Site-Directed Mutagenesis of the Cysteinyl Residues and the Active-Site Serine Residue of Bacterial D-Amino Acid Transaminase†

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ABSTRACT: Each of the three cysteinyl residues per subunit in D-amino acid transaminase from a thermophilic species of *Bacillus* has been changed to a glycine residue (C142G, C164G, and C212G) by site-directed mutagenesis. The mutant enzymes were detected by Western blots and a stain for activity. After purification to homogeneity, each mutant protein had the same activity as the wild-type enzyme. Thus, none of the Cys residues are essential for catalysis. Each protein when denatured showed the expected titer of two SH groups per subunit. In the native state, each of the three mutant proteins exhibited nearly the same slow rate of titration of SH groups as the wild-type protein with about one SH group titratable over a period of 4 h. Conversion of Ser-146, adjacent to Lys-145 to which the coenzyme pyridoxal phosphate is bound, to an alanine residue (S146A) does not alter the catalytic activity but has a significant effect on the SH titration behavior. Thus, three to four of the six SH groups of S146A are titratable by DTNB. The rapid SH titration of S146A is prevented by the presence of D-alanine. This finding suggests that the change of Ser-146 to Ala at the active site promotes the exposure and rapid titration of a Cys residue in that region. The rapid SH titration of S146A by DTNB is accompanied by a loss of enzyme activity. Two of the mutant enzymes, C142G and S146A, lose activity at 4 °C and also upon freezing and thawing. The mutant enzymes C164G and C212G show the same degree of thermostability as the wild-type enzyme.

The peptidoglycan layer of the bacterial cell wall, which contains the D isomers of two amino acids, D-alanine and D-glutamate, is the primary barrier to osmotic lysis. Agents such as penicillin which interfere with the assembly of the peptidoglycan are clinically effective antibacterial agents. We and others have shown that compounds which inhibit enzymes that synthesize the component D-amino acids of the peptidoglycan are also effective antibacterial agents (Gale et al., 1981). For example, a variety of agents such as β-chloro-D-alanine, cycloserine, and gabaculine (Soper et al., 1977, 1985; Manning et al., 1974; Soper & Manning, 1981, 1982) inhibit D-amino acid transaminase. As a result, the supply of bacterial D-glutamate is depleted so the peptide portion of the peptidoglycan cannot be completed and bacterial growth is inhibited. However, the efficiency of these antimicrobial agents is highly variable since their partition ratio, i.e., the ratio of hydrolysis of inactivator to inhibition of the enzyme, varies in a completely unpredictable manner. Therefore, we have aimed at fully characterizing the catalytic properties of D-amino acid transaminase so that a very efficient suicide substrate can be designed and synthesized.

Studies on the reactivity of the SH groups of D-amino acid transaminase (Soper et al., 1985) are part of an effort to identify the active-site residues of this enzyme. Recently, the primary structure of D-amino acid transaminase from a thermostable species of *Bacillus* has been deduced from the sequence of the gene that encodes the protein (Asano, 1987; Tanizawa et al., 1987, 1989a,b). The D-amino acid specific enzyme from the thermostable *Bacillus* and the analogous enzymes from *Bacillus sphaericus* and *Bacillus subtilis* appear to be homologous although the enzyme from the former source contains six SH groups per dimer and the enzymes from the latter two species contain eight SH groups per dimer. Furthermore, the amino acid composition of the tryptic peptide containing the active-site pyridoxal 5′-phosphate (PLP)[1] isolated from the *Bacillus sphaericus* enzyme[2] is identical with that predicted for the analogous peptide from the thermostable enzyme. The availability of the gene encoding for this enzyme permits site-directed mutagenesis experiments aimed at the separate replacement of each of the cysteinyl residues of this enzyme to ascertain their catalytic or structural role, if any. In addition, the residue adjacent to the active-site Lys of D-amino acid transaminase is serine, which is absent in all of the L-amino acid specific pyridoxal enzymes that have been sequenced to date. Therefore, this residue has been changed to Ala, and the effects of this mutation on the properties of the enzyme have been examined in this study.

