ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 12 (2001) 145–150

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

www.elsevier.com/locate/molcatb

Stereochemistry of the hydrogen abstraction from pyridoxamine phosphate catalyzed by alanine racemase of *Bacillus stearothermophilus*

Akira Watanabe [a], Tohru Yoshimura [a], Young Hee Lim [a], Yoichi Kurokawa [a], Kenji Soda [b], Nobuyoshi Esaki [a,*]

[a] *Laboratory of Biofunctional Molecules, Institute for Chemical Research, Kyoto University, Uji, Kyoto 611, Japan*

[b] *Faculty of Engineering, Kansai University, Yamate-cho, Suita, Osaka 564-8680, Japan*

Received 16 September 1999; accepted 22 September 1999

Abstract

Alanine racemase of *Bacillus stearothermophilus* catalyzes transamination as a side reaction. Stereospecificity for the hydrogen abstraction from C-4′ of pyridoxamine 5′-phosphate occurring in the latter half transamination was examined. Both apo-wild-type and apo-fragmentary alanine racemases abstracted approximately 20 and 80% of tritium from the stereospecifically-labeled (4′*S*)- and (4′*R*)-[4′-^{3}H]PMP, respectively, in the presence of pyruvate. Alanine racemase catalyzes the abstraction of both 4′*S*- and 4′*R*-hydrogen like amino acid racemase with broad substrate specificity. However, *R*-isomer preference is a characteristic property of alanine racemase.

Keywords: Alanine racemase; Pyridoxal 5′-phosphate; Transamination; Stereochemistry

1. Introduction

Alanine racemase (EC 5.1.1.1) depending on pyridoxal 5′-phosphate (PLP) catalyzes the interconversion between L- and D-alanine and occurs in various bacteria and several invertebrates [1,2]. The enzyme plays a central role in the metabolism of D-alanine, an essential component of the peptidoglycans in the bacterial cell wall.

The generally accepted mechanism of alanine racemase reaction is shown in Fig. 1 [3]. PLP bound with the active-site lysyl residue (A or E) reacts with a substrate to form an external Schiff base (B or D) through transaldimination. The subsequent α-hydrogen abstraction results in the formation of a resonance-stable deprotonated intermediate (C). If reprotonation occurs at the α-carbon of the substrate moiety on the opposite face of the planar intermediate (C), then an antipodal aldimine (D or B) is formed. The ε-amino group of the lysine residue is substituted for the isomerized amino acid through transaldimination, and the internal aldimine (E or A) is regenerated.

We found that alanine racemase from *Bacillus stearothermophilus* catalyzes transamination as a side reaction [4]. The transamination is probably attained through a sequence A → B → C → F (or G) → H (Fig. 1). An equivalent route can be delineated for

* Corresponding author. Tel.: +81-774-38-3240; fax: 81-774-38-3248.
E-mail address: esaki@scl.kyoto-u.ac.jp (N. Esaki).

