The refolding of mitochondrial aspartate aminotransferase (mAAT; EC 2.6.1.1) has been studied following unfolding in 6 M guanidine hydrochloride for different periods of time. Whereas reactivation of equilibrium-unfolded mAAT is sigmoidal, reactivation of the short term unfolded protein displays a double exponential behavior consistent with the presence of fast and slow refolding species. The amplitude of the fast phase decreases with increasing unfolding times (k ~ 0.75 min⁻¹ at 20 °C) and becomes undetectable at equilibrium unfolding. According to hydrogen exchange and stopped-flow intrinsic fluorescence data, unfolding of mAAT appears to be complete in less than 10 s, but hydrolysis of the Schiff base linking the coenzyme pyridoxal 5'-phosphate (PLP) to the polypeptide is much slower (k ~ 0.08 min⁻¹). This implies the existence in short term unfolded samples of unfolded species with PLP still attached. However, since the disappearance of the fast refolding phase is about 10-fold faster than the release of PLP, the fast refolding phase does not correspond to unfolding of the coenzyme-containing molecules. The fast refolding phase disappears more rapidly in the pyridoxamine and apoenzyme forms of mAAT, both of which lack covalently attached cofactor. Thus, bound PLP increases the kinetic stability of the fast refolding unfolding intermediates. Conversion between fast and slow refolding forms also takes place in an early folding intermediate. The presence of cyclophillin has no effect on the reactivity of either equilibrium or short term unfolded mAAT. These results suggest that proline isomerization may not be the only factor determining the slow refolding of this cofactor-dependent protein.

The folding and unfolding of proteins are complex processes affected by a number of features such as size, chain topology, and oligomeric state and ultimately by the amino acid sequence, which encodes each unique three-dimensional structure (1). Much of our understanding of the protein folding problem arises from in vitro refolding studies of small monomeric proteins (2). However, the molecular mass of the majority of proteins found in prokaryotic or eukaryotic cells is larger than 25 kDa (3). Most of these larger polypeptides fold into several structural domains, and in the case of oligomeric proteins several subunits associate through noncovalent interactions. Folding and unfolding of many large proteins with several folding domains and/or multiple subunits often show multiphasic kinetics with fast and slow folding phases, suggesting the presence of either multiple paths or intermediate states in the process (4). Slow folding phases are often associated with proline isomerization (5, 6), but in some cases the rate-limiting step involves, at least to some extent, the reorganization of misfolded species or metastable kinetic folding intermediates (7–9). The significance of these kinetic intermediates is still a matter of discussion. They are considered to represent either productive intermediates that facilitate folding or kinetic traps that decrease the rate of folding (10, 11). Both types of intermediates are likely to occur in protein folding, depending on the particular protein or the folding conditions.

In addition, many proteins in the cell require the binding of cofactors in their native state to become biologically active. The folding of these proteins must therefore include the binding of cofactor, and analysis of this group of proteins is essential to obtain a better understanding of protein folding in vivo. However, the roles of cofactors in directing the folding pathway and their effect on the kinetics of protein unfolding are not well understood. Most cofactors stabilize the structure of the native protein, often by decreasing its unfolding rate (12–15). In some proteins, the cofactor remains associated with the polypeptide after unfolding. This has been observed with organic cofactors such as FMN (16) or heme (13, 17) as well as metal ions (18). Thus, binding of at least some cofactors may take place before the protein folds in the cell. These cofactors may stabilize local native-like structure in unfolded proteins and thereby facilitate the search for the native state by acting as nucleation sites (16). However, this is not always the case. For instance, misligation of the heme group in unfolded cytochrome c is thought to cause the appearance of slow folding partially folded states (19, 20). In some proteins such as E. coli DNA gyrase B subunit, the metal cofactor binds to the active site after the enzyme reaches its native state, and therefore the overall folding pathway of this enzyme is not perturbed by the presence of metal ions (12).

Mitochondrial aspartate aminotransferase (mAAAT) is a member of the large family of pyridoxal 5'-phosphate (PLP)-dependent enzymes that catalyze many reactions in the metabolism of amino acids. This family includes at least five different

* This work was supported by National Institutes of Health Grant GM-58341. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Slow Refolding of Aspartate Aminotransferase

Among the invariant residues of subgroup I of aminotransferases, two prolines, Pro^138 and Pro^195, are in the cis configuration in all of the known AAT structures. Members of the PLP-dependent family of enzymes are very suitable systems to study the folding of cofactor-containing oligomeric proteins. Different enzyme forms containing coenzyme attached either covalently or noncovalently, as well as coenzyme-free apoenzyme, can be easily prepared in order to study and compare their folding properties. The effect of PLP on folding varies among the different PLP-dependent enzymes analyzed. The isolated β, subunit of E. coli tryptophan synthetase requires coenzyme to reactivate in vitro (24), whereas the refolding of E. coli serine hydroxymethyltransferase can proceed in the absence of coenzyme (25). Binding of PLP imparts considerable structural stability to most PLP-dependent enzymes (see Ref. 26 and references therein). Based on reactivation and fluorescence data, a kinetic model including two consecutive first order rate-limiting steps was proposed to explain the refolding in vitro of mAAT following unfolding in guanidine hydrochloride (GdnHCl) or low pH solvents (27, 28). These steps follow the rapid collapse of the unfolded chain into a molten globule-like intermediate. It has been proposed that isomerization of the two cis-prolines in AAT is responsible for the two slow phases observed during in vitro refolding of chicken mAAT (29) or E. coli AAT (30). However, analysis of the folding properties of P138A and P195A mutants of E. coli AAT indicated that although proline isomerization may play some role, other conformational rearrangements must rule the folding of this protein (31, 32).

Here we extend these studies to the analysis of the relationship between the presence of coenzyme and the properties of the unfolding/refolding reaction of mAAT. The coenzyme PLP is completely released from the active site upon unfolding (33), but its covalent link with the polypeptide persists long after the protein appears to be fully unfolded according to activity, fluorescence, CD and amide hydrogen exchange data. The presence of coenzyme does not perturb the kinetics or pathway of refolding. However, double jump experiments indicate that covalently attached PLP slows down the interconversion between fast and slow folding populations of unfolded states. Although we cannot discard the involvement of proline isomerization on the slow refolding steps of this protein, our results are consistent with additional structural rearrangements occurring both in the unfolded state and in populations of folding intermediates along the folding pathway.