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[1] Abbreviations: PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; DTNB, 5,5′-dithiobis(2-nitrobenzoate); SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; ssDNA, single-stranded DNA; Tris, tris(hydroxymethyl)aminomethane.

[2] Ueno et al., unpublished results.

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Materials and Methods

DNA Phage and Bacterial Strains. Bacterial strains used were *Escherichia coli* BW313 [Hfr, KL16 PO/45 [LysA-(61–62)], dut-1, ung-1, thi-1, relA1] and JM101 [SupE, thi, Δ(lac-proAB), [F′, traD36, proAB, LacI^{q}ZΔM15]]. Helper phage F1 (IR1) (DoHo & Horiuchi, 1981) and plasmid pEMBL 18 were obtained from Drs. P. Model, M. Russel, and M. Kelley of The Rockefeller University. Plasmid pICT 113 was obtained as described previously (Asano, 1987; Tanizawa et al., 1987, 1989a,b).

Enzymes and Chemicals. Polynucleotide kinase was obtained from NEN Biolabs. T4 DNA ligase and restriction endonucleases were from Boehringer. The Klenow fragment and Sequenase were from Boehringer or USB. Deoxynucleoside triphosphates were from Pharmacia. Uridine, fast violet B, and goat anti-rabbit IgG–alkaline phosphatase conjugate were from Sigma.

Oligonucleotides were synthesized at the Rockefeller University Protein Sequencing Facility on an Applied Biosystems DNA synthesizer 380 B by the phosphoramidite method. The mutagenic oligonucleotides used were 22-mers with the mismatched base situated approximately in the center, the italicized bases representing the mismatched ones:

S146A: 5′-CAA GTT CAA AG*C* TTT AAT ATC A-3′

C142G: 5′-TTT AAT ATC AC*C* TCG TAA CCA A-3′

C164G: 5′-T CGC TTC ATA GC*C* GCC TTT TTC-3′

C212G: 5′-TTC ATT TGC AC*C* AGC TAT GAC A-3′

Construction of pAZZI. pAZZI has been constructed by cloning (in both orientations) the 1.7-kb *Eco*RI fragment from pICT 113 (Tanizawa et al., 1987, 1989a,b) into the unique *Eco*RI site of pEMBL 18 (Dente et al., 1983). This fragment contains the entire coding sequence for D-amino acid transaminase with an additional 700 bp upstream and about 200 bp downstream (Asano, 1987). The orientation of the products was determined by DNA sequencing (Sanger et al., 1977). pAZZI+, used as template for site-directed mutagenesis, produces the noncoding strand of the transaminase gene after infection with F1 (IR1).

Site-Directed Mutagenesis. Mutagenesis was carried out as described by Kunkel (Kunkel, 1985; Kunkel et al., 1987). pAZZI+ containing uridine was prepared from *E. coli* BW313 that had been transformed for two cycles in the presence of 100 μg/mL ampicillin and 1 μg/mL uridine. One cycle includes transformation and plasmid extraction.

To obtain single-stranded pAZZI+ (sspAZZI+), 50 mL of medium containing 10 g of tryptone/L, 10 g of yeast extract/L, 5 g of NaCl/L, 100 μg/mL ampicillin, 0.2% glucose, and 1 μg/mL uridine was inoculated with one clone of BW313 carrying the plasmid. The cells were grown at 37 °C with vigorous shaking until the absorbance at 600 nm was 0.2–0.3. F1 (IR1), 200 μL of a solution containing 2×10^{12} phages/mL, was then added and the infection permitted to take place for about 5 h at 37 °C. To 45 mL of the supernatant solution were added 5 mL of a solution containing 25% poly(ethylene glycol) and 2.5 M NaCl. After 1 h on ice, the solution was centrifuged, and the supernatant was discarded. The pellet was treated for single-stranded DNA (ssDNA) purification as described by Dente and Cortese (1985). After precipitation with ethanol, the template was resuspended in 30 μL Tris–EDTA pH 8.0.

