Reversible Dissociation and Unfolding of Aspartate Aminotransferase from *Escherichia coli*: Characterization of a Monomeric Intermediate†

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ABSTRACT: The unfolding and dissociation of the dimeric enzyme aspartate aminotransferase (D) from *Escherichia coli* by guanidine hydrochloride have been investigated at equilibrium. The overall process was reversible, as judged from almost complete recovery of enzymic activity after dialysis of 0.7 mg of denatured protein/mL against buffer. Unfolding and dissociation were monitored by circular dichroism and fluorescence spectroscopy and occurred in three separate phases: $D \rightleftarrows 2M \rightleftarrows 2M^* \rightleftarrows 2U$. The first transition at about 0.5 M guanidine hydrochloride coincided with loss of enzyme activity. It was displaced toward higher denaturant concentrations by the presence of either pyridoxal 5′-phosphate or pyridoxamine 5′-phosphate and toward lower denaturant concentrations by decreasing the protein concentration. Therefore, bound coenzyme stabilizes the dimeric state, and the monomer (M) is inactive because the shared active sites are destroyed by dissociation of the dimer. M was converted to M* and then to the fully unfolded monomer (U) in two subsequent transitions. M* was stable between 0.9 and 1.1 M guanidine hydrochloride and had the hydrodynamic radius, circular dichroism, and fluorescence of a monomeric, compact "molten globule" state.

Aspartate aminotransferase from *Escherichia coli* [eAAT[1] (EC 2.6.1.1)] has a molecular weight of 87 146 and is a dimer of two identical subunits (Kondo et al., 1987; Fotheringham et al., 1986). The crystal structures of both the cytoplasmic and the mitochondrial AAT from chicken are known (Ford et al., 1980; Arnone et al., 1985; Borisov et al., 1985). Although only about 40% of the residues are identical between eAAT and the chicken isoenzymes, Seville et al. (1988) succeeded in modeling the structure of eAAT, based on the coordinates of mitochondrial AAT. Latest X-ray data of crystals of the *Escherichia coli* enzyme show extended structural similarities between the two enzymes (Kamitori et al., 1988; Smith et al., 1988; Jaeger et al., 1989). The enzyme has two identical catalytic centers that are shared between the two subunits. It is therefore active only in the dimeric state. The crystallographic evidence has been confirmed recently by site-directed mutagenesis (Toney & Kirsch, 1987; Cronin & Kirsch, 1988; Hayashi et al., 1989). The mechanism of the catalytic reaction is well-known and follows the ping-pong scheme (Scheme I) [cf. Jansonius and Vincent (1987)].

Scheme I

L-aspartate + AAT–PLP ⇄ oxaloacetate + AAT–PMP

α-ketoglutarate + AAT–PMP ⇄ L-glutamate + AAT–PLP

The enzyme needs the coenzyme pyridoxal 5′-phosphate (PLP) for catalysis, which is covalently bound to the dormant protein by forming a Schiff's base with Lys-258. During the transfer of the amino group, PLP is converted to pyridoxamine 5′-phosphate (PMP), which is bound noncovalently.

AAT from pig heart cytosol can be dissociated into monomers either at extreme pH values (pH 3 or 11) or with 2 M urea [for a review, see Braunstein (1973)]. It is known that both PLP and PMP stabilize the dimeric form of chicken AAT aginst thermal denaturation (Relimpio et al., 1981; Iriarte et al., 1984). The coenzymes also stabilize AAT with regard to unfolding by urea (Ivanov et al., 1973). Although the stability of AAT has been studied intensively, little is known about intermediates in the refolding and reassembly of the enzyme from the unfolded monomers. AAT is a highly interesting candidate for refolding studies not only because of its dimeric state but also because the influence of the two coenzymes on the rate and equilibrium constants of refolding and reassembly can be studied.

Previous work by Giartosio et al. (1973) and West and Price (1989) has shown that cytoplasmic AAT from pig heart can be refolded after denaturation in 6 M GuCl. These authors used low final protein concentrations, and the yield depended critically on the presence of PLP and dithiothreitol in the renaturation buffer. The present work shows that it is possible to refold and reactivate eAAT at high protein concentrations in the absence of coenzyme with high yield after incubation in 6 M GuCl. This is exceptional for oligomeric enzymes (Jaenicke, 1987) and a necessary prerequisite for studying in detail the effects of the coenzymes on the kinetics and yield of reactivation of the dimeric eAAT. The main object of this study was to determine (i) the interrelationship between dissociation and unfolding steps and (ii) the effect of bound coenzyme on these processes and (iii) to characterize intermediates with respect to their CD and fluorescence spectra, enzymic activity, and hydrodynamic radius. Here we report the existence of a monomeric intermediate at about 1 M GuCl. It is compact and enzymically inactive but retains partially some of the spectral properties of the native dimer.

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[1] Abbreviations: eAAT, aspartate aminotransferase from *E. coli* (EC 2.6.1.1); PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; DEAE, diethylaminoethyl; EDTA, (ethylenedinitrilo)tetraacetic acid; DTE, dithioerythritol; GuCl, guanidinium chloride; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CD, circular dichroism.

