Reversible Dissociation/Association of D-Amino Acid Transaminase Subunits: Properties of Isolated Active Dimers and Inactive Monomers†

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Received September 9, 1999; Revised Manuscript Received October 25, 1999

ABSTRACT: The crystal structure of dimeric D-amino acid transaminase shows that the two Trp-139 sites are located in a hydrophobic pocket at the interface between the subunits and that the two indole side chains face one another and are within 10 Å of coenzyme. This enzyme prefers an aromatic character at position 139, as previously demonstrated by the finding that Phe-139 but no other substitution tested provides the maximum degree of thermostability and catalytic efficiency. Here we show that an equilibrium between active dimers and inactive monomers can be demonstrated with the W139F mutant enzyme, whereas with the wild-type enzyme the subunit interface is so tight that a study of this equilibrium is precluded. We show how the processes of dimerization of monomers and dissociation of dimers to monomers are controlled. Lower pH (5.0) favors monomer formation from dimers. Gel filtration and activity analysis show that at higher pH (7.0) the monomers combine to form active dimers with a K_d of 0.17 μM. This assembly process is relatively slow and takes several hours for completion, thereby permitting accurate measurement of kinetics and equilibrium parameters. Absorption and circular dichroism spectra of dimers and monomers are significantly different, indicating that the environment around the cofactor is very likely altered between them. The circular dichroism peak of the W139F dimer at 418 nm is less negative than that of the wild-type enzyme in accordance with its lower visible absorbance; the circular dichroism peak of the W139F monomer at 418 nm is more negative than that of the wild-type enzyme. The dissociation of dimers to monomers has also been studied by taking advantage of these spectral differences, thus permitting the rates of the dissociation and the reassociation to be calculated and compared. 2-Mercaptoethanol assists in the conversion of monomers to dimers. The results here describe dissociation/reassociation in the dimeric enzyme under native conditions without denaturants.

Many pyridoxal phosphate-dependent (PLP)[1] enzymes exist as very tightly associated oligomers (*1*−*6*). In the case of dimeric D-amino acid transaminase, which is composed of two identical subunits, some components of the active site are contributed by either one monomeric subunit or the other (*7*, *8*). Thus, the stereospecificity of this enzyme for D-amino acids is determined in part by Arg98 and His100, which are contributed by the neighboring subunit, to form bonds with the substrate α-carboxyl group. Such shared domains involving subunits make the dimer−monomer equilibrium difficult to study in the wild-type enzyme because of the strength of the interface. Here we report the successful attempt to study both the dissociation and the association processes in a mutant enzyme.

Dissociation of other PLP enzymes upon alkaline treatment, pressure, or addition of denaturant has been reported (*9*−*11*). The tryptophan synthase $\alpha_2\beta_2$ complex is well-studied with respect to the subunit interaction between α and β subunits by use of mutations in the contact region or at the active site (*12*−*14*); e.g., it was shown that some amino acid residues between α and β subunits or at the active site of tryptophan synthase play important roles in intersubunit communication (*13*, *14*). There are few studies on the reversible dissociation/association of subunits of pyridoxal enzymes under native, nondenaturing conditions.

We have previously reported some of the properties of the W139F mutant of D-amino acid transaminase, which bears a substitution at the interface between the monomeric subunits, about 10 Å from the coenzyme PLP (*15*, *16*). We now demonstrate that at low pH this mutant is prone to dissociate into its monomeric subunits, which are enzymatically inactive. This system provides an opportunity to study the absorption and CD spectral properties of inactive monomers and to determine how these change on formation of the active dimeric enzyme. We use these differences to evaluate both the kinetics and equilibria of the dissociation and the reassociation processes.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Expression Enzymes. These experimental procedures have been described previously (*15*, *17*).

† This work was supported by NSF Grant MCB-9727613 (J.M.M.).

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[1] Abbreviations: PLP, pyridoxal 5′-phosphate; CD, circular dichroism; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; FPLC, fast-performance liquid chromatography; Bis-Tris, bis-(2-hydroxyethyl)iminotris(hydroxymethyl)methane; EPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid; PMP, pyridoxamine 5′-phosphate; HPLC, high-pressure liquid chromatography.