Hybridization. Template ssDNA (3 μL), prepared as described above and corresponding to 1–2 μg of DNA, was mixed with 3 μL of 5′-phosphorylated mutagenic oligonucleotide (5 pmol/μL) in 10-μL final volume in buffer A (20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, and 1 mM DTT). Hybridization was done in a siliconized capillary at 100 °C for 90 s, and the solution was cooled slowly at room temperature.

Elongation and Ligation. After transfer of the hybridization mixture to a microtube, elongation and ligation were carried out overnight at room temperature in a final volume of 20 μL in buffer B (20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, and 2 mM each of dATP, dCTP, dGTP, dTTP, and ATP) in the presence of 5 units of DNA polymerase I, Klenow fragment, and 1 unit of T4 DNA ligase. After inactivation of these enzymes at 65 °C for 10 min, dilutions of this solution were used to transform competent JM101.

Screening of Mutants. Screening for mutant clones was done directly by DNA sequencing (dideoxy method) (Sanger et al., 1977) using synthetic oligonucleotide primers. Once identified, the described clone was purified further by infecting fresh JM101 with the supernatant remaining from the ssDNA purification. Plating was done on media containing ampicillin. In this manner, only clones that have been infected with a plasmid can grow, and if extreme dilutions are used, it is almost certain that only a single plasmid will enter the cell. One clone was chosen and used to confirm the sequence around the mutagenic site (about 300 nucleotides on each side). The same clone was used for protein purification.

Expression of DAT. Wild-type and mutant D-amino acid transaminases were purified from cells of transformed *E. coli* JM101 grown to stationary phase in 2.5 L of rich medium (YT) containing 100 μg/mL ampicillin for about 18 h. The expression of the enzyme was tested by collecting cells from 100 μL of a stationary-phase culture and lysing them by boiling for 2–3 min in 100 μL of buffer (50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1% SDS, 15% glycerol, 14 mM 2-mercaptoethanol, and 0.02% bromophenol blue). The sample (5–10 μL) was applied in a 10 or 15% SDS–PAGE system (Laemmli, 1970).

After the gel was stained with Coomassie blue, the intensity of the 32-kDa band, which is not present in JM101 carrying pEMBL18, indicated that D-amino acid transaminase is produced in yields up to 10% of the total soluble protein as previously reported (Tanizawa et al., 1987). The fact that the 32-kDa protein and the enzyme were in the same position was also verified by a Western blot (Towbin et al., 1979) using antibodies against the enzyme developed in a rabbit and anti-rabbit IgG–alkaline phosphatase conjugate for detection (Figure 1). Only one cross-reactive band of approximately 32 kDa was present in the lysate from cells carrying pAZZI+ or its derivatives, and no enzyme was detected in a lysate of JM101 carrying pEMBL 18.

A staining method in nondenaturing PAGE has been developed to detect the activity of D-amino acid transaminase. The purified enzymes, as well as extracts, were subjected to electrophoresis on a 7.5% acrylamide/bis(acrylamide) (30:0.8) gel (0.75 × 7 × 11.5 cm) in Tris–glycine (25 mM Tris/192 mM glycine) for 30 min at 10-mA constant current. The zone of D-amino acid transaminase activity is exhibited by a brilliant red color that develops after soaking the gel for 5 min at 25 °C in a solution of 20 mM D-aspartate, 270 μM α-ketoglutarate, 0.12% fast violet B, and 0.2 mM PLP buffered at pH 7.5 with Trizma base. This gel assay is based upon an assay similar to that described for L-aspartate transaminase (Dekker & Rau, 1963). The assay is useful for the detection of enzymically active protein either in crude extracts or in the purified state. Under these conditions, as little as 0.1 μg of enzyme can be easily detected. The extracts from cells of

Table I: Purification and Some Properties of D-Amino Acid Transaminase Mutants

enzyme	yield of pure protein (mg)	sp act. at 37 °C (units/mg)[a]	SH titrated per dimer[b]	T_m (°C)[c]	ϵ_{420}/dimer	ϵ_{338}/dimer
wild-type	112	80	6.2 ± 0.6	65.5	15150	5500
C212G	25	70	4.0 ± 0.5	65.0	11900	5900
C164G	138	81	3.7 ± 0.1	64.0	14750	3400
C142G	179	79	3.9 ± 0.2	61.0	11900	6000
S146A	60	86	6.5 ± 0.3	62.5	14700	6500

[a] Assayed in the presence of 0.5 mM PLP. [b] Each enzyme was first denatured by exposure to 6.4 M guanidine hydrochloride. [c] The samples (0.1 mg of protein/mL) were heated for 10 min at temperatures from 25 to 75 °C in 0.1 M Tris-HCl, pH 7.5, and 2 mM EDTA. They were then cooled in ice and assayed under standard conditions. T_m is defined as the temperature at which the protein retains 50% of its original activity.