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Experimental Procedures

Materials and Buffers. Malate dehydrogenase was purchased from Boehringer, Mannheim and ultrapure GuCl from Schwarz/Mann. All other reagents were of analytical grade. Buffers for refolding and reactivation studies, including the 10 mM stock solutions of PMP and PLP, were not kept for more than 1 week.

The following buffers were used during purification: buffer A, 20 mM morpholinoethanesulfonic acid, pH 6.0; buffer B, 5 mM sodium phosphate, pH 7.0; buffer C, 100 mM sodium phosphate, pH 7.0; buffer D, 10 mM Hepes, pH 7.4, 5 mM DTE, and 1 mM EDTA. Buffers A–C also contained 10 μM PLP, 1 mM 2-oxoglutarate, and 0.1 mM DTE. Buffer A was further supplemented with 1 mM EDTA.

Determination of Enzymic Activity. The activity of eAAT was measured by coupling the production of oxaloacetate (see Scheme I) to the oxidation of NADH by using malate dehydrogenase. Reactions were carried out at 37 °C in 100 mM sodium arsenate (Velick & Vavra, 1962), pH 7.4, 2 mM 2-oxoglutarate, 20 mM L-aspartate, 0.2 mM NADH, and 10 international units of malate dehydrogenase/mL. The range of subunit concentrations varied between 4 nM and 1 μM. The decrease in the absorbance at 340 nm was recorded by using a Kontron 800 spectrophotometer. After the blank rate, if any, was recorded, the catalytic reaction was initiated by adding eAAT. Apo-eAAT was converted to the holoenzyme by incubation with an excess of at least 10 μM PLP for 40 min at room temperature in the dark. One international unit of enzyme activity is defined as the amount of enzyme required for the formation of 1 μmol of product in 1 min under the above conditions.

Purification of eAAT. The enzyme was isolated from an overproducing strain of *Escherichia coli* (HW 857) harboring the expression plasmid piF 100 (Fotheringham et al., 1986). Bacteria were grown at 37 °C in a 35-L fermenter in Luria–Broth medium (Lennox, 1955) containing 100 μg of ampicillin/mL. All further operations were carried out at 4 °C. Cells were harvested in the stationary phase by centrifugation (15000*g* for 15 min) and washed once with buffer A. The yield was about 250 g. Sixty grams of the pellet was resuspended in 100 mL of buffer A, and the cells were disrupted by sonication. Cell debris was removed by centrifugation (16500*g* for 15 min). The DNA and some of the protein were eliminated by addition of solid poly(ethylene glycol) (PEG 6000) to 20% (w/v), stirring for 30 min, and centrifugation at 18000*g* for 15 min (Humphreys et al., 1975). The supernatant was dialyzed for 12 h against buffer A. The protein solution was then loaded onto a column of DEAE-Sepharose-Cl 6B (2.4 × 32 cm) equilibrated with buffer A. The column was washed with 150 mL of the same buffer, and the proteins were eluted with a linear gradient of sodium chloride increasing from 0 to 500 mM in buffer A (volume, 600 mL). AAT appeared at 180 mM NaCl. The active fractions were pooled and dialyzed against buffer B. The protein solution was loaded onto a column (4.5 × 40 cm) of hydroxylapatite (Atkinson et al., 1973) equilibrated in the same buffer. The column was washed with 300 mL of buffer B followed by 1000 mL of a linear gradient formed from equal volumes of buffers B and C. The enzyme appeared at 36 mM phosphate. The pooled fractions were dialyzed against buffer D containing 10 μM PLP and 1 mM 2-oxoglutarate. The material was typically 95% pure and was stored at −70 °C after the solution was dripped into liquid nitrogen. In this form, the enzyme can be stored for several months without significant loss of activity. Protein concentrations were determined spectrophotometrically at 280 nm. A solution of 1.0 mg of protein/mL has an $A_{280}^{d=1cm}$ of 0.86 (Yagi et al., 1979).

Preparation of Apoenzyme. Apoenzyme was prepared by a procedure similar to that used by Kuramitsu et al. (1985). Twenty milliliters of PLP–enzyme (1–3 mg of protein/mL) was converted to the PMP form by addition of 10 mM cysteine sulfinate and incubation for 10 min at room temperature. Solid GuCl was added to the protein solution to a final concentration of 6 M. After 45 min of stirring, the solution of denatured protein was loaded on a gel filtration column of Sephadex G-25 (3.2 × 40 cm) equilibrated in 6 M GuCl in buffer D. The protein was eluted with the same buffer. The protein peak was detected by its absorption at 280 nm. The pooled fractions with a maximal concentration of 0.7 mg of protein/mL were dialyzed at 4 °C against three changes of at least 50 volumes of buffer D. The absence of the coenzyme was checked by the lack of absorption at 330 nm, and the decrease of a specific activity, usually to 1–2% that of the holoenzyme. Reconstitution with PLP increased the activity to 80–100% that of the native enzyme.