**EXPERIMENTAL PROCEDURES**

Materials and Protein Purification—Aspartic acid, L-ketoglutarate, cysteine sulfinic acid, GdnHCl, guanidine deuterchloride (GdnDCI), PLP, pyridoxamine 5'-phosphate (PMP), and NaBH4 were purchased from Sigma. D2O and DCI were from Aldrich. All other reagents were of the highest purity available. The precursor form of rat liver mAAT was expressed in E. coli and purified as previously described (34). To prepare the mature form (mAAT), the precursor was incubated with tryptophan (precursor ratio 1:1.60 by weight) to remove the prosequence peptide (34). This mature protein differs from the naturally occurring mAAT by having an additional alanine residue at its N-terminal end. Protein concentration was determined spectrophotometrically from the absorbance at 280 nm using an extinction coefficient of ε280 = 1.40. The enzyme activity was measured at 25°C using aspartate and L-ketoglutarate as substrates in a coupled assay with malate dehydrogenase as described previously (35). The specific activity of the mAAT preparation was about 250 μmol·min⁻¹·mg⁻¹. The purified protein was stored in 40 mM HEPES, 0.1 mM EDTA, pH 7.4 (reducing buffer), containing 0.02% sodium azide. The homogeneity of the preparation and the molecular size of the protein were verified both by SDS-PAGE, according to Laemmli (56), and by electrospray mass spectrometry (ESI-MS). The PMP form of the enzyme was prepared by incubating PLP-mAAT with a 20-fold molar excess of cysteine sulfinate in refolding buffer followed by extensive dialysis against refolding buffer (37). Conversion of PLP to PMP was confirmed by monitoring the absorption spectra, which showed a shift in the position of the coenzyme peak from 356 to 334 nm. The apoenzyme was prepared by extensive dialysis of PMP-mAAT against 0.5 mM potassium phosphate buffer, pH 5.7, containing 0.2 mM NaCl and 0.02% NaN3, followed by dialysis against 40 mM HEPES, pH 7.5, containing 0.1 mM EDTA and 0.02% NaN3. The apoenzyme preparation was over 95% free of PLP and recovered 90% of the original activity upon reconstitution of the holoenzyme by incubation with 100 μM PLP. Reduction of the PLP and Lys^258 was carried out by incubation with 5 mM NaBH4 (38). Complete reduction of the Schiff base was confirmed by monitoring the expected shift in the absorption maximum of the bound coenzyme from 356 to 334 nm.

**Unfolding and Refolding Reactions—GdnHCl unfolding and refolding of mAAT were performed following published procedures (27). For unfolding, aliquots of mAAT were incubated with 6 M GdnHCl, 40 mM HEPES, 0.1 mM EDTA, 10 mM dithiothreitol, pH 7.4. The final protein concentration was 2.4 mg/ml. After incubation for different times as indicated in the corresponding figures, refolding was initiated by dilution of the unfolded protein at 10°C in 40 mM HEPES, 0.1 mM EDTA, pH 7.4 (reducing buffer) to a final protein concentration of 40 μg/ml. The dead time of the manual mixing was 15–20 s. When necessary, the refolding buffer was supplemented with 10 μM PLP or PMP. When monitoring reactivation of the apoenzyme, the time aliquots were preincubated for 20 s with 20 μM PLP before assaying. The enzymatic activity was monitored for only 30 s to minimize reactivation during the determination. Reactivation data are expressed as percentage relative to the final activity recovered after incubation for 4 h, which was about 40% of the total protein added to the reaction.

**Refolding Monitored by Intrinsic Fluorescence—**Fluorescence measurements were performed in an SLM 8000 C Amino acid spectrophotometer set on photon counting mode. The recovery of native fluorescence during refolding was followed by monitoring absorption at 295 nm and emission above 309 nm using a 10°C cuvette at pH 7.4. The excitation wavelength was 280 nm, and emission was monitored above 309 nm using a 10°C cuvette at pH 7.4. The excitation wavelength was 280 nm, and emission was monitored above 309 nm using a 10°C cuvette at pH 7.4. The excitation wavelength was 280 nm, and emission was monitored above 309 nm using a 10°C cuvette at pH 7.4. The excitation wavelength was 280 nm, and emission was monitored above 309 nm using a 10°C cuvette at pH 7.4.

**Stopped-flow Fluorescence—**The changes in fluorescence upon unfolding or refolding were monitored using an SPM-4 (Bio-Lyte/Molec-}

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flow fluorescence analysis, mAAT incubated in 3 mM GdnHCl for 1 h was fully unfolded. The dead time of the instrument was determined to be about 1 ms. The temperature was regulated at either 20 °C (unfolding) or 10 °C (refolding) using a thermostatted water bath. Control experiments performed mixing N-acetyl-tryptophanamide with buffer, native protein with buffer, or buffer with GdnHCl confirmed the absence of mixing artifacts beyond the first 10–15 ms following mixing. Typically, 8–10 kinetic traces were averaged.

Deuterium Exchange and Mass Spectrometry Analysis—Following equilibration in deuterated refolding buffer (40 mM HEPES, 0.1 mM EDTA, pH 6.7) for 17 h, aliquots of native mAAT (0.5 mg/mL) were unfolded in 6 M GdnDCI, 40 mM HEPES, 0.1 mM EDTA, pH 6.7, at 20 °C. At different times after mixing with GdnDCI, aliquots of the unfolding reactions (3.2 μL) were mixed with 40 μL of ice-cold 200 mM N,N,N,N-tetramethyl-PO 2.35. Samples were then desalted on a C18 reverse phase chromatography column (Zorbax C18SB Wide Pore Guard Column; Micro-Tech Scientific; 1 cm × 0.32 mm), which was connected online to a valve to direct the flow to either the waste or the mass spectrometer. After washing the column with 0.1% (v/v) trifluoroacetic acid at a flow of 200 μL/min, the flow was diverted to the mass spectrometer, and the protein was eluted using a 0–54% (v/v) acetonitrile gradient in 0.1% (v/v) trifluoroacetic acid at a flow rate of 100 μL/min. The time lag between injection of the sample and the peak of the ionic current in the detector was 4 min. The reverse phase column, solvent delivery line, and injection valve were immersed in an ice-water bath to minimize H/D back exchange during injection of the sample into the mass spectrometer. All ESI-MS data were acquired on a Finnigan LCQ mass spectrometer. The LCQ settings were previously optimized for detection of mAAT in high flow conditions (ion spray voltage 4.5 kV, capillary temperature 200 °C, capillary voltage 10 V). Ions with an m/z ratio below 800 were excluded from the ion trap, and m/z data in the range 800–2000 were collected. The relative abundance versus mass spectra was reconstructed from multiple charge states of mAAT (+23 to +55) using the BIOMASS deconvolution algorithm provided in the Xcalibur™ (Finnigan) software, and molecular weights of untreated (protiated) and deuterated protein were then calculated from the molecular mass of the peaks. Because the chromatographic step was performed in protic solvents, rapidly exchanging deuterium in mAAT was replaced with protium, leaving deuterium only in the peptide amide linkages, so the deuterium levels estimated are a direct measure of the deuteration at amide groups (39–42).

