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Construction and Properties of a Fragmentary D-Amino Acid Aminotransferase[1]

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D-Amino acid aminotransferase [EC 2.6.1.21] catalyzes the inter-conversion between various D-amino acids and α-keto acids. The subunit of the homodimeric enzyme from *Bacillus* sp. YM-1 consists of two domains connected by a single loop, which has no direct contact with the active site residues or the cofactor, pyridoxal 5′-phosphate [Sugio, S., Petsko, G.A., Manning, J.M., Soda, K., and Ringe, D. (1995) *Biochemistry* 34, 9661-9669]. We constructed two plasmids, one encoding a polypeptide fragment corresponding to the N-terminal domain, and the other a fragment corresponding to the C-terminal domain. When both polypeptide fragments were expressed together in the same host cell, an active fragmentary enzyme consisting of two sets of the two polypeptide fragments was produced. When the two polypeptide fragments were expressed separately, each of them provided a soluble protein but with no activity. However, D-amino acid aminotransferase activity appeared upon incubation of a mixture of the two fragments. The active fragmentary enzyme was purified to homogeneity and characterized; it was found to be similar to the wild-type enzyme in various enzymological properties except substrate specificity, inhibition by α-ketoglutarate, and thermostability. The fragmentary enzyme showed higher catalytic activity toward several substrates, such as D-lysine and D-arginine, than the wild-type enzyme.

Key words: D-amino acid aminotransferase, domain, folding, fragmentary enzyme, pyridoxal 5′-phosphate.

D-Amino acid aminotransferase (D-AAT, EC 2.6.1.21) requires pyridoxal 5′-phosphate (PLP) as a cofactor; it catalyzes the transamination between various D-amino acids and α-keto acids. The enzyme participates in the metabolism of D-amino acids, some of which are indispensable for bacteria as components of the peptidoglycan layer of the cell wall. D-AAT has been regarded as a target for the development of novel antibacterial agents serving, for example, as suicide substrates (*1-3*).

We purified and characterized a thermostable D-AAT from an isolated thermophile, *Bacillus* sp. YM-1 (*4*), and cloned and sequenced its gene (*5*). The primary structure of the enzyme shows significant similarity with that of the branched-chain L-amino acid aminotransferase (BCAT, EC 2.6.1.1) of *Escherichia coli* (*5*). D-AAT and BCAT belong to the same family of proteins, which is distinct from other families of proteins including all other aminotransferases (*6, 7*). The dimeric D-AAT differs from the hexameric BCAT in subunit structure, but an X-ray crystallographic study of *E. coli* BCAT demonstrated that the folding of the dimer unit of the enzyme is similar to that of D-AAT (*8, 9*). The subunit of D-AAT is divided into two structural domains connected by a single loop composed of ^{118}Asn-^{119}Pro-^{120}Arg-^{121}Pro (Fig. 1). These residues are located at a distance of 12 to 26 Å away from the cofactor, and from the residues known to participate in catalysis such as ^{145}Lys and ^{98}Arg (*9*). This indicates that the residues are not directly involved in catalysis.

A loop is defined as a region connecting two elements with secondary structures, and is thought to play an important role in the proper folding and functioning of proteins (*10-13*). Site-specific mutations in loops located at a distance from the active site have been shown to modify the properties of the enzyme. For example, replacement of amino acid residues in surface loops near the S1 binding pocket of trypsin by other residues occurring at the corresponding positions of chymotrypsin provides trypsin with a new substrate specificity similar to that of chymotrypsin (*14*). Insertion of a pentapeptide into the ω-loop region of TEM-1 β-lactamase elevates the activity of the enzyme toward ceftazidime *in vivo* (*15*). Similarly, a mutant streptavidin with an altered flexible loop acquires a higher affinity for Strep-tag, an artificial peptide ligand for streptavidine, than for the natural protein (*16*).

We attempted to increase the flexibility of the inter-

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Abbreviations: BCAT, branched chain L-amino acid aminotransferase; D-AAT, D-amino acid aminotransferase; PLP, pyridoxal 5′-phosphate; Tris, tris(hydroxymethyl)aminomethane.

