

Pyridoxal enzymes acting on D-amino acids

Kenji Soda and Nobuyoshi Esaki

Institute for Chemical Research, Kyoto University, Uji, Kyoto-Fu 611,
Japan

Abstract — We isolated a thermophile, *Bacillus* sp. YM-1 which abundantly produced the thermostable D-amino acid aminotransferase (EC 2.6.1.21; D-AAT), and cloned the enzyme gene into *E. coli*. The primary structure of D-AAT was found to be homologous with that of branched-chain L-amino acid aminotransferase (EC 2.6.1.42; BCAT) of *E. coli*. Both enzymes are unique in their stereospecificity of pro-R C-4' hydrogen transfer through the coenzyme-substrate Schiff base intermediates in contrast to various L-amino acid aminotransferases catalyzing the pro-S hydrogen transfer. Thus, D-AAT and BCAT are categorized into the same group on the basis of stereospecificity of the hydrogen transfer, but different from all other aminotransferases. The thermostable alanine racemase (EC 5.1.1.1) of *B. stearothermophilus* has a molecular weight of about 78,000, and consists of two identical subunits (*M_r* 39,000). We generated a mutant which was genetically engineered to produce two polypeptide fragments corresponding to the domains. The fragments associate with each other to form an active structure, which was termed "fragmentary form", and shows about 50% of the specific activity of the wild-type enzyme. The CD study showed that the secondary structure of the fragmentary form is closely similar to that of the wild-type enzyme. We have developed a procedure for the synthesis of various D-amino acids by means of bacterial thermostable D-amino acid aminotransferase, alanine racemase and L-alanine dehydrogenase (EC 1.4.1.1), and yeast formate dehydrogenase (EC 1.2.1.2) with a high yield.

D-AMINO ACID AMINOTRANSFERASE

D-AAT catalyzes transamination between various D-amino acids and α -keto acids, and occurs in bacteria, in particular in the genus *Bacillus*, and in higher plants. We have isolated a thermophile which grows in a medium containing D-amino acids as a nitrogen source and identified as a new *Bacillus* species (ref. 1). The organism (*Bacillus* sp. YM-1) showed a very high activity of D-AAT. The enzyme was purified to homogeneity from cell-extracts of *Bacillus* sp. YM-1. It has a molecular weight of about 62,000, and is composed of two subunits identical in molecular weight (30,000). The gene of the enzyme from *Bacillus* sp. YM-1 was cloned into *E. coli* C600 cells with the vector plasmid pBR322 (ref. 1). The clone cells carrying the plasmid of 4.3-kb

DNA (pICT113) produced the enzyme abundantly: its amount corresponds to about 10% of the total cellular proteins. The enzyme overproduced by the clone cells was purified from cell-extracts about 10-fold to homogeneity in a 60% yield by heat treatment and another three steps. The complete primary structure of the enzyme and the position of the active site lysyl residues were determined from the nucleotide sequence of the gene and the amino acid sequence of tryptic peptides.

Role of Lys-145

Lys-145 of the thermostable D-AAT, which binds PLP, was replaced by Ala or Arg by site-directed mutagenesis (ref. 2). Both mutant enzymes were purified to homogeneity. Their absorption spectra indicated that both mutant enzymes contained PLP bound non-covalently. Even though we found no activity with either mutant enzyme by the standard method, addition of D-alanine to the K145R mutant enzyme by the standard assay method led to a slow decrease in absorption at 392 nm with a concomitant increase in absorption at 333 nm. This result suggests that the enzyme was converted into the pyridoxamine 5'-phosphate (PMP) form. The amount of pyruvate formed was almost equivalent to that of the reactive PLP in the mutant enzyme. Thus, the K145R mutant enzyme is able to catalyze the half-reaction of transamination, though very slowly.

Exogenous amines, such as methylamine, had no effect on the half-reaction with the K145R mutant enzyme (ref. 2). In contrast, the K145A mutant enzyme neither showed the spectral change by addition of D-alanine nor catalyzed pyruvate formation, in the absence of added amine. However, the K145A mutant enzyme catalyzed the half-reaction significantly in the presence of added amine. These findings suggest that a basic amino acid residue such as lysine or arginine is required at the position 145 to catalyze the half-reaction. The exogenous amines are bound non-covalently to the active site to mimic Lys-145, and play a dual role as a base in the formation of Schiff base with PLP, and in the α -hydrogen abstraction.

