Structural Studies on Folding Intermediates of Serine Hydroyxymethyltransferase Using Single Tryptophan Mutants*

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Previous studies showed that during the in vitro folding of *Escherichia coli* serine hydroxymethyltransferase at 4 °C, both monomer and dimer intermediates accumulated and were stable for periods of minutes to hours (Cai, K., Schirch, D., and Schirch, V. (1995) J. Biol. Chem. 270, 19294–19299). To obtain structural information on these intermediates, two of the three Trp residues in the protein were changed to Phe to generate a set of three single Trp mutant enzymes. These mutant enzymes were purified and characterized and shown to retain essentially all of the properties of the wild-type enzyme. The fluorescence and circular dichroism measurements of each mutant enzyme were studied under unfolding-refolding equilibrium conditions and during refolding. In addition, the sensitivity of the protein to digestion by subtilisin during refolding was investigated. The results of these studies show that the unfolded enzyme has two domains that rapidly fold to form a monomer in which the first 55 amino acids and a segment between residues 225 and 276 remain in a largely disordered form. This partially folded enzyme can form dimers and slowly undergoes a rate determining conformational change in which the unstructured segments assume their native state.

In a previous study we demonstrated that eSHMT could be reversibly refolded from 8 M urea after a 10-fold dilution at concentrations as high as 1 mg/ml of protein (2). At 30 °C refolding was essentially complete in 6 min. This study also provided evidence for several intermediates and a mechanism as shown in Equation 1. The most important information was obtained with refolding done at 4 °C, where distinct kinetic intermediates could be shown to exist for periods of several hours and complete refolding took as long as 20 h. U is the unfolded enzyme and rapidly folds in a few seconds to form a monomer M. This monomer forms dimers (labeled as D) during 20 min at 4 °C. The dimer D' does not bind PLP but undergoes a slow rate-determining conformational change to a dimer (apoD) that can bind PLP to form native holoD. At 30 °C the process of converting D' to apoD takes a few minutes, but at 4 °C it takes many hours.

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U \leftrightarrow M \leftrightarrow D' \leftrightarrow \text{apoD} \leftrightarrow \text{PLP} \leftrightarrow \text{native holoD (Eq. 1)}
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The purpose of this study was to obtain structural information about M, D', and apoD. Because these intermediates exist for minutes to hours at 4 °C, a variety of physical measurements can be made to aid in elucidation of their structure. Important information can be obtained on the structure of intermediates by monitoring the fluorescence properties of Trp residues. To simplify the interpretation of results with eSHMT, we made single Trp mutants by changing the remaining two Trp residues in this enzyme to Phe. We also used protease digestion to determine what regions of the protein in each intermediate had been folded into a protease-resistant form. The results provide evidence for the rapid formation of two domains with an NH2-terminal region and a central region of the amino acid sequence remaining largely in a random coil until the final rate-determining step.

EXPERIMENTAL PROCEDURES

Materials—Ultrapure urea, all coenzymes, amino acids, and buffers were obtained from Sigma. A Transformer® site-directed mutagenesis kit was purchased from Clontech (Palo Alto, CA). Restriction enzymes were from DuPont NEN. Site-directed Mutagenesis—All mutants were made by the unique site elimination method of Deng and Nickoloff (3). The oligonucleotides used to generate Trp to Phe mutants and to introduce silent mutations for new restriction sites are as follows: 5'-pGCCGAAACTGTTCCAGGC-CATGGAG-3' was used to change Trp93 to Phe and to introduce a Ncol site (the underlined bases are the positions of the mismatches); 5'-pCGT-GGTGACTCAGCGAAAATGGC-3' was used to change Trp385 to Phe and to introduce a NruI site; 5'-pGCCGAAAGACTGGCGCATGTCGTTGACG-3' was used to change Trp16 to Phe and to introduce a NruI site. Site-directed triple mutants were made by introducing two or three primers in one mutagenic experiment. Mutations were identified by unique restriction enzyme digestion and by sequence analysis of the structural gene.

Expression and Purification of Mutant eSHMTs—All mutant eSHMTs, as well as the wild-type enzyme, were expressed and purified from *E. coli* strain GS1993, which is glyA-, as described previously (4). The purity of each protein was >98% as judged by SDS-PAGE. Apo-eSHMT was prepared by adding l-cysteine and chromatographing on a phenyl-Sepharose column (2). The apoenzyme was stored at 0 °C for no more than 3 days before use.

