S-6-2 B₆-Enzymes Participating in D-Amino Acid Metabolism

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I. INTRODUCTION

Various D-amino acids occur in mammals, plants and microorganisms. In particular, eubacteria contain a variety of D-amino acids in their peptidoglycans: D-alanine is an essential component of peptidoglycans. D-Alanine is produced from L-alanine by alanine racemase (EC 5.1.1.1), which requires pyridoxal 5'-phosphate (PLP) as a coenzyme. The enzyme is distributed widely in prokaryotes. We have cloned the gene of thermostable alanine racemase of *Bacillus stearothermophilus*, purified and characterized the dimeric thermostable enzyme, and determined its primary structure. D-Glutamate occurs also in peptidoglycans of various bacterial strains, and is formed by transamination between α -ketoglutarate and D-alanine by D-amino acid aminotransferase (EC 2.6.1.21). We have cloned the gene of thermostable D-amino acid aminotransferase from thermophilic *Bacillus* sp. YM-1 chromosomes into *E. coli*, purified and characterized the thermostable enzyme. We here describe properties of these B₆-enzymes participating in D-amino acid metabolism in bacteria.

I. THERMOSTABLE D-AMINO ACID AMINOTRANSFERASE

D-Amino acid aminotransferase (D-AAT) was found in several thermophilic *Bacillus* species and purified to homogeneity from the best producer, *Bacillus* sp. YM-1, which was newly isolated from a sauna dust [1]. The enzyme was purified to homogeneity from the cell extract. The thermostable D-AAT has a molecular weight of about 62,000 and consists of two subunits identical in molecular weight (30,000). The native holo enzyme exhibits absorption maxima at 279, 333, and 415 nm at pH 7.4 and contains one mol of PLP per monomer [1]. The active site sequence is markedly different from those of L-amino acid aminotransferases and other PLP enzymes. The addition of D-alanine to the enzyme solution causes a decrease in absorbance at 419 nm and an increase at 335 nm, suggesting the conversion of the PLP form of the enzyme into the pyridoxamine 5'-phosphate (PMP) form. Treatment of the enzyme with phenylhydrazine yields a semiapoenzyme, which is catalytically inactive in the absence of added PLP and no longer exhibited the absorption maximum at 415 nm. Semiapoenzyme still has the peak at 333 nm, which is due to the bound cofactor as can be detected as PLP (about 0.2 mol per mol subunit). The circular dichroism spectrum of the thermostable enzyme indicates

the positive peak at 280 nm, and negative peak at 333 and 418 nm [1]. The *Bacillus* YM-1 enzyme reacts with D-alanine, D-glutamate, D-aspartate, D-asparagine, and D-A-aminobutyrate [1].

The gene for thermostable D-AAT from a thermophile, *Bacillus* sp. YM-1 was cloned and expressed efficiently in *E. coli* [2]. The entire covalent structure of the enzyme was determined from the nucleotide sequence of the cloned gene and mostly confirmed by amino acid sequence of tryptic peptides from the gene product [2]. The polypeptide composed of 282 amino residues is:

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1  GYTLWNDQIV KDEEVKIDKE DRGYQFGDGV YQVVKVYNGG
41 MFTVNEHIDR LYASAEKIRI TIPYTKDKFH QLLHELVEKN
81 ELNTGHIYFQ VTRGTSPRAH QFPENTVKPV IIGYTKENPR
121 PLENLEKGVK ATPVEDIRWL RCDIKSLNLL GAVLAKQEAH
161 EKGCYEAILH RNNTVTEGSS SNVFGIKDGI LYTHPANNMI
201 LKGITRDVVI ACANEINMPV KEIPFTTHEA LKMDELFVTS
241 TTSEITPVIE IDGKLIRDGK VGEWTRKLQK GFETKIPKPL
281 HI

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The calculated molecular weight is 32,226, and PLP is bound with Lys145. As D-AAT is the only transaminase which acts on D-amino acids, the process of evolution of this enzyme is an interesting problem. A computer-aided search of the NBRF protein sequence data bank has revealed that L-branched chain amino acid aminotransferase of *E. coli* [3] has a significant sequence homology to the thermostable D-AAT [2]. If the mutationally allowed substitutions for similar residues are taken into consideration, the similarity score increase to as high as 50, 58, and 43 % for the whole sequence, and C- and N-terminal portions, respectively. It may suggest that the structural genes for both enzymes evolved from a common ancestral gene.