10.1021/bi992111k CCC: $19.00
Published on Web 12/15/1999

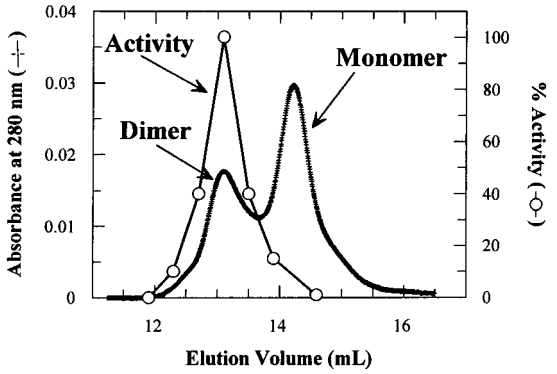

FIGURE 1: Separation and activity of dimer and monomer forms of the W139F mutant of D-amino acid transaminase. Gel filtration was performed as described in the text. Samples were incubated in 100 mM potassium acetate containing 0.01% 2-mercaptoethanol, pH 5.0, at 4 °C overnight and injected onto a Superose 12 10/30 column equilibrated in the same buffer. Activities in several fractions shown in the figure were measured.

Purification of the W139F Mutant Enzyme. The recombinant mutant enzyme was purified according to Merola et al. (*17*) and van Ophem et al. (*18*) with minor modifications. After the heat treatment was performed at 48 °C, the enzyme was chromatographed on a DEAE-Sepharose column equilibrated in 10 mM potassium phosphate buffer (pH 8.0) containing 0.1 mM EDTA. The enzyme was eluted with a linear gradient of 0−0.35 M KCl. The active fraction was rechromatographed on a Superose 12 10/30 column (Pharmacia) equilibrated in 10 mM potassium phosphate buffer (pH 8.0). The final step was performed on a Mono Q 10/10 column (Pharmacia) equilibrated in 10 mM potassium phosphate buffer (pH 8.0). The enzyme was eluted with a linear gradient of 0−0.18 M KCl. The purified enzyme, which gave one major band on SDS−PAGE, was analyzed for amino acid composition and electrophoretic behavior. The purified enzyme was stored at −30 °C.

Isolation of the W139F Monomer. The W139F dimer was put into 100 mM potassium acetate buffer (pH 5.0) containing 0.01% 2-mercaptoethanol using a PD-10 column (Pharmacia) and then incubated at 4 °C overnight to promote its dissociation into monomers. This solution was chromatographed on a Superose 12 10/30 column equilibrated in the same buffer. The peaks eluted at the expected positions for monomer and dimer with 80−90% yield (Figure 1). The monomer fraction was stored at −30 °C.

Absorption and CD Spectra. Absorption and CD spectra were recorded at room temperature with a Shimadzu UV-1601 spectrophotometer and a Jasco J-715 spectropolarimeter, respectively. For the wild-type enzyme, the W139F monomer, and the W139F dimer, each enzyme concentration was 0.31 mg/mL. The wild-type enzyme and W139F dimer were in 10 mM potassium phosphate, pH 8.0. The W139F monomer was in 100 mM potassium acetate containing 0.01% 2-mercaptoethanol, pH 5.0. The absorption at 280 nm was identical for dimer and monomer.

Gel-Filtration Assay. Gel-filtration FPLC was used to estimate the dissociation constant of the W139F mutant enzyme using the methods of Manning et al. (*19, 20*). A 0.64 μM monomer stock solution was prepared in 100 mM potassium acetate buffer (pH 5.0) containing 0.01% 2-mercaptoethanol. To study the association process, dilutions were prepared from this monomer stock using the same buffer. Since both the dissociation and the association processes are so dependent on pH, special care was taken to ensure its control. An aliquot (160 μL) of monomer solution was added to 40 μL of 1 M Bis-Tris buffer (pH 7.5) at final pH of 7.0 and incubated for 6 h at room temperature; during this time the association of a given concentration of monomers was completed (see below). An aliquot (100 μL) of this solution was injected via a 100 μL injection loop onto a Superose 12 10/30 column that had been previously equilibrated with a buffer of the same composition given above.

