Activity and Spectroscopic Properties of Bacterial D-Amino Acid Transaminase after Multiple Site-Directed Mutagenesis of a Single Tryptophan Residue†

A. Martinez del Pozo, M. Merola, H. Ueno, and J. M. Manning*
The Rockefeller University, New York, New York 10021
K. Tanizawa, K. Nishimura, S. Asano, H. Tanaka,‡ and K. Soda
Kyoto University, Uji, Kyoto, Japan
D. Ringe and G. A. Petsko
Massachusetts Institute of Technology, Cambridge, Massachusetts 02139
Received July 1, 1988; Revised Manuscript Received August 30, 1988

ABSTRACT: One of the three tryptophan residues per subunit of thermostable D-amino acid transaminase, Trp-139, is close to the active-site Lys-145 in the sequence of the protein. This tryptophan has been changed to several other types of residues by site-directed mutagenesis. The only mutant protein that was sufficiently active and stable for study had Phe substituted for Trp (W139F). The spectroscopic properties of this mutant enzyme differed from those of the wild-type transaminase. For example, denatured W139F showed the expected decrease in fluorescence emission intensity at 350 nm due to the deletion of one Trp residue, but the fluorescence emission of the wild-type and W139F enzymes in the native state did not differ in intensity. This result suggests that the fluorescence of Trp-139 in the native, wild-type enzyme is not manifested perhaps due to its proximity to the coenzyme, pyridoxal phosphate. Results of energy-transfer studies at several wavelengths could also be interpreted as due to the proximity of Trp-139 and the coenzyme. Circular dichroism studies indicated that the negative Cotton effect at 420 nm due to the coenzyme was still present in W139F. However, the 280-nm optically active band present in the wild-type enzyme was greatly diminished in W139F. The mutant protein with Asp at position 139 (W139D) could not be isolated presumably because it was degraded. The other mutant enzymes, W139P, W139A, and W139H, were isolated with partial activities (15–35%) that were slowly lost upon storage at 4 °C. Overall, these results indicate the importance of Trp-139 in the thermostable D-amino acid transaminase.

We have employed site-directed mutagenesis to replace each of the cysteinyl residues of thermostable D-amino acid transaminase by a glycine residue (Merola et al., 1989). The results indicate that none of the these cysteine residues is essential for catalytic activity since each mutant protein has the same specific activity as the wild-type enzyme. It was also demonstrated that replacement of Ser-146 adjacent to the active-site Lys-145 by an alanine residue led to a fully active enzyme with an enhanced rate of SH titration by DTNB. As shown for the wild-type (Asano, 1987; Tanizawa et al., 1987, 1989a,b), the mutant enzymes are expressed on the order of 10% of the bacterial protein. Hence, this procedure provides us with the opportunity to discover the role of other amino acid residues in this enzyme if judicious selections of mutation sites are made.

In the present report, we describe one such mutation based on the known structure of L-aspartate transaminase. Both solution studies (Iriarte & Martinez-Carrion, 1983) and high-resolution X-ray diffraction analysis (Jansonius et al., 1985; Arnone et al., 1985) of this L-amino acid specific transaminase revealed that Trp-140 interacts strongly with the coenzyme pyridoxal phosphate (PLP)[1] linked to the active-site Lys-258. This interaction and others with the protein render the coenzyme in a fairly rigid orientation. However, the exact role of Trp-140 in catalysis by L-aspartate transaminase is not precisely known, and site-directed mutagenesis of this residue has not yet been reported.

We have chosen to mutate Trp-139 of D-amino acid transaminase since it is close to the active-site Lys-145 in the linear sequence of the protein. It is conceivable that Trp-139 may have a role in D-amino acid transaminase analogous to that of Trp-140 in L-aspartate transaminase. Therefore, it has been changed to a series of other types of amino acid residues including aliphatic, heterocyclic, and aromatic residues. These findings are reported in this paper.

Materials and Methods

DNA, Phage, Bacterial Strains, Enzymes, and Chemicals and Site-Directed Mutagenesis. These experimental procedures are described in the preceding paper (Merola et al., 1989). Oligonucleotides were synthesized at the Rockefeller University Protein Sequencing Facility on an Applied Biosystems DNA 380 B synthesizer using phosphoramidite chemistry. The italicized bases represent the mismatched ones that are located approximately in the center of the sequence. To replace Trp-139 by a Phe residue, we used the following 24-mer oligonucleotide:

W139F: 5′-TC ACA TCG TAA *GA*A ACG GAT ATC T-3′

† This work was supported in part by NSF Grant DMB-86-10269 to J.M.M., in part by Biomedical Research Support Grant BRSG-507-RR-07065 from the Division of Research Resources, National Institutes of Health, to The Rockefeller University, and by the W. R. Grace, Co. (D.R.).
‡ Present address: Laboratory of Applied Gene Chemistry, Faculty of Agriculture, Okayama University, Okayama 700, Japan.

[1] Abbreviations: PLP, pyridoxal 5′-phosphate PMP, pyridoxamine 5′-phosphate; CD, circular dichroism; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)-aminomethane.

