Interaction of Pyridoxal 5′-Phosphate with Tryptophan-139 at the Subunit Interface of Dimeric D-Amino Acid Transaminase†

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Received September 18, 1995; Revised Manuscript Received November 8, 1995⊗

ABSTRACT: The crystal structure of dimeric bacterial D-amino acid transaminase shows that the indole rings of the two Trp-139 side chains face each other in the subunit interface about 10 Å from the coenzyme, pyridoxal 5′-phosphate. To determine whether it has a role in the catalytic efficiency of the enzyme or interacts with the coenzyme, Trp-139 has been substituted by several different types of amino acids, and the properties of these recombinant mutant enzymes have been compared to the wild-type enzyme. In the native wild-type holoenzyme, the fluorescence of one of the three Trp residues per monomer is almost completely quenched, probably due to its interaction with PLP since in the native wild-type apoenzyme devoid of PLP, tryptophan fluorescence is not quenched. Upon reconstitution of this apoenzyme with PLP, the tryptophan fluorescence is quenched to about the same extent as it is in the native wild-type enzyme. The site of fluorescence quenching is Trp-139 since the W139F mutant in which Trp-139 is replaced by Phe has about the same amount of fluorescence as the wild-type enzyme. The circular dichroism spectra of the holo and the apo forms of both the wild-type and the W139F enzymes in the far-ultraviolet show about the same degree of ellipticity, consistent with the absence of extensive global changes in protein structure. Furthermore, comparison of the circular dichroism spectrum of the W139F enzyme at 280 nm with the corresponding spectral region of the wild-type enzyme suggests a restricted microenvironment for Trp-139 in the latter enzyme. The functional importance of Trp-139 is also demonstrated by the finding that its replacement by Phe, His, Pro, or Ala gives mutant enzymes that are optimally active at temperatures below that of the wild-type enzyme and undergo the E−PLP → E−PMP transition as a function of D-Ala concentration with reduced efficiency. The results suggest that a fully functional dimeric interface with the two juxtaposed indole rings of Trp-139 is important for optimal catalytic function and maximum thermostability of the enzyme and, furthermore, that there might be energy transfer between Trp-139 and coenzyme PLP.

D-Amino acid transaminase catalyzes the synthesis of D-glutamate and D-alanine in the peptidoglycan moiety of bacterial cell walls (Soper et al., 1977); alanine racemase also catalyzes the formation of D-alanine in bacteria (Wang & Walsh, 1981). Since these enzymes are absent in mammalian cells, the design of a very specific inhibitor for either enzyme could form the basis for the development of novel antimicrobial agents. In order to understand the mechanism of action of bacterial D-amino acid transaminase, we are using site-directed mutagenesis to substitute several important sites including the active-site Lys-145, which binds the coenzyme pyridoxal 5′-phosphate (PLP)[1] as an internal aldimine structure. It was replaced by several other amino acids that could not act as nucleophiles to abstract the α-proton of substrate, yet the enzyme still displayed features, i.e., spectral shifts of the coenzyme spectrum, identical to that of the wild-type enzyme although on an extended time scale (Futaki et al., 1990; Yoshimura et al., 1992). These "attenuated" active-site mutant enzymes displayed low amounts of activity (i.e., 0.1−1.0%) that was attributed to an alternate catalytic base, Lys-267 (Yoshimura et al., 1992). In another approach, we are investigating the contribution of the amino acid side chains that bind the other functional groups of PLP and thereby influence catalysis (Van Ophem et al., 1995).

Before the structure of the enzyme was known (Sugio et al., 1995), one of the three Trp residues per subunit, Trp-139, was substituted by Phe, Ala, Pro, His, or Asp residues (Martinez del Pozo et al., 1989) in order to evaluate its importance. Those studies indicated that Trp-139 played an important role in catalysis and that some involvement with coenzyme seemed likely, although its precise role could not be determined at that time. Recently, the structure of this enzyme has been solved by Sugio et al. (1995), and the two Trp-139 side chains were found to be opposite one another in the interface between the two subunits, about 10 Å from the coenzyme PLP. In this paper, we evaluate the effect of these different types of replacements for Trp-139 on its spectroscopic properties, its thermostability, and its catalytic efficiency. These criteria provide evidence for involvement of Trp-139 with the coenzyme PLP for optimal catalytic function.