Enzyme Activity. Enzyme activity was usually measured at pH 8.1 at room temperature and calculated as described (*18*). However, when the activity of the enzyme was determined at pH 7.0, Bis-Tris buffer was used in place of EPPS buffer (*18*).

RESULTS

Purified W139F Dimer. The preparation of the W139F mutant enzyme was >95% homogeneous as judged by SDS−PAGE. A single peak of the purified enzyme eluted with a linear gradient of 0−0.18 M KCl on a Mono Q 10/10 column equilibrated in 10 mM potassium phosphate buffer (pH 8.0). Its amino acid composition was in excellent agreement with the predicted values (*21*). The specific activity of the W139F mutant enzyme reported previously was 63% that of wild-type enzyme (*15*). This value is an underestimate because of dissociation to inactive monomers reported here. The current method of enzyme purification gave a specific activity that was increased to 180 units/mg, which is 90% that of the pure wild-type enzyme. The amount of enzyme recovered from 20 g (wet weight) of *Escherichia coli* was >100 mg.

Preparation of W139F Monomer. At pH 5.0 the dimeric enzyme dissociated to monomers as determined by the relative elution positions of dimers and monomers on a Superose 12 10/30 column (Pharmacia) (Figure 1). The wild-type enzyme eluted exclusively as a dimer under these conditions. The dimers of W139F were active whereas the monomers were not when assayed at pH 8.0 for 1 min before dimerization occurs (Figure 1). When the inactive monomer at pH 5.0 (described in detail below) was brought to pH 7.0 or 8.0 at room temperature, the enzyme had almost the same activity as wild-type enzyme after 5−6 h and eluted from a Superose 12 10/30 column as a dimer. Restoration of activity depended on enzyme concentration and pH. This result indicates that the separated inactive monomers could form a fully active dimer. The monomer was stable at −30 °C for at least 3 months as judged by its ability to be completely converted to active dimer with full restoration of activity.

Absorption Spectra. The spectrum of the W139F dimer shows a major absorption maximum at 418 nm, corresponding to the internal aldimine with an extinction coefficient lower than that of wild-type enzyme. Part of the enzyme was in the PMP form absorbing at 330 nm (not shown). The W139F monomer also has an absorption maximum at 418 nm with an extinction coefficient very similar to that of wild-type enzyme. The PLP contents of wild type, W139F dimer, and W139F monomer analyzed directly by HPLC are shown in Table 1. The HPLC results suggest that the W139F mutant enzyme may lose its cofactor more easily than the wild-type enzyme, which may account for the lower specific activity reported previously (*15*).

Table 1: Some Properties of the Wild-Type and Mutant Enzymes

enzyme	PLP content[a] (per monomer)	PMP content[a] (per monomer)	no. of HPLC determinations for coenzyme	A_{418}/A_{280}	specific activity (units/mg)
wild-type	1.0 ± 0.1	not detected	3	0.17	200
W139F dimer[b]	0.52 ± 0.034	0.028 ± 0.002	5	0.13	180
W139F monomer	0.69 ± 0.017	not detected	5	0.17	0

[a] Coenzyme content was calculated from the HPLC analysis (*18*, *31*). [b] No addition of PLP was done throughout the enzyme purification.

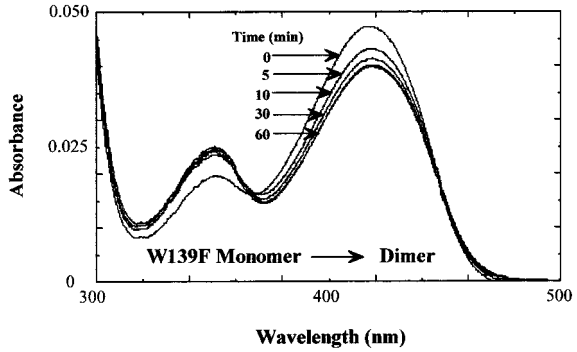

FIGURE 2: Time dependence of spectral changes of the W139F monomer at pH 8.0. Eight hundred microliters of the monomer solution was added to 200 μL of 1 M Bis-Tris solution to adjust the pH value to 8.0. The final enzyme concentration was 0.31 mg/mL. At the indicated times, spectra were recorded. The absorbance of monomers and dimers at 280 nm was identical and did not change on the course of the experiment, indicating that there was no loss of protein.