0006-2960/89/0428-0510$01.50/0

Table I: Properties of D-Amino Acid Transaminase Mutants

enzyme	yield of pure protein (mg)	sp act. at 37 °C[a] (%)	PLP content (per protein subunit)	ϵ_{280} (M^{-1} cm^{-1} per dimer)	ϵ_{338} (M^{-1} cm^{-1} per dimer)	ϵ_{420} (M^{-1} cm^{-1} per dimer)
wild-type	112	100	1.07	81725	5500	15150
W139F	50	63	1.08	59800	5330	12850
W139P	59	14	1.02	57550	4039	16400
W139A	129	18	1.13	56202	5450	13700
W139H	22	33	nd[b]	64040	5130	15000

[a] Assayed in the absence of PLP. [b] Not determined.

To obtain the other mutants containing Asp, Pro, His, or Ala residues, we used the following 27-mer mixed oligonucleotide:

5′-ATC ACA TCG TAA G^{TG}_{GC} ACG GAT ATC TTC-3′

The procedures used for preparation of the plasmid, for the site-directed mutagenesis experiments, and for the expression of the enzyme are described in the preceding paper (Merola et al., 1989).

Protein Purification. D-Amino acid transaminase was purified according to Asano (1987) and to Stoddard et al. (1987), with minor modification as described in the preceding paper (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.

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). The apparent Michaelis constant for PLP with the thermostable enzyme is 3.5 μM.[2] After extensive dialysis to remove excess PLP from each purified protein, there is one PLP per subunit retained by the enzyme as determined by the method of Wada and Snell (1961) (Table I). This amount of PLP per protein subunit provided maximal enzyme activity.

Spectroscopic Characterization. Absorption spectra were recorded at 25 °C on an Aminco DW-2 UV/VIS spectrophotometer, with a recording speed of 0.5 nm/s in cells of 1-cm optical path. Fluorescence spectra were measured on a Hitachi/Perkin-Elmer MPF2A spectrofluorometer at 1 nm/s scanning speed with slit widths of 7 and 5 nm for the excitation and emission wavelengths, respectively. The temperature was maintained at 25 °C with a circulating water bath. To obtain the corrected excitation spectra and the fluorescence polarization values, a Perkin-Elmer MPF-44E spectrofluorometer, equipped with a DCS unit, was used as described previously (Martínez del Pozo et al., 1986). 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 the fluorescence measurements. Under such conditions, the inner filter effect, as well as possible artifacts due to absorption and reemission of fluorescence, was negligible. We ascertained that the energy-transfer processes described below were independent of the optical path employed and were not due to trivial radiative energy-transfer events.

Circular dichroism (CD) spectra were obtained on a Aviv-modified Cary 60 dichrograph at a scanning speed of 0.2 nm/s. Optical path cells of 0.05 and 1.0 cm were used in the far-ultraviolet (below 250 nm) and the near-ultraviolet and visible (500–250 nm) regions, respectively, as described previously (Martínez del Pozo et al., 1988). The results of the CD studies are expressed as molar ellipticities, in units of degrees centimeter squared per decimole, using 32 000 as the molecular weight per enzyme subunit (Tanizawa et al., 1987, 1989a,b). The protein concentrations were 0.4 and 2 mg/mL for the measurements in the far- and in the near-UV and visible regions, respectively.

Other analytical determinations including SDS–PAGE and amino acid analysis are described in the preceding paper (Merola et al., 1989).

Results

Identification of Clones. Mutant W139F was easily identified by using methodology described in the preceding paper (Merola et al., 1989) with a yield >80%. On the other hand, the use of a mixed oligonucleotide to generate different mutations in the same codon gave unexpected results since, in addition to a high background of wild-type (31%), most of the clones were W139P and W139A (34% and 28%, respectively), and only 3.5% were W139H and W139D. Because the former two mutants are generated by an introduction of a C in the second position of the codon (corresponding to a G on the mutagenic oligonucleotide) and the other two by an A (corresponding to a T on the oligonucleotide), it is possible that the GG pair is favored about 10 times more than the TG pair. One positive clone from each was chosen, purified, and checked by DNA sequencing around both sides of the mutagenic point. This same clone was used to isolate the protein. We have not been able to isolate the mutant enzyme W139D although the DNA sequence was correct, as described below.

Purification of Mutant Enzymes. The preparations of the mutant D-amino acid transaminase proteins W139F, W139H, W139A, and W139P were essentially >95% homogeneous as judged by SDS–PAGE. The amino acid compositions were in excellent agreement with the predicted values (Asano, 1987; Tanizawa et al., 1987). The amount of protein recovered from 20 g (wet weight) of bacteria was always greater than 20 mg. The PLP content of the purified W139F, W139A, and W139P mutant proteins was identical with that found for the wild-type enzyme, i.e., two molecules of cofactor per molecule of dimeric protein (Table I) as determined by the method of Wada and Snell (1961). Comparison of the amino acid composition of the wild-type and mutant enzymes was consistent with the replacement of one tryptophan residue in the latter enzymes.

The mutant in which Trp-139 was changed to Asp (W139D) was not isolated since there was no 32-kDa band detected by SDS–PAGE of an extract of this mutant. However, multiple antibody-reactive bands were detected with higher and lower molecular weight mobilities. This result suggests that the change from Trp to Asp at position 139 severely compromises the integrity of the enzyme.

Specific Activity and Stability of Mutant Enzymes. The pure mutant proteins had specific activities that were lower

[2] Tanizawa et al., personal communication.