Stereospecificity of C-4' hydrogen transfer of cofactor

In all reactions catalyzed by aminotransferases studied so far, 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. Comparison of their amino acid sequences shows that all the aminotransferases are considerably homologous with one another (ref. 3). However, D-AAT of *Bacillus* sp. YM-1 and branched-chain L-amino acid aminotransferase (BCAT) of *Escherichia coli* show a significant homology with each other in their primary structures, but are different from all other aminotransferases.

Both PLP and PMP are too tightly bound with the D-AAT protein to dissociate. Accordingly, the stereospecificity 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 coenzymes as poor as pyridoxal (PL), but readily dissociates from the enzyme protein, in the first place (ref. 4). When PL and L-aspartate were incubated with apo-aspartate aminotransferase (AspAT) in deuterium oxide, AspAT catalyzes protonation of PL at the pro-*S* position of the C-4' to produce 4'-S-[4'-²H]PM during transamination. The 4'-S-[4'-²H]PM thus formed was isolated and incubated with α -ketoglutarate and apo-D-AAT in ¹H₂O. The ¹H-NMR spectrum of the PL formed showed no peaks in the region corresponding to the signal of C-4'-¹H of PL. This indicates that the C-4'-¹H was removed from 4'-S-[4'-²H]PM during the D-AAT

reaction. 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.

Incubation of apo-AspAT with [4'-³H]PMP in the absence of an amino acceptor results in a stereospecific exchange of the C-4' *S*-³H with the solvent hydrogen. We observed the similar stereospecific C-4' hydrogen exchange catalyzed by BCAT (ref. 4). When apo-BCAT was incubated with randomly labeled [4'-³H] PMP, the final ratio of the radioactivity released from [4'-³H]PMP to the initial radioactivity was about 50 %. The PMP was isolated and incubated with either apo-AspAT or apo-BCAT. ³H was released only in the reaction with apo-AspAT: the PMP recovered was tritiated specifically at 4'-*S* position. Therefore, BCAT catalyzes the C-4' pro-*R* hydrogen exchange with the solvent hydrogen. When 4'-*R*-[4'-³H]PMP and 4'-*S*-[4'-³H]PMP were prepared and incubated with apo-BCAT, the *R*-specific exchange of ³H of [4'-³H]PMP was catalyzed also by D-AAT.

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. The stereospecificities of D-AAT and BCAT for the hydrogen abstraction were examined by measurement of the ³H release in the reaction of an apo-form of enzyme with the stereospecifically tritiated PMP and α -ketoglutarate (ref. 4). The stereospecificities of the hydrogen abstraction during the overall reactions were also determined with D-alanine and L-valine, respectively by the same procedure (TABLE 1). In the half and overall reactions catalyzed by D-AAT and BCAT, ³H was released exclusively from 4'-*R*-[4'-³H]PMP into the solvent. Thus, D-AAT and BCAT specifically abstract the C4' pro-*R* hydrogen from PMP in both half and overall reactions in contrast to AspAT.

TABLE 1. Stereochemistry of hydrogen withdrawal from PMP in the half and overall reactions by D-AAT and BCAT.

	4'- <i>S</i> -[4'- ³ H]PMP		4'- <i>R</i> -[4'- ³ H]PMP	
	³ H-released dpm	%	³ H-released dpm	%
apo-D-AAT + α -KG	36	1.5	1,681	78.6
apo-D-AAT + α -KG + D-Ala	0	0	1,657	75.9
apo-BCAT + α -KG	229	9.6	1,233	57.6
apo-BCAT + α -KG + L-Val	0	0	1,506	70.4
apo-AspAT + α -KG + L-Asp	1,209	50.9	0	0

Thus, 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: 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.

ALANINE RACEMASE

Alanine racemase (EC 5.1.1.1) catalyzes racemization of L- and D-alanine, providing the D-enantiomer for the synthesis of the peptidoglycan of bacterial cell walls. The enzyme occurs ubiquitously in bacteria. We have cloned the gene of thermostable alanine racemase of *B. stearothermophilus* into *E. coli* with a vector plasmid, pICR301 to produce the enzyme abundantly, and purified it (ref. 5). The enzyme has a molecular weight of about 78,000 and consists of two identical subunits (mol. wt., 39,000), which contain one mole of PLP per subunit. The entire amino acid sequence of the thermostable alanine racemase has been deduced from the DNA sequence of the gene (ref. 6).