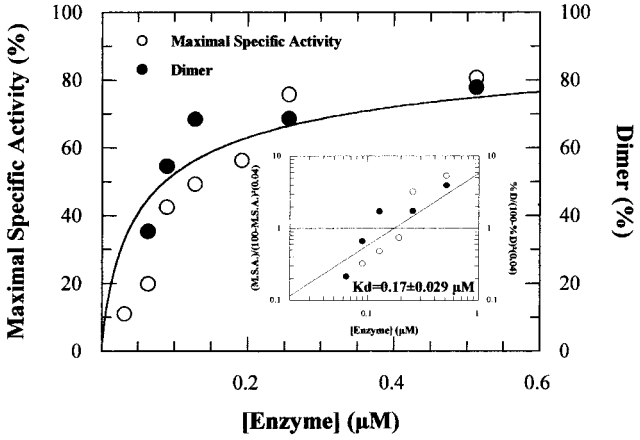

FIGURE 3: Dimer−monomer K_d value of the W139F mutant enzyme. The procedures are described in the text. Maximal activity of the wild-type enzyme under these conditions was determined to be 69 units/mg. (○); Maximal specific activity; (●) percentage of the dimer to total enzyme.

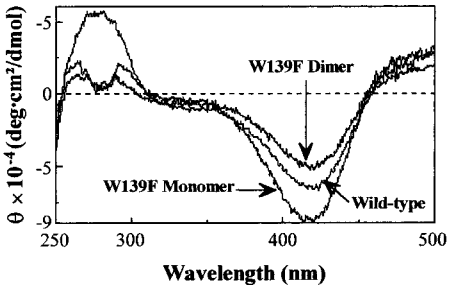

FIGURE 4: CD spectra of the wild-type enzyme, the W139F dimer, and the W139F monomer. Each enzyme concentration was 0.31 mg/mL. The wild-type enzyme and W139F dimer were in 10 mM potassium phosphate, pH 8.0. The W139F monomer was in 100 mM potassium acetate containing 0.01% 2-mercaptoethanol, pH 5.0.

When the spectral change of the monomer was recorded at pH 8.0 as a function of time, a minor shift and a decrease in absorption maximum were observed near 418 nm. The difference in amplitude between 0 and 60 min (Figure 2) was close to the difference between the dimer and monomer. The basis for this effect could be that the conformation around cofactor might be different between the dimer and monomer. This possibility was further evaluated by CD measurements described below. The slow time dependence of the absorbance conversion permitted a detailed study of the kinetics of the monomer to dimer conversion under various conditions. No change at 280 nm was found in the conversion of the W139F monomer to the dimer, indicating that there is no loss of protein under these conditions.

Determination of the Equilibrium Dimer−Monomer Dissociation Constant. Analysis for activity showed that the equilibrium between monomer and dimer was established within hours under the conditions described above. We chose a time of 6 h to ensure completion of the dissociation process. A combination of gel filtration and activity analysis (Figure 1) showed that the proportion of dimer decreased as the overall enzyme concentration was lowered as described below. The eluent of the column was monitored by UV absorption at 280 nm and the elution profiles were analyzed by PeakFit program (SPSS Inc.) to resolve the two partially overlapped peaks. The calculated areas of both peaks were used to calculate the dissociation constant with the equation according to Manning et al. (*19*, *20*). The dissociation constant (K_d) of the W139F mutant enzyme was determined by a plot of enzyme concentration (as dimer) versus percent dimer and maximal specific activity using amounts of the enzyme that spanned either side of the dissociation constant (Figure 3). There was good agreement between the amount of dimer and the increase in activity. The curve was fit to an equation derived from an equilibrium expression according to Manning et al. (*19*, *20*):

$$\%\ \mathrm{D} = [(8[\mathrm{E}] + K_d) - (K_d^2 + 16K_d[\mathrm{E}])^{1/2}]/0.08[\mathrm{E}]$$

where % D is the percentage of dimer to total enzyme and [E] is the total concentration of enzyme (in dimer equivalents). The value of the dimer−monomer dissociation constant can be read directly from the log−log plots in the insets as the corresponding values on the x axis when $y = 1$ (*19*, *20*). The K_d value was independently calculated as described in refs *19* and *20* to be 0.17 μM (Figure 3).