1381-1177/01/$ – see front matter
PII: S1381-1177(00)00215-0

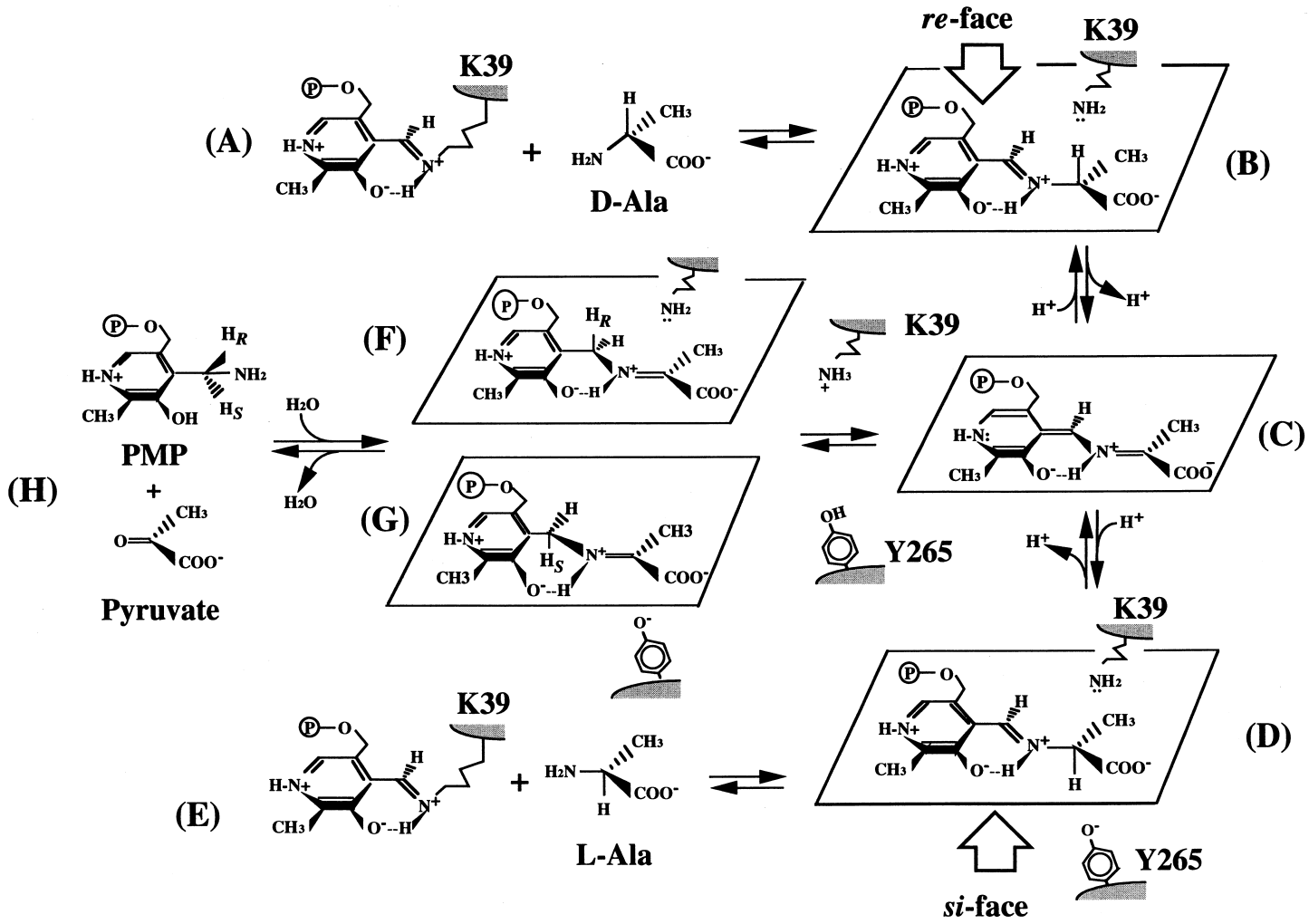

Fig. 1. Reaction mechanism of alanine racemase. The amino acid residues, K39 and Y265, are the proposed catalytic bases of alanine racemase of *B. stearothermophilus*.

the antipode: E → D → C → G (or F) → H. During the transamination, α-hydrogen of the substrate is transferred between the C-2 of the substrate and the C-4′ of the cofactor. In the aminotransferase reaction, the hydrogen is stereospecifically transferred on the *si*- or *re*-face of the plane of the substrate-cofactor complex. Aspartate aminotransferase (AspAT) [5], alanine aminotransferase [6], dialkylamino acid aminotransferase [7], pyridoxamine pyruvate aminotransferase [8], and ornithine aminotransferase [9] catalyze *si*-face-specific hydrogen transfer. Tryptophan synthase [10], glutamate decarboxylase [11], and L-serine hydroxylmethyltransferase [12] also catalyze the *si*-face specific hydrogen transfer during the transamination catalyzed as a side reaction. On the other hand, D-amino acid aminotransferase (D-AAT) [13] and branched chain L-amino acid aminotransferase (BCAT) [13] catalyze *re*-face specific hydrogen transfer. Stereospecificity for the hydrogen transfer probably reflects the geometrical relationship between the catalytic base abstracting the α-hydrogen of the substrate and the cofactor. The idea is supported by the active-site structures of aminotransferases: the catalytic residues (PLP-binding lysyl residues) of D-AAT and BCAT extend their side chain toward the *re*-face of PLP in contrast to the catalytic lysine of AspAT, which lies on the *si*-face of the coenzyme.