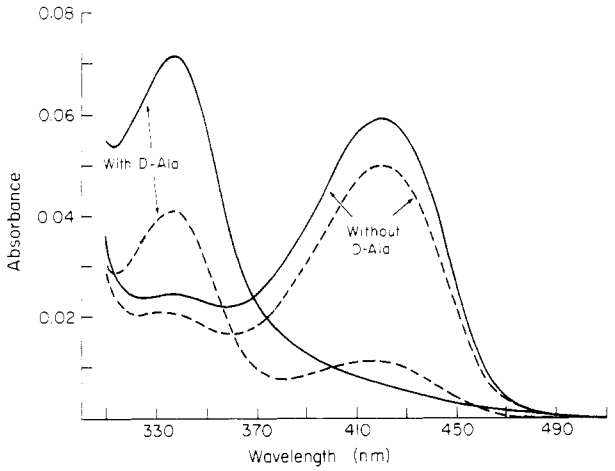

FIGURE 1: Absorption spectra of the wild-type (—) and the W139F mutant (---) D-amino acid transaminase, in the absence and the presence of substrate. The protein concentration was 0.25 mg/mL in 0.1 M Tris-HCl, pH 7.5, and 2 mM EDTA. When present, the concentration of D-Ala was 8 mM.

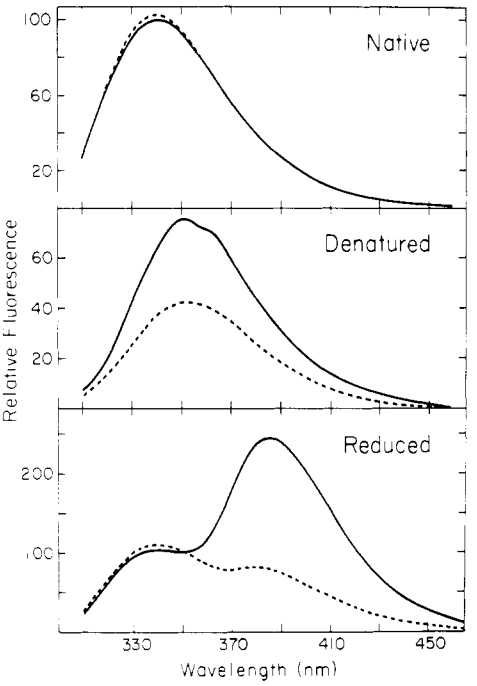

FIGURE 2: Fluorescence emission spectra of the wild-type (—) and the W139F mutant (---) proteins. The excitation wavelength was 295 nm, and the protein concentration was 0.04 mg/mL in 0.1 M Tris-HCl, pH 7.5, and 2 mM EDTA. The temperature was maintained at 25 °C. The results are expressed as emission percentages, considering 100% as the emission at 340 nm of the native wild-type enzyme. (Top panel) Native enzymes; (middle panel) enzymes denatured in the presence of 6.4 M guanidine hydrochloride; (bottom panel) $NaBH_4$-reduced enzymes. This reduction was performed according to Yonaha et al. (1975) but employing 0.1 M Tris-HCl, pH 7.4, containing 2 mM EDTA as the buffer. The efficiency of the reduction was monitored through the disappearance of the absorption band at 420 nm.

than that of the wild-type enzyme. Thus, W139F was 63% as active as the wild-type, W139H was 33% as active, and W139A and W139P were even less active—18% and 14%, respectively (Table I). W139A, W139H, and W139P were fully inactive after 20 days at 4 °C. Other manifestations of the instability of these three mutants were that their PLP contents gradually decreased and then they precipitated. Therefore, the observations presented here for W139H, W139A, and W139P were made during the first week after their isolation, unless otherwise indicated. After purification, only W139F and the wild-type protein retained their original activities for longer than 2 months. Therefore, we focused our attention on these two enzymes. With catalytic amounts of enzyme, no pyruvate production was detected in any of the five proteins studied when concentrations of L-alanine as high as 50 mM were used.

Effect of D-*Alanine on the Absorption Spectra.* Since the tryptophan residue and the coenzyme PLP are major chromophores in D-amino acid transaminase, the characterization of the isolated enzymes has emphasized their spectroscopic properties. The absorption spectrum of the wild-type D-amino acid transaminase, as described in the preceding paper (Merola et al., 1989), has a major spectral band at 420 nm with a minor peak centered at 338 nm (both due to the coenzyme) and a very well-defined protein band at 282 nm (Asano, 1987; Tanizawa et al., 1987, 1989a,b). All the mutant proteins displayed very similar coenzyme absorption spectra with bands at the same wavelength as the wild-type enzyme, but their extinction coefficients were somewhat different (Table I). Significant differences were found at 282 nm between the mutant proteins and the wild-type enzyme. This absorbance is due to the Tyr and Trp residues of the protein and to the coenzyme pyridoxal phosphate and the nature of the environment that surrounds this cofactor. (However, the differences observed at 282 nm are too large to be explained *only* by the deletion of a single tryptophan residue. Factors such as the relative degree of polarity of the microenvironment around the coenzyme in the wild-type and the mutant enzymes must be considered, as described below.)