CD Spectra. The CD spectrum in the coenzyme region of wild-type D-amino acid transaminase differs from those of most PLP enzymes since it is negative whereas most of the other PLP enzymes exhibit positive CD peaks in this region. The CD peak of the W139F dimer at 418 nm is less negative than that of the wild-type enzyme (Figure 4) in accordance with its lower visible absorbance. On the other hand, the

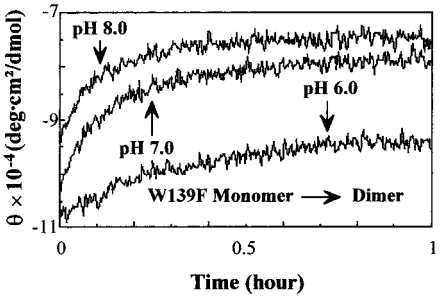

FIGURE 5: Rate of increase of the 418-nm CD band with increasing pH. After 800 μL of the monomer solution was added to 200 μL of 1 M Bis-Tris solution to adjust pH value to that shown in the figure, the CD peak at 418 nm increased. The rate of increase at 418 nm was recorded. Each enzyme concentration was 0.31 mg/mL.

CD peak of the W139F monomer at 418 nm is *more* negative than that of the wild-type enzyme. This result is consistent with the suggestion above from the visible spectral differences that the PLP may be in a different microenvironment in the monomer compared to that in the dimer.

At 280 nm the W139F monomer has a low positive ellipticity similar to that of the dimer and significantly different from the wild-type enzyme due to absence of Trp-139 (*15*). Rowlett et al. (*13*) made a similar observation of a reduced ellipticity at 280 nm for a Trp to Phe mutant of tryptophan synthase, although for their mutant there were only small changes in ellipticity in the coenzyme region, all of which were positive. In the far-UV region of the CD spectrum, no significant differences were detected when the monomer and the dimer were compared (data not shown).

Rate of Association of Monomers to Dimers in the CD. The amplitude of the CD change at 418 nm increased with increasing pH (Figure 5) due to more efficient monomer to dimer conversion as described in Figure 4. A constant value was reached within 1 h, which was taken as the maximum amount of the dimer formed at a given pH. We used this observation to study the kinetics of this process in detail by taking CD readings every 0.05 h during the association process. The progress curve of the conversion of monomers to dimers at each pH (Figure 5) fit a single-exponential time course as shown for pH 8.0 in Figure 6a. The rates of the amplitude changes at 418 nm shown in Figure 5 were also plotted according to Swinbourne (*22, 23*), resulting in an equation of single-exponential decay, $CD_t = Ae^{-kt} + B$, shown for pH 8.0 in Figure 6a inset. This equation is of the form $CD_{t+1} = CD_t e^{-k\Delta t} + a$. The value of the CD peak at 418 nm at each 0.05 h (CD_t) was plotted against the value at 418 nm 0.05 h later (CD_{t+1}). From the above equation the negative ln of the slope of the experimental line in Figure 6a inset is equal to $k\Delta t$, which, when divided by 20, is the rate constant since $\Delta t = 0.05$ h. The kinetic parameters of the monomer to dimer conversion are shown in Table 2. The rates calculated from the Swinbourne equation are consistent with those calculated from the equation of single-exponential decay increase with increasing pH.

Rate of Dissociation of Dimers to Monomers. The changes in CD at the coenzyme absorbance at 418 nm described in Figures 4, 5, and 6a for monomer association to dimer have also been used to study the process of dissociation of dimers to monomers (Figure 6b). This experiment was performed at pH 5.0 rather than at pH 8.0 used for the reassociation

