultrafiltration and purified by two ion-exchange column chromatographies as described later in Experimental Section. The ^{1}H NMR spectrum of the purified PM measured in ^2H_2O with a Varian-200 spectrophotometer showed that the PM contained one proton at the C-4′ carbon. Incubation of 50 mM EPPS buffer (p^{1}H 8.5), 0.92 mM (4′*S*)-[4′-^{2}H]PM and 8 mM α-ketoglutarate with 6 mg of apo-D-AAT in 10 mL of ^1H_2O at 45 °C for 4 h results in formation of either [4′-^{2}H]PL **1b** or [4′-^{1}H]PL. (4′*S*)-[4′-^{2}H]PM (0.87 mM) and α-ketoglutarate (8 mM) were incubated with 3 mg of apo-AspAT in 8 mL of 50 mM EPPS buffer (p^{1}H 8.5) at 37 °C for 3 h to

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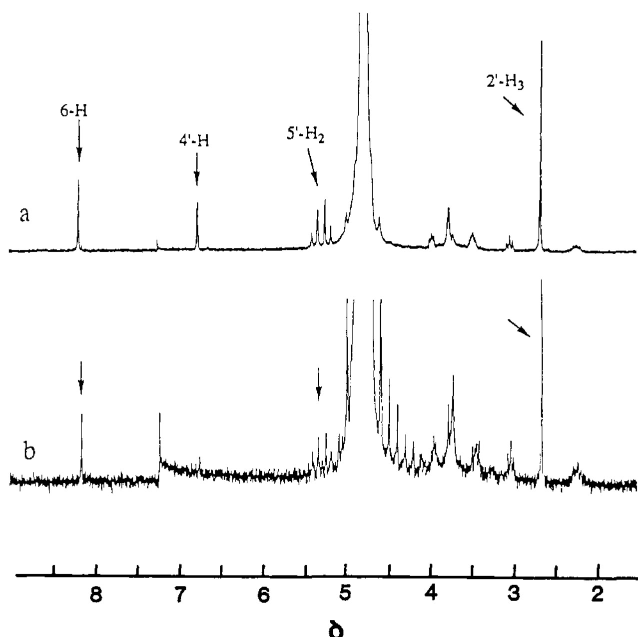


Figure 1. ^{1}H NMR spectra of PL formed: (a) PL formed with apo-AspAT from (4'*S*)-[4'-^{2}H]PM and (b) PL formed with apo-D-AAT from (4'*S*)-[4'-^{2}H]PM.

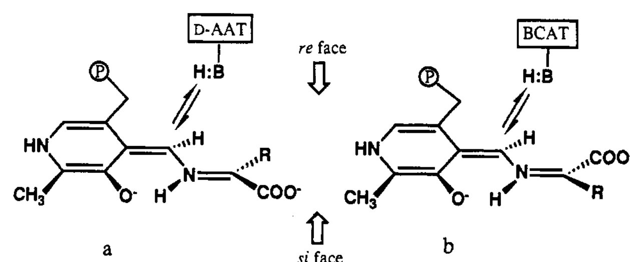


Figure 2. Schematic representation of C-4' hydrogen transfer by D-AAT (a) and BCAT (b).

yield [4'-^{1}H]PL (**2a** → **1a**). PL formed was isolated as described for PM. The ^{1}H NMR spectra of the PL measured in ^2H_2O (p^{2}H 2-3[7]) showed the C-4'-^{1}H peak of the PL produced by AspAT reaction at δ 6.7 ppm (Figure 1a), but no peaks were found in this region in the spectrum of the PL obtained with apo-D-AAT (Figure 1b). This result indicates that the C-4'-^{1}H was removed from (4'*S*)-[4'-^{2}H]PM during the D-AAT reaction (**2a** → **1b**). Thus, hydrogen is removed from the C-4' on the *re* face of the cofactor–substrate imine in the transamination reaction in contrast with the *pro-S* specificity of the hydrogen transfer by other aminotransferases so far studied (Figure 2a). The high catalytic efficiency[8] of D-AAT shows that there is no catalytic advantage for transamination to occur on the *si* face of the cofactor–substrate imine.