Data Analysis—The rate constants of the refolding process were determined by fitting the activity data to either an irreversible two-consecutive first-order reaction mechanism as described previously (27, 28) or to a double exponential process (A = A s (1 − e −k∞) + A f (1 − e −k∞)). In this analysis, irreversibility only means that under conditions favoring refolding of the unfolded protein, the rate constants for the reverse reactions are much lower than those for the forward reactions. Nonlinear least squares fitting of the data was performed using the Levenberg-Marquardt algorithm in SigmaPlot 6.0 for Windows (Jandel Corp.). The temperature dependence kinetic traces were analyzed using either the BioKin software provided with the stopped-flow instrument or SigmaPlot. The quality of the fit was judged by visual analysis of plots of the residuals (deviation of the fitted function from the experimental values). Details on the determination of the rate constants are included in the figure legends. The relative amplitudes of the fast and slow refolding populations in samples unfolded for short periods of time were estimated by simulation of the kinetic traces using the PC version of the kinetic simulation program KINSIM (43).

RESULTS

Reactivation Kinetics of PLP-mAAT after Different Times of Unfolding—It has been reported that the rate of refolding of chicken cAAT and mAAT decreased by a factor of 4 and 25, respectively, when the proteins were denatured in 6 mM GdnHCl for 40 s instead of for 40 min (29). These results were interpreted as indicating that proline isomerization is the rate-limiting step in the slow refolding of these enzymes unfolded for long periods of time. The rate of reactivation of the PLP (holoenzyme) form of rat liver mAAT (PLP-mAAT) also increases after short term unfolding in 6 mM GdnHCl, but the dependence of the reactivation rate on the duration of unfolding is much less pronounced. The observed rate constants estimated by fitting the kinetic data to a single exponential function show that mAAT unfolded for 40 s at 20 °C refolds only about 2-fold faster than when unfolded for 40 min at the same temperature (Fig. 1). More importantly, the reactivation curves for the short and long term unfolded protein are qualitatively very different. The reactivation of mAAT unfolded for 40 min shows the characteristic sigmoidal behavior with an initial lag of about 4 min (at 10 °C) previously described for the enzyme unfolded at low pH (25) or in 4 M GdnHCl (27). A kinetic model including two consecutive first order rate-limiting steps was proposed to explain the refolding in vitro of guanidine- or acid-unfolded mAAT (27, 28).

The data shown in Fig. 1A for the refolding at 10 °C of 40-min unfolded mAAT can be fit by such a sequential model with rate constants of 0.30 and 0.025 min⁻¹, essentially identical to those reported earlier (27). By contrast, when mAAT was incubated only briefly (40 or 10 s) in 6 mM GdnHCl, no lag was observed, but the reaction still showed complex reactivation kinetics. Reactivation was best fit to a double exponential function consistent with the presence of fast and slow refolding species in the unfolded sample (Fig. 1A). For instance, after a short 10-s unfolding incubation at 20 °C, about 37% of the protein reactivated with a rate constant of 0.58 min⁻¹. The remaining 63% of the activity was recovered very slowly, with a rate constant of 0.02 min⁻¹, almost identical to the rate constant of the slower rate-limiting step of long term unfolded protein. This result was at first surprising. After such a short incubation, the unfolded protein is expected to be homogeneous with regard to backbone configuration, and therefore a monophasic reactivation reaction should be observed.

A possible explanation could be that slow folding molecules were formed during the 10-s unfolding period by some extremely rapid backbone isomerization. To address this issue, we followed the kinetics of interconversion between fast and slow folding species in the unfolded protein by monitoring the decrease in the amplitude of the fast refolding phase with time of unfolding. The amplitude of the fast phase was difficult to estimate accurately by fitting the data to a double exponential function using standard fitting routines, particularly at times of unfolding longer than 40 s, where its relative amplitude is less than 20% of the total. Furthermore, when short term unfolded protein was refolded at 0 °C, we detected a small plateau between the fast and slow reactivation phases (Fig. 1B, inset), which is consistent with the existence of a lag phase in the refolding of the slow folding species. This plateau is not detected during refolding at 10 °C, probably because the lag period is considerably shorter (about 4 min at 10 °C and 20 min at 0 °C). Thus, reactivation of the slow folding molecules in the short term unfolded protein follows the sequential mechanism described before for the long term unfolded protein. For these reasons, we estimated the amplitude of the slow and fast phases by simulating the reactivation data for short unfolding times using KINSIM (43) and a model in which, after the rapid collapse into an early folding intermediate (U → I in Reaction 1), reactivation of the slow folding population of unfolded molecules (U) follows a sequential mechanism (I₁ → I₂ → N in Reaction 1, where I and N represent intermediate and native states, respectively) and the fast refolding species (U) reactivate in a single rate-limiting step (I₁ → N in Reaction 1).

\[
\begin{align*}
U & \rightarrow I₁ \rightarrow N \\
I₁ & \rightarrow I₂ \rightarrow N
\end{align*}
\]