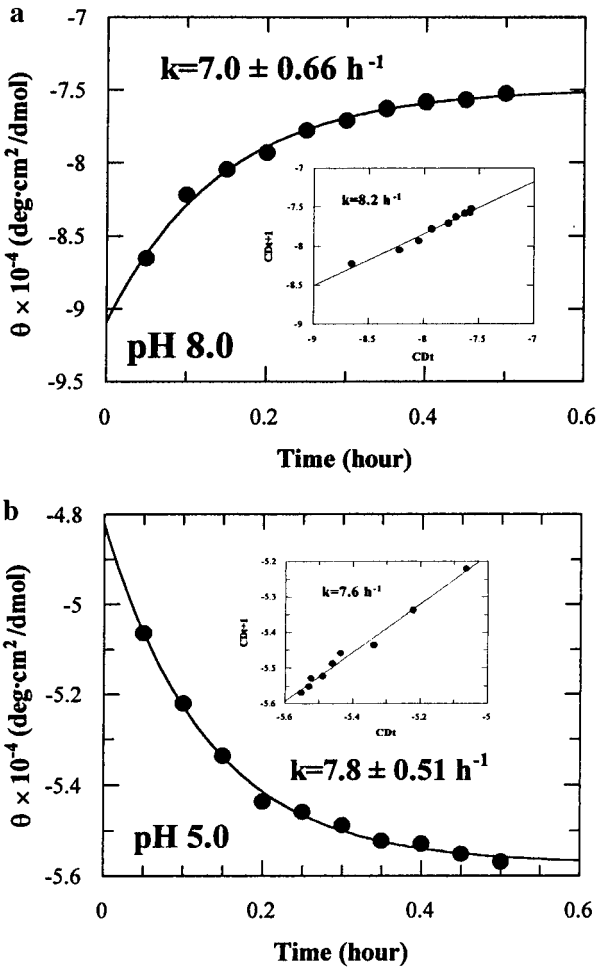

FIGURE 6: Kinetics of conversion in the CD. (a) Monomer to dimer at pH 8.0. Data were from Figure 5. (b) Dimer to monomer at pH 5.0. Enzyme concentration was 0.31 mg/mL in 100 mM potassium acetate containing 0.01% 2-mercaptoethanol, pH 5.0. The rate of the decrease at 418 nm was recorded. The rate of CD changes was plotted according to Swinbourne in each inset.

Table 2: Kinetic Parameters of Conversion of Monomers to Dimers in CD Experiments[a]

pH	k^b (h^{-1})	k^c (h^{-1})
	Association	
6.0	2.5	3.1 ± 0.90
7.0	5.2	4.9 ± 0.29
8.0	8.2	7.0 ± 0.66
	Dissociation	
5.0	7.6	7.8 ± 0.51

[a] All buffers consist of 80 mM acetate, 200 mM Bis-Tris, and 0.008% 2-mercaptoethanol. [b] Determined with the equation according to Swinbourne (*22, 23*). [c] Determined with the equation of single-exponential decay, $CD_t = Ae^{-kt} + B$.

process of monomer to dimer (Figure 6a) since pH 5.0 favors monomer formation as described above. It is evident from the symmetrically opposite profiles in Figure 6 panels a and b that both processes can be studied with a high degree of precision. The data in Figure 6b were analyzed by the Swinbourne equation to give the values in the inset and in Table 2.

Effect of 2-Mercaptoethanol on the Conversion of Monomers to Dimers. The conversion of monomers to dimers was greatly facilitated by addition of 2-mercaptoethanol. To study this effect more thoroughly, 2-mercaptoethanol was first

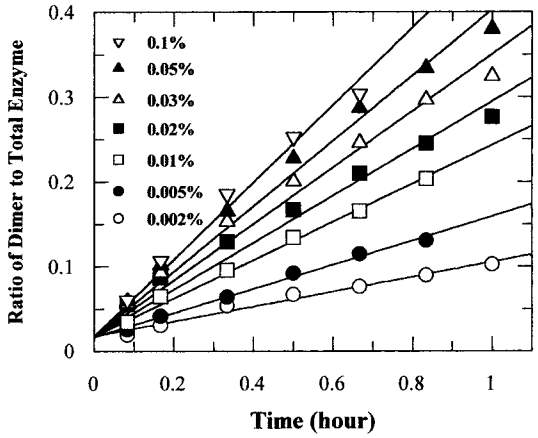

FIGURE 7: Effect of different percentages of 2-mercaptoethanol on the conversion of monomers to dimers. 2-Mercaptoethanol in the preparation of the monomer was removed with a PD-10 column. Eighty microliters of the monomer solution was added to 20 μL of 1 M Bis-Tris solution to adjust pH value to 8.0 containing the indicated percentage of 2-mercaptoethanol. Each final enzyme concentration was 0.031 mg/mL. A 10 μL of aliquot was withdrawn from each mixture at the indicated times and subjected to activity assay at pH 8.0. Maximal activity of the wild-type enzyme under each condition was determined to be 200 units/mg. Ratio of the dimer to total enzyme when the specific activity was 200 units/mg was defined to be 1.0.