Hydrogen Addition at C-4' of PLP by BCAT. Neither PL nor PM serves as a coenzyme of BCAT. Accordingly, the stereospecificity of hydrogen addition at C-4' of the coenzyme in the transamination reaction catalyzed by BCAT was examined with PLP as follows.[9] We incubated BCAT (a PLP form, 40 mg) with 50 mM EPPS (p^{2}H 8.5) and 50 mM L-cysteinesulfinate in 6 mL of ^2H_2O at 30 °C for 90 min to convert the PLP-form of enzyme to the PMP-form. After the protein was denatured with 3 M urea and removed by ultrafiltration, the PMP released was isolated by ion exchange chromatography and hydrolyzed with alkaline phosphatase to yield PM. PM thus obtained (0.22 mM) and α-ketoglutarate (8 mM) were incubated with 3.5 mg of apo-AspAT in 5 mL of 50 mM EPPS (p^{1}H 8.5) at 37 °C for 1 h. After purification, PL formed was subjected to ^{1}H NMR analysis. The NMR spectrum obtained was similar to that shown in Figure 1b, indicating the absence of a peak in the region of δ 6.7 ppm (data not shown). This suggests that a deuteron was added to C-4' of the bound PLP from the *re* face in the BCAT region (**3** → **4**) (Figure 2b). To confirm these results, we employed another method to determine the stereospecificity of D-AAT and BCAT for the C-4' hydrogen transfer.

(7) PL forms hemiacetal structure in this pH range (pH 2–3), see: Korytnyk, W.; Ahrens, H. *Methods Enzymol.* **1970**, *18*, 475–483.

(8) (a) The rate constant of AspAT is 220 s^{-1} (Inoue, Y.; Kuramitsu, S.; Inoue, K.; Kagamiyama, H.; Hiromi, K.; Tanase, S.; Morino, Y. *J. Biol. Chem.* **1989**, *264*, 9673–9681). (b) The rate constant of D-AAT is 180 s^{-1} (Nishimura, K.; Tanizawa, K.; Yoshimura, T.; Esaki, N.; Futaki, S.; Manning, J. M.; Soda, K. *Biochemistry* **1991**, *30*, 4072–4077).

(9) Because neither PL nor PM functions as a coenzyme of BCAT, the same method as described for D-AAT is not applicable to the stereochemical analysis of the BCAT reaction.

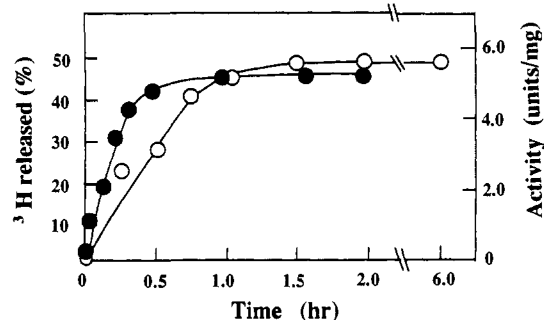


Figure 3. Exchange of C-4' ^{3}H of randomly labeled [4'-^{3}H]PMP with the solvent hydrogen by apo-BCAT. The reaction mixture (0.5 mL) containing 50 μmol of Tris-HCl buffer (pH 8.0), 30 nmol of [4'-^{3}H]-PMP, and 60 nmol of apo-BCAT was incubated at 30 °C. At the time indicated, a 50-μL aliquot of the mixture was withdrawn and mixed with 50-μL of 1 M HCl. The sample was used for determination of the vaporable tritium (○) as described in Experimental Section. The amount of tritium released from PMP is equal to that of the vaporable tritium. At the same time, 5 μL of the reaction mixture was withdrawn and diluted with 495 μL of 50 mM Tris-HCl buffer (pH 8.0) and then used for determination of the enzyme activity (●).