The efficient titration of the 415-nm band by D-alanine has been reported previously for D-amino acid transaminase both from *Bacillus subtilis* (Martinez-Carrion & Jenkins, 1965) and from *Bacillus sphaericus* (Yonaha et al., 1975). The part of the absorption spectra that corresponds to the coenzyme of the wild-type enzyme and of the mutant enzyme W139F is shown in Figure 1. The spectrum of the wild-type protein was readily shifted from 420 to 338 nm by a low concentration (0.5 mM) of D-alanine. In contrast, the spectral shift of W139F is incomplete even at a 10-fold higher concentration of D-Ala. This result suggests that either the binding of D-alanine to W139F or its catalytic turnover is not as efficient as it is with the wild-type enzyme. Spectral titration studies were also performed with the other mutant proteins in the presence of increasing amounts of D-Ala (data not shown). In each case, a fairly high concentration of D-Ala (0.1 M) was required to achieve the spectral shift from 420 to 338 nm. For the mutant enzymes W139A, W139H, and W139P, this spectral titration required several minutes whereas with the wild-type and W139F enzymes it occurred within the time of mixing the solutions in the cuvette. Furthermore, the PMP absorbance of W139H, W139A, and W139P is shifted to shorter wavelengths (333 nm), indicating a different microenvironment around the coenzyme for these mutant enzymes compared with the wild-type and the W139F enzymes.

Fluorescence Intensity. The emission fluorescence spectra of the wild-type and mutant enzymes in their native states were measured at an excitation wavelength of 295 nm. At this wavelength, only tryptophan residues but not PLP become fluorescent. The wild-type enzyme as well as the W139F, W139A, and W139P mutants showed almost the same emission spectrum, which was centered at 340 nm, a usual emission wavelength of Trp in proteins. Unexpectedly, the *native* wild-type and each of the mutant proteins displayed the same emission *intensity* although the mutant proteins contain one tryptophan less than the wild-type enzyme (Figure 2, top

panel). The results that are shown for the W139F enzyme are representative of those found for the other mutant enzymes. Thus, it appears that the fluorescence of Trp-139 is not evident in the wild-type enzyme.

The emission fluorescence spectra of the wild-type and mutant enzymes in the denatured state were also recorded in the presence of 6.4 M guanidine hydrochloride so that specific interactions between residues would be eliminated as a consequence of the unfolding of the protein. The quantum yield of the denatured mutant W139F was 59% that of the denatured wild-type enzyme (Figure 2, middle panel). This result is consistent with the replacement of one of the three Trp residues per subunit of this enzyme by the site-directed mutagenesis. In this case, the use of emission fluorescence is preferable to measurement of the absorbance spectra since the fluorescence measures only the Trp emission without interference from other chromophores in the protein. The observed red shift in the spectra of the denatured proteins with respect to that in their native states, as well as the decrease in the emission intensity, is due to the interaction of the newly exposed tryptophan residues with the solvent in the denatured state.

Energy-Transfer Studies. Churchich (1965) has indicated that the requirement for spectroscopic overlapping in energy transfer is conveniently met by the Trp–PMP pair. In fact, it has been shown how radiationless energy transfer occurs from the tryptophan residues to pyridoxal chromophores covalently attached to several proteins. However, attempts to detect this kind of energy transfer with native L-aspartate aminotransferase have been unsuccessful (Churchich, 1965) in spite of the fact that the coenzyme ring interacts strongly with the indole ring of Trp-140 (Arnone et al., 1985; Jansonius et al., 1985).

For D-amino acid transaminase, fluorescence emission spectra of the reduced enzyme have been carried out with an excitation wavelength of 295 nm (Figure 2, bottom panel). We have also performed the same fluorescence measurements for the native transaminases in the presence of 0.1 M D-Ala. A high concentration of D-Ala was used to ensure that all of the enzyme was present in the PMP form. The results for the PMP form of the enzyme were very similar to those for the reduced enzyme, so only the results for the latter sample are shown in Figure 2. The emission of free PMP itself, when excited at 295 nm, was subtracted from the spectra shown in Figure 2. There is a large emission centered at 390 nm when the reduced or PMP form of the wild-type enzyme is excited at 295 nm. The 390-nm emission is almost abolished in the W139F mutant (Figure 2) and practically absent in the W139P and W139A mutant proteins (data not shown). The emission spectra of the PMP form of the wild-type and mutant enzymes in the presence of 6.4 M guanidine hydrochloride did not show the 390-nm emission. Thus, this energy transfer disappears when the proteins are denatured. On the other hand, the emission intensities at 340 nm for the PMP form of the four proteins in the absence of denaturing agents are identical with each other and similar to those for the PLP forms of the enzymes (Figure 2, top panel).

When the emission of these samples is recorded with an excitation wavelength of 338 nm, only the emission due to the cofactor is observed (data not shown). These spectral bands are centered at 390 nm for the wild-type and W139F enzymes but shifted to 393 nm for the W139A and W139P enzymes. These findings agree with the absorption spectra of the mutant proteins in their PMP form as described above and indicate a different environment for the cofactor. Although each