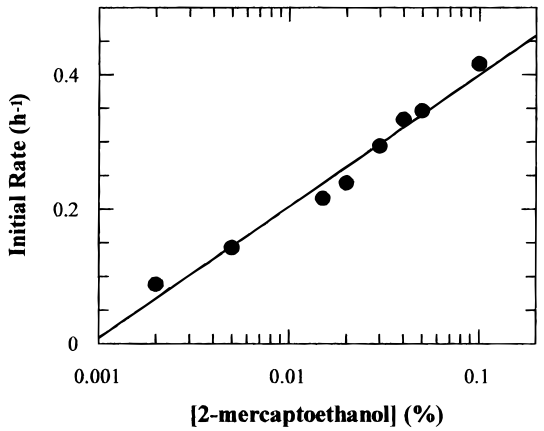

FIGURE 8: Correlation of the conversion of monomers to dimers as a function of percentage of 2-mercaptoethanol.

removed from the preparation of monomers on a PD-10 column and then added back in a concentration-dependent manner to monomer. The conversion of monomer to dimer was then shown to be dependent on the amount of 2-mercaptoethanol present (Figure 7). Figure 8 shows that a plot of initial rate (Figure 7) versus the log of the percentage of 2-mercaptoethanol gives a straight line. This result indicates that the conversion of monomers to dimers accelerated by 2-mercaptoethanol has a maximum rate of about 0.5 h^{-1} at a percentage of about 0.2% 2-mercaptoethanol. Further studies on the details of this effect are in progress.

DISCUSSION

There have been a substantial number of amino acid substitutions made in various PLP enzymes, most of which have been at their active sites in order to elucidate their mechanisms. Trp-140 of L-aspartate aminotransferase, which is near the cofactor but not in the subunit interface, has been substituted by Phe or Gly (*24*). As compared to the wild-type enzyme, both mutant enzymes showed a dramatic increase in K_m for natural dicarboxylic substrates but not for aromatic substrates and the k_{cat} values for dicarboxylic and aromatic substrates were greatly decreased, suggesting that N(1) of Trp-140 may be involved in catalysis to some extent. Converting either Lys-69 or Cys-360 to alanine in mammalian ornithine decarboxylase was demonstrated to distinguish between the possibilities of shared versus independent active sites (*25*), suggesting that these residues are located in two distinct regions of each subunit that interact to form shared active sites in the active dimer.

There are few reported studies on the dynamics of the subunit dissociation/reassociation process in pyridoxal 5′-phosphate-dependent enzymes. The results reported here elucidate some of the details of this process. Experimental conditions were found whereby the inactive monomers could be obtained in high yield and in the native state as judged by the ability to form the fully active enzyme with about 95% recovery of activity. These findings permit a detailed study of the properties of the monomers themselves and the changes that occur in the process of its dimerization. The reverse process of dimer dissociation was also readily measured at a pH (5.0) that favored monomer formation. These processes have the symmetrically opposite profiles expected of a true equilibria process (Figure 6 panels a and b). The subject of dissociation/reassociation is of interest because these processes are a potential mechanism to control activity since the dimer is active but the monomer is not. Rowlett et al. (*13*) measured the dissociation constants between the two different types of subunits (α and β) of tryptophan synthase by mixing different ratios of the separate types of subunits and then measuring the amount of activity generated by the subunit complex. 4-Aminobutyrate transaminase also undergoes a reversible process of dissociation/association that is pH-dependent but occurs at rates much faster than for D-amino acid transaminase (*26*). At low pH (pH 5.0) the rate of dissociation of dimers to monomers was reported to be 56 s^{-1} for 4-aminobutyrate transaminase compared to 7.8 h^{-1} for D-amino acid transaminase (Figure 6b). However, the association of monomers to form active dimers at pH 7.0 for 4-aminobutyrate transaminase was too fast to be detected even for stopped-flow instruments (*26*). In our experiments monomer association to dimer was slow enough so that kinetics and equilibria could be accurately determined by spectral differences as well as activity measurement. The reason(s) for the time dependence of these events is under investigation. Furthermore, these spectral differences between dimers and monomers have allowed us to make conclusions about differences in the PLP cofactor environment of monomer vs dimer.