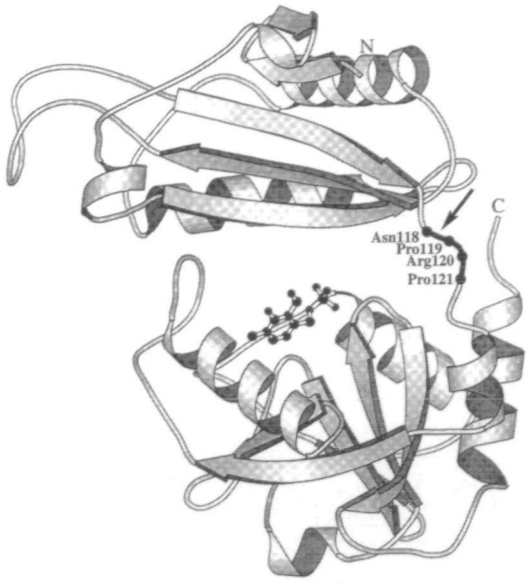

Fig. 1. **Three-dimensional structure of the subunit of D-AAT (*9*).** The cleavage site between Asn118 and Pro119 is indicated by an arrow.

domain loop of D-AAT by replacement of ^{119}Pro-^{120}Arg-^{121}Pro in the loop by Gly-Gly-Gly. The resultant mutant enzyme showed higher k_{cat} values than those of the wild-type enzyme for bulky substrates such as D-leucine and D-arginine (*17*). In this study, we constructed a fragmentary D-AAT, which is a mutant enzyme genetically cleaved between ^{118}Asn and ^{119}Pro in the loop, to provide an enzyme with low constraint freed from the conjunction. The mutant enzyme constructed has been characterized.

MATERIALS AND METHODS

Materials—Plasmid pICT113p containing the D-AAT gene of *Bacillus* sp. YM-1 was prepared as described previously (*18*). The oligonucleotides used for mutagenesis were synthesized by means of phosphoamidite chemistry. The enzymes used for DNA manipulation were from Takara Shuzo, Kyoto. All other reagents and chemicals were of analytical grade.

Construction of Plasmids pMDAAT-ND, pKDAAT-CD, and pKDAAT-ND—We constructed plasmids, pMDAAT-ND and pKDAAT-CD, which encode the polypeptides named the N- and C-fragments corresponding to the N- and C-terminal domains of D-AAT, respectively. Plasmid pMDAAT-ND was designed to produce an N-fragment with the same sequence as that of D-AAT from the N-terminus to Asn118. The C-fragment was designed to have an N-terminal methionine followed by 163 residues with the same sequence as ^{120}Arg-^{282}Ile of D-AAT. The DNA fragment encoding the N-fragment was amplified by polymerase chain reaction (PCR) with pICT113p (*18*) as a template, and primers DN-1 (5′-AAGGGTGGGATCCGGCAGTTT-AT-3′) and DN-2 (5′-TCAAGATTTTCTAAGCTTCGTTA-ATTTTCTTTCGT-3′). The design of DN-1 was based on the sequence upstream of the promoter region of D-AAT and contained a *Bam*HI site, as shown above with underlining. The design of DN-2 was based on the sequence around the C-terminal region of the N-domain; DN-2 contained a stop codon and a *Hin*dIII site, which are also underlined in the above sequence. The DNA fragment obtained on PCR was digested with appropriate restriction enzymes and then ligated into plasmid pMW219. A DNA fragment encoding the C-fragment was also obtained with the same template, and primers DC-1 (5′-CAGTAATCATGAATTCTAGGAA-AGAAAATATGCGACCATTAG-3′; *Eco*RI site, SD sequence and stop codon, underlined) and DC-2 (5′-CAAAC-TATTCAAGCTTCAGATTAT-3′; *Hin*dIII site and stop codon, underlined). The amplified fragment was digested with appropriate restriction enzymes and then ligated to pKK223-3. The resulting plasmid, pKDAAT-CD, was compatible with pMDAAT-ND in the same host cells. We found an unexpected base substitution only in the C-fragment gene, *i.e.* a mutation from ^{190}Ile to Met. The tertiary structure of D-AAT (*9*) revealed that ^{190}Ile is located on the surface of the protein, and is distant from not only the active site but also the interdomain loop. Therefore, we used the plasmid without further modification. We also constructed plasmid pKDAAT-ND by inserting the DNA-fragment encoding the N-fragment to the downstream of the *tac* promoter of plasmid pKK223-3 in order to express the N-fragment under the control of the *tac* promoter.