Based on the result of gene analysis, the correlation between structure and function of D-AAT has been studied by site-directed mutagenesis. D-AAT contains six SH groups per dimer [4]. A possibility has been suggested that one or more SH groups may have some catalytic role. To clarify the role of cysteinyl residues of the enzyme, each of the three cysteinyl residues per subunit in the thermostable D-AAT changed to a glycine residue [4]. Two of the three Cys mutants (C164G and C142G) are as active as the wild-type, and the third mutant (C212G) retained 90% activity to that of wild type enzyme. Therefore, it is clear that SH groups of the enzyme are not essential for the catalytic activity. Conversion of Ser146, adjacent to Lys145 to which PLP is bound, to alanine residue does not alter the catalytic activity but has a significant effect on the SH titration behavior with DTNB [4]. With the wild type enzyme, only one SH per dimer is titrated after 4h-exposure to 1 mM DTNB. However, under the same conditions, three to four Cys residues are titrated in the Ser mutant (S146A) with a concomitant loss in activity. In the presence of substrate D-alanine, the titration behavior of the S146A mutant is similar to that of the wild type enzyme. This suggests that the Cys residue(s) which is titrated in the S146A mutant could be at or near the active site, and the presence of thionitrobenzoate

moiety at (or near) the active site disturbs the enzyme. The accessibility of the SH group in the wild type enzyme is relatively low. Perhaps the thermostable D-AAT adopts a compact structure, and Ser146 is important for this structure. Site-directed mutagenesis has been also employed to study the role of Trp139, which is close to the active site Lys145. This tryptophan residue has been changed to Phe, Pro, Ala, His, and Asp. The only mutant protein that has Phe substituted for Trp (W139F) is sufficiently active and stable (63% as active as wild type). The results of the fluorescence emission studies indicate that there is a clear energy transfer between the Trp in the wild type enzyme, which is almost absent in the W139F mutant. The fluorescence polarization studies has revealed that in the PMP form of the enzyme, the rotational freedom of the cofactor is higher in the W139F mutant enzyme than in the wild type. These results suggest the importance of an aromatic side chain at the position 139 in D-AAT and the influence of this residue on the behavior of the coenzyme PLP. These findings will be confirmed by the x-ray crystallographic study which has already been started. The availability of large amounts of pure enzyme achieved by molecular cloning of the enzyme gene leads to interesting findings. Recent study has demonstrated that D-serine, which is a relatively poor substrate compared with D-alanine, serves as an unique type of inhibitor of D-amino acid aminotransferase. Incubation of D-serine with the enzyme leads to the rapid generation of an absorption band at 493 nm. This band is represents the quinonoid intermediate, and is also observed but with a short half life during the transamination of D-alanine. In the reaction with D-serine, the quinonoid intermediate is extremely stable to bring its accumulation. After accumulation of significant amount of the intermediate, it slowly disappears concomitant with an irreversible inactivation of the enzyme. During the reaction, D-serine undergoes turnover to yield a compound which is considered to be B-hydroxypyruvate. The presence of D-alanine provides the protection of the enzyme against the inactivation by D-serine. These results suggest that D-serine acts as an enzyme-activated inhibitor (suicide substrate) of D-amino acid aminotransferase. The lysyl residue (Lys145) that binds PLP in the thermostable D-AAT has been converted into Arg. This mutant enzyme was enzymatically inactive, but PLP formed a Schiff base with an amino donor added. This suggests that an anionic intermediate is formed, but does not proceed further in the reaction by the mutant enzyme.

II. THERMOSTABLE ALANINE RACEMASE

The primary structures of alanine racemases of *B. stearothermophilus*, *B. subtilis*, and two isozymes (DadB and Alr) of *Salmonella typhimurium* show considerable homology to each other; the average homology of the four sequences is about 33%.³ Galakatos and Walsh reported that both isozymes of *S. typhimurium* (Mr39,000 each) are cleaved at homologous positions by achymotrypsin and subtilisin to produce the non-overlapping peptides with Mr28,000 and 11,000. They briefly mentioned that the *B. stearothermophilus* enzyme is also suffered from proteolysis by subtilisin to form two peptide fragments similar to those from the *S. typhimurium* isozymes. The amino acid sequences of the cleavage site and its vicinity between the NH₂-terminal and CO₂H-terminal regions corresponding to the two fragment peptides formed proteolytically of both *S.*

typhimurium enzymes are highly homologous to that of the short region around 264Tyr of the *B. stearothermophilus* enzyme. Accordingly, it was expected for the *B. stearothermophilus* enzyme to be possibly cleaved around 264Tyr by subtilisin.

We constructed a plasmid pARSD2 containing a termination codon (TAA), a ribosome-binding site (AGGAG), and an initiation codon (ATG) at the site corresponding to the site between Ala270 and Gln271. The recombinant cells carrying the plasmid pARSD2 showed considerable alanine racemase activity. The mutant enzyme is designated hereafter as the fragmentary alanine racemase on the basis of its polypeptide composition. Specific activity of the fragmentary enzyme was about 40% of that of the wild-type alanine racemase. The molecular weight of the fragmentary enzyme is almost the same as that of the wild-type enzyme. The far-UV CD spectrum study of the fragmentary enzyme showed that the enzyme shows the closely similar secondary structure to that of the wild-type enzyme. In contrast, a single polypeptide corresponding to each domain was attempted to be expressed separately in *E. coli* cells, but no proteins reactive with the antibody against the wild-type alanine racemase were produced. Therefore, it is suggested that the two polypeptide fragments associate with each other and fold to form an active structure only when they are co-translated, and that they correspond to structural folding units in the parental polypeptide chain.

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