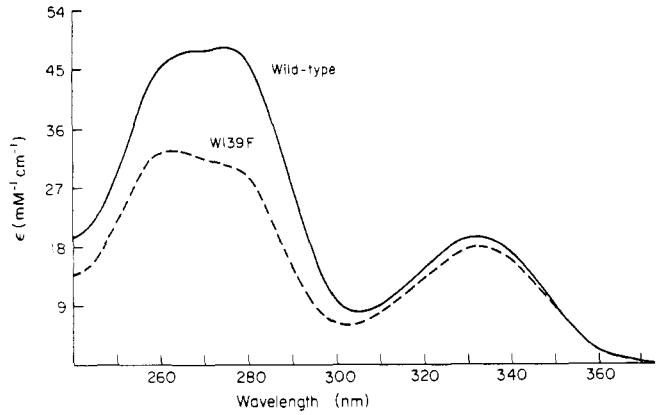

FIGURE 3: Fluorescence excitation spectra of D-amino acid transaminase. The reduced wild-type (—) and W139F (---) enzymes with an emission of 390 nm. The temperature was maintained at 25 °C. Protein concentration was 0.04 mg/mL in 0.1 M Tris-HCl, pH 7.5, containing 2 mM EDTA. These spectra were corrected by employing the DSC unit of the spectrofluorometer, and the figure was constructed by considering the absorbance values of the reduced enzymes at 340 nm, as described in the text.

mutant enzyme contains the same amount of PLP (Table I), their emission intensities were not identical. For example, the quantum yield for the W139F mutant was 25% lower than that of the wild-type enzyme, whereas W139P and W139A displayed even lower emissions with quantum yields of 50%. These different emissions are also consistent with the existence of somewhat different environments for the PLP coenzyme. Hence, it is conceivable that in the absence of Trp-139 the cofactor could be exposed to the solvent or to some other residue of the protein to result in some quenching. Furthermore, these lowered quantum yields cannot explain the difference in energy transfer shown in Figure 2, since the emission at 390 nm of W139F is only 24% of that of the wild-type and it is even lower in the other two mutant proteins, W139A and W139P.

The fluorescence excitation spectra of the reduced wild-type and the reduced W139F enzymes have also been recorded with the emission set at 390 nm (Figure 3). These excitation spectra should be qualitatively the same as the absorption spectrum of the reduced PLP bound to the protein. For the wild-type enzyme, three main peaks are detected at 265, 275, and 335 nm, as described by Yonaha et al. (1975) for the enzyme from *B. sphaericus*. For the spectrum of W139F, the absorbance in the 250–300-nm range is greatly diminished. The spectra shown in Figure 3 were calculated by considering the extinction coefficients at 340 nm for both enzymes in their reduced forms. If the ϵ_{340} values are considered to be equivalent, then the ϵ for the excitation spectra at other wavelengths can be calculated to derive the absorption spectra of all the fluorophores contributing to the emission at 390 nm of the reduced PLP in both enzymes. When this difference is calculated, the entire spectrum is that of tryptophan absorbance with the peak centered at 275 nm and a $\Delta\epsilon_{280}$ of 16 900 M^{-1} cm^{-1} for the dimeric protein. Considering that the ϵ_{280} for a Trp residue is 6000 M^{-1} cm^{-1} (Metzler et al., 1972), this result indicates that the difference between both enzymes is due to the contribution of 2.8 Trp per dimer of protein. These results agree with the removal of Trp-139 in the W139F mutant, and they clearly demonstrate that the energy transfer is due to the tryptophan residue.

Fluorescence Polarization of the Cofactor. The fluorescence polarization values at 390 nm for an excitation of 338 nm for the wild-type and W139F enzymes are shown in Table II. These values, which are in good agreement with those reported

Table II: Polarization Values of Wild-Type and W139F Mutant D-Amino Acid Transaminases

	polarization	α^0
wild-type + 0.1 M D-Ala	0.159	40.6
W139F + 0.1 M D-Ala	0.124	43.9
reduced wild-type	0.354	17.1
reduced W139F	0.351	17.8
PMP alone	0.033	51.9

for some other transaminases (Churchich, 1984), indicate that in both enzymes the movement of pyridoxal phosphate is restricted. This parameter can also be expressed as the angle of rotation of the coenzyme (Table II) as calculated according to Kim and Churchich (1987). In the presence of 0.1 M D-Ala, these values are lower, as observed with many other PLP-dependent enzymes (Churchich, 1984). Low polarization could have been due to loss of PLP from the protein at such high D-Ala concentrations. However, this possibility is considered unlikely since other transaminases, such as the 4-aminobutyrate aminotransferase (Kim & Churchich, 1987), exhibit an angle of rotation of 46°, which is even higher than the 41° found for the wild-type D-amino acid transaminase. Indeed, the polarization value is lower in W139F than in the wild-type enzyme. This result indicates that Trp-139 could contribute to the immobilization of the cofactor in the PMP form of the enzyme.

Circular Dichroism Spectra. The circular dichroism spectrum of bacterial D-amino acid transaminase from *B. sphaericus* was first determined by Yonaha et al. (1975), who clearly demonstrated that there was a *negative* Cotton effect in the region corresponding to the absorption of the cofactor. The enzyme from the thermophilic *Bacillus* has a very similar spectrum (Tanizawa et al., 1987, 1989a,b). This finding indicated that the coenzyme was in an optically active environment that was of a different type than that for the coenzyme in L-aspartate transaminase, which had a *positive* Cotton effect in this region. Yonaha et al. (1975) also demonstrated that the spectrum corresponding to tyrosine and tryptophan residues of the protein had a positive Cotton effect at 280 nm. The wild-type and the W139F mutant D-amino acid transaminases from the thermophilic *Bacillus* have about the same circular dichroism spectra in the region of the coenzyme absorbance (420 nm) (Figure 4) as the D-amino acid transaminase described by Yonaha et al. (1975) and Tanizawa et al. (1987). However, the spectrum of W139F is significantly different at 280 nm. These results suggest that the Cotton effect at 280 nm is due predominantly to Trp-139 which may be highly restricted in its movement in the native wild-type enzyme. Yonaha et al. (1975) also showed that the denatured, reduced, and carboxymethylated D-amino acid transaminase did not have active Cotton effects in the regions of the coenzyme absorption or the aromatic amino acid residue absorption. The CD spectra in the far-UV range of the wild-type enzyme and the W139F mutant revealed that both enzymes contain a very similar secondary structure.