Characterization of Mutant eSHMTs—ε280 nm for each holoenzyme was calculated from the amino acid composition by the method of Gill and von Hippel (5). The original molar absorption coefficient for wild-type eSHMT was determined by weighing a dried protein sample of known absorbance. This value was in close agreement with the predicted value determined by the method calculated from the amino acid composition.
composition and the value determined from the Bio-Rad protein kit using bovine serum albumin as a standard. The experimentally deter-
mined molar absorption coefficient values for the mutant proteins were obtained only by the Bio-Rad method.

$K_m$ and $V_{max}$ values were determined at 30 °C with serine and tetrahydroyalolate as substrates (1). Tetrahydroyalolate was used at 0.15 mM in each assay, which is six times its $K_m$ value, and serine was varied in concentration between 0.2 and 3.2 mM.

Thermograms for the denaturation of each protein were obtained with an MC-2 scanning calorimeter from Microcal, Inc. (Amherst, MA). Protein samples were dialyzed for 24 h against several changes of either 20 mM Tris-HCl, pH 7.5, 5 mM 2-mercaptoethanol, and 1 mM EDTA or the same buffer with 20 mM potassium phosphate replacing the Tris-HCl. Protein concentrations were near 3.0 mg/ml for all samples. Data analysis was performed using the software supplied with the instrument. The $T_m$ was the average value of three individual measurements for each sample.

Fluorescence and CD Measurements—Fluorescence spectra were taken on a Shimadzu 5000 fluorimeter with 5-nm slits for both excita-
tion and emission. A circulation bath was used to maintain the desired temperature. Fluorescence quenching experiments were performed by adding 10-μl aliquots of an 8 mM solution of acrylamide to 2 ml of the enzyme solution. Data were corrected for dilution and inner filter effects (6) as well as any change in fluorescence intensity of the sample during the measurement. Constants for collisional and static quenching effects (6) were obtained by linear extrapolation of the base lines for native and unfolded protein concentrations as the refolding samples and digested by the same procedure. All samples were concentrated 10-fold at low temperature in a speed-vac. 20 μl of SDS-PAGE sample buffer, which had been adjusted to pH 3 with HCl, was added to each concentrated protein. All samples were placed in a boiling water bath for 1 min and after cooling adjusted to neutral pH by the addition of 10 μl of 2 M NaOH. Samples were analyzed by SDS-PAGE on a 15% acrylamide gel (10).

Urea-Gradient Gel Electrophoresis—A 0–8 M linear urea gradient polyacrylamide gel was made from stock solutions of 50 mM Tris-HCl, pH 8.0, and 8 M urea in the same buffer (11). To compensate for the change in pore size of the polyacrylamide with increasing urea a gradient of acrylamide was made from 15 to 11%. Each form of eSHMT, 75 μg, in Tris buffer, pH 8.0, was layered on top of the gel in a 6-cm slot, and electrophoresis was performed at 300 V for 2 h at room temperature. The gel was then stained with Coomassie Blue R-250.

Amino Acid Sequence Determination—The amino-terminal and carboxyl-terminal sequences of proteolytic-resistant fragments were determined as follows. Protease-treated enzyme was analyzed by SDS-PAGE as described above and stained with Coomassie Blue R-250. After destaining, the protein bands were cut from the gel with a razor blade. The protein was eluted from the gel by electrophoresis. The protein was precipitated with acetonitrile in acetic acid solution, dissolved in 50 mM pH 6.0, MES, and analyzed against this buffer. After digestion the proteins were separated by HPLC on a C18 column using an aqueous gradient elution. Amino acids were identified by absorption at 278 nm, and aliquots were subjected to amino-terminal sequencing by cycles of conventional Edman degradation on a G1000 A HP sequencer. Each aliquot of eluted protein (about 0.2 mg) was incubated with 4 μg of carboxypeptidase Y, and aliquots were removed at 10, 30, 60, 90, and 180 min. After removing protein with trichloroacetic acid and centrifugation, the supernatant was analyzed for released amino acids on an amino acid analyzer.

RESULTS

Characterization of Mutant eSHMTs—Single Trp mutant eSHMTs were purified by site-directed mutagenesis and analyzed by DNA sequencing. The wild-type enzyme contains 3 Trp residues at positions 16, 183, and 385. The E. coli expression of each single Trp mutant proteins yield the wild-type enzyme positions at 16, 183, and 385, respectively. The Trp residue at position 385 was mutated to arginine. The apparent mol wt of each mutant protein was determined by SDS-PAGE and shown in Table 1. Each mutant protein was expressed and purified as described previously for wild-type eSHMT (1). The mutants enzymes were analyzed for kinetic constants, thermal stability, and absorption properties at 278 nm. These results are recorded in Table 1.