\text{REACTION 1}
The solid line in Fig. 1B is a representative example of such a simulation for the 10-s unfolded protein in which 60% of slow folding molecules follow the sequential mechanism (rate constants of 0.3 and 0.025 min⁻¹) and the remaining 40% regain activity in a single exponential process with k = 0.5 min⁻¹. As shown in Fig. 2 (data for PLP-mAAT), the relative amplitude of the fast refolding phase decreases with increasing unfolding times in a single exponential process with an apparent rate constant of 0.75 min⁻¹ at 20 °C. Interestingly, the fraction of fast refolding molecules at zero unfolding time obtained by extrapolation of the exponential decay to 0 was not 100% as expected but only 42% of the total unfolded protein. The rate of disappearance of the fast refolding phase changes with the temperature of the unfolding reaction (data not shown) and exhibits an activation energy of 24 ± 3 kcal/mol, but it is not affected by changes in the temperature of refolding. By contrast, the amplitude of the fast phase at zero unfolding time is insensitive to changes in the conditions of the unfolding reaction such as temperature but decreases slightly (by about 7%) when the temperature of the refolding reaction is lowered to 0 °C. These results indicate that the slow refolding species detected during refolding of short term unfolded mAAT are formed by the isomerization of the population of fast folding intermediates If to slow refolding intermediates (Is1) similar to those arising directly from Uf. This isomerization would compete with the direct conversion of If to N. Using KINSIM and the model depicted in Reaction 1, we calculated that during refolding at 10 °C (k_f = 0.3 min⁻¹, k_u = 0.025 min⁻¹), even when starting with 100% Uf, only 42% of If would follow the direct reactivation track to N with k_f = 0.5 min⁻¹ if If converted to Is1 with a rate constant of 0.75 min⁻¹. The reactivation kinetics observed at 0 °C (k_f = 0.08 min⁻¹, k_u = 0.005 min⁻¹) could be reproduced using a rate constant of 0.24 min⁻¹ for the If → Is1 conversion and 35% of If refolding directly to N with k_f = 0.16 min⁻¹. The activation energies of the If → Is1 and Is1 → Is2 reactions are about 16–18 kcal/mol, comparable with that typical of cis-trans proline isomerization (20 kcal/mol). However, the activation energy of the direct If → N reaction is also in the same range (17 kcal/mol), and this step is not expected to be limited by proline isomerization. The addition of cyclophilin does not alter the rate of any of these reactions (data not shown). The activation energy of k_u is considerably larger, about 36 kcal/mol. Thus, the slow refolding of mAAT may be the result of a kinetic trap that can arise from an isomerization occurring either in the unfolded state (Uf → U) or at an early folding intermediate (Is1). For PLP-mAAT, the rate of this conversion is faster in the folding intermediate than in the unfolded state (0.75 and 0.25 min⁻¹, respectively, at 10 °C; see Fig. 3A).
Characterization of the Unfolding Reaction—Since folding kinetics can be affected by the structural properties of the denatured state from which a folding reaction is initiated, we analyzed the unfolded state of samples of mAAT incubated with GdnHCl for different periods of time by deuterium exchange and ESI-MS. The deconvolved mass spectrum of the native protein under the conditions used in our experiment (Fig. 3A, top trace) shows two peaks with masses of 44,597 and 44,826 Da. The first peak (NH in Fig. 3A) matches the predicted mass of the rat liver mAAT subunit (44,597 Da) (44). The difference in mass between the two peaks is about 229 Da, which corresponds closely to the calculated mass of the phosophyrindoxyl group (231 for the fully protonated group). Thus, the second peak must represent the polypeptide chain containing one molecule of PLP still attached (N-PLP in Fig. 3A). It appears that a substantial fraction of the covalently bound coenzyme survives the mild conditions used for the high pressure liquid chromatography separation and subsequent ESI-MS analysis of the samples. According to the relative areas of the two peaks in the mass spectrum, we estimated that about 60% of the PLP originally attached to native mAAT remained bound at the time the sample reached the MS detector. We used this estimate to correct subsequent determinations of the PLP content of unfolded mAAT.

When native mAAT was incubated for 17 h in deuterated buffer under native conditions (refolding buffer, pH 6.7), both the PLP-free and PLP-containing peaks shifted toward the higher mass region of the spectrum by 139 Da (Fig. 3A, second trace from the top). This result indicates that a minimum of about 139 amide hydrogens have been exchanged in the native protein. Since some back exchange is expected to occur during analysis in protic buffers (see below), the actual total number of amide hydrogens exchanged is probably higher. As expected, the loss of structure during incubation of deuterated native mAAT in a deuterated medium in the presence of 6 M GdnDCI for 10 s results in a further increase in the mass of both peaks (Fig. 3A, third trace from the top) by an additional 200 Da (196

FIG. 2. Time-dependent changes in the population of fast refolding species during unfolding of different forms of mAAT in 6 M GdnHCl. Refolding of PLP-mAAT (●), PMP-mAAT (○) or apo-mAAT (■) was performed as described in the legend to Fig. 1 following unfolding in 6 M GdnHCl for different periods of time. Relative amplitudes were calculated by simulation to the model depicted in Reaction 1, using the rate constants estimated by fitting the data to a double exponential or two-consecutive first order reaction mechanism as described in the legends to Figs. 1 and 5 and the initial relative amounts of fast and slow refolding species as independent variables. The lines represent fits of data to a single exponential function with rate constants for the disappearance of the fast refolding species of 0.75 ± 0.10 min⁻¹ for PLP-mAAT, 3.88 ± 0.33 min⁻¹ for PMP-mAAT, and 3.90 ± 1 min⁻¹ for apo-mAAT. By extrapolation to zero unfolding time, we estimated the fraction of fast refolding species at zero unfolding time as 42 ± 2.0, 46 ± 3.6, and 35 ± 6.0%, for PLP-mAAT, PMP-mAAT, and apo-mAAT, respectively.

FIG. 3. Time course of deuterium exchange and PLP release during unfolding of PLP-mAAT in 6 M GdnDCI. A. Representative ESI-MS spectra of native and unfolded mAAT. Aliquots of native mAAT diluted in either protiated buffer (pH 7.1) or incubated for 17 h in deuterated buffer (pH 6.7) at room temperature were injected into the mass spectrometer. The 44,597-Da peak represents the protiated mAAT polypeptide without PLP (NH), N-PLP represents the mAAT polypeptide deuterated in the native state with or without PLP attached, respectively. In unfolding experiments, mAAT was mixed (0.5 mg/ml) with 6 M GdnDCI, 40 mM HEPES, 0.1 mM EDTA, pH 6.7, at 20 °C, and at the times indicated, 3.2-μl aliquots of the unfolding reaction were diluted with 40 μl of 200 mM (NH₄)₃PO₄, pH 2.35, to slow down further deuterium exchange, desalted in a C18 reverse phase column and injected in the mass spectrometer. Uₙ and U-PLPD represent the protein deuterated in the unfolded state with or without PLP attached, respectively. B. PLP release during unfolding of PLP-mAAT. The fraction of PLP that remained attached to the unfolded protein was estimated from the area of the U-PLPD peak after correction for the release of PLP during the analysis (see top spectrum of native mAAT). Data are expressed as percentage relative to the combined areas of the Uₙ and U-PLPD peaks for each sample, which was taken as 100%. The solid line represents the best fit of the data to a single exponential decay with k = 0.087 ± 0.007 min⁻¹.
Fig. 4. Stopped-flow unfolding trace of PLP-mAAT. Fluorescence stopped-flow unfolding data were obtained using an SFM-4 stopped-flow instrument with a 9-μl FC-08 fluorescence cuvette (λem = 320 nm, λex > 309 nm). The unfolding transition was initiated by a GdnHCl jump from 0 to 6 M at 20 °C at a final protein concentration of 30 μg/ml. Because of mixing artifacts persisting for at least 15–20 ms, the first 20 ms of the recording were ignored for data fitting, and the actual amplitude and rate of the fast fluorescence decay observed could not be estimated with accuracy (rate constants of 90 and 10.5 s–1 were obtained for the exponential decay and rise, respectively, when the data were fit to a double exponential function (solid line). The residuals for the fit are shown in the inset. The dashed line is the kinetic trace calculated for a single exponential rise with k = 10.5 s–1.

and 199 for the difference between U-PLP/N-PLP, and U/ml NPL, respectively). No further shift in the position of the peaks is observed after longer times of unfolding (Fig. 3A, third to seventh trace from the top), suggesting that complete exposure of the amide backbone hydrogen atoms to exchange with the deuterated solvent is achieved during the dead time of manipulation of the sample (about 10 s). The maximum total of deuterons incorporated in the unfolded protein is about 340 (139 ± 200), whereas theoretically the actual number of exchangeable amide hydrogens in this protein is 381. Therefore, about 40 deuterons are probably lost through back exchange during the chromatographic steps preceding injection in the mass spectrometer.