Protein Folding Monitored by CD Spectroscopy. Circular dichroism spectra at a concentration of 0.17 mg of protein/mL were recorded on a Cary Model 60 instrument at 20 °C in buffer D with cuvettes of 0.1-cm path length. Either PLP or PMP was added to the apoenzyme to a final concentration of 10 μM to reconstitute the holoenzyme. All measurements were repeated 3 times. Attempts to measure protein folding using 0.017 mg of protein/mL and d = 1 cm cuvettes were unsuccessful due to the high background absorption of buffers containing GuCl.

Protein Folding Monitored by Gel Filtration. The principle of GuCl gradient gel filtration has been described by Endo et al. (1983). All experiments were conducted at 23 ± 2 °C. The column of Superose 12 (1 × 30 cm; volume, 23 mL; Pharmacia) was calibrated with the following proteins of known molecular weight in buffer D plus 100 mM sodium chloride: cytochrome *c* (12300), myoglobin (17000), carbonic anhydrase (30000), ovalbumin (45000), bovine serum albumin (67000), phosphorylase B (94000), and lactate dehydrogenase (140000). A linear gradient of GuCl was formed by mixing 100 mL of buffer D containing 100 mM sodium chloride with 100 mL of buffer D containing 6 M GuCl (flow rates, 0.1 or 0.3 mL/min) using an FPLC chromatography system. This resulted in a gradient of GuCl concentration corresponding to 23 mM GuCl per centimeter column length. Successive samples of 50 μL of native apo-eAAT (1 mg of protein/mL) were applied to the column after each increment of GuCl concentration of 0.12 M (i.e., 4-mL eluted volume). The enzyme remained on the column for periods of 32–40 min, depending on the GuCl concentration. The elution profile was monitored by recording the absorbance of 280 nm (d = 1 cm), and each gradient run was repeated 3 times.

Hydrodynamic Measurements. Ultracentrifugation was performed with a Beckman Model E analytical ultracentrifuge that was equipped with ultraviolet absorption optics and a photoelectric scanning system. Sedimentation velocity studies were carried out at 6 °C, using the An-D rotor and 12-mm double-sector Kel-F cells at 52000 rpm for a period of 5 h. The protein concentration corresponded to about 0.3 A_{280}. Buffer D containing protein and GuCl was preincubated for 12 h at 4 °C prior to the runs. The sedimentation coefficients were calculated from the midpoint of the moving boundary and represent the average sedimentation rate. They were finally corrected to water at 20 °C (Kawahara & Tanford, 1966). Sedimentation equilibrium experiments were carried

out with the same solution composition as above at 8 ± 2 °C in the An-F rotor at 13000 rpm and repeated with 18000 rpm. Molecular weights were evaluated from ln A versus r^2 plots, where A is the absorbance at 280 nm and r is the distance from the rotor center.

Protein Unfolding Monitored by Enzyme Activity. The solutions of holoenzyme and GuCl in buffer D were incubated at 4 °C in the dark for 24 h and then at 23 ± 2 °C for 1 h. The concentration of active enzyme was determined by adding aliquots (10–50 μL) of these enzyme plus GuCl solutions to assay solution (see above) at 37 °C to a final volume of 1.0 mL. The following controls were performed to ascertain that the shift of temperature did not affect the distribution between inactive and active protein species: (i) The progress curves at 37 °C were linear for at least 1 min. Reactivation during the assay would have led to a concave-up progress curve. (ii) Variation of the final concentration of denaturant in the assay solution (0.0017 mg of protein/mL, 0.06 M < [GuCl] < 0.2 M; 0.017 and 0.17 mg of protein/mL, 0.2 M < [GuCl] < 0.4 M) had no effect on either the linearity or the slope of the progress curves. Thus, the shift in temperature to that of the standard assay does not affect the activity profiles.

Unfolding and Refolding Measurements by Fluorescence. Fluorescence measurements were carried out with an SLM 8000 single photon counting fluorometer. For equilibrium studies, the protein was preincubated in buffer D plus GuCl for 24 h at 4 °C. The measurements were performed at 20 °C after prewarming the protein samples for 1 h. All solutions were filtered to remove dust particles directly before use. The measured protein fluorescence values were corrected by the small apparent fluorescence of the solutions of GuCl in buffer D. The change of the native protein fluorescence was monitored at an emission wavelength of 335 nm (excitation at 280 nm), using 8-nm bandwidths on both sides.