Discussion

The results of the present studies are consistent with the proposal that Trp-139 influences the spectral behavior of the coenzyme pyridoxal phosphate in D-amino acid transaminase. This is true with respect to its fluorescence spectra and polarization, the energy transfer, and the circular dichroism spectra of the wild-type and the phenylalanine mutant, W139F. However, the results do not indicate definitively whether Trp-139 has a role analogous to that of Trp-140 in stabilizing the active-site pyridoxal phosphate of L-aspartate transaminase.

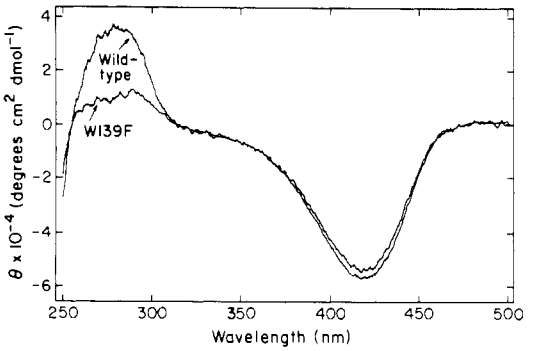

FIGURE 4: Near-ultraviolet circular dichroism spectra of D-amino acid transaminase. The native wild-type and W139F mutant enzymes were in 0.1 M Tris-HCl, pH 7.5, containing 2 mM EDTA. The results, which were obtained in triplicate, are expressed as degrees centimeter squared per decimole.

This conclusion must await the results of X-ray diffraction analysis currently in progress. Nevertheless, the findings indicate that Trp-139 plays some important role in D-amino acid transaminase since its replacement by a variety of other nonaromatic amino acid residues including Ala, Pro, and His produces an enzyme with very low initial activity that is subsequently lost under conditions where the wild type and the mutant W139F are stable. In addition, when an aspartate is introduced at position 139, no active protein can be isolated. Presumably, it is degraded rapidly by the bacteria. Since the only mutant protein (W139F) that we have found to be stable over a period of time is that with another aromatic residue (Phe) replacing Trp-139, it appears that the *nature* of residue 139 is crucial for the stability of the transaminase.

The absorption spectra corresponding to the coenzyme of the wild-type and the mutant enzymes show the same bands as the D-amino acid transaminases from *Bacillus subtilis* (Martinez-Carrion & Jenkins, 1965), from *Bacillus sphaericus* (Yonaha et al., 1975), and from the thermophilic *Bacillus* (Tanizawa et al., 1987, 1989a,b). However, whereas the intensity of the 330-nm band is about twice that for the 415-nm band for these enzymes from the first two sources, the latter absorbance band is more intense both for the wild-type enzyme from the thermophilic *Bacillus* (Asano, 1987) and for the mutant enzymes reported in this paper.

There is a large difference in the absorption intensity at 280 nm for the wild-type and the mutant enzymes, but the reason for this is not apparent at this time. This finding is consistent with the difference in the excitation spectra of the wild-type and the W139F mutant enzymes. It is conceivable that changes in the environment of the PLP attached to this transaminase, i.e., the presence or absence of a tryptophan nearby, could explain the small differences observed at 420 nm as well as the larger differences of the extinction coefficients at 280 nm. This latter absorption band not only would be due to Trp and Tyr but also would be influenced by the PLP and its microenvironment. Hence, it has been shown that the absorption behavior of PLP Schiff bases is affected by some organic solvents. Thus, the relative intensities of the absorption bands of the Schiff base of PLP with *n*-hexylamine are highly dependent on the medium polarity in mixtures of dioxane–water (Shaltiel & Cortijo, 1970). These bands can be attributed to the different tautomers of the PLP Schiff bases, whose stability also depends on the pH and polarity of the solvent.

The differences in the fluorescence spectra between the wild-type and the W139F enzymes when an excitation wavelength of 295 nm is employed (corresponding to Trp) are clearly indicative that the different energy-transfer efficiencies

are due not only to changes in the microenvironment of the PLP but also that Trp-139 is directly involved. However, there is also some energy transfer in some of the mutants, but these contributions are very small (24% of that of the wild-type for W139F). This residual energy-transfer fluorescence for W139F is probably due to some nonspecific interaction of some of the other Trp residues. In fact, Churchich (1965) has also observed that some energy transfer can be observed in L-aspartate transaminase in the presence of excess PMP or a protein denaturant such as 8 M urea. Indeed, the existence of energy transfer between the other two Trp residues and the cofactor is also possible.

The results of the fluorescence emission studies indicate that there is a clear energy transfer between the Trp in the wild-type transaminase which is almost absent in the W139F mutant, whereas the emission at 340 nm due to Trp remains unchanged both before and after reduction with $NaBH_4$. Since the only differences between these two proteins is the substitution of Trp-139 by a Phe residue in the mutant enzyme, we can conclude that there is an energy-transfer process between Trp-139 and the cofactor.