| Property                  | Wild type | Trp<sup>16</sup> | Trp<sup>183</sup> | Trp<sup>385</sup> | Trp<sup>?</sup> |
|---------------------------|-----------|------------------|------------------|------------------|------------------|
| Relative specific activity| 1.0       | 0.86             | 0.95             | 0.86             | 0.78             |
| $T_m$ (°C)                | 67.6      | 63.8             | 64.9             | 60.9             | nd               |
| $K_m$ (μM)                | 0.3       | 0.3              | 0.2              | 0.2              | 0.4              |
| $e_{SIFT - cal}$ (mM<sup>-1</sup> cm<sup>-1</sup>) | 43        | 32               | 32               | 32               | 26               |
| $e_{SIFT - exp}$ (mM<sup>-1</sup> cm<sup>-1</sup>) | 44        | 32               | 33               | 34               | 27               |

*a* Trp<sup>16</sup>, Trp<sup>183</sup>, and Trp<sup>385</sup> refer to eSHMT mutants that contain a single Trp residue at positions 16, 183, and 385, respectively. The mutant enzyme contains no Trp residues, nd, not determined.

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**TABLE I**

Characterization of Mutant eSHMTs with Wild-type eSHMT

| Property                  | Wild type | Trp<sup>16</sup> | Trp<sup>183</sup> | Trp<sup>385</sup> | Trp<sup>?</sup> |
|---------------------------|-----------|------------------|------------------|------------------|------------------|
| Relative specific activity| 1.0       | 0.86             | 0.95             | 0.86             | 0.78             |
| $T_m$ (°C)                | 67.6      | 63.8             | 64.9             | 60.9             | nd               |
| $K_m$ (μM)                | 0.3       | 0.3              | 0.2              | 0.2              | 0.4              |
| $e_{SIFT - cal}$ (mM<sup>-1</sup> cm<sup>-1</sup>) | 43        | 32               | 32               | 32               | 26               |
| $e_{SIFT - exp}$ (mM<sup>-1</sup> cm<sup>-1</sup>) | 44        | 32               | 33               | 34               | 27               |

*a* Trp<sup>16</sup>, Trp<sup>183</sup>, and Trp<sup>385</sup> refer to eSHMT mutants that contain a single Trp residue at positions 16, 183, and 385, respectively. The mutant enzyme contains no Trp residues, nd, not determined.
Trp16 and Trp385 enzymes, which exhibited relative specific activity values of 0.86. The properties of the mutant eSHMTs, as recorded in Table I, suggest that each mutant enzyme has only minor structural differences compared with the wild-type enzyme.

K_m values for serine were essentially identical for all of the single Trp mutants and wild-type enzymes (Table I). Each mutant enzyme exhibited considerable catalytic activity as well as the enzyme, which had all three Trp residues replaced by Phe. The single Trp mutants with the least activity were the Trp16 and Trp385 enzymes, which exhibited relative specific activity values of 0.86. The properties of the mutant eSHMTs, as recorded in Table I, suggest that each mutant enzyme has only minor structural differences compared with the wild-type enzyme.

The solvent exposure of the Trp residue in each single Trp mutant apoenzyme was estimated by analyzing its fluorescence spectra (7). The Trp fluorescence emission maxima at both 4 and 30 °C for the Trp16, Trp183, and Trp385 mutant apoenzymes were 338 nm, 325 nm, and 334 nm, respectively (Fig. 1). Evidence that the individual Trp residues in the mutant proteins are very close to their environment in the wild-type enzyme is suggested by the combined fluorescence spectra of the mutant enzymes. If the Trp residues in the mutant apoenzymes are in the same environment as they are in the native apoenzyme, then their combined emission spectra should be the same as the emission spectrum of the wild-type apoenzyme. As shown in Fig. 1, the sum of the fluorescence spectra for the three Trp mutant apoenzymes is about 5% lower than the fluorescence spectrum of the wild-type apoenzyme. Errors in calculating protein concentrations for the mutant enzymes could easily account for the 5% difference. The sum of the three emission spectra of the mutant apoenzymes has the same 333 nm wavelength maximum observed for the native enzyme (Fig. 1).

Equilibrium Refolding—The equilibrium fraction folded ratio (F_{eq}) was determined in increasing urea concentrations for mutant and wild-type apo-eSHMTs by both fluorescence and CD spectroscopy. Fig. 2A compares the CD signals of the apoenzymes. The urea concentration resulting in 50% folding is shifted from 2.5 M urea for the wild-type enzyme to about 2.3–2.4 M urea for the three mutant enzymes. The results suggest that with respect to secondary structure there is little difference in the unfolding pattern between the mutant and wild-type apoenzymes. Although each set of data was fit to a two-state model, the data points are not of sufficient accuracy to rule out a three-state model. These CD results are in agreement with the thermal denaturation studies that the mutant enzymes are slightly less stable than the wild-type enzyme.