MATERIALS AND METHODS

DNA, Phage, Bacterial Strains, Enzymes, and Chemicals and Site-Directed Mutagenesis. These experimental proce-

† This work was supported in part by NSF Grant DMB-94-04332.

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⊗ Abstract published in *Advance ACS Abstracts,* February 1, 1996.

[1] Abbreviations: PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; EDTA, ethylenediaminetetraacetic acid.

0006-2960/96/0435-2112$12.00/0

dures, including those for preparation of the plasmid, for the site-directed mutagenesis experiments, and for the expression of the wild-type and mutant enzymes, have been described previously (Merola et al., 1989; Martinez del Pozo et al., 1989). Oligonucleotides were synthesized at the Rockefeller University Protein Sequencing Facility on an Applied Biosystems DNA Synthesizer 380 B using phosphoramidite chemistry.

Protein Purification. D-Amino acid transaminase was purified according to Asano (1987) and to Stoddard et al. (1987), with minor modifications as described previously (Merola et al., 1989). Before use, each purified protein was exhaustively dialyzed against 0.1 M Tris-HCl, pH 7.5, containing 2 mM EDTA, to remove excess PLP used in some of the purification steps.

Preparation of Apoenzyme. Coenzyme was removed using the procedure described by Yonaha et al. (1975) in which the enzyme is treated with phenylhydrazine at neutral pH. Under these conditions, a native apoenzyme is obtained whose activity can be nearly completely restored by addition of PLP. The amount of coenzyme remaining on the apoenzyme is 3% of the original amount as determined by its residual activity, by its absorption spectrum, and by HPLC analysis (unpublished).

The native apoenzyme retains the dimeric structure of the holoenzyme as ascertained by gel filtration on a Superose-12 10/30 column attached to a Pharmacia FPLC unit; the buffer was 0.1 M potassium phosphate containing 0.01% 2-mercaptoethanol. This system, which is capable of separating 30 kDa from 60 kDa (monomer and dimer molecular masses of the enzyme, respectively), indicated the presence of only the dimeric species of freshly prepared wild-type apoenzyme, which gave the same molecular mass as the native holoenzyme.

Enzyme 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 NADH and lactate dehydrogenase or with salicylaldehyde, as described previously (Martinez-Carrion & Jenkins, 1965; Soper et al., 1977; Jones et al., 1985). PLP was not added in these assays since nonspecific and reversible inhibition of enzyme activity was found in the presence of excess PLP as reported previously by Martinez-Carrion and Jenkins (1965) and by us (Martinez del Pozo et al., 1989). In some instances, protein concentration was determined by amino acid analysis or most often by the absorption spectrum of the enzyme. After extensive dialysis to remove excess PLP from each purified protein, one PLP was retained per subunit as determined by the method of Wada and Snell (1961) (Martinez del Pozo et al., 1989). This amount of PLP per protein subunit provided maximal enzyme activity.

Spectroscopic Characterization. Absorption spectra were recorded at 25 °C on a Cary 219 UV−Vis spectrophotometer with a recording speed of 0.5 nm/s in cells of 1-cm optical path. Fluorescence spectra were measured with a Hitachi/Perkin Elmer MPF2A spectrofluorometer at a scanning speed of 1 nm/s with slit widths of 7 and 5 nm for the excitation and emission wavelengths, respectively. The temperature in the cuvette compartment was maintained at 25 °C with a circulating water bath. The optical density of the samples at the corresponding excitation wavelengths was always lower than 0.05. Cells with optical path lengths of 1.0 and 0.4 cm were used for all fluorescence measurements. Under such conditions, the inner filter effect, as well as possible artifacts due to absorption and re-emission of fluorescence, was negligible.