The deuterium exchange data indicate that mAAT is fully unfolded after incubation in 6 M GdnHCl for 10 s. The change in the far UV CD signal is also complete during the dead time of the experiment (data not shown), and rapid kinetic analysis of the unfolding process by fluorescence stopped flow indicates that the unfolding reaction is essentially complete in less than 1 s (Fig. 4). The slowest step detected in the stopped-flow trace is a single exponential increase in intrinsic fluorescence with a rate constant of 10 s–1. This fluorescence enhancement is not related to the disruption of the PLP environment, since identical results were obtained with apoenzyme (data not shown). Thus, the effect of the duration of unfolding on the kinetics of reactivation of mAAT does not seem to be due to major differences in the global structure of the starting material.

In contrast to the fast unfolding of the polypeptide chain, hydrolysis of the internal Schiff base between PLP and the protein is much slower. As shown in Fig. 3A, the intensity of the PLP-containing peak in the mass spectrum of mAAT (labeled as U-PLP) progressively decreases with increasing unfolding times, whereas the intensity of the PLP-free peak (U/ml) concomitantly increases. As detailed above for the native enzyme, the PLP content at each time point was estimated from the relative area under the U-PLP peak corrected for the fraction of PLP that is lost during the analysis (40% of the total). Similar results were obtained when the Schiff base linking the PLP to the polypeptide was reduced with 10 mM NaBH4 prior to analysis by ESI-MS (data not shown). Reduction of the Schiff base irreversibly traps bound PLP and therefore prevents its loss during analysis. The release of PLP takes place over a period of minutes with a rate constant of about 0.087 min–1 (Fig. 3B). Since the removal of PLP is much slower than the U/ml → U/ml conversion (0.75 min–1), the loss of PLP should occur almost exclusively from U-PLP (see scheme in Fig. 9A). The presence of PLP bound to the already unfolded polypeptide chain implies the existence of mAAT unfolding intermediates containing PLP attached. The presence of covalently attached PLP, however, does not seem to be responsible for the fast refolding phase detected in the reactivation experiments. From the data presented in Figs. 2 and 3B, it is evident that the disappearance of the fast refolding phase is much faster than the release of PLP.

Reactivation Kinetics of Apo- and PMP-mAAT Unfolded for Different Periods of Time—Although there is no correlation between the kinetics of U/ml isomerization and loss of PLP, we cannot rule out an effect of the bound coenzyme on the rate of this isomerization or on the single step reactivation of I/ml. To address this question, we analyzed the reactivation kinetics of apo- and PMP-mAAT, two mAAT forms lacking covalently attached coenzyme. PMP is unable to form a Schiff base with Lys258 (it contains an amino group instead of an aldehyde group at the 4′ position) and therefore associates with the active site exclusively through noncovalent interactions. The reactivation kinetics of PMP-mAAT (Fig. 5) or apo-mAAT (data not shown) are very similar to that described for PLP-mAAT, including the presence of an initial fast phase during reactivation of protein unfolded for 10 s (Fig. 5). Thus, fast refolding species also appear during unfolding of coenzyme-free polypeptide chains. The rate constant of the I/ml → N fast phase is also comparable (0.6 ± 0.2 min–1) with that of PLP-mAAT, which indicates that PLP bound to an early intermediate does not accelerate reactivation. Interestingly, the apparent rate constant for the decrease in the amplitude of the fast phase with unfolding time is considerably faster for PMP-mAAT and apo-mAAT (k ≈ 4 min–1 for unfolding at 20 °C) than for PLP-mAAT (Fig. 2). Thus, the presence of covalently bound PLP is not responsible for the fast refolding phase but increases the kinetic stability of the fast refolding unfolded species (see Fig. 9A for a summary). On the other hand, the amplitude of the fast phase extrapolated to zero unfolding time is very similar for the three forms of the enzyme (Fig. 2), which suggests that the presence of coenzyme attached to I/ml has no effect on the rate of its conversion to I/ml. Thus, for PMP- and apo-mAAT (i.e. coenzyme-free chains), the rate of conversion between fast and slow
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Fig. 5. Reactivation kinetics of PMP-mAAT unfolded for different periods of time. Protein unfolding and refolding were performed as described in the legend to Fig. 1 following unfolding in 6 M GdnHCl for 10 s (C), 40 s (D), or 40 min (E). Data are expressed relative to the final activity recovered after a 4-h incubation, which was taken as 100%. The solid lines represent best fits of the data to a double exponential function ($\gamma t$, $k_{\text{low}} = 0.6 \pm 0.20 \text{ min}^{-1}$, $k_{\text{high}} = 0.020 \pm 0.004 \text{ min}^{-1}$) or a two-consecutive first order reaction mechanism ($\square$, rate constants of $0.26 \pm 0.022$ and $0.031 \pm 0.009 \text{ min}^{-1}$). The data for the 40-s unfolded protein (■) could not be fit to either model but rather to a single exponential function ($R^2 = 0.99$) with a rate constant of $0.02 \pm 0.007 \text{ min}^{-1}$. This is probably because the amplitude of the fast phase was too small to be detected when fitting the data to a double exponential, but nonetheless its contribution is enough to conceal the lag phase characteristic of the slow folding reaction.

Refrolding species is faster in the unfolded protein (1.8 min$^{-1}$ at 10 °C) than in the folding intermediate (0.75 min$^{-1}$ at 10 °C).

The release of PMP during unfolding could not be followed by ESI-MS, because no protein-bound cofactor was detected during analysis of the native protein (data not shown). This was to be expected, since associations involving noncovalent interactions do not survive the conditions of the ESI-MS analysis. However, indirect fluorescence evidence (see below) suggests that PMP indeed dissociates very rapidly during unfolding so that the unfolding reactions incubated for 10 s contain exclusively unfolded protein free of PMP.