Fig. 2B compares the fluorescence signals of Trp residues at equilibrium as a function of urea concentration. The data points were fit to either a two-state or a three-state model and are represented by the solid lines. A three-state model suggests that an intermediate (I) exists at a significant concentration between the unfolded state U and the folded state N as shown in Equation 4. The wild-type apoenzyme (solid circles) is fit by...
Trp mutant apoenzymes are very different (Fig. 2). The equilibrium fluorescence properties of the three single Trp mutant apoenzymes are very different from either the fully folded or the unfolded state. The equilibrium fluorescence properties of Trp16, Trp183, and Trp385 show a rapid change in fluorescence spectra to native values following a 10-fold dilution into refolding buffer at 30°C (Fig. 4, A and B, solid lines). However, the Trp16 apoenzyme showed a much slower return of the fluorescence signal, taking about 500 s to reach 90% of the native enzyme value (Fig. 4C). The rate of return of native-like fluorescence for the Trp16 enzyme was concentration-dependent increasing about 3-fold from 0.004 to 0.1 mg/ml enzyme. Above this concentration there was no fur-

| Enzyme | Curve fit | ΔG° | ΔH° |
|--------|-----------|-----|-----|
| Wild type | Three-state | 4.1 ± 0.6 kcal/mol | 4.4 ± 0.7 kcal/mol |
| Trp16 | Three-state | 4.2 ± 0.2 kcal/mol | 5.5 ± 1.5 kcal/mol |
| Trp385 | Two-state | 5.9 ± 0.4 kcal/mol | 6.0 ± 0.2 kcal/mol |

The equilibrium fluorescence properties of the three single Trp mutant apoenzymes are very different (Fig. 2B). Because these mutant apoproteins all have similar stabilities, the differences in Trp fluorescence with increasing urea concentration are not the result of differences in the stability of the intermediate (I) for each mutant apoenzyme but reflect the differences in the fluorescence properties of the individual Trp residue in the intermediate. Inspection of the curves suggests that most of the change in fluorescence for the Trp16 apoenzyme occurs in the N to I transformation, whereas for Trp385 apoenzyme virtually all of the change in fluorescence occurs in the I to U transition. This shows that in the intermediate Trp16 is nearly fully exposed and Trp385 is still fully buried. The Trp16 fluorescence change occurs in both reactions, suggesting that in the intermediate Trp16 experiences an environment that is different than in either the native state or the unfolded state. The equilibrium fluorescence curve for Trp16 apo-eSHMT did not fit either a two-state or three-state model. It is shown in Fig. 2B as a two-state fit only for visual effect.

Each refolding curve was determined over a 10-fold concentration range of enzyme. Only the Trp16 mutant apoenzyme showed any concentration dependence. A 25-fold increase in protein concentration shifted the midpoint of the urea unfolding curve from 1.1 to 1.6 M urea. This suggests that changes in the fluorescence of Trp16 apo-eSHMT may be associated with the formation of dimers in the equilibrium unfolding pathway.

The kinetic refolding of each mutant apoenzyme was first investigated at 30°C by a 10-fold dilution of apoenzyme, which had been unfolded in 8 M urea for at least 2 h, into buffer containing excess PLP. Active holoenzyme is formed quantitatively in about 6–10 min for each mutant enzyme at essentially the same rate previously observed for wild-type enzyme (data not shown). This suggests that changing two of the Trp residues to Phe has not significantly altered the kinetics of the refolding reaction.

As shown previously, the rate of refolding at 4°C is dramatically different than at 30°C. For the wild-type enzyme less than 10% of the catalytic activity returns within 100 min after initiation of refolding. Furthermore, the previous study showed that for wild-type apo-eSHMT refolded at 4°C, all of the secondary structure had returned within 2 min and that the enzyme had completely formed dimers after about 100 min (2). This dimer could not bind PLP and is listed as structure D' in Equation 1. When the single Trp mutant enzymes were tested for refolding at 4°C, the return of activity was the same slow rate as observed for the wild-type enzyme. Although we did not check for the rate of dimer formation, the results of urea gradient PAGE suggests that the mutations have not altered the unfolding and separation of dimers. We assume that each mutant enzyme forms dimers at close to the same rate as observed previously for the wild-type enzyme (2). The results suggest that the rate-controlling step in each single Trp mutant enzyme is the conversion of D' to apoD as shown in Equation 1.