Cultivation of Transformant Cells—Both pMDAAT-ND and pKDAAT-CD were introduced together into *E. coli* JM109 cells. The transformant cells were grown at 37°C in LB medium supplemented with 50 μg/ml each of ampicillin and kanamycin. Production of the recombinant protein was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when the optical density of the culture at 660 nm reached 0.9. The cells were cultured for another 6 h, and then harvested by centrifugation. The cells were suspended in 20 mM potassium phosphate buffer (pH 7.2) containing 0.1 mM phenylmethylsulfonylfluoride (PMSF), and then disrupted by sonication.

Purification of the Enzyme—Both the fragmentary and wild-type D-AATs were purified in essentially the same manner as described previously (*19*), except that the fragmentary enzyme was purified with replacement of the heat-treatment by hydrophobic chromatography on Butyl-TOYOPEARL (Tosoh, Tokyo). The homogeneity of the final preparation was confirmed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Protein bands were stained with Coomassie Brilliant Blue R and analyzed on a Macintosh computer using the public domain NIH Image program, which was developed at the U.S. National Institutes of Health and is available on the Internet at <http://rsb.info.nih.gov/nih-image/>. Protein concentrations were determined based on the absorbance at 280 nm ($A^{1\%}_{1cm}$, 12.3) or with a Bio-Rad protein assay kit with bovine serum albumin as a standard.

Enzyme Assay—The overall reaction of D-AAT with D-alanine and α-ketoglutarate as substrates was assayed at 37°C with lactate dehydrogenase and NADH. The assay mixture (1 ml) contained 100 mM Tris-HCl buffer (pH 8.0), 0.2 mM NADH, 50 μM PLP, 5 units of lactate dehydrogenase, 100 mM D-alanine, 10 mM α-ketoglutarate, and D-AAT. The rate of decrease in absorbance at 340 nm due to oxidation of NADH to NAD was followed at 37°C.

The reaction was also assayed with salicylaldehyde. The reaction mixture (400 μl), containing 100 mM Tris-HCl buffer (pH 8.0), 100 mM D-alanine, 10 mM α-ketogluta-

rate, and D-AAT, was incubated at 37°C for 10 min. The reaction was stopped by the addition of 125 μl of 2% salicylaldehyde and 400 μl of 60% KOH, and then the solution was incubated at 37°C for another 10 min. After the addition of 1 ml of water to the mixture, the increase in absorbance at 480 nm was measured.

The overall reaction between D-amino acid and α-keto acid was routinely followed using a Beckman amino acid analyzer System 7300 to determine the amount of D-amino acid formed from the α-keto acid. The reaction mixture (1 ml), consisting of 100 mM Tris-HCl (pH 8.0), 50 mM D-amino acid, 20 mM pyruvate (or α-ketoglutarate), 50 μM PLP, and the wild-type (or the fragmentary) D-AAT, was incubated at 37°C for 5 min.

Spectrophotometric Measurements—Absorption spectra were taken with a Shimadzu MPS-2000 recording spectrophotometer. CD was measured with a Jasco J-600 recording spectropolarimeter at 25°C under a nitrogen atmosphere.

RESULTS

Expression and Purification of Fragmentary D-AAT—The cell extract of *E. coli* JM109 containing two plasmids, pMDAAT-ND and pKDAAT-CD, showed D-AAT activity. We purified the enzyme from about 40 g of wet cells by DEAE- and Butyl-TOYOPEARL column chromatographies, and obtained 9 mg of a homogeneous preparation of the fragmentary D-AAT. The specific activity of the cell extract (about 1.8 units/mg) increased about 80-fold with the purification. The molecular weight of the fragmentary enzyme estimated by gel filtration was 64,000, which is similar to that of the wild-type enzyme. On SDS-PAGE, the fragmentary enzyme gave two protein bands corresponding to approximate molecular weights of 20,000 and 14,000 (Fig. 2). These values are consistent with the calculated molecular weights of the C- and N-fragments, respectively. These results indicate that the fragmentary enzyme has an $\alpha_2\beta_2$ structure. The N-terminal sequence of the large fragment was determined to be Met-Arg-Pro-Leu-Glu-Asn-Leu-Glu-Lys by the method of Matsudaira (*20*). The sequence from ^{2}Arg to ^{9}Lys was the same as that from ^{120}Arg to ^{127}Lys of the wild-type D-AAT. The N-terminal sequence of the small fragment was demonstrated to be Met-Arg-Pro-Gly-Tyr-Thr-Leu-Trp-Asn-Asp-Glu-Glu-Ile, which is exactly the same as that of the wild-type D-AAT. The absorption spectrum of the fragmentary enzyme was nearly the same as that of the wild-type enzyme (Fig. 3). The CD spectrum of the fragmentary enzyme in the far UV region was very similar to that of the wild-type enzyme (data not shown). The PLP content of the fragmentary enzyme was determined to be about 2 mol per mol of enzyme by the fluorometric method (*21*).