JM101 carrying pAZZI or its derivatives showed a single band of enzyme activity (Figure 2).

Protein Purification. D-Amino acid transaminase was purified according to Asano (1987) and to Stoddard et al. (1987) with minor modifications. After the heat treatment step, the protein was chromatographed on DEAE-Sephadex A-50 (2 × 20 cm column) equilibrated in 0.01 M potassium phosphate buffer (pH 7.6) containing 50 μM pyridoxal phosphate, 0.2 mM EDTA, and 0.01% 2-mercaptoethanol. The protein was eluted with a linear gradient of 0–0.15 M KCl. The active fractions were rechromatographed on Sephadex G-100 (2.5 × 40 cm column) equilibrated in 0.1 M potassium phosphate, pH 7.6, with 50 μM pyridoxal phosphate and 0.01% 2-mercaptoethanol. The active fractions were pooled and analyzed for amino acid composition and electrophoretic behavior. The purified enzymes were stored at 4 °C.

Activity Assay. Enzyme activity was determined by measuring the rate of pyruvate production from D-alanine and α-ketoglutarate. Pyruvate was determined either by an assay employing an NADH and lactate dehydrogenase system or with salicylaldehyde as described previously (Soper et al., 1977).

DTNB Titrations. The kinetic profile of the reaction of the transaminase with DTNB was followed as described earlier (Soper et al., 1977). The absorption at 412 nm was recorded periodically at the indicated temperature, and the number of SH groups that had reacted was determined by using a molar absorbance coefficient of 14000 M^{-1} cm^{-1} at 412 nm for the nitrothiophenylate anion (Collier, 1973).

Other Analytical Determinations. SDS–PAGE electrophoresis of samples that had been previously reduced with 0.05% 2-mercaptoethanol was carried out according to Laemmli (1970). Amino acid analyses were carried out on a Beckman Model 6300 automatic analyzer with an IBM-AT based System Gold enhancement. Absorption spectra were recorded on an Aminco DW-2 UV/VIS spectrophotometer with a recording speed of 0.5 nm/s.

The PLP content of the proteins was measured with 2% (w/v) phenylhydrazine in 10 N H_2SO_4 according to the method of Wada and Snell (1961). The samples were heated at 60 °C for 10 min before the absorbance at 410 nm was read.

The T_m, which is defined as the temperature at which 50% of the initial activity remained, was determined by incubating each enzyme for 10 min over a temperature range from 25 to 75 °C. The temperature at which inactivation began was indicated by a sharp break in the profile of activity versus temperature.

Results

Protein Purification. Extracts of cells containing both wild-type and mutant enzymes showed that the predominant band corresponded to the position of D-amino acid transaminase both in an enzyme activity assay (Figure 1) and in a Western blot assay (Figure 2). Extracts of cells that did not contain the plasmid did not show a predominant 32-kDa band either by Coomassie blue staining, by activity assay, or by Western blot analysis. After purification, the preparations of the wild-type and the four mutant D-amino acid transaminase proteins were nearly homogeneous (>95%) as judged by SDS–PAGE. The amino acid compositions of the samples were in excellent agreement with the predicted values (Tanizawa et al., 1987). The amount of enzyme purified from 20 g (wet weight) of bacterial cells was variable, but it was always greater than 25 mg (Table I). The wild-type and S146A enzymes in the denatured state gave the expected titer of six SH groups per dimer with DTNB. The denatured C142G, C164G, and C212G mutant enzymes titrated for four SH groups per dimer in agreement with the expected mutagenesis (Table I).