The rates of change in fluorescence was investigated with each of the mutant apoenzymes at 30°C after initiation of refolding. Both the Trp183 and Trp385 mutant apo-eSHMTs showed a rapid change in fluorescence spectra to native values following a 10-fold dilution into refolding buffer at 30°C (Fig. 4, A and B, solid lines). However, the Trp16 apoenzyme showed a much slower return of the fluorescence signal, taking about 500 s to reach 90% of the native enzyme value (Fig. 4C). The rate of return of native-like fluorescence for the Trp16 enzyme was concentration-dependent increasing about 3-fold from 0.004 to 0.1 mg/ml enzyme. Above this concentration there was no fur-
ther increase in rate.

The fluorescence emission properties for each apo-eSHMT during refolding at 4 °C are shown as dashed lines in Fig. 4. For wild-type apo-eSHMT, refolding at 4 °C in the presence of PLP results in almost no active enzyme being formed in the 1000-s time period recorded in this figure. For the Trp385 and Trp183 mutant apoenzymes, the fluorescence signal returned to native values by 120 s (Fig. 4, A and B, dashed lines). However, the return of native fluorescence for the Trp16 mutant eSHMT took as long as 15 h to reach final native values. As shown in Fig. 4C about 50% of the fluorescence change has returned in 20 min after refolding. As observed at 30 °C, only Trp16 eSHMT showed any observable concentration dependence. The fluorescence emission properties of the wild-type eSHMT during refolding at 4 °C as shown in Fig. 4D is a composite of the properties of the mutant enzymes.

We had previously shown that in the refolding of wild-type apo-eSHMT the CD spectrum returned rapidly even at 4 °C (2). In this study, we determined the CD spectrum from 200 to 240 nm after initiation of refolding for each mutant apo-eSHMT at 4 °C. Within the 120 s it took to record the spectra, the CD signals were the same as for the native apoenzymes, confirming that for each mutant enzyme the secondary structure returns very rapidly (data not shown).

To further characterize the environments of the three Trp residues during refolding at 4 °C, the susceptibility of each mutant enzyme to acrylamide quenching was investigated (Fig. 5). The results are presented as the collisional quenching constant ($K_{SV}$) and the static quenching constant (V) as recorded in Table III. $K_{SV}$ is obtained from the initial slope of the quenching curve, and V is obtained by applying Equation 2 (7). The collisional quenching constant values show that each Trp is partially solvent-exposed with values ranging from 1.6 m$^{-1}$ for Trp183 eSHMT to 5.8 m$^{-1}$ for Trp16 eSHMT, suggesting that Trp183 is the most buried and Trp16 is the most solvent exposed of the three Trp residues. The collisional quenching constant values are in agreement with the values of the fluorescence emission maximum for each mutant enzyme as shown in Fig. 1. In general, the shorter the wavelength of maximum emission the more buried the Trp residue (Trp183, 325 nm; Trp385, 334 nm; and Trp16, 338 nm) with fully exposed Trp residues in the unfolded state having an emission wavelength of about 354 nm.

The quenching by acrylamide was used to follow each Trp residue during refolding at 4 °C to determine what the status of each Trp residue was during the formation of intermediates M, D', and apoD (Equation 1). Quenching by acrylamide was followed for each single Trp mutant enzyme as a function of time after initiation of refolding and compared with the quenching properties of the native mutant enzymes as shown in Fig. 5D. For the Trp183 apoenzyme, acrylamide quenching was native-like within the 5 min it took to determine a quenching curve (Fig. 5A and Table III). After 5 min the quenching of Trp385 apo-eSHMT was close to native values, but both the values of $K_{SV}$ and V were larger than the native enzyme. By 5 min the CD spectra of these proteins show that all of the secondary structure has returned and the fluorescence emission of both Trp183 and Trp385 have returned. Previous studies using size exclusion chromatography show that the enzyme is mostly the monomer (M) after 5 min of refolding. These results suggest that both Trp183 and Trp385 are close to their native environments. However, Trp16 is far from its native value, as indicated by the large value of 0.93 for the static quenching constant (V) (Table III). This shows that Trp16 is largely solvent exposed in structure M. After 2 h of refolding the enzyme will be mostly D' with some apoD. Both Trp183 and Trp385 apoenzymes have values for the quenching constant that are the same as the native enzyme. Trp16 apo-eSHMT may still be slightly more solvent exposed than the native enzyme.