Refolding Followed by Monitoring Intrinsic Fluorescence—To investigate further the effect of bound coenzyme on the folding process, we analyzed the refolding kinetics of mAAT unfolded in 6 M GdnHCl for different periods of time by monitoring the changes in intrinsic fluorescence at 338 nm after excitation at 280 nm. Due to the well known dramatic quenching of intrinsic fluorescence caused by binding of PLP (or PMP) to the apoenzyme (45, 46), the fluorescence intensity of native holo-enzyme is lower than that of native apoenzyme (Fig. 6A, inset). Unfolding of the apoenzyme in 6 M GdnHCl results in a red shift of the emission maximum from 338 to 350 nm and in a decrease in the fluorescence intensity due to an increase in the accessibility of aromatic residues to the aqueous solvent (47). The increase in fluorescence caused by the relief of the quenching of PLP produces an unfolded state with fluorescence levels intermediate between those of the native holo- and apoenzyme.

As expected from the fluorescence properties of native and unfolded holoenzyme, a monophasic decrease in fluorescence was observed during refolding of PLP-mAAT samples unfolded for 90 min (Fig. 6A). The first order rate constant of this decay (0.036 min$^{-1}$) is close to the rate constant of the slower reactivation step. Analysis of the refolding reaction by fluorescence stopped-flow reveals the existence of a very rapid fluorescence increase, which is completed during the dead time of the instrument (mixing artifacts usually persist for about 15 ms under the experimental conditions used) (Fig. 7). This is followed by a slower fluorescence enhancement ($k = 0.6 \text{ s}^{-1}$ for the major component of the biphasic increase) and a very slow decay to the fluorescence levels of the native protein (Fig. 7, inset). The amplitude of the initial burst phase represents about 80% of the total increase in fluorescence. The fluorescence enhancement stages were completed during the first 5 s of the refolding reaction and therefore could not be detected during manual mixing experiments (dead time, 10–15 s), where only the slow decay in fluorescence mentioned above was observed (Fig. 6). Refolding of the apoenzyme in the absence of coenzyme includes the two fluorescence enhancement steps plus a single decay with a rate constant of 0.26 min$^{-1}$ (data not shown), similar to that of the faster reactivation step. Probably because of its small amplitude (only about 10% of the decrease in fluorescence observed with the holoenzyme), this phase is usually not detected when following refolding of the holoenzyme by either stopped-flow or manual mixing methods. The slow fluorescence decay observed during refolding in the presence of PLP has been ascribed to fluorescence quenching of the refolding protein upon binding of PLP (27, 28). Together, these results indicate that refolding can proceed in the absence of PLP and that refolding of PLP-free unfolded mAAT involves the rapid formation of an intermediate with higher fluorescence than the native apoenzyme.

By contrast, no high fluorescence intermediate appears during refolding of the PLP-mAAT holoenzyme unfolded for 40 s (Fig. 6A), and the refolding protein shows native-like fluorescence at the shortest time resolvable by manual mixing (about 15 s). This cannot be explained by incomplete unfolding of the sample, since the fluorescence changes associated with PLP-mAAT unfolding reach a plateau in less than 1 s after mixing with GdnHCl (Fig. 4). The amplitude of the observable decay in fluorescence increases with the time of unfolding with a rate constant (0.067 min$^{-1}$) comparable with that estimated for the release of PLP from the unfolded protein (0.087 min$^{-1}$). As shown in the inset of Fig. 8, the two processes appear to be inversely correlated. The most likely explanation of these results is that the coenzyme bound to the unfolded chain quenches the fluorescence of the folding intermediates $I_1$ and $I_2$ as it does when binding to the active site of the fully folded protein; i.e. the reconstitution of holoenzyme in short term unfolded PLP-mAAT is a monomolecular process, whereas during folding of a PLP-free unfolded chain, the formation of holoenzyme is a bimolecular process in which free PLP binds to the already folded apoprotein (27). What is somewhat surprising is that the quenching effect induced by the attached PLP on the earliest folding intermediate detected during manual mixing is of a magnitude comparable with that expected when the coenzyme binds to the native protein. This quenching is thought to be due partially to an energy transfer between PLP and tryptophan residues in the protein and partially to a small conformational change induced by the binding of the coenzyme (48). This observation suggests that the intermediate $I_1$ has elements of native-like structure, at least in terms of the overall positioning of the coenzyme in its binding pocket relative to those aromatic residues whose fluorescence is most affected by the coenzyme. Analogous intermediate states generated in the absence of covalently attached coenzyme do not bind free coenzyme (see results with PMP-mAAT in Fig. 6B). A possible explanation for these findings is that PLP forces such a fold when attached to the chain undergoing the collapse transition. In order to explore this possibility, we are currently studying the structural properties of PLP-free and PLP-containing $I_1$ by H/D exchange and ESI-MS.

On the other hand, a slow fluorescence decay consistent with the formation of a high fluorescence intermediate is always
observed during refolding of the PMP-mAAT holoenzyme, regardless of the duration of its incubation with GdnHCl prior to the initiation of refolding (Fig. 6B). This confirms that short term unfolded PMP-mAAT does not contain protein-bound co-enzyme able to quench the fluorescence of the high fluorescence refolding intermediate. The fluorescence decay observed during refolding of long term unfolded PMP-mAAT is monophasic ($k = 0.03 \text{ min}^{-1}$), whereas refolding of samples unfolded for only 9 s

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**Fig. 6. Refolding kinetics of mAAT followed by monitoring changes in intrinsic fluorescence.** Refolding was initiated by manual mixing of aliquots of the proteins unfolded in 6 M GdnHCl for 40 s (2) or 90 min (1) (A, PLP-mAAT) or for 9 s (2) or 120 min (1) (B, PMP-mAAT) with refolding buffer at 10 °C as indicated in Fig. 1. The final protein concentration was 40 μg/ml. Fluorescence was measured at 338 nm with $\lambda_{ex} = 280$ nm. The traces in B have been offset slightly for easier visualization. The arrows indicate the approximate fluorescence level of the unfolded proteins. The solid lines represent best fit of the data to a single exponential (A and B, trace 1, rate constants $0.036 \pm 0.003$ and $0.03 \pm 0.002 \text{ min}^{-1}$ for PLP- and PMP-mAAT, respectively) or a double exponential decay (trace 2 in B, $k_{fast} = 0.7 \pm 0.1 \text{ min}^{-1}$ and $k_{slow} = 0.035 \pm 0.02 \text{ min}^{-1}$). The residuals for the fit are indicated in the lower panel of each trace. Inset, fluorescence emission spectra of native and unfolded (6 M GdnHCl) apo- or PLP-mAAT as indicated. The spectra were recorded using $\lambda_{ex} = 280$ nm and 60 μg/ml protein. AU, arbitrary units.
is clearly biphasic (Fig. 6B, rate constants 0.70 and 0.035 min$^{-1}$ for the fast and slow steps, respectively). The amplitude of the fast step represents 15% of the total decay in fluorescence. Both the rate and relative amplitudes of the fast and slow phases are in good agreement with the values estimated when monitoring recovery of enzyme activity (Fig. 5, 10 s unfolded protein, where about 17% of the population reactivate with a rate constant of 0.6 min$^{-1}$ and the remaining 83% with an observed rate constant of 0.02 min$^{-1}$). The rate of the decrease in the amplitude of the fast refolding phase detected by fluorescence with unfolding time was 6 $\times$ 1.5 min$^{-1}$, identical within experimental error to that estimated in reactivation experiments.