The fluorescence polarization studies indicate that the Trp-139 does not influence the degree of mobility of the cofactor in the reduced enzyme probably because of the covalent linkage to the enzyme. On the other hand, in the PMP form of the enzyme, the rotational freedom of the cofactor is higher in the mutant enzyme than in the wild-type, indicating that Trp-139 has a role in limiting this rotational freedom of the coenzyme.

The negative Cotton effect in the region of the coenzyme absorbance was first observed by Yonaha et al. (1975) for D-amino acid transaminase from *Bacillus sphaericus*. In the present study, the results from the circular dichroism studies clearly indicate that the positive Cotton effect in the region of 280 nm is *not* due to a cumulative effect of tryptophan and tyrosine residue but only to a limited number of these residues that are present in an environment that makes them optically active. The results are consistent with the suggestion that Trp-139 in the wild-type enzyme is in some type of rigid conformation.

A major conclusion of these studies is that Trp-139 greatly influences the spectroscopic behavior of the coenzyme PLP. We have demonstrated the existence of a strong energy-transfer process between this Trp-139 and the coenzyme. This phenomenon is only possible when adequate requirements of distance and orientation are met. When the secondary structure is predicted according to the method of Gibrat et al. (1987), Trp-139 and Lys-145 are found to be part of a long α-helix in which they could interact closely. The results presented in this paper are also consistent with a direct interaction between Trp-139 and the PLP molecule, although this is not the only possible explanation. In fact, it is also known that Trp-140 in the L-aspartate transaminase forms a very tight interaction with the coenzyme and no energy transfer was found (Churchich, 1965). However, the fluorescence of PMP in this enzyme is highly quenched as Churchich has reported, and this quenching could mask the energy-transfer process.

The choice of residues for site-directed mutagenesis studies to obtain information about a given enzyme is of obvious importance. The location of the site to be mutated is also critical since a change in an important site for enzyme conformation could have drastic effects on the stability of the protein. Some changes near the active site may be tolerated but still might affect the protein in other, more subtle ways. Thus, for D-amino acid transaminase, we have found that substitution of Cys-142 and Ser-146 near the active-site Lys-145 has no effect on the specific activity of the purified enzyme (Merola et al., 1989). However, substitution of Gly and Ala, respectively, at these sites affects the stability of the protein as determined by their rates of inactivation at elevated temperatures. Likewise, mutation of Trp-139 in all cases leads to a less active protein. We also noted that the choice for the substitution also dictated whether an enzyme usable for study could be obtained. Thus, the aspartate mutant (W139D) could not be isolated, presumably because the presence of a negative charge at residue 139 disrupted the protein structure and the bacterium degraded it. Aliphatic and heterocyclic residues were tolerated better, but even these did not generate an enzyme that could be readily studied since their PLP moieties were gradually lost and they then precipitated. The most informative mutant was that in which part of the aromatic character of position 139 was retained by a Phe residue. Thus, even though the studies in this paper do not elucidate the precise role of Trp-139 in D-amino acid transaminase, they clearly point to the importance of an aromatic side chain at position 139 in this enzyme and the influence of this residue on the behavior of the coenzyme PLP.

ACKNOWLEDGMENTS

The expert assistance of Judith Gallea with the manuscript is very much appreciated.

Registry No. Trp, 73-22-3; Phe, 63-91-2; DAT, 50864-42-1.

REFERENCES

Arnone, A., Roger, P. H., Hyde, C. C., Briley, P. D., Metzler, C. M., & Metzler, D. E. (1985) in *Transaminases*, p 138, Wiley, New York.

Asano, S. (1987) Thesis, Kyoto University.

Churchich, J. (1965) *Biochemistry 4*, 1405.

Churchich, J. (1984) in *Transaminases*, p 185, Wiley, New York.

Gibrat, J. F., Garnier, J., & Robson, B. (1987) *J. Mol. Biol. 198*, 425.

Iriarte, A., & Martinez-Carrion, M. (1983) *J. Biol. Chem. 258*, 2217.

Jansonius, J. N., Eichele, G., Ford, G. C., Picot, D., Thaller, C., & Vincent, M. G. (1985) in *Transaminases*, p 110, Wiley, New York.

Jones, W. M., Soper, T. S., Ueno, H., & Manning, J. M. (1985) *Methods Enzymol. 113*, 108.

Kim, D. S., & Churchich, J. (1987) *J. Biol. Chem. 262*, 14250.

Kuramitsu, S., Inoue, K., & Kagamiyama, H. (1987) in *Biochemistry of Vitamin B_6*, p 153, Birkhauser, Boston.

Martínez-Carrion, M., & Jenkins, W. T. (1965) *J. Biol. Chem. 210*, 3538.

Martínez del Pozo, A., Olmo, N., Oñaderra, M., Lizarbe, M. A., & Gavilanes, J. G. (1986) *Int. J. Pept. Protein Res. 28*, 173.

Martínez del Pozo, A., Gasset, M., Oñaderra, M., & Gavilanes, J. G. (1988) *Biochim. Biophys. Acta 953*, 280.

Merola, M., Martínez del Pozo, A., Ueno, H., Recsei, P., Di Donato, A., Manning, J. M., Tanizawa, K., Masu, Y., Asano, S., Tanaka, H., Soda, K., Ringe, D., & Petsko, G. A. (1989) *Biochemistry* (preceding paper in this issue).