Protase Digestion—Structural information on the intermediates trapped at 4 °C during refolding was also obtained by
observing if domains had become protease-resistant. A variety of proteases were used to digest the refolding enzyme at 4°C. The best results were obtained with subtilisin, which did not digest the native enzyme with a digestion period of 2 min at either 4 or 30°C. A slightly smaller band representing about 5% of the protein was seen in the native enzyme both with and without subtilisin during SDS-PAGE (Fig. 6, lanes 8 and 9). We assume this was due to a proteolytically nicked form of the enzyme. When the unfolded enzyme was added to refolding buffer containing subtilisin, no native enzyme was observed on analysis by SDS-PAGE. Three clear bands of smaller size were observed, however (Fig. 6, lane 2). A doublet was seen at about 23 kDa, and two other bands were observed at 20 and 17 kDa. If the protease was added 0.17 min (10 s) after initiation of refolding, the 20-kDa band disappeared, but the 23-kDa doublet increased in intensity (Fig. 6, lane 3). Still no native enzyme was observed. The appearance of native enzyme did not appear until 17 min (Fig. 6, lane 5) after initiation of refolding and even at 60 min only a small fraction of the enzyme existed with native-like resistance to protease digestion. The major bands at 23 and 17 kDa were eluted from the gel and analyzed for their amino-terminal and carboxyl-terminal sequences. The 17-kDa band showed an NH₂-terminus sequence of KEAME-, which is consistent with residues 277–281. Carboxypeptidase Y digestion released Ala, Tyr, and Val, consistent with the sequence of the carboxyl terminus of the enzyme. NH₂-terminal sequencing of the 23-kDa fragment was consistent with the sequence AEGYP-, which suggests protease cleavage at Tyr⁵⁵. Carboxypeptidase Y digestion was complex, releasing rapidly two equivalents of Val, two equivalents of Leu, and one equivalent each of Thr, Ile, and Tyr. These results suggest that subtilisin had cleaved the protein about equally in at least two positions. The major sites were Thr²²⁴ and Leu³⁴⁰. The origin of the single Tyr is not clear.

**DISCUSSION**

Several other researchers have cited the many advantages of using single Trp mutants to study the mechanism of protein folding (12–15). *E. coli* SHMT offers several unique advantages for using single Trp mutants. First, this enzyme has three Trp residues that are widely spaced in the sequence of the enzyme. There is no conservation of any of the Trp residues in a list of 14 different SHMT sequences from a variety of sources, suggesting that they do not play a critical catalytic role in this
enzyme (16). Second, eSHMT refolds rapidly at high protein concentration. Third, monomeric and dimeric intermediates accumulate at 4 °C for periods that allow physical and chemical probes to be used.

The single Trp mutant forms of the enzyme used in this study appear to fold by the same mechanism and to form essentially the same structure as previously observed for the wild-type enzyme. Both equilibrium and kinetic folding studies suggest that at 4 °C intermediates accumulate on the refolding pathway. Kinetic studies suggest that upon dilution of unfolded enzyme at 4 °C, two domains rapidly fold to structures characteristic of the native state. These are listed as domains 1 and 2 in Fig. 7. This rapid folding is supported by the fluorescence characteristic of the native state. These are listed as domains 1 and 2 in Fig. 7. This rapid folding is supported by the fluorescence studies that show that Trp^163, which is in domain 1, is rapidly buried within a few seconds and exhibits characteristics of fluorescence emission, CD spectrum, and acrylamide quenching of its environment in the native enzyme. Almost the same conclusions can be stated for Trp^185, which is a part of domain 2. Only the acrylamide quenching studies suggest that the enzyme does not completely reach its native environment by 5 min. Protease digestion studies show that these two domains have become resistant to digestion immediately upon dilution of the unfolded enzyme into refolding buffer, further supporting the view that two domains exist in a largely condensed state.

Kinetic studies show that Trp^16 is outside of domains 1 and 2 and appears not to be part of any ordered structure during the first stages of folding. It is removed by protease treatment and is solvent exposed as indicated by fluorescence emission and acrylamide quenching (Figs. 4 and 5). It appears to reach its native state only when the enzyme reaches its catalytically active form. The fact that the equilibrium refolding studies and the rate of burial, as determined by fluorescence emission of this residue in the Trp^16 eSHMT, both show some concentration dependence suggests that this residue is in a different environment in the dimers D' and apoD as compared with the monomer intermediate M (Equation 1). The observation that the CD signal of all mutant eSHMTs are fully formed after a few seconds and that the equilibrium refolding curves are nearly the same by this technique (Fig. 2) suggests that most of the secondary structure of eSHMT is present in domains 1 and 2.

Protease digestion studies also suggest that an amino acid segment between domains 1 and 2 is unordered, being accessible to protease digestion during the period when D' is the dominant intermediate. This segment includes the active site Lys^229, which binds PLP (Fig. 7). In our previous study we concluded that the rate-determining step in forming active eSHMT involved the formation of the active site that includes Lys^229 (2). The protease digestion studies confirm that this section of the protein remains in a nonnative state until the rate determining final isomerization of D' to apoD (Equation 1). Unfortunately there is no Trp residue in this segment of the molecule to monitor the environment during folding studies. However, we have used NaCNBH₃ to reduce the bound PLP of holo-eSHMT to form a stable secondary amine to Lys^229. This now places a fluorescent probe in this region of the protein. Preliminary studies show that the PLP is not buried in its native state until late in the folding process.