The circular dichroism spectra of D-amino acid transaminase and branched-chain L-amino acid transaminase show negative Cotton effects in the coenzyme region, in contrast to most other PLP enzymes, which show positive Cotton effects in this region. The induced optical activity is conveniently measured by the dissymmetry factor, which can be expressed as the ratio of the molar circular dichroism to the molar absorptivity at a fixed wavelength (*14*). The dissymmetry factor for the W139F monomer is 1.4-fold greater than those for the wild-type enzyme and W139F dimer (these dissymmetry factors are negative values because of their negative ellipticity), suggesting that the orientation

of PLP at the active site of the W139F monomer is more asymmetric or more rigid than that of the wild-type enzyme or the W139F dimer and that the mutation of Trp-139 to Phe does not affect the asymmetry or rigidity of the PLP internal aldimine in dimeric D-amino acid transaminase. It cannot be excluded that this might be because of the difference in pH; the W139F monomer is at pH 5.0, whereas the wild-type enzyme and the W139F dimer are at pH 8.0. Banik et al. (*27*) proposed the interdomain bridge of PLP in the β subunit of tryptophan synthase stabilizes the interaction of the β subunit with α subunit (*27*). Our results may indicate that interaction of subunits in the W139F dimer is likely similar to that of the wild-type enzyme.

For D-amino acid transaminase, 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. In an earlier report, we have assessed the importance of Trp-139, and we have found that its replacement by Phe (W139F) but not by other amino acids nearly completely satisfied the requirement of a hydrophobic interaction at this site (*15, 16*). The specific activity (180 units/mg) of the W139F mutant enzyme by the purification procedure reported here is higher than that (120 units/mg) reported previously, perhaps because the W139F mutant enzyme has a tendency to dissociate to monomers.

Theoretically, if an associating interaction between monomer and dimer is very slow, like the W139F mutant D-amino acid transaminase described here or uncomplemented M15 β-galactosidase (*28*), two distinct peaks should elute, whereas if there is very rapid interaction as for hemoglobins (*19*), only a single intermediate peak is observed. It is possible that some domain rearrangement(s) is needed in monomers of D-amino acid transaminase before association to dimers takes place in a process that is slower than the separation procedure. This possibility is supported by the kinetics in Figures 5 and 6, although more study is clearly needed. The results can be explained by either of two mechanisms:

$$2M^* \rightleftarrows 2M \rightleftarrows D$$

or

$$2M \rightleftarrows D^* \rightleftarrows D$$

where the asterisks on either the monomer or the dimer refer to a somewhat different conformation around the PLP coenzyme compared to a second conformational state representing monomers that can assemble to dimers or to dimers with slightly different conformations similar to that described by Cai et al. (*29*) for serine hydroxymethyltransferase. At present we cannot distinguish between these possibilities.

We show here that a thiol group may be a good candidate to study the mechanism of subunit dissociation/reassociation. The crystal structure of D-amino acid transaminase, which contains six SH groups per molecule dimer, shows no S−S bonds (*30*). When wild-type or W139F dimer is denatured by exposure to guanidine hydrochloride, six SH groups per molecule dimer were titrated instantaneously. This result is consistent with the absence of S−S bonds in D-amino acid transaminase. It has recently been reported that tetrahydrobiopterin inhibits monomerization of nitric oxide synthase (*31*). Nitric oxide synthase dimers slowly dissociate into inactive monomers, a process that is inhibited when tetrahydrobiopterin is added during the catalysis. Tetrahydrobiopterin has been reported to keep thiol groups in a reduced state to stabilize the enzyme structure (*32*). Our results may show that the role of 2-mercaptoethanol in the dimerization of the mutant D-amino acid transaminase may be similar to that of tetrahydrobiopterin in the dimerization of nitric oxide synthase. Further study is needed to establish the role of thiols in both the dissociation and the assembly events.

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BI992111K