Stereospecific Exchange of C-4' Hydrogen of the Coenzyme with the Solvent Hydrogen by BCAT. It was reported that incubation of apo-AspAT with [4'-^{3}H]PMP in the absence of an amino acceptor results in a stereospecific exchange of the C-4' (*S*)-^{3}H with the solvent hydrogen.[10] We observed the similar stereospecific C-4' hydrogen exchange catalyzed by BCAT. When apo-BCAT was incubated with randomly labeled [4'-^{3}H]PMP, reconstitution of the enzyme[11] and a release of ^{3}H into the solvent, which was shown by an increase in vaporable radioactivity of the solvent, were observed (Figure 3). The absorption spectrum of the enzyme reconstituted by incubation for 30 min was not changed even after a 6-h incubation, and only PMP was found in the enzyme denatured after a 15-h incubation (data not shown). Therefore, the ^{3}H-release did not result from conversion of PMP into PLP but from the C-4' tritium exchange with the solvent hydrogen as observed for the AspAT reaction.[10] The final ratio of the radioactivity released from [4'-^{3}H]PMP to the initial radioactivity was about 50%. This suggests the stereospecific release of ^{3}H. After the ^{3}H release ceased, PMP was recovered from the enzyme by acid and heat treatment as described in the Experimental Section. The PMP was purified by a reversed-phase column chromatography and incubated with apo-AspAT or apo-BCAT. As shown in Figure 4, both enzymes were reconstituted with the PMP, but ^{3}H was released only in the reaction with apo-AspAT. This shows that the PMP recovered was tritiated specifically at the 4'*S* position. Therefore, BCAT catalyzes the C-4' *pro-R* hydrogen exchange with the solvent hydrogen. We have prepared both (4'*R*)-[4'-^{3}H]PMP and (4'*S*)-

(10) Tobler, H. P.; Christen, P.; Gehring, H. *J. Biol. Chem.* **1986**, *261*, 7105–7108.

(11) Reconstitution means the formation of a holo enzyme (a PMP form of enzyme here) and is reflected by the recovery of activity.

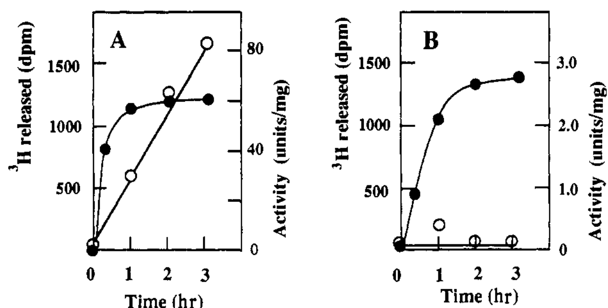


Figure 4. Release of tritium from [4'-^{3}H]PMP treated with apo-BCAT during incubation with apo-AspAT and apo-BCAT. The reaction mixture (450 μL) contained 45 μmol of Tris-HCl buffer (pH 8.0), 43 nmol of apo-AspAT or 58 nmol of apo-BCAT, and 15 nmol of stereospecifically labeled [4'-^{3}H]PMP obtained by incubation of the randomly labeled [4'-^{3}H]PMP with apo-BCAT as described in the legend for Figure 3. At the time indicated, 100 μL of the mixture was withdrawn and used for the determination of vaporable tritium. Other conditions were the same as described in the legends for Figure 3: (○) released (vaporable) tritium and (●) activity of the enzyme.

Table I. Stereochemistry of Hydrogen Withdrawal from PMP in the Half and Overall Reactions by D-AAT and BCAT[a]

	(4'S)-[4'-^{3}H]PMP ^{3}H-released[b]		(4'R)-[4'-^{3}H]PMP ^{3}H-released	
	dpm	%[c]	dpm	%
apo-D-AAT + KG[d]	36	1.5	1681	78.6
apo-D-AAT + KG + D-Ala	0	0	1657	75.9
apo-BCAT + KG	229	9.6	1233	57.6
apo-BCAT + KG + L-Val	0	0	1506	70.4
apo-AspAT + KG + L-Asp	1209	50.9	0	0

[a] Details are described in the Experimental Section. No tritium was released in the absence of enzyme. [b] Vaporable radioactivity. [c] Ratio of the radioactivity released to that initially added in the reaction mixture. [d] α-Ketoglutarate.