Metzler, D. E., Harris, C., Yang, I.-Y., Siano, D., & Thomson, J. A. (1972) *Biochem. Biophys. Res. Commun. 46*, 1588.

Shaltiel, S., & Cortijo, M. (1970) *Biochem. Biophys. Res. Commun. 41*, 594.

Soper, T. S., Jones, W. M., Lerner, B., Trop, M., & Manning,

J. M. (1977) *J. Biol. Chem. 252*, 3170.
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).
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.

^{13}C, ^{15}N, and ^{31}P NMR Studies on 6-Hydroxy-L-nicotine Oxidase from *Arthrobacter oxidans*†

Stefan Pust,‡ Jacques Vervoort,§ Karl Decker,*,‡ Adelbert Bacher,‖ and Franz Müller§

Biochemisches Institut der Albert-Ludwigs-Universität, D-7800 Freiburg, FRG, Department of Biochemistry, Agricultural University, Wageningen, The Netherlands, and Department of Organic Chemistry and Biochemistry, Technical University of Munich, D-8046 Garching, FRG

Received May 26, 1988; Revised Manuscript Received August 11, 1988

ABSTRACT: The interaction between the apoprotein of 6-hydroxy-L-nicotine oxidase from *Arthrobacter oxidans* and the prosthetic group FAD has been investigated by ^{13}C, ^{15}N, and ^{31}P NMR techniques. The FAD prosthetic group was selectively enriched in ^{13}C and ^{15}N isotopes by adding isotopically labeled riboflavin derivatives to the growth medium of riboflavin-requiring mutant cells. In the oxidized state the chemical shift of the C(7) and C(8) atoms indicates that the xylene moiety of the isoalloxazine ring is embedded in a hydrophobic environment. The polarization of the isoalloxazine ring as a whole is, however, much more comparable to that of free flavin in a polar and protic environment than to free flavin in an apolar environment. The polarization of the ring system can be ascribed to strong hydrogen bonds between the apoprotein and the two carbonyl groups. The binding of the competitive inhibitor, 6-hydroxy-D-nicotine, influences the resonances of the C(4a) and the N(5) atoms strongly. It is suggested that these shifts are due to a strong hydrogen-bonding interaction between the N(5) atom and the inhibitor. On reduction all resonances, except those of the C(10a) and the N(1) atoms, shift upfield, indicating the increased electron density in the ring system. In the dithionite-reduced enzyme, the ring system is bent at the N(5) position. Due to the bending of the N(5) atom and the sp^2 hybridized N(10) atom, electron density from the N(10) atom is reallocated at the C(4) carbonyl group. In contrast, in the substrate-reduced enzyme the N(5) atom is almost completely sp^2 hybridized, yielding a rather planar isoalloxazine ring. As a consequence, the electron density from the N(5) atom is reallocated at C(6) and C(8) positions in the isoalloxazine ring. It can unambiguously be concluded from the chemical shift of the N(1) atom that the reduced flavin is anionic. The doublet character of the N(3) and N(5) resonances suggests that bulk water has no access to the active center. The strong downfield shift of the N(1) position indicates that this atom is embedded in a polar environment, but it does not indicate the presence of a positively charged residue. The "red" anion radical form of the flavin prosthetic group is obtained from the dithionite-reduced enzyme in the presence of 6-hydroxy-D-nicotine and oxygen. EPR measurements support this finding. The ^{31}P NMR spectra show that the resonances of the pyrophosphate group of the bound FAD differ slightly from those of free FAD. Besides the ^{31}P resonances from FAD, four peaks around 0 ppm are observed that belong to bound phosphorus residues. The residues are not located close to the isoalloxazine ring.

6-Hydroxy-L-nicotine oxidase from *Arthrobacter oxidans* is a FAD-containing enzyme that catalyzes the conversion of 6-hydroxy-L-nicotine to the optically inactive product 6-hydroxy-*N*-methylmyosmine and γ-(methylamino)propyl 6-hydroxy-3-pyridyl ketone ("ketone"), respectively (Gries et al., 1961). The enzyme is induced when the aerobic bacterium is grown on D,L-nicotine as its sole carbon and nitrogen source (Decker & Bleeg, 1965). The D,L-nicotine is converted to 6-hydroxy-D,L-nicotine by a nonstereospecific nicotine dehydrogenase (Gloger & Decker, 1969). Besides the stereospecific 6-hydroxy-L-nicotine oxidase, there is also a stereospecific 6-hydroxy-D-nicotine oxidase that catalyzes the conversion of 6-hydroxy-D-nicotine to the same ketone (Decker & Bleeg, 1965). The latter enzyme has a covalently bound FAD that was shown to be linked to the enzyme by a 8α-*N*-(3)-histidyl linkage (Möhler et al., 1972). In contrast, the FAD molecule in 6-hydroxy-L-nicotine oxidase can be removed reversibly (Decker & Dai, 1967). Most studies performed so far were directed toward the biochemistry and genetics of the

† Part of this study has been carried out under the auspices of the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO). The work carried out in Freiburg was supported by grants from the Deutsche Forschungsgemeinschaft, Bonn (FRG), through SFB 206. The work carried out in Munich was supported by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie, Frankfurt (FRG).
‡ Albert-Ludwigs-Universität.
§ Agricultural University.
‖ Technical University of Munich.

0006-2960/89/0428-0516$01.50/0