We expressed either the N- or C-fragment by introducing only pKDAAT-ND or pKDAAT-CD into *E. coli* JM109. The occurrence of the corresponding fragment in the soluble fraction of the clone cells was confirmed by SDS-PAGE and Western blotting. However, the expression level of the N-fragment was much higher (about 10-fold, as analyzed with NIH Image) than that of the C-fragment, even though they were expressed under the same *tac* promoter (data not shown). When the preparation of the N-fragment was mixed with that of the C-fragment, D-AAT activity appeared. The activity increased with an increase in the amount of the counterpart fragment added (Fig. 4). These results suggest that each fragment separately expressed interacts with the counterpart fragment to form the active fragmentary enzyme. The same amount (100 nmol) of pyruvate was produced with about 25 μg of the C-fragment preparation (Fig. 4A), and about 1.5 μg of the N-fragment preparation (Fig. 4B), respectively, in the

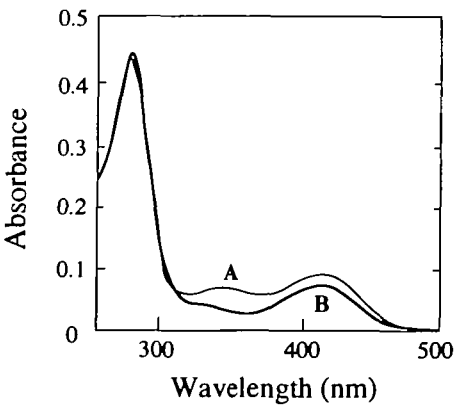

Fig. 3. Absorption spectra of the wild-type (A) and fragmentary (B) D-AATs. Absorption spectra were taken in 20 mM potassium phosphate buffer (pH 7.3).

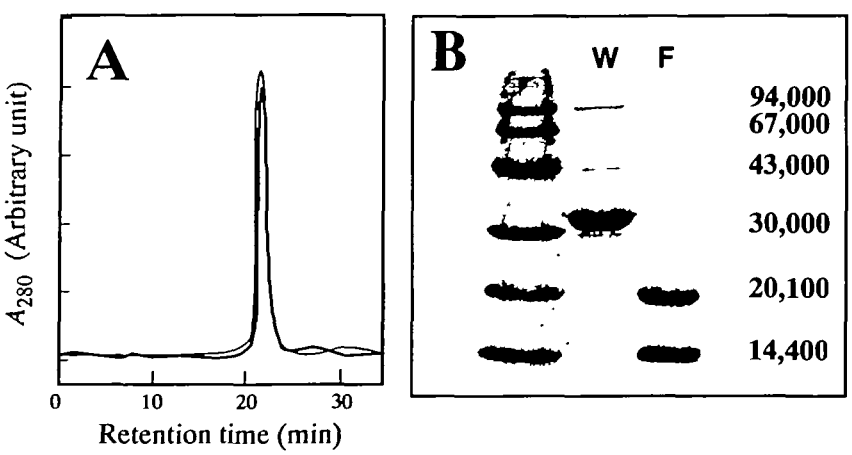

Fig. 2. Gel filtration chromatography and SDS-PAGE of the fragmentary enzyme. Elution profiles of the wild-type (thick line) and fragmentary (thin line) D-AATs on gel filtration (A). Each protein (0.1 mg) was applied to a Superose 12 column and eluted with 20 mM potassium phosphate buffer (pH 7.3) containing 0.2 M KCl at the flow rate of 0.5 ml/min. SDS-PAGE of the wild-type (W) and fragmentary (F) D-AATs (B). Ten micrograms of each protein was analyzed by SDS-PAGE with 16% acrylamide. Standard proteins are shown on the left side.

presence of an excess amount of the counterpart. These values enabled us to calculate the apparent specific activity of each fragment preparation: C-fragment, 1.6 units/mg; N-fragment, 27 units/mg. These values indicate that the relative content of the N-fragment was about 17 times higher than that of the C-fragment in the corresponding preparation. The value for the C-fragment preparation is close to the specific activity (about 1.8 units/mg) of the extract of *E. coli* cells expressing both the N and C-fragments simultaneously (see above). SDS-PAGE demonstrated that the relative amount of the C-fragment in this extract was similar to that in the extract of the cells expressing only the C-fragment; the relative intensity of the corresponding protein band was about 1.4%, as analyzed with NIH Image (data not shown). These results suggest that a fragmentary enzyme with significant activity probably comparable to that of the natural fragmentary form was reconstituted from the N- and C-fragments separately folded.