Results

Preparative Reactivation Yield. Only apo-eAAT was used in the first set of experiments to avoid any possible complication by unspecific interaction of the coenzymes with the unfolded polypeptide chains. Incubation of the enzyme in 6 M GuCl in buffer D for as long as 120 min at room temperature had no effect on the reactivation yield, but longer incubation times led to lower yields. Consequently, all incubation periods in 6 M GuCl at 20 °C were kept between 30 and 60 min. Because precipitation was observed at final protein concentrations higher than 1 mg of protein/mL, the concentration during refolding never exceeded 0.7 mg of protein/mL. For preparative refolding, the denatured protein was dialyzed at 4 °C against at least 50 volumes of buffer D, which was changed twice after 2-h intervals each. For studying the effect of the coenzyme on the yield of reactivation, the dialysis buffer was supplemented with 10 μM either PLP or PMP. The recovery of active enzyme was usually between 80–90% (refolding in the absence of coenzyme) and 90–100% in the presence of 10 μM coenzyme. It was also possible to refold the denatured enzyme by dilution 1:1000-fold with buffer D containing 10 μM coenzyme (Giartosio et al., 1973; West & Price, 1989). The yield of active enzyme was usually about 10% lower in this case than that after dialysis.

Spectroscopic Studies. The unfolding of apo-eAAT and holo-eAAT by GuCl was studied by CD spectroscopy at 221 nm, a measure of the α-helix content of proteins (Labhardt, 1986). In order to reach the folding equilibrium at each GuCl concentration, all measurements were performed after incubation of the protein solution for 24 h at 4 °C, followed by a shift to 20 °C for 1 h. Control measurements showed no

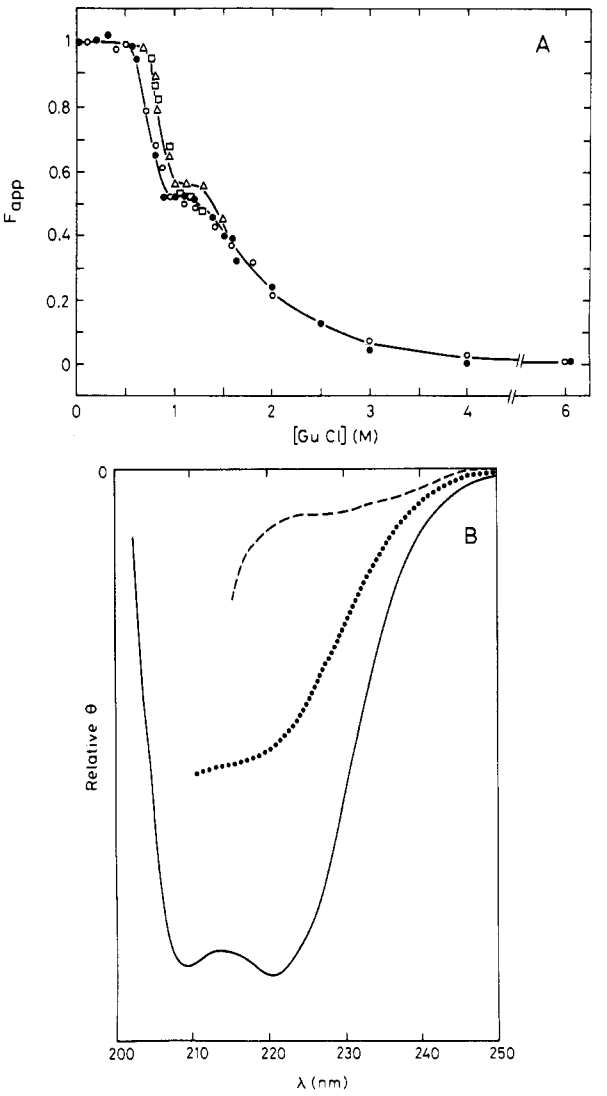

FIGURE 1: GuCl-dependent reversible unfolding of apo-eAAT measured by CD spectroscopy. 10 mM Hepes buffer containing 5 mM DTE/1 mM EDTA (buffer D), pH 7.4, at 20 °C. Concentration: 0.17 mg of protein/mL. (A) Reversible biphasic transition monitored at 221 nm. The linear change of θ_{221} above 4.5 M was extrapolated to 0 M GuCl concentration. F_{app} is the apparent fraction of native protein and represents the normalized deviation from this base line. (●) Unfolding of apo-eAAT; (○) refolding of apo-eAAT; (△) unfolding of PLP–eAAT; (□) unfolding of PMP–eAAT. (B) Relative CD spectra at the following concentrations of GuCl: (—) native dimeric protein at 0 M GuCl; (···) monomeric partially folded intermediate at 1 M GuCl; (- - -) unfolded monomers at 6 M GuCl.

change of the signal after 1-h incubation at 20 °C. In contrast, incubation of the protein samples at GuCl concentrations between 0.7 and 1.2 M for more than 5 h at room temperature led to protein precipitation.

Figure 1A shows that the mean residue ellipticity, θ_{221}, decreased reversibly at 0.17 mg of protein/mL as the concentration of GuCl was increased. The overall process was biphasic, and an intermediate state appeared to be stable at about 1 M [GuCl]. The transition midpoints defined by the horizontal base lines and the plateau were at 0.8 M for the first phase and at 1.7 M GuCl for the second phase. The presence of either PLP or PMP had a slightly stabilizing effect on the first unfolding phase; that is, the midpoint was shifted upward to 0.9 M GuCl.