Recently, we showed that PLP-dependent amino acid racemase with broad substrate specificity catalyzed non-stereospecific hydrogen transfer during the transamination catalyzed as a side reaction [14]. We speculate that the hydrogen transfer is mediated by the two catalytic residues located opposite across the cofactor. However, neither the three-dimensional structures nor the primary structure of the enzyme have been clarified yet. To confirm the relationship between the stereospecificity and active-site structure of the enzyme, we studied the stereospecificity for the hydrogen transfer catalyzed by the alanine racemase of *B. stearothermophilus*. The alanine racemase reaction has been proved to proceed through a two-base mechanism with PLP-binding lysine 39 (K39) and tyrosine 265 (Y265), which are situated opposite across the cofactor [15–19]. We expected that the enzyme would catalyze the non-stereospecific hydrogen transfer during the transamination.

2. Materials and methods

2.1. Materials

The wild-type [4] and fragmentary [20] alanine racemases of *B. stearothermophilus* and the D-AAT of *Bacillus* sp-YM-1 [21] were prepared as described previously. L-Alanine dehydrogenase was a gift from Dr. Yonekazu. Sakamoto of Unitika (Osaka, Japan). BCAT of *Escherichia coli* K-12 was kindly supplied by Professor Hiroyuki Kagamiyama and Dr. Katsura. Inoue of Osaka Medical College, Takatsuki, Japan [22]. AspAT and L-lactate dehydrogenase were purchased from Boehringer Mannheim (Germany). All other reagents and chemicals were of analytical grade.

2.2. Enzyme and protein assays

Alanine racemase was assayed as described previously [4]. Protein concentrations were determined by measurement of absorbance at 280 nm or by the method of Bradford with bovine serum albumin as a standard [23]. The absorption coefficient at 280 nm, $\varepsilon_M = 4.78 \times 10^4\ M^{-1}\ cm^{-1}$, was estimated from the amino acid composition of the enzyme [24].

2.3. Preparation of apoenzymes

The wild-type and fragmentary alanine racemases were dialyzed against a 100 mM potassium phosphate buffer (pH 8.0) containing 30 mM hydroxylamine and 0.01% 2-mercaptoethanol for 24 h at 4°C and then dialyzed against a 100 mM potassium phosphate buffer (pH 8.0) containing 0.01% 2-mercaptoethanol overnight at 4°C [4]. Formation of apoenzyme was confirmed by measurement of the activity in the presence or absence of 20 μM PLP. Apo-AspAT, apo-BCAT, and apo-D-AAT were prepared as described in our previous paper [13].

2.4. Preparation of (4′*S*)- and (4′*R*)-[4′-^{3}H]pyridoxamine 5′-phosphate

(4′*S*)- and (4′*R*)-[4′-^{3}H]pyridoxamine 5′-phosphate (PMP) were prepared from randomly labeled [4′-^{3}H]PMP with apo-AspAT and apo-BCAT, respectively, as described previously [13]. Radioactivity was determined with a Beckman LS-6500 scintillation

counter using Clear-sol II (Nacalai Tesque, Japan) as a scintillator. The specific radioactivity of the prepared (4′*S*)- and (4′*R*)-[4′-^{3}H]PMP were 1.54×10^6 dpm/μmol and 1.35×10^6 dpm/μmol, respectively. Stereospecificities for the labelling were confirmed by measuring the tritium released from each prepared PMP by the incubation with apo-AspAT and apo-BCAT in the presence of α-ketoglutarate.