Catalytic Properties of the Fragmentary Enzyme—The specific activity of the purified fragmentary enzyme was about 145 units/mg protein, *i.e.* about 60% of that of the wild-type enzyme (232 units/mg). The apparent K_m and V_{max} values for D-alanine were 4.0 mM and 156 s^{-1} for the fragmentary enzyme, and 1.8 mM and 340 s^{-1} for the wild-type enzyme, respectively. The apparent K_m and V_{max} values for α-ketoglutarate were 2.0 mM and 254 s^{-1} for the fragmentary enzyme, and 6.7 mM and 260 s^{-1} for the wild-type enzyme, respectively.

We studied the substrate specificities of the wild-type and fragmentary enzymes in the overall reactions. The activity toward D-alanine decreased with the mutation, but those toward D-arginine, D-lysine, and D-phenylalanine increased when α-ketoglutarate was used as the amino acceptor (Table I). However, when pyruvate was used as the amino acceptor, the fragmentary enzyme was slightly less active than the wild-type enzyme (Table I).

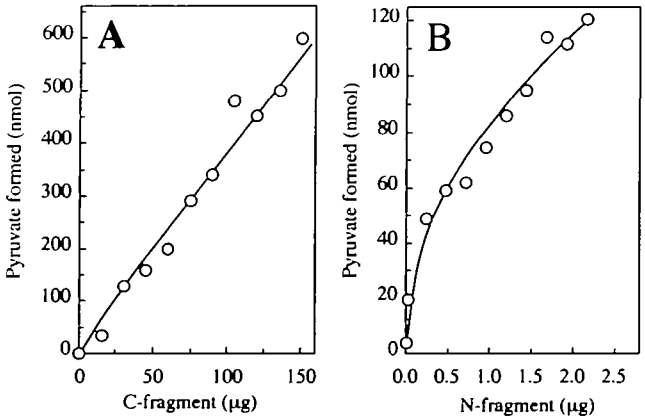

Fig. 4. **Formation of the fragmentary D-AAT from the N- and C-fragments.** The reaction mixture consisted of 20 mM potassium phosphate buffer (pH 7.3), 0.15 mg (as protein) of the cell extract containing the N-fragment (A) or 0.32 mg of that containing the C-fragment (B), and the indicated amount of cell extract containing the counterpart fragment. The reaction mixture, whose final volume was adjusted to 40 μl with the extract of *E. coli* JM109 cells containing no plasmid, was incubated at 37°C for 30 min. A 10-μl aliquot was withdrawn from the mixture and subjected to the D-AAT assay with salicylaldehyde.

α-Ketoglutarate has an inhibitory effect on the wild-type D-AAT. We have suggested that the inhibition is caused by competition between an amino donor and an amino acceptor for binding to the same site, Arg98, the common binding site for the α-carboxyl group of substrates (*22*). Figure 5 shows a $1/S$-$1/V$ plot of the reactions of the wild-type and fragmentary enzymes with various concentrations of D-alanine and 5 or 10 mM α-ketoglutarate. The plots for the fragmentary enzyme yielded parallel lines in contrast to those for the wild-type enzyme, which showed intersecting ones. The inhibitory effect of α-ketoglutarate was reduced in the reaction catalyzed by the fragmentary enzyme.

Thermostability—D-AAT of *Bacillus* sp. YM-1 is a thermostable enzyme (*4*). The wild-type enzyme retained the full original activity when it was incubated at 50°C for 55 min. However, the fragmentary enzyme lost 50% of its initial activity on incubation at the same temperature for 30 min (Fig. 6).