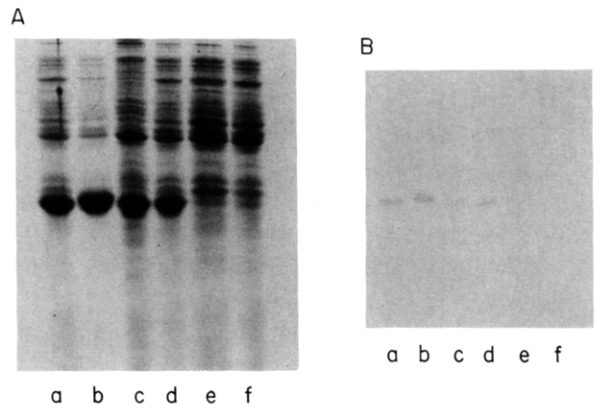

FIGURE 1: (A) Coomassie blue stain. (B) Western blot. (a) C164G extract; (b) C142G extract; (c) S146A extract; (d) wild-type extract; D-amino acid transaminase is at 32 kDa; (e) (pEMBL-18) *E. coli* JM101 extract; (f) *E. coli* JM101 extract. The gel was prepared under denaturing conditions as described in the text.

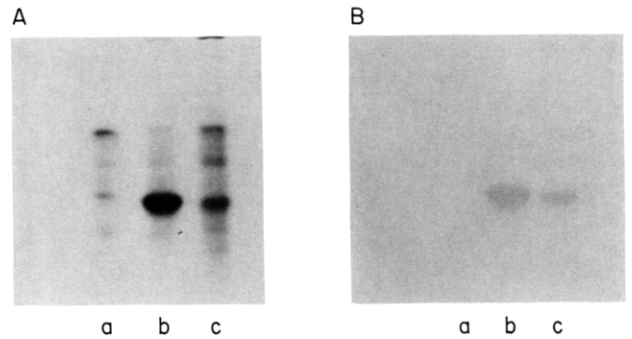

FIGURE 2: (A) Coomassie blue stain. (B) Activity stain. (a) pEMBL-18 *E. coli* JM101 extract; (b) S146A, partially purified; (c) C164G extract. The gel was prepared under nondenaturing conditions as described in the text.

Specific Activity of Wild-Type and Mutant Enzymes. Wild-type D-amino acid transaminase has a specific activity at 37 °C of 80 units/mg (in the presence of 0.5 mM PLP), which is nearly the same found for C142G, C164G, and

S146A mutant transaminases (Table I). Purified C212G had just a slightly lower specific activity. Both the wild-type enzyme and the four mutant enzymes were found to contain 2 mol of PLP/mol of dimeric enzyme by the method of Wada and Snell (1961) (Table I).

Thermostability of Enzymes. Initial observations on the freshly purified S146A and C142G mutant enzymes suggested that they were less stable than the wild-type, C164G, or C212G mutant proteins. Thus, S146A loses 25% of its activity after 1 month at 4 °C, and this inactivation is even more pronounced with the C142G protein. The wild-type and the other two cysteine mutant enzymes are quite stable under these conditions. In a more systematic study, we have estimated the thermostability of these five enzymes as shown in Table I. The wild-type enzyme, the C164G mutant enzyme, and the C212G mutant enzyme remain fully stable at 62 °C whereas at that temperature only 10% of the C142G enzyme activity is retained. The S146A mutant is of intermediate thermostability since at 62 °C it retains about 50% of its specific activity. Thus, it is evident that both Ser-146 and Cys-142, which are located near the active-site Lys-145, play a role in the stability of D-amino acid transaminase.

Spectrophotometric Characterization. The absorption spectrum of wild-type enzyme shows a major band at 420 nm and a minor absorbance at 338 nm both of which remain constant over the pH range of 5.0–8.5. This behavior has been also observed for other transaminases (Yonaha et al., 1975; Wada & Snell, 1961). The A_{420}/A_{338} ratio for the wild-type protein decreases very slowly as the enzyme ages, and there is a gradual loss in activity over a period of 5 months of 4 °C. The four mutant proteins possess very similar spectra. However, the intensities of the absorbance at 420 nm for C142G and C212G were lower than those for the other proteins (Table I). The reason for this is not known at present, even though each of them contains one molecule of PLP per monomer.