Subunit dissociation and unfolding

The guanidine hydrochloride (GdnHCl)-induced subunit dissociation and unfolding of thermostable alanine racemase from *B. stearothermophilus* were studied by circular dichroism (CD), fluorescence and absorption spectroscopies, and gel filtration (ref. 7). The overall process was found to be reversible: more than 75% of the original activity was recovered upon reduction of the denaturant concentration. In the range of 0.6-1.5 M GdnHCl, the dimeric enzyme was dissociated into a monomeric form, which was catalytically inactive. The monomeric enzyme appeared to bind the cofactor pyridoxal-P by a non-covalent linkage, although the native dimeric enzyme binds the cofactor through an aldimine Schiff base linkage. The monomer was mostly unfolded with a transition occurring in the range of 1.8 to 2.2 M GdnHCl.

We examined limited proteolysis of the thermostable alanine racemase with subtilisin (ref. 8). SDS-PAGE showed that the enzyme is composed of two identical subunits with M_r 43,000, and it was cleaved into two major fragments with M_r 14,000 (fragment, F-1) and 29,000 (F-2). The amino acid compositions of F-1 and F-2 were determined and the result suggests that the enzyme is first cleaved by proteolysis around 264Tyr. The limited proteolysis was monitored at intervals by densitometric determination of the stained protein band in polyacrylamide gel. Approximately 85% of the native protein disappeared after incubation for 72 hr, but about 50% of the original activity remained. This suggests that about 15% of the native enzyme was unchanged, and the activity corresponding to 35% of the original activity was probably derived from the peptide fragments produced. Therefore, it is conceivable that the peptide fragments of the thermostable alanine racemase interact with each other more strongly to form an active structure than those of usual thermolabile enzymes.

Construction of fragmentary alanine racemase

Limited proteolysis studies on alanine racemases suggested that the enzyme subunit is composed of two domains. We constructed a mutant gene which tandemly encodes the two polypeptides of the *B. stearothermophilus* enzyme subunit cleaved at the position corresponding to the predicted hinge region (ref. 9). The mutant gene product purified was shown to be composed of two sets of the two polypeptide fragments, and was immunologically identical to the wild-type enzyme. The mutant enzyme, i.e. the fragmentary alanine racemase was active in both directions of the racemization of alanine: the maximum velocity (V_{max}) was about a half of that of the wild-type enzyme; the K_m value was about twice as high as that of the wild-type enzyme. Absorption and CD spectra of the fragmentary enzyme were similar to those of the wild-type enzyme. A single polypeptide corresponding to each domain was attempted to be expressed separately in *E. coli*, but no protein that is reactive with the antibody

against the wild-type alanine racemase was produced. Therefore, it is suggested that the two polypeptide fragments can fold into an active structure only when they are produced simultaneously, and that they correspond to structural folding units in the parental polypeptide chain.

We isolated the large and the small fragments from the fragmentary alanine racemase after denaturation with 6 M GdnHCl. The two fragments were reconstituted and reactivated after the concentration of the denaturant reduced as judged from the result of gel-filtration analysis and the activity measurement. The spectra obtained by summation of the CD spectra of the isolated subunits were close to those of the native fragmentary enzyme. The lysine residue to which PLP is bound in the wild-type enzyme occurs in the large peptide of the fragmentary enzyme. The absorption spectrum of the large peptide indicates that PLP is not bound to it. The large peptide alone showed no appreciable activity, but it was activated by incubation with the small peptide. These indicate that the two fragments are a unit of the folding, and that both fragments are needed to be folded together in order to form an active structure.

ENZYMATIC SYNTHESIS OF D-AMINO ACIDS

We have developed a procedure for the synthesis of D-amino acids on the basis of the stereoselectivity of D-AAT. Various D-amino acids are produced from D-alanine and the keto analogues, and pyruvate formed is aminated to L-alanine by alanine dehydrogenase (EC 1.4.1.1), which is also a thermostable enzyme of *E. coli* clone cells carrying the plasmid that contains the dehydrogenase gene from *B. stearothermophilus*. L-Alanine is racemized by alanine racemase to form D-alanine. NADH is regenerated with formate by catalysis of formate dehydrogenase (EC 1.2.1.2) of a *Candida* yeast.