TABLE I. **Overall reactions catalyzed by the wild-type and fragmentary D-AATs.**

Amino donor	Amino acceptor: α-Ketoglutarate, Enzyme WT (A)	Fr (B)	Ratio (B/A)	Amino acceptor: Pyruvate, Enzyme WT (A)	Fr (B)	Ratio (B/A)
	Specific activity (μmol/mg/min)			Specific activity (μmol/mg/min)		
D-Glutamate	–	–	–	190	150	0.79
D-Alanine	220	160	0.73	–	–	–
D-Asparagine	84	110	1.3	110	120	1.1
D-Methionine	65	73	1.1	82	73	0.89
D-Glutamine	56	72	1.3	49	75	1.5
D-Serine	17	18	1.1	23	16	0.70
D-Norleucine	15	14	0.90	21	13	0.62
D-Ornithine	13	16	1.2	15	16	1.1
D-Valine	12	17	1.4	17	12	0.71
D-Histidine	4.9	7.1	1.4	7.7	7.4	0.96
D-Phenylalanine	3.2	4.6	1.4	4.8	3.4	0.71
D-Arginine	3.2	5.9	1.8	4.3	6.1	1.4
D-Lysine	1.6	3.2	2.0	2.5	3.6	1.4

WT, wild-type enzyme; Fr, fragmentary enzyme.

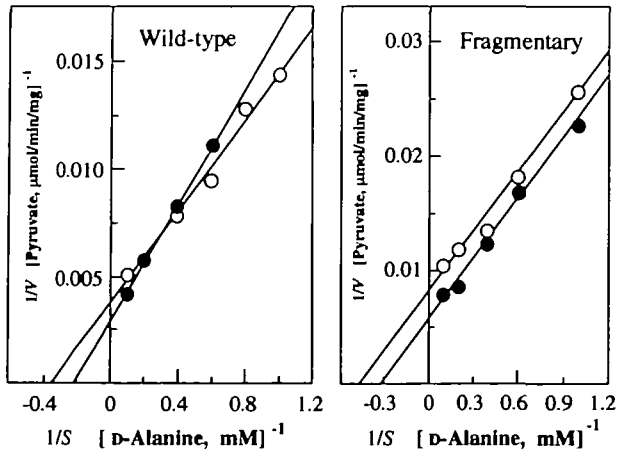

Fig. 5. **Double reciprocal plots of the rates of the overall reactions catalyzed by the wild-type and mutant enzymes against the concentrations of D-alanine.** Both enzymes were assayed with various concentrations of D-alanine, and 5 mM (○) or 10 mM (●) α-ketoglutarate. Other conditions are described in the text.

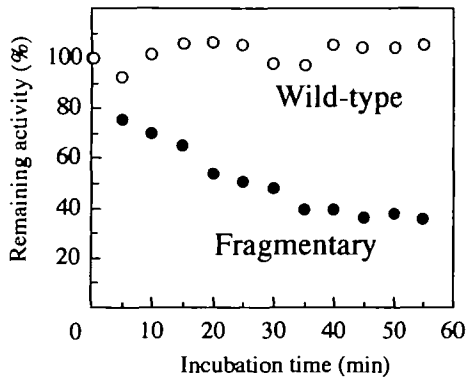

Fig. 6. **Thermostability of the wild-type and fragmentary enzymes.** The enzymes were incubated at 50°C in 20 mM potassium phosphate buffer (pH 7.3). The remaining activity at the indicated times was assayed at 37°C.

DISCUSSION

Wetlaufer (*23*) and Levitt and Chothia (*24*) have proposed that domains and subdomains behave as independent folding units which assemble to form the native molecules. This proposal has been verified by studies on folding of the domains of the FII fragments of thermolysine (*25*), phosphoglycerate kinase (*26*), γII-crystallin (*27*), and aspartate aminotransferase (*28*).

We previously constructed the fragmentary alanine racemase of *Bacillus stearothermophilus*. The enzyme was genetically cleaved into two peptide fragments at the site cleaved on limited proteolysis (*29*). The active fragmentary alanine racemase was obtained when both fragments were expressed in the same host cell. When each fragment was expressed separately, the N-terminal fragment was obtained as an inclusion body, and the C-terminal fragment was not detected in the cell extract on SDS-PAGE (*29*). Alanine racemase activity was not recovered on incubation of a mixture of the two fragment preparations (*29*). This may be due to the fact that the C-terminal fragment (120 residues) is shorter by about 30 residues than the region corresponding to the actual C-terminal domain observed on X-ray crystallography (*30*): the site of fragmentation was about 30 residues away from the actual region connecting the two structural domains. In contrast, the N- and C-fragments of the fragmentary D-AAT were produced in the soluble fractions of the cell extracts, and interacted with each other to form an active D-AAT. Since D-AAT was fragmented in the region connecting the two structural domains, the fragments can be regarded as isolated domains. If the structure of each fragment folded independently is similar to that of the corresponding domain in the native enzyme, then one can consider this as evidence that each domain of D-AAT acts as an independent folding unit. Alternatively, if the structures of the fragment and the domain are markedly different from each other, then one can assume that the N- and C-fragments folded independently interact with one another to form a complex, which is ultimately transformed to the active fragmentary enzyme, probably through complicated processes. Whichever is the case, our system has provided us with a unique tool for studying the roles of domains in protein folding. Therefore, the structure of each fragment folded in the absence of its partner fragment needs to be clarified, and attempts are being made to obtain a homogeneous preparation of each fragment.