As observed with L-amino acid specific transaminases (Christen & Metzler, 1985), the analogous spectral changes occur with D-amino acid transaminase in the presence of D-Ala and D-Glu (1.5 mM). The absorption maximum at 420 nm disappears with a corresponding increase in the band at 338 nm. Since these experiments were performed in the absence of any α-keto acid, these spectra in the presence of D-amino acids can be assigned to the pyridoxamine 5′-phosphate (PMP) form of the enzyme. The changes in absorbance at the two wavelengths are a function of the amino acid concentration. D-Glu and D-Ala have their maximum effect at about 0.5 mM concentration.

L-Ala (3 mM) can also lead to spectral changes in the enzyme although these are minor compared to those that occur in the presence of D-amino acids. With catalytic amounts of enzymes, the L isomer is not a substrate for either the wild-type or the mutant enzymes since even at concentrations of L-Ala as high as 50 mM no pyruvate production is observed.

DTNB Titration. D-Amino acid transaminase from the thermostable *Bacillus* is a dimer which contains six free SH groups. Since the titration of some Cys residues in the enzyme from *B. sphaericus* led to its inactivation (Soper et al., 1977), it was considered possible that one or more of these SH groups may have some catalytic role. That question can now be answered more definitively by studies on each of the three mutant Cys enzymes separately.

We have also examined the DTNB titration of the wild-type and mutant enzymes as a function of temperature. There is a very clear temperature dependence of the titration rates, which are faster as the temperature is increased (data not shown). Distinct DTNB titrations of these four enzymes could be obtained at 30 °C (Figure 3). With the wild-type, the C142G, and the C212G mutant enzymes only about one SH per dimer was titrated even after 4-h exposure to 1 mM DTNB. The extremely slow titration of C164G suggests that Cys-164 may be the most reactive of the Cys residues in the enzyme. However, the DTNB titration of the S146A enzyme is clearly different as shown in Figure 3. After 4 h, three to four Cys residues per molecule of dimer were titrated in this mutant enzyme. Subtraction of the kinetic profiles of either the wild-type or the other three cysteine mutant proteins from that of the S146A indicates that two Cys residues per dimer were titrated preferentially in the latter mutant. In the presence of 25 mM D-Ala, the S146A mutant protein titrates like the wild-type, the C142G, and C212G, or the C164G mutant proteins (Figure 4). This result suggests that the Cys residues which are being titrated rapidly in the S146A enzyme could be at or near the active site.

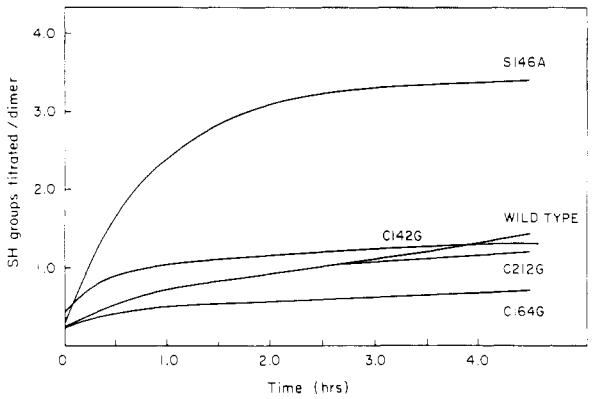

FIGURE 3: DTNB titration of SH groups of wild-type, mutant Cys enzymes, and mutant S146A enzyme at 30 °C. The protein concentrations were 0.4 mg/mL in 0.1 M Tris-HCl, pH 7.5, and 2 mM in EDTA.