Figure 1B shows that the intermediate state at 1 M [GuCl] possesses about 50% of the native CD spectral amplitude. Since complete denaturation must involve dissociation of the dimeric apoenzyme to monomers, it was of interest whether

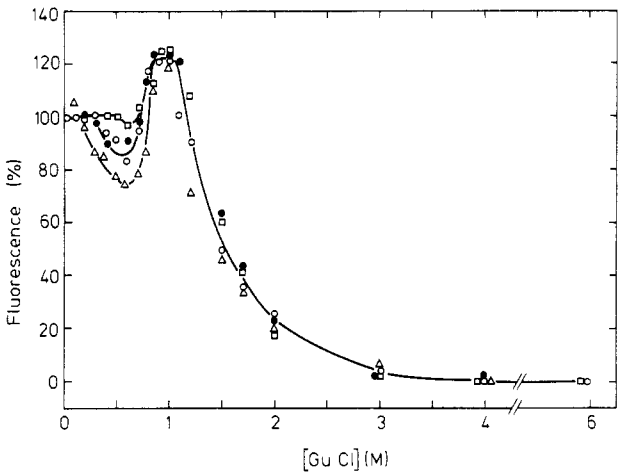

FIGURE 2: Reversible GuCl-dependent unfolding of apo-eAAT measured by fluorescence emission at 335 nm. Excitation at 280 nm. Relative fluorescence normalized to the difference between 0 and 6 M GuCl. Buffer as for Figure 1, at 20 °C. Protein concentration for unfolding: (□) 0.17 mg/mL; (○) 0.017 mg/mL; (△) 0.004 mg/mL. Squares and triangles omitted when superimposed on circles. Protein concentration for refolding: (●) 0.017 mg/mL.

the intermediate is dimeric or monomeric. Variation of protein concentration would have settled the matter, but CD spectroscopy proved to be too insensitive. We therefore turned to fluorescence spectroscopy in the following.

eAAT has five tryptophan residues, one of them (Trp-140) being in the active center (Kamitori et al., 1988; Jaeger et al., 1989). For that reason, fluorescence spectroscopy should be a sensitive tool to monitor conformational rearrangements during the unfolding and refolding of the enzyme, even in the active site.

Figure 2 shows the dissociation and unfolding of apo-eAAT as measured by changes of the relative fluorescence quantum yield at three different protein concentrations. The overall transition occurred reversibly and in three distinct phases: (1) a cooperative decrease of fluorescence emission between 0.0 and 0.6 M GuCl concentration that became relatively more pronounced as the concentration decreased from 0.17 via 0.017 to 0.004 mg of protein/mL; (2) an increase of the fluorescence emission between 0.6 and 0.9 M GuCl concentration to an intermediate plateau value that was about 25% higher than the fluorescence of native apo-eAAT; and (3) a final decrease of fluorescence from the plateau value at 1.1 M to the plateau value at 6 M GuCl concentration. The last two transitions appeared to be independent of protein concentration and coincided with the two transitions observed with CD (Figure 1A). This dependence on protein concentration indicates that apo-eAAT dissociates during the first transition at about 0.5 M GuCl, followed by two rearrangement processes involving monomers.

Figure 3 shows the fluorescence emission spectra of native apo-eAAT (0 M GuCl), the intermediate (1 M GuCl), and the unfolded molecule (6 M GuCl). The fluorescence maximum was shifted under these conditions from 335 to 339 nm and finally to 355 nm, respectively. Moreover, at 1 M GuCl, the fluorescence quantum yield was increased significantly, whereas in the presence of 6 M GuCl it diminished to about 70%. These effects must be due to alterations of the environment of one or more tryptophan residues (Teipel & Koshland, 1971).

Transitions Monitored by Enzymic Activity. Enzymic activity is the property of native proteins that often responds most sensitively toward denaturants (Tsou, 1986). Therefore, we decided to monitor the dissociation and unfolding of

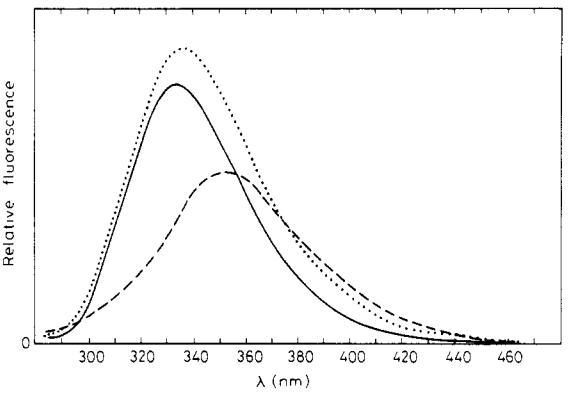

FIGURE 3: Fluorescence emission spectra of apo-eAAT. Fluorescence was excited at 280 nm. Buffer as for Figure 1, at 20 °C. Concentration: 0.1 mg of protein/mL; (—) 0 M GuCl; (···) 1 M GuCl; (---) 6 M GuCl.

