Pergamon

Bioorganic & Medicinal Chemistry, Vol. 2, No. 7, pp. 605-607, 1994

0968-0896/94 $7.00 + .00

0968-0896(94)00056-5

A Simple Method for Determination of Stereospecificity of Aminotransferases for C-4' Hydrogen Transfer of the Coenzyme§

Katsushi Nishimura,[a] Junko Ito,[b] Tohru Yoshimura,[b] Nobuyoshi Esaki[b] and Kenji Soda[b,*]

[a]*Department of Applied Chemistry and Biotechnology, Yamanashi University, 4-3-11 Takeda, Kofu, Yamanashi 400, Japan*

[b]*Institute for Chemical Research, Kyoto University, Uji, Kyoto-Fu 611, Japan*

Abstract—A simple method was established for determination of the stereospecificity of C-4' hydrogen transfer of the coenzymes (pyridoxal and pyridoxamine). The method is based on the findings that aspartate aminotransferase of pig heart and D-amino acid aminotransferase of *Bacillus* sp. YM-1 catalyze the abstraction of the *pro-S* and *pro-R* proton at C-4' of pyridoxamine, respectively. Pyridoxal is a poor coenzyme, but readily released from the enzyme. It reacts in ^3H_2O with a substrate amino acid and an apo-aminotransferase whose stereospecificity for C-4' hydrogen transfer is to be determined. The resultant pyridoxamine which is tritiated at C-4' is incubated with an apo form of aspartate aminotransferase or D-amino acid aminotransferase and a substrate, α-keto acid. The stereospecificity for the C-4' hydrogen transfer examined is determined by measurement of radioactivity retained in the pyridoxal formed. We showed by means of this method that C-4' hydrogen transfer of coenzyme occurs on the *si* face of the external Schiff base in the transamination reactions of two aspartate aminotransferases of *Bacillus* sp. YM-2 and *Escherichia coli*, and aromatic amino acid aminotransferase of *E. coli*.

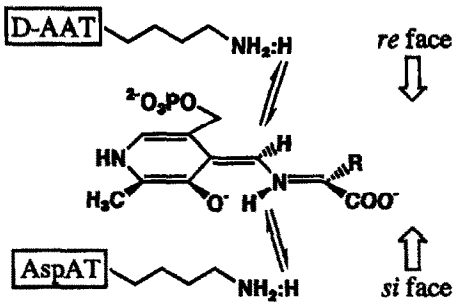
Introduction

In transamination reactions catalyzed by aspartate aminotransferase(AT)s and other pyridoxal 5'-phosphate (PLP) dependent enzymes catalyzing an abortive transamination, a hydrogen is stereospecifically transferred on either the *si* or *re* face at C-4' of the coenzyme moiety of the external Schiff base (Scheme I). This stereospecificity is one of the important characteristics of PLP enzymes catalyzing transamination from the view point of molecular evolution and relationship between the enzyme structure and reaction mechanism. The hydrogen transfer occurs on the *si* face in the transamination reactions by most of the aminotransferases such as aspartate aminotransferase(AspAT)s.[1-3] However, D-amino acid aminotransferase (D-AAT) which belongs to the different group from those of AspAT and other aminotransferases in the primary structures,[4] catalyzes the C-4' hydrogen transfer on the *re* face as reported recently.[5] We describe here a simple method for the determination of the stereospecificity of aminotransferases on the C-4' hydrogen transfer of the coenzyme in enzymatic transamination reactions by combination of apo-AspAT of pig heart and apo-D-AAT of *Bacillus* sp. YM-1.