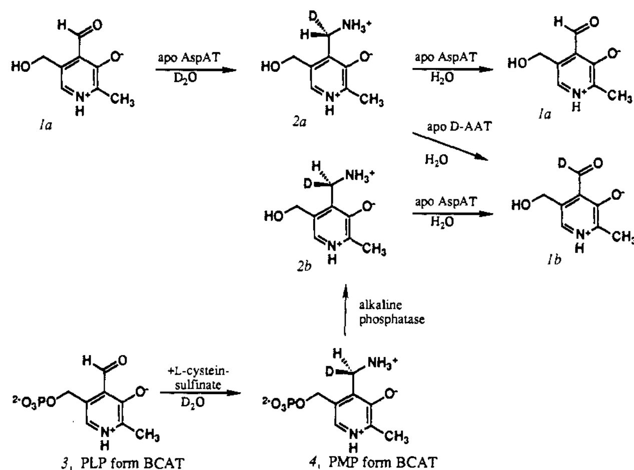
[4'-^{3}H]PMP by incubation of randomly labeled [4'-^{3}H]PMP in ^1H_2O with apo-AspAT and apo-BCAT, respectively. We found the *R*-specific exchange of ^{3}H of [4'-^{3}H]PMP with the solvent hydrogen in the D-AAT reaction as well (data not shown).

Stereospecificity of D-AAT and BCAT for the C-4' Hydrogen Withdrawal from PMP in the Half and Overall Reactions. When a PMP form of aminotransferase is converted to the PLP-form by incubation with an amino acceptor (a half reaction), one hydrogen is withdrawn from C-4' of PMP.[12] We examined the stereospecificities of D-AAT and BCAT for the hydrogen abstraction by measurement of the ^{3}H release in the reaction of an apo-form of enzyme with the stereospecifically tritiated PMP and α-ketoglutarate.[13] The stereospecificities of D-AAT and BCAT for the hydrogen abstraction during the overall reactions were also determined with D-alanine and L-valine, respectively by the same procedure (Table I). In the half and overall reactions by D-AAT and BCAT, ^{3}H was released exclusively from (4'*R*)-[4'-^{3}H]PMP into the solvent but was released from only (4'*S*)-[4'-^{3}H]PMP by AspAT. Thus, D-AAT and BCAT specifically abstract the C4' *pro-R* hydrogen from PMP in both half and overall reactions in contrast to AspAT.

(12) The hydrogen is theoretically considered to be transferred to the α-carbon of a substrate moiety of keto acid–PMP Schiff base. However, most of ^{3}H was actually released into the solvent in this experiment. This is probably due to the hydrogen exchange between the catalytic residue and the solvent water during the transfer reaction. See ref 2a.

(13) The C-4' hydrogen exchange with the solvent hydrogen may occur during the half and overall reactions of transamination. Under the conditions used, the rate of the hydrogen exchange was less than 5% of that of the hydrogen abstraction in the presence of α-ketoglutarate (data not shown). The stereospecificity for hydrogen exchange and hydrogen withdrawal is the same. Therefore, influence of the hydrogen exchange on the results shown in Table 1 is probably negligible.