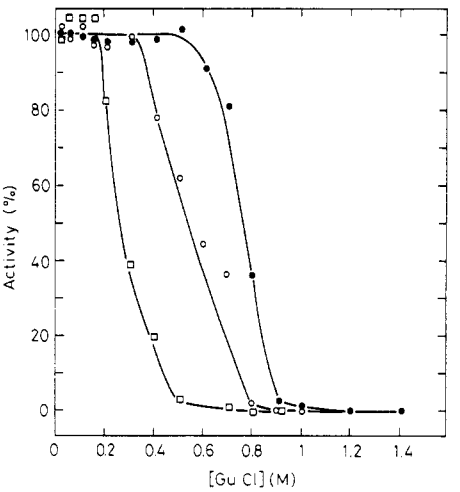

FIGURE 4: Holo-eAAT loses enzymic activity with increasing concentrations of GuCl. Buffer as for Figure 1, at 23 °C. Protein concentration: (●) 0.17 mg/mL; (○) 0.017 mg/mL; (□) 0.0017 mg/mL. Initial substrate turnover measured in assay buffer at 37 °C, as described under Experimental Procedures.

holo-eAAT as a function of protein concentration by this method. Figure 4 shows that enzyme activity at 0.17 mg of protein/mL was lost via a strongly cooperative transition, which coincides roughly with the first transition observed by fluorescence at the same protein concentration (cf. Figure 2). Moreover, the midpoint of the transition decreased dramatically with decreasing protein concentration. Therefore, dissociation leads to inactive monomers.

Hydrodynamic Measurements. We used ultracentrifugation to confirm that dimeric apo-eAAT dissociates at low concentrations of GuCl. For reasons of sensitivity and protein stability, the protein concentration was about as high (0.29 mg/mL) as the highest concentration in Figures 1A, 2, and 4, but the long duration of the experiment required lowering of the temperature from 20 to 6 °C. The sedimentation coefficient decreased from 5.4 S at 0 M GuCl to 2.2 S at 3 M GuCl (Figure 5). The greatest change of s value occurred between 0.5 and 1 M GuCl, consistent with dissociation of the dimer to monomers within this concentration range. The second transition was more gradual and coincided roughly with the final transitions in Figures 1A and 2. We also performed sedimentation equilibrium runs at 8 ± 2 °C. Unfortunately, the long time interval (24 h) required for equilibration led to uninterpretable protein concentration distributions due to aggregation (data not shown). This side reaction was particularly acute at concentrations of GuCl between 0.5 and 1.5 M.

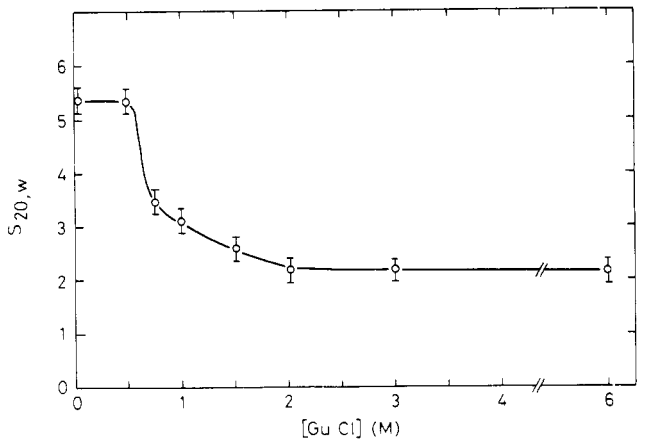

FIGURE 5: Unfolding of apo-eAAT by GuCl as monitored by changes of the sedimentation coefficient. Buffer as for Figure 1, at 6 °C. Concentration: 0.29 mg of protein/mL. The data are averages from two independent measurements.

A more sensitive and rapid method was needed to separate the processes of dissociation of the dimer from unfolding of the monomer at 20 °C. This goal required use of a relatively low protein concentration, and therefore we used the method of gel filtration through a gradient of increasing GuCl concentration that was developed by Endo et al. (1983). As seen in Figure 6A, the protein eluted as a symmetric peak at various concentrations of GuCl. The elution volume first increased to a broad maximum between 0.7 and 1.1 M GuCl (Figure 6B). The CD and fluorescence transitions attained the intermediary plateau in the same range (cf. Figures 1A and 2). The absence of a second peak or shoulder in the elution diagrams (Figure 6A) indicates that dissociation and unfolding were rapid compared to the retention time on the column (32–40 min). When the elution rate was decreased from 0.3 to 0.1 mL/min, superimposable elution profiles were obtained at all concentrations of GuCl. Thus, the protein was in thermodynamic folding equilibrium throughout the unfolding transition.