Equilibrium unfolding-refolding studies are in agreement with the model developed from the kinetic refolding studies as shown in Fig. 7. Equilibrium fluorescence studies of the wild-type enzyme containing all three Trp residues suggest that an intermediate structure I dominates the population of protein molecules at 2 M urea (Fig. 3). Equilibrium fluorescence studies of the single Trp mutants show that Trp^16 becomes disordered at a much lower urea concentration than the other two Trp mutants. This would suggest that the conversion of the native form N to the intermediate I may not represent a significant energy barrier. However, as shown in Table II, ω for the conversion of N to I is significant and in the same range as the conversion of the intermediate I to the unfolded form U that takes place at a much higher urea concentration. The relationship of ω to the concentration of urea is given by Equation 5 (17). For the conversion of the native apoenzyme N to I, the value of m is ~ 3.1 kcal-mol⁻¹-M⁻¹, and its value for the conversion of I to the unfolded form U is ~ 1.6 kcal-mol⁻¹-M⁻¹. When plots of ω versus [urea] are extrapolated to zero urea concentration to obtain ω, the two transitions give nearly the same values for ω (Table II).

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\Delta G = \Delta G^0 + m[\text{urea}] \quad \text{(Eq. 5)}
$$

However, it is clear that in I the amino-terminal segment is

![Fig. 6. SDS-PAGE of proteolytic digestion during the refolding of wild-type apo-eSHMT. Unfolded enzyme was diluted 10-fold into Tris buffer at 4 °C, and at various times aliquots were removed and incubated with subtilisin for 2 min at 4 °C. The digestion was stopped by the addition of phenylmethanesulfonyl fluoride. These digestions were then submitted to SDS-PAGE. Lane 1 is subtilisin. Lanes 2-6 are refolding solutions that were incubated from 0, 0.17, 1.7, 17, and 60 min, respectively, before incubation with subtilisin. Lane 7 is a sample that had been refolded for 60 min at 4 °C, and then the temperature was increased to 30 °C for 10 min before protease digestion. Lane 8 is native apoenzyme and subtilisin incubated at 4 °C for 2 min. Lane 9 is native enzyme without protease. Lane 10 shows the molecular mass markers.

![Fig. 7. Model showing the structure of an early intermediate in the folding of apo-eSHMT. Protease digestion experiments show that folding intermediates representing both monomer and dimer forms of apo-eSHMT are digested at Tyr^55, Thr^224, Leu^240, and Leu^276. A ~23-kDa domain is resistant to protease digestion. This domain contains Trp^183 (circle), which is the most buried of the three Trp residues. Also, a ~17-kDa domain at the carboxyl terminus of the protein containing Trp^163 is resistant to protease digestion. Trp^16 remains exposed to solvent during the early stages of refolding and appears to reach its native state only during the rate-determining step of forming native apoenzyme. Lys^229, which is the PLP binding site, also remains protease-sensitive until the final stages of forming native enzyme.](image-url)
largely disordered and Trp\textsuperscript{16} is solvent-exposed. It is not clear from the fluorescent studies if the residues between the two domains are also being exposed and disordered between 1 and 2 \textmu m urea. The urea gradient gel suggests that two distinct sizes of molecules do exist in the range of stability where 1 is formed (Fig. 3). The band at the left of the gel (0 \textmu m urea) represents the migration of the dimer apoD, and the band above 6 \textmu m urea represents the migration of the unfolded monomer (U). There is not much difference in the size of these two species. In the range where the intermediate 1 is stable (1.5–3.0 \textmu m urea), there is a transition between these two extremes. In this range of urea concentration, we observed for all apoenzyme forms a distinct band that migrated much more slowly than either native or unfolded enzyme (Fig. 3). We suggest that this could be an expanded dimer in which the NH\textsubscript{2}-terminal sequence and the section between the two domains have become disordered. The smear of protein between the upper and lower bands in the 2–4 \textmu m urea range of the gel suggests that there is equilibrium between species of different sizes during the 2 h it took to run the gel.