Cleavage of D-AAT at the interdomain loop reduced its susceptibility to inhibition by α-ketoglutarate, and increased its catalytic efficiency towards several substrates, such as D-arginine and D-lysine, when α-ketoglutarate was used as the amino acceptor. The enzyme acquired similar properties on substitution of Gly-Gly-Gly for ^{119}Pro-^{120}Arg-^{121}Pro in the interdomain loop (*17*). Both fragmentation and glycine substitution probably affect the conformation of the active site of D-AAT through increased flexibility in the interdomain interaction. Mutant enzymes with increased reactivity toward some selected amino acids, such as D-arginine, may be applicable to the production of these D-amino acids, which are obtained inefficiently by means of the wild-type enzyme, from the corresponding α-keto acids (*31*).

REFERENCES

1. Soper, T.S., Jones, W.M., Lerner, B., Trop, M., and Manning, J.M. (1977) Inactivation of bacterial D-amino acid transaminase by β-chloro-D-alanine. *J. Biol. Chem.* **252**, 3170-3175
2. Soper, T.S., Manning, J.M., Marcotte, P.A., and Walsh, C.T. (1977) Inactivation of bacterial D-amino acid transaminase by the olefinic amino acid D-vinylglycine. *J. Biol. Chem.* **252**, 1571-1575
3. Soper, T.S. and Manning, J.M. (1978) β-Elimination of β-halo substrates by D-amino acid transaminase associated with inactivation of the enzyme. Trapping of a key intermediate in the reaction. *Biochemistry* **17**, 3377-3384
4. Tanizawa, K., Masu, Y., Asano, S., Tanaka, H., and Soda, K. (1989) Thermostable D-amino acid aminotransferases from a thermophilic *Bacillus* species. *J. Biol. Chem.* **264**, 2445-2449
5. Tanizawa, K., Asano, S., Masu, Y., Kuramitsu, S., Kagamiyama, H., Tanaka, H., and Soda, K. (1989) The primary structure of thermostable D-amino acid aminotransferase from a thermophilic *Bacillus* species and its correlation with L-amino acid aminotransferases. *J. Biol. Chem.* **264**, 2450-2454
6. Mehta, P.K., Hale, T.I., and Christen, P. (1993) Aminotransferases: demonstration of homology and division into evolutionary subgroups. *Eur. J. Biochem.* **214**, 549-561
7. Grishin, N.V., Phillips, M.A., and Goldsmith, E.J. (1995) Modeling of the spatial structure of eukaryotic ornithine decarboxylases. *Protein Sci.* **4**, 1291-1304
8. Okada, K., Hirotsu, K., Sato, M., Hayashi, H., and Kagamiyama, H. (1997) Three-dimensional structure of *Escherichia coli* branched-chain amino acid aminotransferase at 2.5 Å resolution. *J. Biochem.* **121**, 637-641
9. Sugio, S., Petsko, G.A., Manning, J.M., Soda, K., and Ringe, D. (1995) Crystal structure of a D-amino acid aminotransferase: how the protein controls stereoselectivity. *Biochemistry* **34**, 9661-9669
10. Sibanda, B.L. and Thornton, J.M. (1985) β-Hairpin families in globular proteins. *Nature* **316**, 170-174
11. Sibanda, B.L. and Thornton, J.M. (1991) Conformation of β-hairpins in protein structure: classification and diversity in homologous structures. *Methods Enzymol.* **202**, 59-82
12. Sibanda, B.L., Blundell, T.L., and Thornton, J.M. (1989) Conformation of β-hairpins protein structures. A systematic classification with applications to modelling by homology, electron density fitting and protein engineering. *J. Mol. Biol.* **206**, 759-777
13. Greer, J. (1990) Comparative modeling methods: application to the family of the mammalian serine proteases. *Proteins* **7**, 317-334
14. Hedstrom, L., Perona, J.M., and Rutter, W.J. (1994) Converting