The apparent molecular weight of the protein, which was interpolated from the elution volumes of standard proteins, decreased from about 80 000 (elution volume 11.2 mL) at 0.0 M GuCl to about 48 000 (elution volume 12.1 mL) at 0.9 M GuCl. Since the molecular weight of the monomer is 43 573 (Kondo et al., 1987; Fotheringham et al., 1986), we conclude that, at a concentration of about 0.05 mg of protein/mL, the dimeric apo-eAAT is converted to relatively compact monomers at 0.9 M GuCl. Further increase of the GuCl concentration beyond 1.1 M led to a gradual decrease of elution volume (i.e., an increase of the apparent Stokes radius; cf. Figure 6A,B), which is expected for complete unfolding of the monomeric protein. The transition midpoint of this second phase (1.7 M GuCl) coincides with the transition midpoint of the terminal unfolding phase detected by CD spectroscopy (Figure 1A).

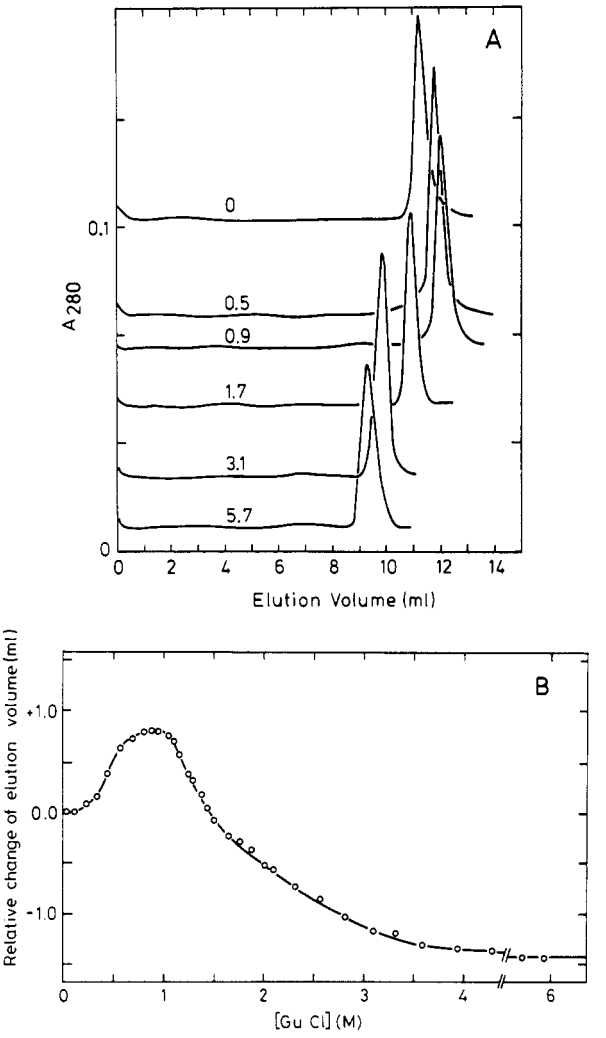

FIGURE 6: Dissociation and unfolding of apo-eAAT as monitored by GuCl gradient gel filtration. Buffer as for Figure 1, at 23 ± 2 °C. Quantitative gel filtration was performed as described under Experimental Procedures. (A) Elution profiles of 0.05-mg samples of apo-eAAT each at the indicated concentrations of GuCl. (B) Change of elution volume as a function of GuCl concentration. Final half-width concentration about 0.05 mg of protein/mL. The curve was normalized by extrapolating the linear change of the elution volume above 4.5 M GuCl to zero GuCl concentration. The normalized change of the elution volume is the apparent deviation from this base line.

Discussion

At 20 °C, the unfolding of eAAT is reversible and clearly triphasic. The simplest mechanism that accounts for all experimental observations is

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

where D is the native dimer, M and M* are two distinct "structured" monomers, and U is the unfolded chain.

The decrease of both the Stokes radius (Figure 6A,B) and the sedimentation constant (Figure 5) identifies an intermediate M* that is thermodynamically stable between 0.9 and 1.1 M GuCl. It is monomeric, retains about 50% of the native CD signal at 221 nm (Figure 1B), and has a fluorescence quantum yield that is 25% higher than that of the native enzyme (cf. Figure 3), but is enzymically inactive. Judged by the sedimentation constant (3.1 S, Figure 5), M* is somewhat less compact than native monomeric proteins of about the same molecular weight. Two examples are concanavalin B (M_r 42 000, 3.5 S) (Svedberg & Pedersen, 1949) and α_1-trypsin inhibitor (M_r 45 000, 3.4 S) (Bundy & Mehl, 1959). Assuming a spherical shape, and the same hydration and partial specific volume as the native dimer, the s value of the native eAAT monomer is 3.4 S [for the calculation procedure, see Cantor and Schimmel (1980)]. Thus, the equilibrium transition from D (native dimer) to M* involves both dissociation and a partial loss of secondary and tertiary structure.

However, the dependence of both enzymic activity (Figure 4) and fluorescence emission (Figure 2) on the concentration of protein reveals yet another, necessarily monomeric intermediate. M is less well characterized than M*, but it is enzymically inactive and has a lower fluorescence quantum

yield than either D or M*. The simplest explanation of these properties is that the enzymic activity of M is lost because dissociation simultaneously destroys both shared active sites (Ford et al., 1980; Kamitori et al., 1988; Jaeger et al., 1989).