2.5. C-4′-hydrogen abstraction from PMPs during the transamination

The reaction mixture with alanine racemase contained a 10 μmol potassium phosphate buffer (pH 8.0), 1.5 nmol of (4′*S*)-[4′-^{3}H]PMP or 1.2 nmol of (4′*R*)-[4′-^{3}H]PMP, each 5.0 nmol of apo-wild-type or apo-fragmentary enzymes, and 5.0 nmol of sodium pyruvate in a final volume of 100 μl. The reaction was started by the addition of pyruvate, carried out at 30°C for 3 h, and terminated by the addition of 100 μl of 1 M HCl. The reaction mixture was dried for the removal of the liberated tritium with a Speed Vac Concentrator. Then, the residue was dissolved in 200 μl of H_2O and subjected to the radioactivity assay. The reaction mixture with AspAT or D-AAT contained 10 μmol Tris–HCl (pH 8.0), 1.0 nmol of (4′*S*)-[4′-^{3}H]PMP or (4′*R*)-[4′-^{3}H]PMP, 10 nmol sodium α-ketoglutarate, and 168 μg of apo-AspAT or apo-D-AAT. The reaction was started by the addition of α-ketoglutarate, carried out at 30°C for 15 min, and terminated by the addition of 100 μl of 1 M HCl. Subsequent steps were the same as those for the reactions with alanine racemases.

3. Results and discussion

During the latter half reaction of transamination, the conversion of PMP to PLP, one of the two hydrogens at C-4′ of PMP is abstracted and transferred to the C-2 of the amino acceptor. We have established a simple method to determine the stereospecificity for the C-4′ hydrogen abstraction catalyzed by PLP enzymes by means of stereospecifically labeled [4′-^{3}H]PMP [9,13,14]. This is based on the finding that tritium abstracted from [4′-^{3}H]PMP is eventually released to solvent water through exchange with solvent hydrogen [13]. Therefore, we can readily determine the stereospecificity by measuring the radioactivity in solvent water.

We prepared stereospecifically labeled (4′*R*)- and (4′*S*)-[4′-^{3}H]PMP according to the stereospecific exchange of the C-4′ tritium of [4′-^{3}H]PMP with solvent hydrogen catalyzed by apo-AspAT and apo-BCAT, respectively. The prepared (4′*R*)- and (4′*S*)-[4′-^{3}H]PMP released tritium by incubation with apo-D-AAT and apo-AspAT, respectively, in the presence of α-ketoglutarate (Table 1). These results confirmed the stereospecificities of the labelling of prepared PMPs, because apo-AspAT and apo-D-AAT specifically abstracted the 4′*S*- and 4′*R*-hydrogen from C-4′ of PMP, respectively, under the conditions [13].

We studied the tritium liberation from (4′*S*)- or (4′*R*)-[4′-^{3}H]PMP catalyzed by the apo-wild-type and apo-fragmentary alanine racemases. Fragmentary alanine racemase is a mutant enzyme consisting of two polypeptides of the alanine racemase subunit cleaved near the hinge region between the domains, showing

Table 1
Release of tritium from stereospecifically labeled [4′-^{3}H]PMPs catalyzed by wild-type and fragmentary alanine racemases

	(4′*R*)-[4′-^{3}H]PMP ^{3}H released		(4′*S*)-[4′-^{3}H]PMP ^{3}H released	
	(dpm)	(%)	(dpm)	(%)
Apo-wild-type ar[a]	1190[b]	74	513[c]	22
Apo-fragmentary ar[a]	1410[b]	87	526[c]	23
Without enzyme	0[d]	0	0[e]	0
Apo-aspAT	0[d]	0	1210[e]	79
Apo-D-AAT	860[d]	64	36[e]	2.3