Scheme I



Discussion

In α-aminotransferase reactions, α-hydrogen of an amino donor is transferred to the C-4' carbon of PLP through an internal Schiff base intermediate and an external Schiff base intermediate, and one of the C-4' hydrogens of PMP is transferred to the C-2 carbon of an amino acceptor in reverse.[14,15] This suggests an intramolecular prototropic shift by a single base and a suprafacial proton transfer. In all the aminotransferase reactions including the abortive transamination[16] so far studied, only a *pro-S* hydrogen is added or withdrawn at the C-4' of coenzyme;[1] the proton transfer occurs between a substrate and the coenzyme on the *si* face of the external Schiff base. We show here for the first time the *pro-R* hydrogen transfer at a C-4' of the coenzyme in the D-AAT and BCAT reactions. If these reactions proceed through a single base mechanism like other aminotransferase reactions, the *pro-R* hydrogen is transferred on the *re* face of the coenzyme–substrate Schiff base (Figure 2). The stereochemistry of this hydrogen transfer reflects the topographical situation of the coenzyme–substrate Schiff base and the catalytic base of enzyme. The identity of D-AAT with BCAT in the stereospecificity indicates that stereostructures of their active sites are homologous with each other but different from those of other aminotransferases. This is compatible with the classification of aminotransferases according to their primary structures: both D-AAT and BCAT belong to the same group which is independent of other three groups of aminotransferases.[3b] In addition, both aminotransferases show negative CD bands in the wavelength region where the absorption maxima occur although the relationship between the primary structures, CD bands, and stereospecificities of the C-4' hydrogen transfer of aminotransferases has not been elucidated.

In this work, we have found that BCAT catalyzes exchange of the *pro-R* C-4' hydrogen of the bound PMP with the solvent hydrogen in the absence of an amino acceptor. The stereospecificities of BCAT and D-AAT and AspAT for the hydrogen exchange of the bound PMP are identical with those of their respective C-4' hydrogen transfer in the half and overall transamination reactions. The catalytic role and also mechanism of the hydrogen exchange of the enzyme bound PMP remains unsettled. The stereospecific hydrogen exchange of the bound PMP by AspAT and BCAT or D-AAT is applicable to preparation of (4'*R*)-[4'-^{3}H]PMP and (4'*S*)-[4'-^{3}H]PMP, respectively. We

(14) (a) Snell, E. E.; Di Mari, S. J. *Enzymes*, 3rd ed.; 1970; Vol. 2, pp 335–370. (b) Braunstein, A. E. *Enzymes*, 3rd ed.; 1973; Vol. 9, pp 387–392.

(15) See refs 1c, 1d, and Gehring, H. *Biochemistry* **1984**, *23*, 6335–6340.

(16) Abortive transamination means the transamination catalyzed by PLP-dependent enzymes different from aminotransferases as side reactions (Miles, E. W. *Transaminases*; Christen, P., Metzler, D. E., Eds.; John Wiley & Sons: New York, 1985; pp 470–481).

can readily determine stereospecificity of an aminotransferase for both the C-4′ hydrogen transfer and the hydrogen exchange of the bound PMP by means of these stereospecifically tritiated PMP.

Conclusion

D-AAT and BCAT, which show a significant sequence similarity catalyze the *pro-R* hydrogen transfer at C-4′ of the plane of the conjugated π-system of the cofactor–substrate Schiff base intermediate, that is, they catalyze the *re*-face proton transfer in contrast with all other aminotransferases so far studied catalyzing the *si* face proton transfer. The conformation of the active sites of D-AAT and BCAT, in particular the topographical situation of the external Schiff base and the catalytic base that abstracts the amino donor α-hydrogen, are probably homologous with each other but different from those of other aminotransferases. Both D-AAT and BCAT are unique not only in the primary structure and CD spectrum but also in stereospecificity of the C-4′ hydrogen transfer of the external Schiff base intermediate.