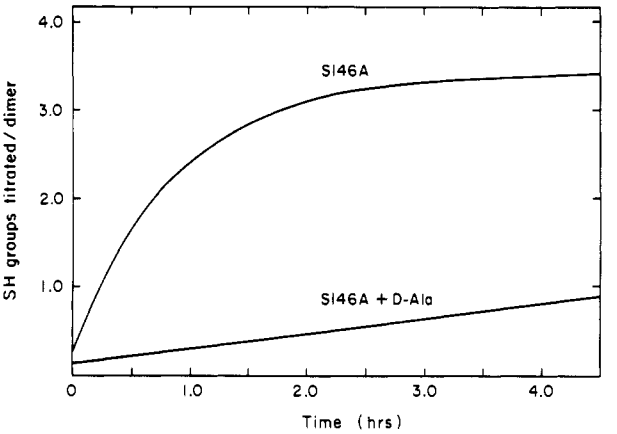

FIGURE 4: DTNB titration of SH groups of S146A in the presence and absence of D-alanine (25 mM) at 30 °C. The experimental conditions were the same as those described in Figure 3.

Finally, we have also studied the effect of the SH titrations on the activity of the S146A enzyme as shown in Figure 5. In part A, the activity of this mutant enzyme in the presence or in the absence of DTNB is plotted as a function of time. The enzyme remains more than 85% active after 2 h at 30 °C in the absence of DTNB. In the presence of DTNB, S146A is inactivated rapidly. After 2 h, only 30% of the activity remains. If the activity is represented as a function of SH groups titrated per dimer, a linear dependence of this inactivation is found (Figure 5B). Extrapolation indicates that full inactivation occurs when four of the SH groups of S146A are titrated by DTNB. Under the same conditions, the activity

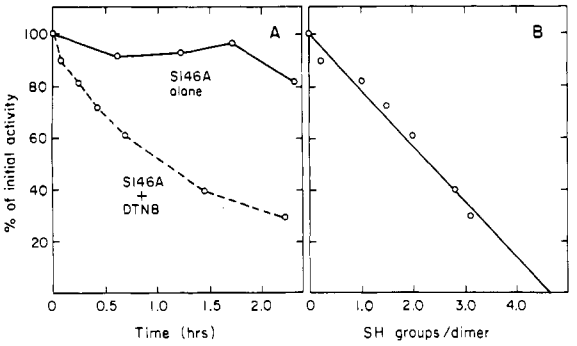

FIGURE 5: (A) Effect of DTNB on activity of S146A. The DTNB titrations were performed as described in Figure 3. Assays for enzyme activity were performed at the times designated by the open circles. (B) Correlation of activity with number of SH groups modified.

of the wild-type enzyme is not affected.

Discussion

In previous studies on purified D-amino acid transaminase from *Bacillus sphaericus*, we found that this enzyme contains eight cysteinyl residues per molecule of dimeric enzyme (Soper et al., 1977). In the denatured enzyme, all eight sulfhydryl groups are titrated by DTNB instantaneously. The native enzyme has two SH groups that react very rapidly with DTNB with very little loss in enzymic activity. Another two SH groups are slowly titrated by DTNB with a corresponding partial loss in enzyme activity. The remaining four SH groups are not titratable by DTNB in the native enzyme. However, the actual role of the reactive cysteinyl residues in the catalytic function of the enzyme could not be determined by those studies.

The studies described in this paper further our knowledge about the cysteinyl residues of D-amino acid transaminase. Two of the three Cys mutants studied in this work are as active as the wild-type protein, and the third mutant is 90% as active. Therefore, it is clear that the SH groups of the enzyme are not required for catalytic activity. Of particular interest is the effect of substitution of Ser-146 by Ala on the titration behavior of some of the cysteine residues. It appears that mutagenesis of this serine residue leads to a more open structure that reveals two SH groups that are very accessible to DTNB but are protected by D-alanine. Another possibility is that Ser-146 participates in some kind of bond, i.e., hydrogen bonding, which restricts the accessibility of DTNB to some Cys residues near the active center. Furthermore, there is a difference in the activity of the wild-type and the S146A enzymes after treatment with DTNB. This reagent reacts with only one SH group per wild-type dimer, and the derivatized enzyme remains fully active. However, four SH groups of the Ser-146 mutant react with DTNB, and the derivatized enzyme is inactive. Since none of the individual Cys mutants are inactive, we conclude that the presence of two thionitrobenzoate moieties on the protein disturbs in some way the active center of the mutant enzyme S146A.