Results and Discussion

Evaluation of the method with AspAT and D-AAT

The method for determination of the stereospecificity for the C-4' hydrogen transfer described in the Experimental Section was evaluated with AspAT (pig heart) and D-AAT (*Bacillus* sp. YM-1). The specific radioactivities (dpm/μmol) of pyridoxamine (PM) prepared with apo-AspAT and apo-D-AAT were 300,000 and 80,000, respectively. PM prepared with apo-AspAT was converted into pyridoxal (PL), whose specific radioactivities were 18,000 and 230,000, by using apo-AspAT and apo-D-AAT, respectively. The specific radioactivity of PL obtained from PM produced with apo-D-AAT were 70,000 (with apo-AspAT) and 24,000 (with apo-D-AAT). The results are summarized in Table 1. When the same enzyme was used for both protonation and deprotonation of C-4' of the coenzyme, most radioactivity of PM was released into the solvent during the conversion of PM to PL. In contrast, when the mutually different enzymes were used, the radioactivity of PM was kept in the PL formed from PM. These results indicate that the C-4' hydrogen transfer in D-AAT and AspAT reactions occurs on the mutually opposite side of the bound coenzyme. This is compatible with the results obtained by other methods,[5] and verifies the present method for determination of the stereospecificity described here.



Scheme I.

§Dedicated to Professor Bryan Jones on the occasion of his 60th birthday.

Table 1. Determination of stereospecificity of ATs

AT examined	$\frac{\text{dpm/}\mu\text{mol of PL}}{\text{dpm/}\mu\text{mol of PM}} \times 100$ (%) prepared with		face of C-4' Protonation
	AspAT	D-AAT	
AspAT (pig heart)	6.2	76	*si*
D-AAT (*Bacillus* sp. YM-1)	87	29	*re*
AspAT (*Bacillus* sp. YM-2)	14	75	*si*
AspAT (*E. coli*)	11	81	*si*
AroAT (*E. coli*)	28	85	*si*

Release and retention of the radioactivity were found incomplete as described above. We examined whether or not the hydrogen exchange occurs in the absence of enzymes. Some degree of radioactivity was detected in both PM and PL after incubation of 2.5 mM PL, 2.5 mM PM, 2.5 mM pyruvate, and 7.5 mM D-alanine for 3 h in the same buffer containing ^3H_2O as described in the Experimental Section. Dunathan[6] also reported a similar finding; hydrogen exchanges with deuterium of the solvent happened not only at the 4-methylene group but also at the 2-methyl group and 6-position of the pyridine ring during the incubation of PM and PL without enzymes. These nonenzymatic and non-site-specific proton exchanges must have resulted in the incomplete release and retention of the radioactivity found in our experiments (Table 1).

*Determination of the stereospecificity of AspAT (*Bacillus *sp. YM-2), AspAT (*E. coli*), and AroAT (*E. coli*) for the C-4' hydrogen transfer*

The above method was applied to the determination of stereospecificity of AspAT (*Bacillus* sp. YM-2), AspAT (*E. coli*), and aromatic amino acid aminotransferase (AroAT, *E. coli*) for the C-4' hydrogen transfer. As shown in Table 1, the C-4' hydrogen transfer of the coenzyme occurred on the *si* face in all three transamination reactions; these three enzymes are classified into the same group as AspATs. This agrees with the results deduced from the comparison of their primary structures.[7–9]

Conclusion

We established a simple method for determination of stereospecificity of aminotransferases for C-4' hydrogen transfer of the coenzyme. The method is reliable because two enzymes whose stereospecificities are mutually opposite are used for the analysis. The method demonstrated *S*-specific protonation at C-4' of PL by AspAT of *Bacillus* sp. YM-2 and *E. coli*, and AroAT of *E. coli*.