**DISCUSSION**

A kinetic model summarizing the results on the refolding of mAAT is presented in Fig. 9B. As observed in many other large multidomain proteins, refolding of mAAT proceeds through slow rate-limiting steps. Upon switching to refolding conditions, long term unfolded mAAT ($U_n$) folds rapidly into an intermediate (or population of intermediates) $I_{s1}$, in a process that may involve multiple steps that are not fully resolvable by stopped flow (notice in Fig. 7 that 80% of the increase in fluorescence occurs within the dead time of the experiment). $I_{s1}$ shows properties characteristic of a molten globule-like intermediate. As we reported earlier (27), $I_{s1}$ contains most of the secondary structure of the native protein, binds ANS, and shows stronger intrinsic fluorescence than either the unfolded or native states. Further transformation of this intermediate into the fully folded protein involves two consecutive first order rate-limiting steps ($I_{s1} \rightarrow I_{s2}$ and $I_{s2} \rightarrow N$ in Fig. 9B). Since the faster step (0.3 min$^{-1}$ at 10 °C) is associated with desorption of ANS (27), it could correspond to the packaging of the tertiary structure of the protein. The slower kinetic step (0.025 min$^{-1}$ at 10 °C) may represent a further conformational rearrangement toward a native-like state able to bind coenzyme and display catalytic activity (27). Dimerization is very fast and not rate-limiting under the experimental conditions used and probably involves the association of $I_{s2}$-type intermediates. For the sake of simplicity, it has not been included as a separate step in Fig. 9B.
The nature of these conformational rearrangements and the possible involvement of proline isomerization are unclear. Double-jump experiments were performed in order to address this issue, but refolding of short term unfolded mAAT is also complex. After a short 10-s pulse in GdnHCl, the population of unfolded molecules \( U_f \) is expected to contain only native backbone isomers. However, more than 50% of short term unfolded mAAT refolds slowly with kinetics similar to the long term unfolded protein. It appears that \( U_f \) goes quickly to a folding intermediate \( I_f \) which then either folds to the native state \( N \) in a single rate-limiting step (0.5 min\(^{-1}\)) or isomerizes to an intermediate identical or similar to \( I_{s1} \). The behavior of the fraction of short term unfolded molecules diverted to \( I_{s1} \) resembles that of the long term unfolded protein. Why is this partitioning taking place instead of all of \( I_f \) proceeding toward the native state? From the amplitude of the fast phase, which reflects the fraction of \( I_f \) molecules that go directly to \( N \) (about 40%), we estimated that the rate constant for the \( I_f \rightarrow I_{s1} \) isomerization should be about 0.75 min\(^{-1}\). Thus, because of the similar rates of the two processes, the \( I_f \rightarrow I_{s1} \) isomerization can effectively compete with folding of \( I_f \) to \( N \).

Fast and slow refolding steps in protein folding often are caused by heterogeneity in the population of unfolded molecules. In some cases, slow folding steps arise from kinetic partitioning early during refolding (49–51). In hen lysozyme, the origin of the kinetic heterogeneity does not appear to be associated with proline residues (52) but rather with the generation of species with different conformations early in the folding reaction. In human carbonic anhydrase II (50) and an antibody fragment (scFv) (51), the kinetic heterogeneity observed in the refolding of short term unfolded proteins results from cis-trans isomerization of proline peptide bonds to the wrong configuration in a folding intermediate. In the case of scFv, a “single chain” Fv fragment, isomerization of a proline residue in an early folding intermediate can compete with folding because of the formation of a kinetic trap in which the two domains associate prematurely (53, 54). The escape from this kinetic trap slows down the folding of short term unfolded protein relative to that of the isolated \( V_1 \) and \( V_2 \) domains.

An analogous situation may apply to the refolding of mAAT. Each subunit of dimeric mAAT also folds into distinct domains, with the active sites located at the interface between the small and large domains (23). The relatively slow reactivation kinetics of the fast refolding population of short term unfolded mAAT indicates that folding intermediates arise even in the absence of backbone isomerization constraints. The observed rate constants and activation energies of this fast track route toward the native state (\( I_f \rightarrow N \)) and the faster reactivation step of the slow folding population (\( I_{s1} \rightarrow I_{s2} \)) are very similar (about 0.5 and 0.3 min\(^{-1}\), respectively). These two steps may therefore involve a similar structural rearrangement. Since the direct refolding of \( I_f \) to \( N \) should not involve proline isomerization, it is likely that neither does the \( I_{s1} \) to \( I_{s2} \) step. The rate-determining step in these conversions may involve further folding of the individual domains or the docking of partially folded domains. Whether \( I_f \) and \( I_{s1} \) represent productive intermediates that direct the folding reaction or kinetic traps involving misfolded species with perhaps nonnative domain interactions is unknown at this point. The main difference between the fast and slow folding tracks resides in the presence in the latter of an additional slow step (\( I_{s2} \rightarrow N \) in Fig. 9B, \( k = 0.02 \) min\(^{-1}\)). This slow folding trajectory arises either directly from \( U_s \) through isomerization in the unfolded state (\( U_f \rightarrow U_s \)) or by isomerization within the \( I_i \) intermediates (\( I_f \rightarrow I_{s1} \)). Given that these isomerizations lead to the same product (\( I_{s1} \)) through two alternative pathways, they must involve analogous structural changes.