Experimental Section

Materials. D-AAT of *Bacillus* sp. YM-1[4] was purified from the recombinant cells of *E. coli* HB101 bearing the plasmid pICT113p as described previously.[8] BCAT of *E. coli* K-12[5] was kindly supplied by Dr. K. Inoue of Osaka Medical College, Takatsuki, Japan. Both enzymes were shown to be homogeneous by SDS-polyacrylamide gel electrophoresis. Pig heart AspAT, calf intestine alkaline phosphatase, horseradish peroxidase (grade I), pig heart malate dehydrogenase, and rabbit muscle lactic dehydrogenase were obtained from Boehringer Mannheim (Germany); L-glutamate oxidase was kindly gifted from Dr. H. Kusakabe of Yamasa Shoyu, Choshi, Japan; ^2H_2O (99.75%) from Commissariat a L'Energie Atomique (France); ^3H_2O (3.7 GBq/g) from Du Pont (U.S.A.). The other chemicals were of analytical grade.

Enzyme and Protein Assays. AspAT was assayed at 30 °C by a modification of the malate dehydrogenase-coupled method.[17] The reaction mixture contained 100 μmol of tris(hydroxymethyl)aminomethane (Tris)-HCl buffer (pH 8.0), 30 μmol of L-aspartate, 5 μmol of α-ketoglutarate, 0.2 μmol of NADH, 8 units of malate dehydrogenase, and AspAT in a final volume of 1 mL. The reaction was monitored by measurement of a decrease in the absorbance at 340 nm with a Shimadzu MPS-2000 recording spectrophotometer. D-AAT was assayed with D-alanine as a substrate according to the method of Tanizawa et al.[4] except that 5 mM α-ketoglutarate was used, and the reaction temperature was 30 °C. One unit of AspAT and D-AAT is defined as the amount of enzyme that catalyzes the formation of 1 μmol of NAD/min. BCAT was assayed by determination of the L-glutamate formed with L-glutamate oxidase. The reaction mixture contained 100 μmol of Tris-HCl buffer (pH 8.0), 10 μmol of L-valine, 5 μmol of α-ketoglutarate, and BCAT in a final volume of 1 mL. The reaction was performed at 30 °C for 30 min and stopped by addition of 50 μL of 12 M HCl. After centrifugation, 500 μL of the supernatant solution was neutralized with NaOH and incubated with 0.5 unit of L-glutamate oxidase in the mixture (1 mL) containing 100 μmol of Tris-HCl buffer (pH 8.0), 24 μmol of *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-*m*-toluidine, 24 μmol of 4-aminoantipyrine, and 3 units of horse radish peroxidase. The reaction was carried out at 30 °C for 1 h, and an increase in the absorbance at 555 nm was measured. One unit of BCAT was defined as the amount of enzyme that produces 1 μmol of L-glutamate/min. Enzyme concentrations were determined from their absorption coefficients at 280 nm ($A^{1\%}_{280nm}$ = 15.1, 12.3, and 15.0 for AspAT,[18] D-AAT,[6] and BCAT,[19] respectively).

Preparation of Apoenzymes. Apo-AspAT and apo-BCAT were prepared according to the procedure by Scardi et al.[20] AspAT or BCAT (5–10 mg) was incubated with 30 mM L-cysteinesulfinate in 100 mM Tris-HCl (pH 8.5) at 30 °C for 90 min. The reaction mixture was then dialyzed against 0.5 M NaH_2PO_4 at pH 5.0 (for AspAT) or pH 6.5 (for BCAT), followed by dialysis against 10 mM HEPES buffer (pH 8.0). Formation of the apo enzymes was followed by determination of the enzyme activity in the presence or absence of 10 μM PLP. Apo-D-AAT was prepared as follows: D-AAT (about 0.5 mg/mL) was dialyzed against 6 M guanidium HCl for 12 h and then dialyzed against 10 mM HEPES buffer (pH 8.0) at 4 °C for 16 h. The resulting apo-D-AAT was fully active (95–100%) in the presence of 10 μM PLP.