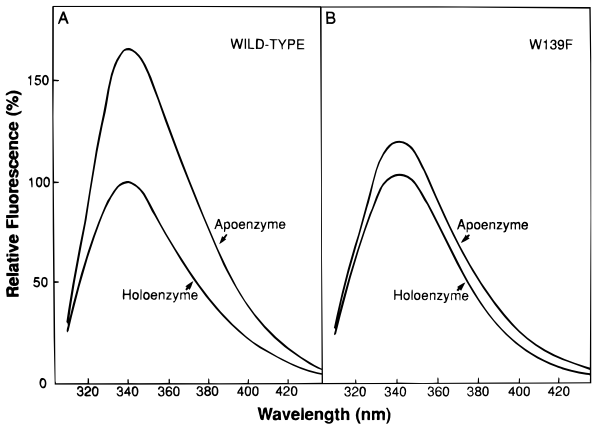

FIGURE 1: Quenching of tryptophan fluorescence in apo and holo forms of D-amino acid transaminase. (A) Wild-type enzyme; (B) W139F mutant enzyme. The protein concentration was 0.04 mg/mL in 0.1 M Bis-Tris chloride, pH 7.2, and the temperature was maintained at 25 °C. The excitation wavelength was 295 nm. The results are expressed as percentages, and the emission of the holo-wild-type enzyme at 340 nm was taken as the 100% value.

Thermal Activity Assay. This assay measures the temperature at which each enzyme shows maximum specific activity. The tubes, containing 0.5 mL of assay mixture (but no enzyme) and 0.49 mL of water, were allowed to reach the designated temperature (ranging from 25 to 80 °C), and they were permitted to remain at that temperature for 10 min. Then 10 μL of the pure protein was added to each test tube, and the amount of pyruvate formed for 2 min at that temperature was determined. Thermostability was measured by maintaining the wild-type or mutant enzyme at the designated temperature for 10 min before the addition of substrate.

Analytical Methods. After hydrolysis in 6 N HCl, the amino acid composition was obtained by a Beckman 6300 amino acid analyzer with a System Gold data handling attachment. From these results, which were consistent with those expected for the known DNA sequence (Tanizawa et al., 1989a), the protein concentration was calculated. Other analytical determinations including SDS−PAGE and SH titrations have been described previously (Merola et al., 1989; Martinez del Pozo, 1989).

RESULTS

Quenching of Tryptophan Fluorescence in Wild-Type Enzyme. The emission fluorescence of the three Trp residues in each subunit of native dimeric wild-type D-amino acid transaminase is increased by about 60% when the coenzyme PLP is removed (apoenzyme) compared to the enzyme with PLP bound (holoenzyme) (Figure 1A). This quenching, approximately one-third of the tryptophan fluorescence, is attributable to Trp-139 since in the W139F native holoenzyme, where Trp-139 is replaced by Phe, the tryptophan fluorescence is practically identical to that of the wild-type holoenzyme, indicating that Trp-139 is completely quenched in the latter; the other two Trp side chains fluoresce to the same extent in both holo and apo forms of the W139F enzyme and are not significantly quenched (Figure 1B). The one-third decrease in the tryptophan fluorescence of the apoenzyme form of the W139F mutant compared to the wild-type apoenzyme is also clearly evident in Figure 1B and

