Structural Studies on Folding Intermediates of Serine Hydroxymethyltransferase Using Fluorescence Resonance Energy Transfer

Kang Cai and Verne Schirch‡

From the Department of Biochemistry and Molecular Biophysics, Virginia Commonwealth University, Richmond, Virginia 23298

Previous studies have demonstrated that the in vitro folding pathway of Escherichia coli serine hydroxymethyltransferase has both monomer and dimer intermediates that are stable for periods of minutes to hours at 4 °C (Cai, K., Schirch, D., and Schirch, V. (1995) J. Biol. Chem. 270, 19294–19299). Single Trp mutant enzymes were constructed and used in combination with other methods to show that on the folding pathway of this enzyme two domains rapidly fold to form a monomer in which the amino-terminal 55 amino acid residues and a segment around the active site region of Lys229 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 (Cai, K., and Schirch, V. (1996) J. Biol. Chem. 271, 2987–2994). To further assess the kinetics and structural details of the intermediates during folding, fluorescence energy transfer and fluorescence anisotropy measurements were made of the three Trp residues and pyridoxyl 5'-phosphate, attached covalently to the active site by reduction to a secondary amine by sodium cyanoborohydride. These studies confirmed that the basic kinetic folding pathway remained the same in the reduced enzyme as compared to the earlier studies with the apo enzyme. Both equilibrium and kinetic intermediates were identified and their structural characteristics determined. The results show that the active site Lys229-bound pyridoxyl 5'-phosphate remains more than 50 angstroms from any Trp residues until the final rate-determining conformational change when it approaches each Trp residue at the same rate. The environment of each Trp residue and the pyridoxyl phosphate in both an equilibrium folding intermediate and a kinetic folding intermediate are described.

Escherichia coli serine hydroxymethyltransferase (eSHMT)†

* This work was supported by Grant GM 28143 from the National Institutes of Health. 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.
‡To whom correspondence should be addressed. Tel.: 804-828-9482; Fax: 804-828-3093; E-mail: schirch@gems.vcu.edu.

†The abbreviations used are: eSHMT, E. coli serine hydroxymethyltransferase; PLP, pyridoxal 5'-phosphate; PyP-eSHMT, pyridoxyl 5'-phosphate attached to Lys229 of eSHMT; PAGE, polyacrylamide gel electrophoresis; Trp16, Trp183, and Trp385, mutant forms of eSHMT that contain a single Trp residue at positions 16, 183, and 385, respectively; Trpmut, mutant eSHMT that contains no Trp residues; In, a folding intermediate observed under equilibrium conditions; Iref, a folding intermediate observed in kinetic folding studies; FRET, fluorescence resonance energy transfer; RM, Förster distance.

(Received for publication, May 23, 1996, and in revised form, August 8, 1996)

Fluorescence studies during refolding of the three single Trp mutants clearly identified the changes in environment occurring for Trp16 in the amino-terminal segment, Trp183 in domain 1, and Trp385 in domain 2. The region between the two domains (residues 225–276) appeared to remain disordered in both intermediates M and D* as judged by susceptibility to protease digestion. However, the segment between the two domains has no fluorescent probe to monitor what is occurring during refolding.

In addition to these kinetic studies, the refolding of apo-eSHMT was also studied by equilibrium methods in increasing concentrations of urea. An intermediate was identified that is
intermediate (activesiteLyso) of the practical difficulty of applying this method to protein folding of this transfer depends on the inverse sixth power of the distance relationships in macromolecules (5–7). The efficiency of energy donors and acceptors has been widely used to determine mechanism had occurred. this reduced eSHMT to determine whether a change in folding procedure places a fluorescent probe in the region of the protein remains attached in the protein in the unfolded state. This structure of both an equilibrium intermediate (the PyP moiety and Trp residues in each intermediate. These measurements were used to monitor the rotational flexibility of fluorescence to determine distance relationships in each folding intermediate of PyP-eSHMT. Also, fluorescence anisotropy measurements were used to monitor the rotational flexibility of the PyP moiety and Trp residues in each intermediate. These two techniques, together with the single Trp mutants constructed previously, provide important information about the structure of both an equilibrium intermediate \( I_e \) and a kinetic intermediate \( I_k \) in the refolding pathway of eSHMT.