Protease digestion by subtilisin gives some evidence about the structure of D’. Protease digestion fails to distinguish between M and D’ as evidenced by the same pattern of forming 17- and 23-kDa fragments from the first few seconds of refolding until 1000 s after refolding. By 1000 s the apoenzyme is a dimer (2). These results suggest that the dimer D’ is greatly expanded, having disordered segments at the amino terminus and between domains 1 and 2. For most oligomeric proteins, forming dimers occurs only after the formation of nearly native monomers (18, 19). This does not seem to be the case with apo-eSHMT.

Subtilisin will probably not digest the full length of the amino acid residues between domains 1 and 2. Only the portion of the sequence that is readily accessible to the protease and meets its specificity requirements will be digested. It is likely that the unfolded segment starts at the sequence 214\textsuperscript{a}PnPVP and extends to -PEP\textsuperscript{284} (16). This portion of the amino acid sequence contains Lys\textsuperscript{229}, which binds PLP and 8 of the 20 Pro residues in eSHMT. The lack of native structure of this part of the molecule helps explain why D’ does not bind PLP (2). A highly conserved sequence in all SHMT enzymes lies next to the active site Lys\textsuperscript{229}. In almost all sequences there are 4 Thr and 2 Val residues between Val\textsuperscript{222} and Thr\textsuperscript{227} (16). This stretch of 6 \beta-branched amino acids would be predicted to exist in an unordered state. The rate determining step may involve the folding in of this stretch of amino acids to form the active site of the apoenzyme.

Several studies have suggested that eSHMT has the same fold as several other PLP enzymes, including aspartate aminotransferase (16, 20). One of these studies aligned the amino acid sequence of eSHMT with aspartate aminotransferase (16). The three-dimensional structure of aspartate aminotransferase has been solved and shown to consist of two domains. These two domains coincide with domains 1 and 2 of eSHMT as determined from the protease digestion studies. In mitochondrial aspartate aminotransferase the large domain starts at residue 47 and the small domain starts at residue 329. These are only a few residues different than the digestion sites found for our subtilisin digestion of eSHMT. It has been shown previously that the large domain of aspartate aminotransferase can be expressed and folded independently of the small domain (21). This large domain also bound PLP but did not form dimers and showed no catalytic activity. Our studies suggest that the small domain (domain 2 in Fig. 7) may also fold by itself.

REFERENCES
1. Schirch, V., Hopkins, S., Villar, E., and Angelaccio, S. (1985) J. Bacteriol. 163, 1–7
2. Cai, K., Schirch, D., and Schirch, V. (1995) J. Biol. Chem. 270, 19294–19299
3. Deng, W. P., and Nickoloff, J. A. (1992) Anal. Biochem. 200, 81–88
4. Delle Fratte, S., Iurescu, S., Angelaccio, S., Bossa, F., and Schirch, V. (1994) FEBS Lett. 325, 395–403
5. Gill, S. C., and von Hippel, P. H. (1989) Anal. Biochem. 182, 319–326
6. Parker, C. A. (1968) Photoluminescence of Solutions, pp. 220–222, Elsevier Science Publishing Co., Inc., New York
7. Eftink, M. R., and Ghiron, C. A. (1976) Biochemistry 15, 672–680
8. Cupo, J. F., and Pace, C. N. (1983) Biochemistry 22, 2654–2658
9. Matthews, C. R., and Crisanti, M. M. (1981) Biochemistry 20, 784–792
10. O’Farrell, P. H. (1975) J. Biol. Chem. 250, 4007–4021
11. Crichton, T. E. (1979) J. Mol. Biol. 129, 235–264
12. Royer, C. A., Mann, C. J., and Matthews, C. R. (1993) Protein Sci. 2, 1844–1852
13. Mann, C. J., Royer, C. A., and Matthews, C. R. (1993) Protein Sci. 2, 1853–1861
14. Mårtensson, L. G., Jonsson, B. H., Freskgård, P. O., Svensson, M., Carlsson, U., and Jonsson, B. H. (1995) Biochemistry 34, 1011–1021
15. Irace, G., Balestrieri, C., Parlati, G., Servillo, L., and Colonna, G. (1981) Biochemistry 20, 792–799
16. Pascarella, S., Schirch, V., and Bossa, F. (1993) FEBS Lett. 311, 145–149
17. Pace, C. N. (1975) Crit. Rev. Biochem. 3, 11–20
18. Price, N. C. (1994) in Mechanisms of Protein Folding (R. H. Pain, ed) pp. 160–193, IRL Press, New York
19. Zetina, C. R., and Goldberg, M. E. (1982) J. Mol. Biol. 157, 133–148
20. Alexander, F. W., Sandmeier, E., Mehta, P. K., and Christen, P. (1994) Eur. J. Biochem. 219, 953–960
21. Herold, M., Leistler, Hage, A., Lugger, K., and Kirschener, K. (1991) Biochemistry 30, 3612–3621