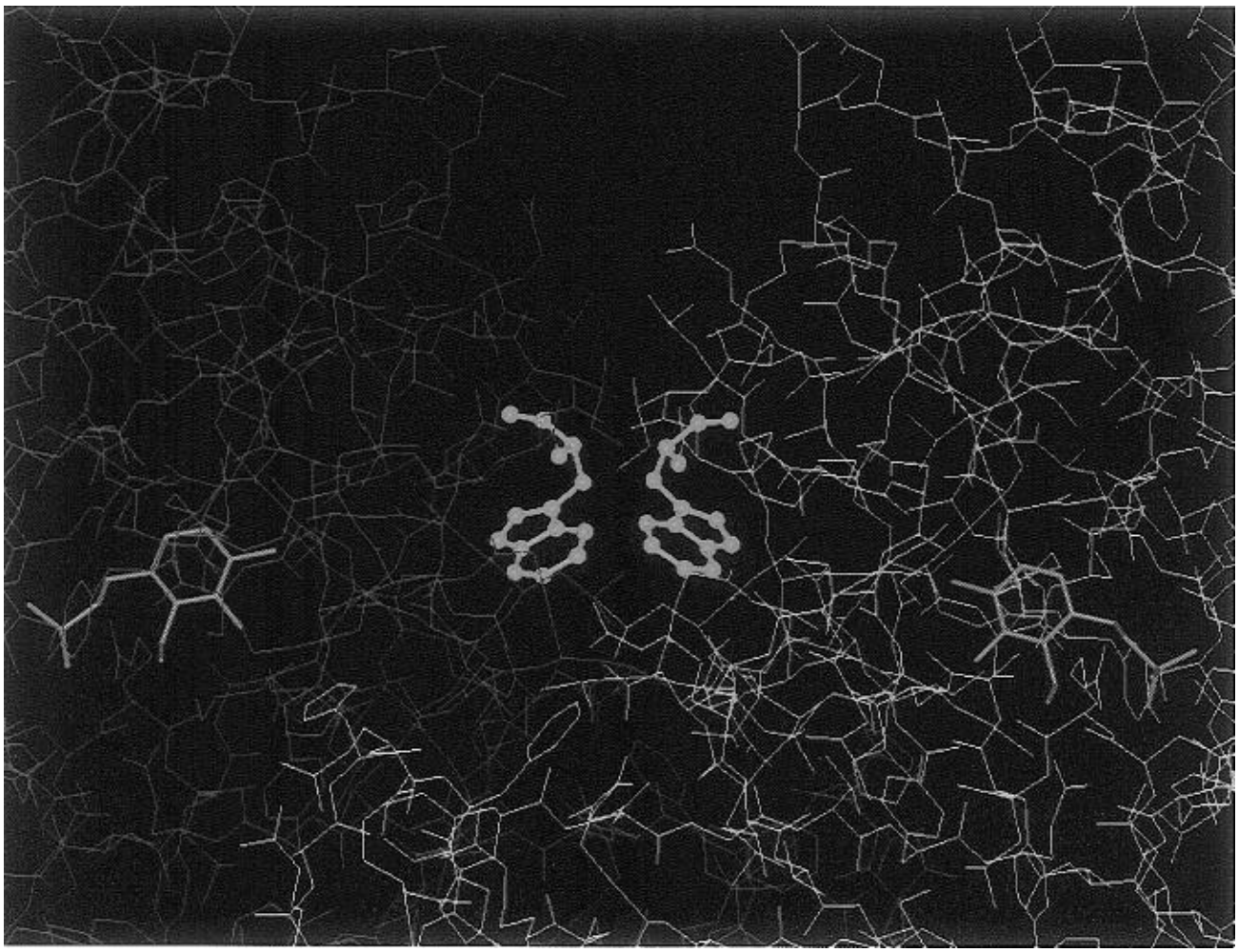

FIGURE 2: Structure of the subunit interface. From coordinates published by Sugio et al. (1995). One subunit is red, and the other is yellow. Each Trp-139 is blue, and each PLP is orange.

Figure 1A, respectively, consistent with the difference of one Trp per subunit.

The structure of D-amino acid transaminase, which has recently been solved (Sugio et al., 1995), indicates that the indole side chains of the two Trp-139 residues face each other at the subunit interface of the enzyme, about 10 Å away from the coenzyme PLP (Figure 2). Thus, the quenching of tryptophan fluorescence in the holoenzyme form of the wild-type enzyme could be due to interaction of the two juxtaposed Trp-139 side chains with each other and/or with the nearby PLP coenzyme. In the studies described next, we attempt to distinguish between these possibilities.

Reconstitution of Apoenzymes and Effect on Activity and Fluorescence. The procedure used for preparation of the apoenzyme involves removal of pyridoxal phosphate with phenylhydrazine in neutral phosphate buffer as described under Materials and Methods. This mild procedure has been shown by Yonaha et al. (1975) to yield a native, wild-type apoenzyme that is readily reconstituted with added PLP with restoration of full activity. We have confirmed these results. Furthermore, employing gel filtration on Superose-12, we have found that the apoenzyme is dimeric, indicating that the phenylhydrazine treatment does not disrupt the dimeric structure of the enzyme. Hence, these results suggest that the quenched tryptophan fluorescence of the wild-type holoenzyme compared to its apoenzyme is due mainly to the presence of the coenzyme and not the protein. Direct evidence for fluorescence quenching by addition of PLP to the apoenzyme is provided below.