[a] Alanine racemase from *B. stearothermophilus*.
[b] The initial radioactivity in the reaction mixture was 1620 dpm.
[c] The initial radioactivity in the reaction mixture was 2310 dpm.
[d] The initial radioactivity in the reaction mixture was 1340 dpm.
[e] The initial radioactivity in the reaction mixture was 1540 dpm.

about 25% of the catalytic efficiency (V_{max}/K_m) of the wild-type enzyme [20]. As shown in Table 1, the wild-type alanine racemase abstracted 22% and 74% of tritium of the (4′*S*)- and (4′*R*)-[4′-^{3}H]PMP, respectively. This was also the case with the fragmentary enzyme: it abstracted 23% and 87% of tritium of the (4′*S*)- and (4′*R*)-[4′-^{3}H]PMP, respectively. Alanine racemase abstracts both 4′*S*- and 4′*R*-hydrogens from PMP during transamination, like amino acid racemase with broad substrate specificity. However, the *R*-isomer preference is characteristic of alanine racemase: amino acid racemase with broad substrate specificity abstracts the 4′*R*- and 4′*S*-hydrogen with similar rates. This probably reflects the difference in the active site structure between the two enzymes.

Crystallographic studies of the alanine racemase from *B. stearothermophilus* [15–17] have suggested that the enzyme uses two individual base catalysts, K39 and Y265, specifically abstracting α-hydrogen from each alanine enantiomer. We have studied the role of K39 by means of site-directed mutagenesis and chemical rescue analyses, obtaining clear evidence that K39 acts as the base catalyst specific to D-alanine [18]. We have found that the enzyme uses another residue functioning as the base catalyst specific to L-alanine, which is most probably Y265. Recently, Sun and Toney presented evidence supporting our proposal by site-directed mutagenesis studies of arginine 219 [19], which interacts with Y265 via a hydrogen-bonding network. According to crystallographic studies, the topographical situation of K39 of alanine racemase is identical to that of the catalytic residue abstracting α-hydrogen of D-alanine of D-AAT (K145) with respect to the relationship with the bound cofactor [15,25]. D-AAT catalyzes the hydrogen transfer between the *pro-R* position of the C-4′ of PMP and C-2 of D-alanine on the *re*-face of the substrate-cofactor complex [13]. K39 of alanine racemase, the base catalyst specific to D-alanine, probably catalyzes the abstraction of *pro-R* hydrogen of PMP; the counterpart residue, Y265, being specific to L-alanine, abstracts *pro-S* hydrogen (Fig. 1). The *R*-isomer preference of alanine racemase in the C-4′ hydrogen abstraction probably derives from the asymmetry of the catalytic residues.

The stereospecificity for the hydrogen transfer reflects the structure of the active site of pyridoxal enzymes, especially the topographical relationship between the catalytic base for the hydrogen abstraction and the bound coenzyme. Therefore, stereospecificity has been discussed in relation to the molecular evolution of the pyridoxal enzymes [12,13,26]. Alanine racemase belongs to the same family of proteins containing mammalian ornithine decarboxylase and meso-α,ε-diaminopimelate (DAP) decarboxylase with little similarity to other PLP enzymes [27]. In this family, alanine racemase is the only enzyme whose three-dimensional structure has been clarified: the folding of alanine racemase is completely different from those of other PLP-enzymes. It may be interesting to examine whether DAP decarboxylase and ornithine decarboxylase catalyze the removal of tritium non-stereospecifically from C-4′ of both (4′*S*)- and (4′*R*)-[4′-^{3}H]-PMP in the same manner as alanine racemase. We have shown that the decarboxylation of DAP catalyzed by DAP decarboxylase proceeds through inversion of the configuration of the α-carbon of DAP [28]. This indicates that the enzyme function must be conducted on both sides of the plane of the substrate-PLP complex so as to decarboxylate on one side and introduce a proton on the other side: the DAP decarboxylase reaction is homologous to the racemase reaction in this respect. It would be interesting to clarify the relationship between the stereospecificity for hydrogen abstraction and structure of PLP enzymes from the viewpoint of molecular evolution.