This procedure is based both on the low stereospecificity and very high substrate specificity of alanine racemase, the high stereoselectivity and low structural specificity of D-AAT. Thus, various D-amino acids are produced from their corresponding keto analogues by the aminotransferase reaction with a consumption of an equimolar amount of ammonia and formate. The reactions of D-AAT, alanine racemase and L-alanine dehydrogenase are reversible, but the formate dehydrogenase reaction is irreversible. The D-amino acids produced are not racemized by alanine racemase.

The standard reaction mixture contained α -ketoglutarate, sodium formate, NH_4Cl / NH_4OH buffer (pH 8.1), DL-alanine, NAD^+ , PLP, and the four enzymes (FDH: AlaR: D-AAT: AlaDH=1:1:1:5 in units) in a total volume of 0.5 ml. After incubation at 50°C for various times, an aliquot was mixed with 12% trichloroacetic acid. Glutamate was shown in the supernatant solution with an automatic amino acid analyzer. On incubation of the reaction mixture for 4 h, nearly 100% of the α -ketoglutarate initially added was converted to D-glutamate, whose e.e. was 97%. To optimize the reaction conditions for the production of D-glutamate, we studied the effect of concentrations of substrates and found that the initial rate of D-glutamate production was highest when 0.2 M α -ketoglutarate, 1 M sodium formate, and 1 M NH_4OH were used. The most appropriate ratio of amounts of the four enzymes was FDH: AlaR: D-AAT:AlaDH=1:3:3:5 in units of each enzyme activity.

Under the optimum conditions thus established, the optically pure D-glutamate was produced from α -ketoglutarate in a large scale reaction mixture (30 ml) with a molar yield of about 100%. D-Methionine, D-leucine, and several other D-amino acids were produced with a high yield in the same manner (TABLE 2). Keto analogues of

histidine, arginine, phenylalanine and tyrosine serve as a poor amino acceptor of D-amino acid aminotransferase and these amino acids were produced in a lower yield

TABLE 2. Enzymatic synthesis of D-amino acids.

Product	D-AAT (units)	Reaction Time (h)	Yield (%)
D-Glutamate	3	4	100
D- α -Aminobutyrate	3	4	93
D-Norvaline	3	4	92
D-Methionine	3	4	100
D-Valine	3	10	90
D-Leucine	3	10	74
D-Isoleucine	60	10	90
D-Histidine	60	4	62
D-Arginine	60	4	88
D-Phenylalanine	60	4	67
D-Tyrosine	60	10	100
D-Phenylglycine	60	10	2
D-Tryptophan	60	4	13

under the same conditions. However, the yields were increased to above 90% by addition of a large amount of D-AAT (about 60 units). In the synthesis of D-serine, D-aspartate, and D-tryptophan a larger amount of D-AAT is needed to attain the high yield.

REFERENCES

1. K. Tanizawa, Y. Masu, S. Asano, H. Tanaka and K. Soda, *J. Biol. Chem.* **264**, 2445-2449 (1989).
2. K. Nishimura, K. Tanizawa, T. Yoshimura, N. Esaki, S. Futaki, J. M. Manning and K. Soda, *Biochemistry* **30**, 4072-4077 (1991).
3. K. Tanizawa, S. Asano, Y. Masu, S. Kuramitsu, H. Kagamiyama, H. Tanaka and K. Soda, *J. Biol. Chem.* **264**, 2450-2454 (1989).
4. T. Yoshimura, K. Nishimura, J. Ito, N. Esaki, H. Kagamiyama, J. M. Manning and K. Soda, *J. Am. Chem. Soc.* **115**, 3897-3900 (1993).
5. K. Inagaki, K. Tanizawa, B. Badet, C. T. Walsh, H. Tanaka and K. Soda, *Biochemistry* **25**, 3268-3274 (1986).
6. K. Tanizawa, A. Ohshima, A. Scheidegger, K. Inagaki, H. Tanaka and K. Soda, *Biochemistry* **27**, 1311-1316 (1988).
7. H. Toyama, N. Esaki, T. Yoshimura, K. Tanizawa and K. Soda, *J. Biochem.* **110**, 279-283 (1991).
8. H. Toyama, K. Tanizawa, M. Wakayama, Q.-L. Lee, T. Yoshimura, N. Esaki and K. Soda, *Agric. Biol. Chem.* **55**, 2881-2882 (1991).
9. H. Toyama, K. Tanizawa, T. Yoshimura, S. Asano, Y.-H. Lim, N. Esaki and K. Soda, *J. Biol. Chem.* **266**, 13634-13639 (1991).