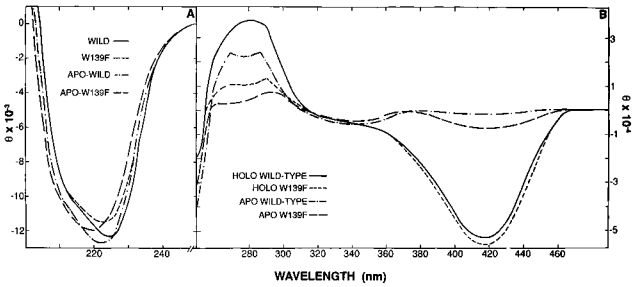

FIGURE 3: Circular dichroism spectra of holo and apo forms of wild-type and W139F mutant D-amino acid transaminases. Experimental details are given in the text.

Addition of PLP to the wild-type apoenzyme in Bis-Tris buffer results in the rapid quenching of tryptophan fluorescence (Figure 4). The extent of quenching is approximately in the same range as found for the holo-wild-type enzyme (Figure 1A). Addition of PMP in the same buffer also leads to quenching of fluorescence but at a rate considerably slower than that for PLP, probably because PMP binding is not facilitated by the internal aldimine linkage that PLP forms with Lys-145. The slow rate of fluorescence quenching by PLP in phosphate buffer is likely due to phosphate acting as competitive inhibitor of reconstitution with PLP. The differences between the three profiles in Figure 4 indicate that the PLP must be bound in the proper relationship to Trp-139 in order for fluorescence quenching to occur since free PLP in the phosphate buffer leads to quenching only very slowly. These results agree with those in Figure 1A,B

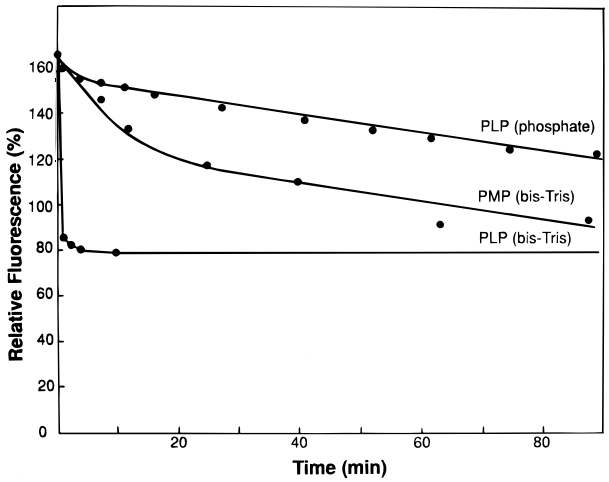

FIGURE 4: Reconstitution of apoenzymes. The native apoenzyme was prepared and characterized as described in the text. Reconstitution with PLP or PMP was performed, and the fluorescence measurements were performed as described in the text.

and indicate that the major contributor to the quenching of Trp-139 is the coenzyme PLP at the active site.

Circular Dichroism Studies. The degree of ellipticity at 222 nm is an indicator of the secondary structure content of a protein. The finding of nearly identical values for the holo and the apo forms of the wild-type as well as the corresponding forms of the W139F mutant at this wavelength provides additional evidence that the apoenzyme forms are not denatured (Figure 3). Similar studies have been reported in barnase and carbonic anhydrase II where the contributions of Trp in the far-UV region have been documented in detail (Vuillema et al., 1993; Freskgard et al., 1994). In our experiments, the circular dichroism measurements are used to provide some indications of global changes, if present, due either to the mutation or to any of the procedures employed but are not employed to calculate the absolute secondary structure content of the protein.

Evidence for the complete removal of coenzyme PLP in the apoenzyme forms of both wild-type and W139F mutant enzymes is provided by the absence of the negative circular dichroism ellipticity found for the holoenzyme forms at 420 nm, due to the optically active environment of the coenzyme. Comparison of the spectra of the wild-type and W139F mutant enzymes in the near-ultraviolet at 280 nm (Figure 3) suggests a restricted microenvironment for Trp-139; the positive ellipticity value at this wavelength in the wild-type enzyme is also consistent with this conclusion since it is absent in the W139F mutant enzyme. In addition, most of this ellipticity is retained in the apo form of the wild-type enzyme in good agreement with the fluorescence emission of the apoenzyme (Figure 1).