Our results do not provide direct evidence as to the nature of
these structural rearrangements. Since prolyl isomerase (human cyclophilin A) had no effect on the folding of short or long term unfolded mAAT, the role of proline isomerization can be neither directly proved nor discarded. The failure of cyclophilin to catalyze these reactions may be due to the inaccessibility of the enzyme to the isomerizing proline peptide bonds. Several pieces of evidence suggest that proline isomerization might be involved. First, during refolding of short term unfolded PMP-mAAT, when there is not bound coenzyme to prematurely quench the protein intrinsic fluorescence, Iₜ shows an enhanced fluorescence similar to that observed in Iₜ coming directly from Uₜ (see Fig. 6B). Moreover, preliminary results from H/D exchange studies indicate that Iₜ and Iₜ are loosely packed and share almost identical H/D exchange properties.² Thus, these two populations of intermediates seem to have very similar global conformations, and they may just differ in the configuration of one or more proline residues. Second, the rates of conversion between fast and slow folding species in the unfolded state (Uₜ → Uₜ, 1.8 min⁻¹ at 10 °C in the absence of coenzyme) or in folding intermediate states (Iₜ → Iₜ, 0.75 min⁻¹ at 10 °C) as well as their estimated activation energies (17–24 kcal/mol) are consistent with proline isomerization. Relaxation rates near 1 min⁻¹ have been reported for proline isomerization in unfolded ribonuclease A at 15 °C (55). In general, proline isomerizations in small peptides are characterized by relaxation rates in the order of 10⁻³ s⁻¹ at 4 °C (56) and activation energy values in the range of 16–20 kcal/mol (57, 58). However, it is difficult to conclude that proline isomerization is rate-limiting in protein-folding reaction steps solely on the basis of their apparent rate constants and activation energies. A number of local effects in partially folded polypeptides can either accelerate or slow down proline isomerization (see Ref. 56 and references therein), and activation energies as low as 5.7 kcal/mol have been estimated for the isomerization around the Pro⁷⁴ peptide bond in ribonuclease A (59).

Proline mutants have in many instances provided valuable information about the implications of these residues in slow folding reactions. However, analysis of alanine mutants of the two cis proline residues in E. coli AAT, which are conserved in all AATs, produced unexpected results. Whereas the reactivation of the P193A mutant, where the alanine bond adopts the trans configuration, refolds more slowly than wild type, the P195A mutant, which retains the cis configuration, refolds faster than wild type (31). As a consequence, a direct connection between cis-trans isomerization at these positions and refolding of E. coli AAT could not be proposed. It was concluded that although cis-trans isomerization at these positions might play some role, other types of conformational rearrangements might govern the folding process of this protein under strongly folding conditions.

In the absence of an unambiguous explanation at the molecular level of the nature of the kinetic heterogeneity observed in double-jump experiments, we can only speculate about possible interpretations of the effect of PLP on the distribution between Uₜ and Uₜ during unfolding. If the Uₜ → Uₜ conversion is governed by the isomerization of the Pro³⁸⁴ and/or Pro⁹⁵⁸ cis peptide bonds, the effect of PLP cannot be sequential because the two proline residues are far away from Lys²⁵⁸. However, folding into the native state brings Pro³⁸⁴ and Pro⁹⁵⁸ close to each other and near the coenzyme-binding site (Asn¹⁹⁴ and Trp⁴⁴⁰ are substrate binding sites and interact with the coenzyme) (23). Thus, it is possible that the presence of PLP may indirectly affect the isomerization rate of these proline residues by slowing down the loss of native interactions in the active site region, although we lack experimental evidence in support of these slow unfolding events. In our unfolding studies, we did not detect structural perturbations in the time range of the Uₜ → Uₜ equilibration, probably because they are confined to a small area of the protein and do not produce noticeable changes in the properties being observed (intrinsic fluorescence and H/D exchange of peptide amide groups). Local interactions in partially structured unfolded states have been shown to perturb the populations of native and nonnative isomers of proline peptide bonds (20, 60). Since the configuration of a proline residue in a native protein is thought to be determined by the three-dimensional structure, the transient persistence of local native interactions in the unfolding chain would favor the native cis configuration of these proline peptide bonds. Alternatively, assuming that Uₜ represents a population of incompletely unfolded molecules retaining local native interactions, the onset of the slow refolding process might be explained by the complete unfolding to the Uₜ state without the involvement of proline isomerization. The complete loss of transient structure would be much faster in the coenzyme-free apo or PMP forms of the polypeptide chain. Upon transfer to refolding conditions, the local interactions in the Uₜ unfolded state might lead to a collapsed intermediate able to recover its native state in a single rate-limiting step. Once the residual fluctuating structure is lost, additional or alternative nonnative interactions may occur either in the unfolded state or in the initial collapsed state populated upon transfer to folding conditions. The reorganization of this intermediate may become the slowest rate-limiting step of folding. With the data at hand, this scenario, although not very plausible, cannot be entirely ruled out.

In summary, double-jump experiments are in most cases a reliable test for the involvement of proline isomerization in rate-limiting steps of protein folding. However, as shown in the present work, in cofactor-dependent proteins, the release of cofactor during unfolding should not be ignored, since it may influence the rate of equilibration of unfolded species and, as a consequence, the dependence of the refolding kinetics on the duration of unfolding. Taken together, our results indicate that proline isomerization is most likely responsible, at least in part, for the slower rate-limiting step in the reactivation of long term unfolded mAAT, but probably it is not associated with the faster step. A detailed study of the refolding pathway of mAAT by H/D exchange and ESI-MS should provide much needed insight into the nature of the folding intermediates detected in the kinetic studies described here. These studies are in progress in our laboratory.

Acknowledgment—We thank John Bellin for the purification of mAAT and for technical assistance.

REFERENCES

1. Dobson, C. M., and Karplus, M. (1989) Curr. Opin. Struct. Biol. 9, 92–101
2. Mathews, C. R. (1993) Annu. Rev. Biochem. 62, 653–683
3. Kiehn, E. D., and Holland, J. J. (1970) Nature 236, 544–545
4. Dobson, C. M., Sali, A., and Karplus, M. (1998) Angew. Chem. Int. Ed. 37, 868–893
5. Kim, P. S., and Baldwin, R. L. (1992) Annu. Rev. Biochem. 51, 459–489
6. Wedemeyer, W. J., Welker, E., and Scheraga, H. A. (2002) Biochemistry 41, 14637–14644
7. Dalesio, P. M., and Rogson, I. J. (2000) Biochemistry 39, 860–871
8. Radford, S. E., Dobson, C. M., and Evans, P. A. (1992) Nature 358, 302–307
9. Mataga, A., Radford, S. E., and Dobson, C. M. (1997) J. Mol. Biol. 270, 1068–1074
10. Baldwin, R. L. (1995) J. Biol. Chem. 270, 103–109
11. Becker, H., and Colon, W. (1997) Curr. Opin. Struct. Biol. 7, 15–28
12. Goedken, E. R., Keck, J. L., Berger, J. M., and Marqusee, S. (2000) Protein Sci. 9, 1914–1921
13. Robinson, C. R., Lin, Y., Thomson, J. A., Sturtevant, J. M., and Shiga, S. G. (1997) Biochemistry 36, 16141–16146
14. Leckner, J., Bonander, N., Wittung-Stafshede, P., Malmstrom, B. G., and Karlsson, B. G. (1997) Biochim. Biophys. Acta 1342, 19–27

² J. A. Oses-Prieto, A. Artigues, A. Iriarte, and M. Martinez-Carrion, manuscript in preparation.