Isolation and Purification of PL, PM, and PMP. PL and PM were isolated and purified from D-AAT and BCAT as follows. After the enzyme protein was removed by ultrafiltration with Amicon Centriprep-10 concentrator, a reaction mixture containing PL and PM was subjected to an Amberlite IRA401 column chromatography (Japan Organo, Cl^- form, 2.8 × 9.5 cm). PL and PM were washed out with H_2O and 5 mM HCl, respectively. PL and PM were further purified with an SP-Toyopearl column (Tosoh, Japan, H^+ form, 1.6 × 12.5 cm) on an LKB 2150PU-2151UV HPLC system. A 70-min gradient from 0–0.5 M HCl in H_2O was used to elute PL (retention time, 29 min) and PM (52 min). PMP was purified by an ion-exchange column chromatography as follows. A reaction mixture containing PMP was applied onto an Amberlite IRA401 column (OH^- form). After the column was washed with water, PMP was eluted with 0.2 M CH_3COOH. PMP was also purified by a reverse-phase column chromatography, with an Ultron SC-18 column (Shinwa-Kako, Japan, 0.46 × 15 cm) equipped on a Shimadzu LC-6A HPLC system. PMP was eluted at 4.3 min with 0.1% trifluoroacetic acid with the flow rate of 1 mL/min. The concentrations of PL, PM, and PMP were determined by HPLC with measurement of the absorption at 290 nm.

Preparation of [4′-^{3}H]PMP. [4′-^{3}H]PMP randomly labeled was prepared according to the published method[10] with a minor modification. The reaction mixture (pH 4.5) containing 16.5 μmol of PMP and 5.7 μmol of PLP in 150 μL of ^3H_2O (555 MBq) was incubated at room temperature in the dark for 4 days. The reaction mixture was then applied onto a Dowex 1 column (acetate form, 1 × 7 cm) equilibrated with 0.1 M NaOH. After the column was washed with 0.1 M NaOH and water, PMP was eluted with 0.1 M HCl. PMP was further purified by a reversed-phase column chromatography. Radioactivity was determined with a Packard Tri-Carb scintillation spectrometer with Clear-sol I (Nacalai Tesque, Japan) as a scintillator. The specific radioactivity of [4′-^{3}H]-PMP prepared was 2.44 × 10^6 dpm/μmol.

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 labeled [4′-^{3}H]PMP with apo-BCAT and apo-AspAT, respectively. The reaction mixture (0.5 mL) containing 50 μmol of Tris-HCl buffer (pH 8.0), 87.9 μmol of [4′-^{3}H]PMP, and 147 μmol of apo-AspAT or 176 nmol of apo-BCAT was incubated at 30 °C for 15 h. The unlabeled PMP (8.9 nmol) was added to the mixture as a carrier, and the reaction was stopped by addition of 18 μL of 12 M HCl. The mixture was further incubated at 30 °C for 3 h followed by incubation at 75 °C for 15 min. After centrifugation, the supernatant solution was dried with a Speed Vac Concentrator (Savant, U.S.A.), and the residue was dissolved in 0.5 mL of H_2O. (4′*S*)-[4′-^{3}H]PMP (1.20 × 10^6 dpm/μmol) and (4′*R*)-[4′-^{3}H]PMP (1.08 × 10^6 dpm/μmol) thus obtained were purified by reversed-phase column chromatography.

Stereochemistry of the C-4′ Hydrogen Abstraction from PMP During Half and Overall Reactions by D-AAT and BCAT. The reaction mixture (100 μL) for the half reaction contained 10 μmol of Tris-HCl buffer (pH 8.0), 0.5 μmol of α-ketoglutarate, 1.98 nmol of (4′*S*)-[4′-^{3}H]PMP or (4′*R*)-[4′-^{3}H]PMP, and 5 nmol of apo-enzyme. The reaction was carried out at 30 °C for 15 min and terminated by addition of 100 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 subjected to radioactivity assay. The tritium released from PMP was expressed as a vaporable radioactivity, which was obtained by subtraction of the radioactivity finally remaining from the radioactivity initially added to the reaction mixture. The reaction conditions for the overall transamination reactions were the same as those for the half reaction except that the reaction mixture of AspAT, D-AAT, and BCAT contained 3 μmol of L-aspartate, 1 μmol of D-alanine, and 1 μmol of L-valine, respectively.

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