References

[1] C.T. Walsh, J. Biol. Chem. 264 (1989) 2393.
[2] E. Fujita, E. Okuma, H. Abe, Comp. Biochem. Physiol. 116A (1997) 83.
[3] W.S. Faraci, C.T. Walsh, Biochemistry 27 (1988) 3267.
[4] Y. Kurokawa, A. Watanabe, T. Yoshimura, N. Esaki, K. Soda, J. Biochem. 124 (1998) 1163.
[5] P. Besmer, D. Arigoni, Chimia 23 (1969) 190.
[6] E. Austermuhle-Bertrola, Ph.D. Dissertation, ETH, Zurich, 1973.
[7] G.B. Bailey, T. Kusamrarn, K. Vuttivej, Fed. Proc. 29 (1970) 857.
[8] J.E. Ayling, H.C. Dunathan, E.E. Snell, Biochemistry 7 (1968) 4532.
[9] K.H. Jhee, T. Yoshimura, N. Esaki, K. Soda, Biochemistry 35 (1996) 9792.
[10] H.C. Dunathan, J.G. Voet, Proc. Natl. Acad. Sci. U. S. A. 71 (1974) 3888.
[11] B.S. Sukhareva, H.C. Dunathan, A.E. Braunstein, FEBS Lett. 15 (1971) 241.

[12] J.G. Voet, D.M. Hindenlang, T.J.J. Blanck, R.J. Ulevitch, R.G. Kallen, H.C. Dunathan, J. Biol. Chem. 248 (1973) 841.
[13] T. Yoshimura, K. Nishimura, J. Ito, N. Esaki, H. Kagamiyama, J.M. Manning, K. Soda, J. Am. Chem. Soc. 155 (1993) 3897.
[14] Y.H. Lim, T. Yoshimura, Y. Kurokawa, N. Esaki, K. Soda, J. Biol. Chem. 273 (1998) 4001.
[15] J.P. Shaw, G.A. Petsko, D. Ringe, Biochemistry 36 (1997) 1329.
[16] G.F. Stamper, A.A. Morollo, D. Ringe, Biochemistry 37 (1998) 10438.
[17] A.A. Morollo, G.A. Petsko, D. Ringe, Biochemistry 38 (1999) 3293.
[18] A. Watanabe, Y. Kurokawa, T. Yoshimura, T. Kurihara, K. Soda, N. Esaki, J. Biol. Chem. 274 (1999) 4189.
[19] S. Sun, M.D. Toney, Biochemistry 38 (1999) 4058.
[20] H. Toyama, K. Tanizawa, T. Yoshimura, S. Asano, Y.-H. Lim, N. Esaki, K. Soda, J. Biol. Chem. 266 (1991) 13634.
[21] K. Tanizawa, Y. Masu, S. Asano, H. Tanaka, K. Soda, J. Biol. Chem. 264 (1989) 2445.
[22] K. Inoue, S. Kuramitsu, K. Aki, Y. Watanabe, T. Takagi, M. Nishigai, A. Ikai, H. Kagamiyama, J. Biochem 104 (1998) 777.
[23] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[24] S. Kuramitsu, K. Hiromi, H. Hayashi, Y. Morino, H. Kagamiyama, Biochemistry 29 (1990) 5469.
[25] S. Sugio, G.A. Petsko, J.M. Manning, K. Soda, D. Ringe, Biochemistry 34 (1995) 9661.
[26] H.C. Dunathan, Adv. Enzymol. Relat. Areas Mol. Biol. 35 (1971) 79.
[27] N.V. Grishin, M.A. Phillips, E.J. Goldsmith, Protein Sci. 4 (1995) 1291.
[28] Y. Asada, K. Tanizawa, S. Sawada, T. Suzuki, H. Misono, K. Soda, Biochemistry 20 (1981) 6881.