It is possible to extrapolate the primary dissociation process of D to M (illustrated in Figure 4) to zero concentration of GuCl. The value of the equilibrium constant K of this reaction ($K = [M]^2/[D]$) at the transition midpoint is equal to the total monomer concentration and corresponds to that particular concentration of GuCl. K decreases 10-fold for each decrement of 0.25 M GuCl concentration between 0.75 and 0 M, and, therefore, the value of K extrapolated to 0 M GuCl is approximately 4 nM. This value agrees well with the finding of Cournil et al. (1975), that the dimer dissociates in the presence of coenzymes and substrates below 5 nM.

This value is 10-fold smaller than that found for the dissociation of another dimeric, PLP-dependent enzyme, namely, the β_2-subunit of tryptophan synthase from *E. coli* (Chafotte & Goldberg, 1987). Using the extrapolated value, it is possible to estimate roughly from Figure 2 the apparent fluorescence quantum yield of M at about 50% relative to that of D. Since none of the tryptophan residues (cytosolic pig AAT numbering 134, 140, 217, 205, and 309) are located at the intersubunit interface (Ford et al., 1980; Kamitori et al., 1988; Jaeger et al., 1989), M must have a somewhat different tertiary structure than the native subunit.

The difference of the tertiary structure of the intermediate M compared to that of the dimeric state D is not surprising. Janin et al. (1988) have shown that the homologous mitochondrial AAT from chicken liver has a relatively large normalized subunit interface by comparison to other dimeric proteins: 20% of the total accessible subunit surface is located at the interface. It is thought that isolated monomers with large intersubunit interfaces are unstable and therefore must undergo a major structural change upon dissociation. Unfortunately, it was not possible to measure CD spectra at low protein concentrations due to the high background absorption of buffer containing GuCl. Thus, it is unknown to what extent the dissociation of D to 2M leads to changes of overall secondary structure.

Because the first transition monitored by CD (Figure 1A) includes both the dissociation and the first isomerization step, that is, $D \rightleftarrows 2M \rightleftarrows 2M^*$, it is not clear whether the effect of the coenzymes is restricted to the dissociation step. Unfortunately, the intrinsic fluorescence of the coenzymes precluded fluorescence measurements of unfolding and refolding in their presence. By analogy to the β_2-subunit of tryptophan synthase (Chafotte & Goldberg, 1987), it is likely that mainly the dissociation of D to 2M is affected by bound PLP and PMP. However, Iriarte et al. (1984) have suggested that the stabilizing effect of PLP on the thermal denaturation of chicken mitochondrial AAT is due to PLP binding to folded monomers.

At the relatively high concentration (0.17 mg of protein/mL) used in Figures 1A, 2, and 4, the intermediate M represents only a very small fraction of the total population of protein species. However, from the data at low protein concentrations (Figure 2) it is clear that M is converted to M* in a strongly cooperative manner that is independent of protein concentration between 0.6 and 0.9 M GuCl concentration. Both gel chromatography (Figure 6A,B) and sedimentation velocity measurements (Figure 5) have shown qualitatively that M* has a compact structure, albeit not as tightly packed as other native monomeric proteins of comparable molecular weight. Since M* itself is unfolded further by increasing the concentration of GuCl from 1.1 M to 4 M, it possesses many properties of an "intermediate denatured" (Shortle & Meeker, 1989) or of a "molten globule" state (Dolgikh et al., 1981; Ohgushi & Wada, 1983; Baum et al., 1989).

The aggregation of the protein between 0.6 and 1.2 M GuCl at 25 °C is consistent with the notion of nonspecific aggregation of the partially folded intermediate M* under entropically favorable conditions. Analogous behavior was observed by Zetina and Goldberg (1980) with the β_2-subunit of tryptophan synthase, and with dimeric intermediates of lactate dehydrogenase at 0.75 M GuCl by Jaenicke et al. (1981). The latter enzyme, which is tetrameric in the native state, forms metastable intermediates only at low temperature (3 °C). They aggregate irreversibly at higher temperatures (25 °C). Partially unfolded tetramers of aspartokinase I–homoserine dehydrogenase I also aggregate nonspecifically at concentrations between 0.2 and 2.0 M GuCl, probably after dissociation to subunits (Müller & Garel, 1984).

To our knowledge, this work is the first where uncoupled dissociation and unfolding of an oligomeric enzyme have been shown by the method of denaturant gel filtration. It is unusual to observe two different monomeric intermediates during the reversible dissociation and unfolding of a dimeric protein (Jaenicke, 1987). Because one of these intermediats is a stable partially folded monomer at about 1 M GuCl, it is feasible to study the kinetics of refolding of $U \rightarrow M^*$ separately from that of rearrangement and reassociation of $2M^* \rightarrow 2M \rightarrow D$.

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