EXPERIMENTAL PROCEDURES

Materials—\( \gamma \)-Butyrobetaine-Lys was purchased from Advanced Chemtech (Louisville, KY). All other coenzymes, amino acids, buffers, and reagents were obtained from Sigma and were of the highest grade available.

Single Trp mutant eSHMTs were constructed by site-directed mutagenesis as described previously (4). Wild-type and single Trp mutant eSHMTs were overexpressed in \( E. \) coli and purified to near homogeneity (4). Thermograms for the denaturation of each protein were obtained with an MC-2 scanning calorimeter as described previously in 20 mM potassium Phosphate (potassium Ph) buffer, pH 7.3, containing 5 mM 2-mercaptoethanol (4).

Synthesis of PyP-eSHMT—Protein samples (1 mg/ml) were dialyzed in 20 mM potassium Ph, 5 mM 2-mercaptoethanol, pH 7.0, with 0.1 mM PLP. Each enzyme was then dialyzed against the potassium Ph buffer without PLP for 4 h to remove excess PLP. The reduction of the PLP aldime was achieved by dialyzing each protein in potassium Ph buffer with 0.1% NaCNBH₃ for 5 h. This was followed by exhaustive dialysis in 20 mM potassium Ph buffer overnight. Reduction of PLP was verified by observing the spectral shift of bound PLP from 422 to 335 nm and the loss of more than 95% of its catalytic activity. Treatment of the apo-enzyme by the same reducing conditions resulted in fully active eSHMT after removal of NaCNBH₃ and reconstitution with PLP. The spectroscopy of the reduced eSHMTs was determined to be 1 PyP in each subunit in all cases, using an \( \varepsilon_{335} = 8700 \text{ cm}^{-1} \text{ M}^{-1} \) to determine the concentration of PyP (10). Protein concentration was determined from its A₂₇₈nm as described previously (4).

e-Lys-PyP was synthesized by the following procedure. One ml of 2 mM \( \gamma \)-butyrobetaine-Lys was mixed with 1 ml of 1 mM PLP and 2 ml of 0.5% NaCNBH₃. The resulting solution was incubated at 23°C for 2 h. During this period, the 390 nm peak of PLP was completely shifted to 325 nm. The \( \gamma \)-butyrobetaine-Lys-PyP was then deblocked by incubation in 30% trifluoroacetic acid for 15 min. The resulting sample was neutralized by NaOH and chromatographed on a 1.5 × 100-cm Bio-Rad P2 column equilibrated with water. The fractions containing material having an absorbance maximum at 325 nm and fluorescence emission at 386 nm (excitation at 335 nm) were pooled and used as the free Lys-PyP control in fluorescence anisotropy studies.

Fluorescence Measurements—Fluorescence spectra were taken on a Shimadzu 5000 fluorimeter with 5-nm slits for both excitation and emission. The concentration of eSHMT was 0.15 mg/ml for all experiments unless noted otherwise. For kinetic studies, the initiation of refolding by manual mixing of samples took about 9 s. The scanning of spectra from 300 to 450 nm took 6 s. A circulation bath was used to maintain the desired temperature.

Fluorescence anisotropy measurements were performed on a SLM AB-2 Fluorescence Polarimeter at the Center of Fluorescence Spectroscopy, University of Maryland at Baltimore. Fluorescence lifetime measurements were also performed on a frequency-domain instrument in the above center as described previously (11). Briefly, a rhodamine 6G dye laser was used as the excitation source at 290 nm, and the modulated emission was detected with a Hamamatsu R1564U microchannel plate photomultiplier tube with a 340-nm interference filter and 10-nm bandwidth. The lifetimes of Trp residues were resolved by a computer program for multieponential intensity decay analysis developed by Dr. M. L. Johnson (University of Virginia, Charlottesville, VA).