Activity of Trp-139 Mutant Enzymes as a Function of Temperature. Wile-type D-amino acid transaminase is a thermostable enzyme (Tanizawa, 1989a,b). Trp-139 appears to be an important site in the interface between the two subunits (Figure 2) since its replacement by nonaromatic side chains led to a dimunition in catalytic ability and stability (Merola et al., 1989; Martinez del Pozo, 1989). The W139F mutant retained 63% of the catalytic competence of the wild-type enzyme but enzymes with nonaromatic substitutions did not; i.e., replacement of Trp-139 by His, Ala, and Pro led to mutant enzymes with specific activities that were 33%, 18%, and 14%, respectively, of the wild-type enzyme. These differences in specific activities are also reflected in the

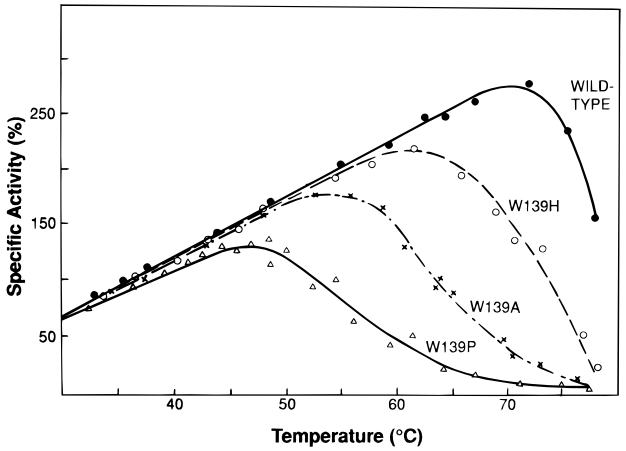

FIGURE 5: Maximal activity of wild-type of W139 mutant enzymes. The specific activity of each enzyme was determined with D-alanine and α-ketoglutarate at the indicated temperatures. The value obtained at 37 °C was set at 100%.

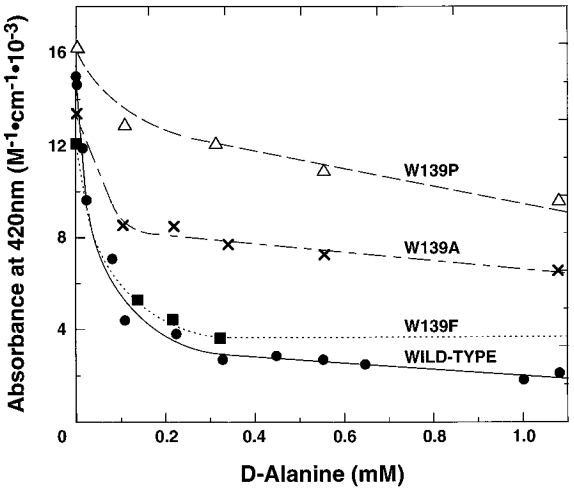

FIGURE 6: Efficiency of wild-type and W139 mutants to undergo the spectral conversion from E−PLP to E−PMP. To each enzyme was added D-alanine at the indicated concentrations and the spectrum recorded immediately.

temperature profile seen in Figure 5. For example, the W139A and W139H mutants showed optimal specific activities of 55 and 60 °C, respectively, compared to about 72 °C for the wild-type enzyme. The substitution of a Pro at position 139 resulted in an even lower thermostability of the enzyme with a decrease in activity at temperatures higher than 45 °C. The heat stability of the W139H and the wild-type enzyme did not differ significantly in the presence or absence of D-alanine. In either case, the same activity was maintained up to 50−55 °C and 60−65 °C, respectively, when each was heated for 10 min at elevated temperature. Hence, the properties of these W139 mutant enzymes are another indication of the importance of the Trp-139 interaction for both catalytic efficiency and stability.