The accessibility of the SH groups in the wild-type enzyme from the thermostable *Bacillus* is much lower than that for the enzyme from *B. sphaericus* (Soper & Manning, 1981, 1982), since for the enzyme from the former source only one Cys per dimer is titrated even after 4 h of titration. Perhaps this thermostable enzyme adopts a more compact structure, and so the accessibility of some of its residues is diminished. Finally, the replacement of the Ser located close to the PLP binding Lys residue by Ala leads to a decreased thermostability of the enzyme. Matthews et al. (1987) described how the replacement of only one amino acid residue can affect the thermostability of some proteins by modifying the configurational entropy of unfolding. Thus, replacing Ser-146 by Ala could destabilize the protein, even without changing its catalytic activity. According to the principles outlined by Matthews et al. (1987), the substitution by a Gly residue might be expected to lead to a more thermolabile protein. The results of the present study confirm this prediction for one of the three Cys mutant proteins. For the other two Cys mutants, substitution by a Gly residue is without effect, presumably because of their locations in the protein.

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Registry No. Cys, 52-90-4; Ser, 56-45-1; D-amino acid transaminase, 50864-42-1.

References

Asano, S. (1987) Thesis, Kyoto University.
Christen, P., & Metzler, D. E. (1985) in *Transaminases*, Wiley, New York.
Collier, H. B. (1973) *Anal. Biochem 56*, 310.
Dekker, L. E., & Rau, E. M. (1963) *Proc. Soc. Exp. Biol. Med. 112*, 144.
Dente, L., Cesarini, G., & Cortese, R. (1983) *Nucleic Acids Res. 11*, 1645.
DoHo, G. P., & Horiuchi, K. (1981) *J. Mol. Biol. 153*, 169.
Gale, E. F., Cundliffe, E., Reynolds, P. E., Richmond, M. H., & Waring, M. J. (1981) in *The Molecular Basis of Antibiotic Action*, Wiley, New York.
Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488.
Kunkel, T. A., Roberts, J. D., & Zakour, R. A. (1987) *Methods Enzymol. 154*, 367.
Kuramitsu, S., Inoue, K., & Kagamiyama, H. (1987) in *Biochemistry of Vitamin B_6*, p 153, Birkhauser, Boston.
Laemmli, U. (1970) *Nature (London) 227*, 680.
Manning, J. M., Merrifield, N. E., Jones, W. M., & Gotschlich, E. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 417.
Matthews, B. W., Nicholson, H., & Becktel, W. J. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 6663.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5653.
Soper, T. S., & Manning, J. M. (1981) *J. Biol. Chem. 256*, 4263.
Soper, T. S., & Manning, J. M. (1982) *J. Biol. Chem. 257*, *13930*.
Soper, T. S., Jones, W. M., Lerner, B., Trop, M., & Manning, J. M. (1977) *J. Biol. Chem. 252*, 3170.
Soper, T. S., Ueno, H., & Manning, J. M. (1985) *Arch. Biochem. Biophys. 240*, 1.
Stoddard, B., Howell, L., Asano, S., Soda, K., Tanizawa, K., Ringe, D., & Petsko, G. A. (1987) *J. Mol. Biol. 196*, 141.
Tanizawa, K., Masu, Y., Asano, S., Tanaka, H., & Soda, K. (1987) in *Biochemistry of Vitamin B_6*, p 43, Birkhauser, Boston.
Tanizawa, K., Masu, Y., Asano, S., Tanaka, H., & Soda, K. (1989a) *J. Biol. Chem.* (in press).
Tanizawa, K., Asano, S., Masu, Y., Kuramitsu, S., Kagamiyama, K., Tanaka, H., & Soda, K. (1989b) *J. Biol. Chem.* (in press).
Towbin, H., Sthaehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6350.
Wada, H., & Snell, E. E. (1961) *J. Biol. Chem. 236*, 2089.
Yonaha, K., Misono, H., Yamamoto, T., & Soda, K. (1975) *J. Biol. Chem. 250*, 6983.