Experimental Section

Materials

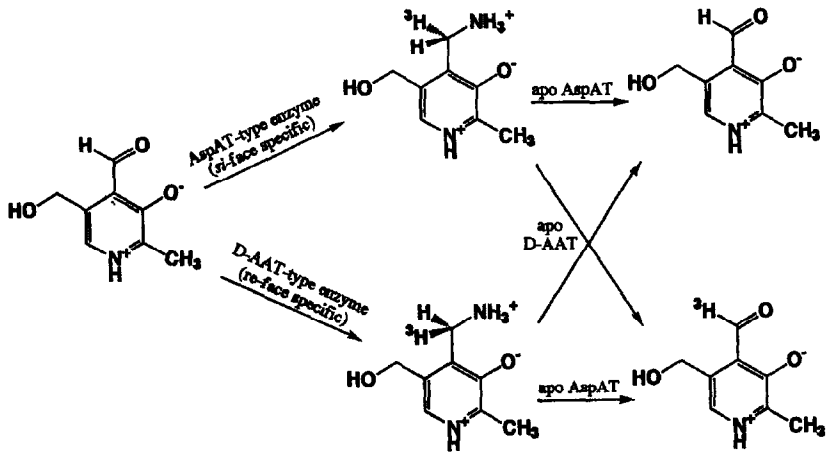
AspAT of pig heart was purchased from Boehringer Mannheim (Germany). D-AAT of *Bacillus* sp. YM-1 was purified from the recombinant cells of *E. coli* HB101 bearing the plasmid pICT113p as described previously.[10] The enzyme purified was shown to be homogeneous by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. D-Alanine, and SP-grade HCl were obtained from Nacalai Tesque (Japan). ^3H_2O (3.7 GBq/g) was purchased from Du Pont (U.S.A.). The other chemicals were the highest grades commercially available. AspAT of *Bacillus* sp. YM-2 was prepared as described previously.[11] AspAT (*E. coli*) and AroAT (*E. coli*) were kindly supplied by Dr K. Inoue and Prof. H. Kagamiyama, Osaka Medical College, Japan.

Preparation of apoenzymes

Apo-AspAT (pig heart), apo-AspAT (*Bacillus* sp. YM-2), apo-AspAT (*E. coli*), and apo-AroAT (*E. coli*) were prepared according to the procedures by Scardi *et al.*[12] The enzymes (2 mg/mL) were incubated with 30 mM L-cysteinesulfinate in 100 mM Tris–HCl buffer (pH 8.5) at 30 °C for 100 min. The coenzyme was thoroughly resolved from the enzyme protein by dialysis against 0.5 M NaH_2PO_4 (AspATs) or 1 M potassium phosphate buffer (pH 7.5, AroAT). The apo-ATs thus obtained were used after dialysis against 10 mM HEPES buffer (pH 7.3) containing 0.01 % 2-mercaptoethanol. Apo-D-AAT was prepared as described previously.[5] D-AAT (about 0.5 mg/mL) was dialyzed against 6 M guanidine hydrochloride for 12 h, then refolded by dialysis against 10 mM HEPES buffer (pH 8.0) containing 0.01 % 2-mercaptoethanol. The resulting apo-D-AAT had full activity (95–100 %) by addition of PLP.

Isolation, purification, and determination of PL and PM

PL and PM were isolated and purified from the ATs as follows. After the enzyme protein was removed by ultrafiltration with a Millipore Molcut II concentrator (U.S.A.), a reaction mixture containing PL and PM was subjected to an SP-Toyopearl column (Tosoh, Japan, H^+ form, 1.6 × 12.5 cm) chromatography on an LKB 2150PU–2151UV HPLC system (Pharmacia, Sweden). A 70 min gradient from 0 to 0.5 M HCl in H_2O was used to elute PL (retention time, 29 min) and PM (52 min) after washing out ^3H_2O with H_2O. Elution of PL and PM was monitored by measurement of absorbance at 290 nm. For the quantitative analysis, PL and PM were appropriately diluted with 0.2 M CH_3COONa buffer (pH 4.8) and applied to a Pharmacia Mono-S column (0.5 × 5 cm, Sweden) equilibrated with 0.1 M $NH_4H_2PO_4$ equipped on a Shimadzu LC-6A HPLC system (Japan). PL (4.5 min)



Scheme II.

and PM (9 min) eluted were monitored and determined by measurement of absorbance at 320 nm. Radioactivity of PL or PM was determined with a Packard Tri-Carb 3320 liquid scintillation spectrometer (U.S.A.) in 10 mL of Clear-sol I (Nacalai Tesque, Japan). The counting efficiency was about 53 %.