Ability of the Mutant Enzymes To Undergo Conversion of E−PLP to E−PMP. An important factor in the catalytic competency of the wild-type D-amino acid transaminase is its ability to abstract the α-proton of substrate rapidly. Concomitant with this process, the aldimine structure is converted to the ketimine form of the enzyme, which can be readily measured by the coenzyme absorbance change from 420 to 330 nm in the presence of D-amino acid substrate alone, i.e., the half-reaction of transamination. As shown in Figure 6, this transformation is accomplished at very low concentrations of D-alanine with the wild-type enzyme as

well as the W139F mutant enzyme. However, the initial response of the W139P and W139A mutants to low concentrations of D-alanine is lower than that of the wild-type and the W139F mutant enzymes, and these two mutants show a diminished tendency to undergo the transformation of the E−PLP form to the E−PMP form even with increasing concentrations of D-alanine. Taken together with the results above, it can be reasonably concluded that a hydrophobic interaction of some type at position 139 with the coenzyme is needed for optimum catalytic ability.

DISCUSSION

The crystal structure of dimeric D-amino acid transaminase shows that the two Trp-139 sites are located in a hydrophobic pocket at an interface between the subunits (Figure 2) (Sugio et al., 1995) and that its two indole side chains face one another and are within 10 Å of coenzyme PLP. In an earlier report, we assessed the importance of Trp-139, and we found that its replacement by Phe (W139F) but not other amino acids nearly completely satisfied the requirement of a hydrophobic interaction at this site (Martinez del Pozo, 1989). In the present work, we report that histidine is also accommodated at position 139 (W139H) to provide a partially active mutant enzyme. The alanine and the proline mutant enzymes (W139A and W139P, respectively) are progressively less active, and the mutant enzyme with a negatively-charged aspartate residue at this position (W139D) could not be isolated perhaps because the bacterium degraded the detached subunits. The general conclusion made from the several different types of techniques employed in this study is that the *nature* of residue 139 plays an important role in the catalytic efficiency of D-amino acid transaminase brought about by a stable dimeric structure.

The quenching of Trp-139 fluorescence described here could arise from at least three different sources or possibly a combination of several of them: static quenching in which a nonfluorescent ground-state complex is formed between a fluorophore, such as Trp, and a quencher, such as PLP or PMP. This possibility is probably excluded because the distance of 10 Å between Trp-139 and PLP is too large for direct contact. The second possibility is that there is self-quenching by the two juxtaposed Trp-139 side chains but this does not seem likely since the apoenzyme, which we demonstrated in this study to be dimeric and hence has its two Trp-139 side chains opposite one another as in the native enzyme (Figure 2), actually shows an *increased* fluorescence. The third possibility is that there is radiationless energy transfer from Trp to the coenzyme PLP at the active site of the enzyme. The tryptophan fluorescence spectra of both the apo- and holo-wild-type enzymes are consistent with an energy transfer process between the Trp-139 and the PLP cofactor, as suggested earlier (Martinez del Pozo et al., 1989). The results of Sugio et al. (1995) show that the coenzyme PLP is about 10 Å from the Trp at the subunit interface; this distance is compatible with energy transfer.

Radiationless energy transfer from Trp to chloramphenicol has recently been reported for chloramphenicol acetyltransferase using resolution of individual excited-state lifetimes by site-directed mutagenesis and multifrequency phase fluorometry (Ellis et al., 1995). In this case, quenching of Trp fluorescence upon binding of chloramphenicol (which decreases 51% and 39% for two individual Trp residues of the enzyme) is explained in terms of energy transfer from those Trp at distances of 17.2 and 16.6 Å from the substrate. These distances are greater than between PLP and Trp-139 in each subunit of D-amino acid transaminase. Churchich (1965) had earlier shown that the requirement for spectroscopic overlapping in energy transfer is conveniently met by the Trp−PMP pair. The results in this paper not only provide support for the requirement of an aromatic character at position 139 in the interface between the two subunits but also provide evidence for its involvement with the coenzyme and thereby in the maximum degree of thermostability and catalytic efficiency of D-amino acid transaminase.

ACKNOWLEDGMENT

We are grateful to Wanda Jones for her help in various aspects of this work. The skillful assistance of Adelaide Acquaviva and Judith A. Gallea in the preparation of the manuscript is very much appreciated.

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BI9522211