trypsin to chymotrypsin: residue 172 is a substrate specificity determinant. *Biochemistry* **33**, 8757-8763
15. Hayes, F., Hallet, B., and Cao, Y. (1997) Insertion mutagenesis as a tool in the modification of protein function. Extended substrate specificity conferred by pentapeptide insertions in the ω-loop of TEM-1 β-lactamase. *J. Biol. Chem.* **272**, 28833-28836
16. Vossa, S. and Skerra, A. (1997) Mutagenesis of a flexible loop in streptavidin leads to higher affinity for the Strep-tag II peptide and improved performance in recombinant protein purification. *Protein Eng.* **10**, 975-982
17. Gutierrez, A., Yoshimura, T., Fuchikami, Y., Soda, K., and Esaki, N. (1998) A mutant D-amino acid aminotransferase with broad substrate specificity: construction by replacement of the interdomain loop Pro119-Arg120-Pro121 by Gly-Gly-Gly. *Protein Eng.* **11**, 53-58
18. Nishimura, K., Tanizawa, K., Yoshimura, T., Esaki, N., Futaki, S., Manning, J.M., and Soda, K. (1991) Effect of a lysyl residue that binds pyridoxal phosphate in thermostable D-amino acid aminotransferase by arginine and alanine. *Biochemistry* **30**, 4072-4077
19. Merola, M., Martinez del Pozo, A., Ueno, H., Recsei, P., Di Donato, A., and Manning, J.M. (1989) Site-directed mutagenesis of the cysteinyl residues and the active-site serine residue of bacterial D-amino acid transaminase. *Biochemistry* **28**, 505-509
20. Matsudaira, P. (1990) Limited N-terminal sequence analysis. *Methods Enzymol.* **182**, 602-613
21. Adams, E. (1979) Fluorometric determination of pyridoxal phosphate in enzymes. *Methods Enzymol.* **62**, 407-410
22. Kishimoto, K., Yoshimura, T., Soda, K., and Esaki, N. (1997) Mutation of arginine 98, which serves as a substrate-recognition site of D-amino acid aminotransferase, can be partly compensated for by mutation of tyrosine 88 to an arginyl residue. *J. Biochem.* **122**, 1182-1189
23. Wetlaufer, D.B. (1973) Nucleation, rapid folding, and globular intrachain regions in proteins. *Proc. Natl. Acad. Sci. USA* **70**, 697-701
24. Levitt, M. and Chothia, C. (1976) Structural patterns in globular proteins. *Nature* **261**, 552-558
25. Dalzoppo, D., Vita, C., and Fontana, A. (1985) Folding of thermolysin fragments. Identification of the minimum size of a carboxyl-terminal fragment that can fold into a stable native-like structure. *J. Mol. Biol.* **182**, 331-340
26. Pecorari, F., Minard, P., Desmadril, M., and Yon, J.M. (1993) Structure and functional complemention of engineered fragments from yeast phosphoglycerate kinase. *Protein Eng.* **6**, 313-325
27. Sharma, A.K., Minke-Gogl, V., Gohl, P., Siebendritt, R., Jaenicke, R., and Rudolph, R. (1990) Limited proteolysis of γII-crystallin from calf eye lens. *Eur. J. Biochem.* **194**, 603-609
28. Herold, M., Leistler, B., Hage, A., Luger, K., and Kirschner, K. (1991) Autonomous folding and coenzyme binding of the excised pyridoxal 5′-phosphate binding domain of aspartate aminotransferase from *Escherichia coli*. *Biochemistry* **30**, 3612-3620
29. Toyama, H., Tanizawa, K., Yoshimura, T., Asano, S., Lim, Y., Esaki, N., and Soda, K. (1991) Thermostable alanine racemase of *Bacillus stearothermophilus*. *J. Biol. Chem.* **266**, 13634-13639
30. Shaw, J.P., Petsko, G.A., and Ringe, D. (1997) Determination of the structure of alanine racemase from *Bacillus stearothermophilus* at 1.9-Å resolution. *Biochemistry* **36**, 1329-1342
31. Galkin, A., Kulakova, L., Yoshimura, T., Soda, K., and Esaki, N. (1997) Synthesis of optically active amino acids from α-keto acids with *Escherichia coli* cells expressing heterologous genes. *Appl. Environ. Microbiol.* **61**, 4651-4656