Procedure for determination of stereospecificity of C-4' hydrogen transfer

PL and PM are poor coenzymes, but readily released from the enzyme. PL (6 mM) and substrate amino acid (12 mM) were incubated with apo-AT (0.5–1 mg), whose stereospecificity is to be determined, in 100 mM bis–tris propane buffer (pH 8.5) in 0.5 mL of ^3H_2O (740 MBq/mL) at 37 °C for 2 h. After enzyme protein was removed by ultrafiltration with a Millipore Molcut II concentrator, PM formed was isolated and purified by SP-Toyopearl column chromatography. A part of the purified PM was subjected to a Mono-S column for a quantitative analysis. Radioactivity of PM eluted was determined as described above. A half amount of the remaining PM was incubated with apo-AspAT (pig heart) and the rest with apo-D-AAT; about 1 mM PM and 6 mM α-ketoglutarate were incubated with apo-AspAT (0.4 mg, 37 °C, 120 min) or apo-D-AAT (0.8 mg, 45 °C, 150 min) in 0.5 mL of 100 mM bis–tris propane buffer (pH 8.5). PL formed was isolated, purified, and determined. Its radioactivity was measured by the same methods as described for PM. When the stereospecificity of the AT examined was identical to that of the authentic AT (either AspAT or D-AAT), the radioactivity of PM was released to the solvent (Scheme II). In contrast, when they showed different stereospecificity to each other, the radioactivity of PM was found exclusively in the PL formed (Scheme II).

(*Received* 4 *January* 1994; *accepted* 28 *February* 1994)

References

1. Dunathan, H. C. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1971**, *35*, 79.

2. Rétey, J.; Robinson, J. A. In *Stereospecificity in Organic Chemistry and Enzymology*, pp. 151–184, Verlag Chemie; Basel, 1982.

3. Palcic, M. M.; Floss, H. G. In *Vitamin B6 Pyridoxal Phosphate*, pp. 25–68, John Wiley & Sons; New York, 1986.

4. Tanizawa, K; Asano, S.; Masu, Y.; Kuramitsu, S.; Kagamiyama, H.; Tanaka, H.; Soda, K. *J. Biol. Chem.* **1989**, *264*, 2450.

5. Yoshimura, T.; Nishimura, K.; Ito, J.; Esaki, N.; Kagamiyama, H.; Manning, J. M.; Soda, K. *J. Am. Chem. Soc.* **1993**, *115*, 3897.

6. Dunathan, H. C.; Davis, L.; Gilmer Kury, P.; Kaplan, M. *Biochemistry* **1968**, *7*, 4532.

7. Sung, M. H.; Tanizawa, K.; Tanaka, H.; Kuramitsu, S.; Kagamiyama, H.; Hirotsu, K.; Okamoto, A.; Higuchi T.; Soda, K. *J. Biol. Chem.* **1991**, *266*, 2567.

8. Mehta, P. K.; Hale, T. I.; Christen, P. *Eur. J. Biochem.* **1989**, *186*, 249.

9. Mehta, P. K.; Hale, T. I.; Christen, P. *Eur. J. Biochem.* **1993**, *214*, 549.

10. Nishimura, K.; Tanizawa, K.; Yoshimura, T.; Esaki, N.; Futaki, S.; Manning, J. M.; Soda, K. *Biochemistry* **1991**, *30*, 4072.

11. Sung, M. H.; Tanizawa, K.; Tanaka, H.; Kuramitsu, S.; Kagamiyama, H; Soda, K. *J. Bacteriol.* **1990**, *172*, 1345.

12. Scardi, V.; Scotto, P.; Iaccarino, M.; Scarano, E. *Biochem. J.* **1963**, *88*, 172