Distance Calculations between Trp Residues and PyP—Since there is no available Förster distance (\( R_0 \)) for Trp and PyP, the \( R_0 \) was determined as follows. The deconvoluted absorbance spectrum of each single Trp mutant PyP-eSHMT between 300 and 400 nm was integrated with 2-nm intervals by a spreadsheet computer program (Cricket Graph III, Version 1.5.1) and converted to an acceptor extinction coefficient as a function of wavelength (\( \varepsilon_\lambda(A) \)). The donor fluorescence (\( F_\lambda(\lambda) \)) was obtained by the same integration method on the fluorescence emission spectrum of each single Trp mutant apo-eSHMT. The spectral overlap integral (\( J \)) of donor fluorescence and acceptor absorption was calculated by Equation 2 (6).

\[
J = \int \frac{F_\lambda(\lambda)\varepsilon_\lambda(A)}{\lambda^4} d\lambda \quad \text{(m}^{-1} \text{ cm}^{-1} \text{ nm}^3) \quad \text{(Equation 2)}
\]

Since \( J \) had units of \( \text{cm}^{-1} \text{ nm}^{-1} \), \( R_0 \) was determined for each mutant eSHMT by using Equation 3, where \( R_0 \) is the Förster critical distance (in Å) at which transfer efficiency (\( E \)) is 50% and \( n \) is the refractive index (\( n = 1.4 \) for water) (6).

\[
R_0 = (8.79 \times 10^{-10}) \left( \frac{\phi_D}{\lambda^2 \cdot \phi_A \cdot J \cdot n^{-1}} \right)^{1/6} \quad \text{(Equation 3)}
\]

Donor quantum yield in the absence of acceptor (\( \phi_D \)) was determined on single Trp mutant apo-eSHMTs based on a value of 0.14 for tryptophan at 25°C (12). A value of two-thirds was used for the orientation factor (\( \tilde{e} \)) by assuming that the Trp and PyP groups rotate in a shorter time relative to the excited state lifetime of the donor (7). In addition, the minimum and maximum values of \( \tilde{e} \) were calculated according to the method of Dale et al. (13).

The transfer efficiency (\( E \)) was calculated by donor fluorescence quantum yields of each single Trp mutant in the presence (\( \phi_D(\lambda) \)) and absence (\( \phi_D(\lambda) \)) of acceptor according to the method of Wu and Brand (6).

Single Trp mutant PyP-eSHMTs were used to obtain the values of \( \phi_D(\lambda) \)
and single Trp mutant apo-eSHMTs were used to obtain $\phi_w$. The apparent distance ($R$) between Trp and enzyme-bound PyP was then calculated for each single Trp mutant eSHMT (6).

**Equilibrium Unfolding-Refolding—**Twenty mM Tris-HCl, pH 7.5, 5 mM 2-mercaptoethanol, and 1 mM EDTA buffer was used throughout the unfolding-refolding experiments and will be referred to as the Tris buffer. Equilibrium unfolding-refolding was performed by adding a concentrated solution of either wild-type or mutant PyP-eSHMTs to a series of urea concentrations in the Tris buffer (4). These solutions were incubated for 5 h at 30 °C before analysis by fluorescence spectrometry. The fluorescence equilibrium unfolding-refolding data were converted to the apparent fraction of native protein ($F_{app}$) and plotted versus urea concentration, as defined by Equation 4.

$$F_{app} = \frac{Y_U - Y_{lab}(Y_U - Y_N)}{Y_N} \quad \text{(Eq. 4)}$$

$Y_{obs}$ is the observed value of the fluorescence signal at a defined wavelength and a particular urea concentration in the region where the protein is unfolding. The fluorescence values for $Y_{obs}$ (native PyP-eSHMT) and $Y_{obs}$ (unfolded PyP-eSHMT) were obtained by linear extrapolation of the base lines for native and unfolded protein into the region where the protein was unfolding (14, 15).

The equilibrium unfolding-refolding plots were fit to either a two-state or a three-state model. In a three-state model, an equilibrium intermediate ($I_1$) accumulates as shown in Equation 5. For a two-state model, only $U$ and $N$ are present. The equations used to fit each model are as described by Matthews and Crisanti (15).

$$U \Leftrightarrow I_1 \Leftrightarrow N \quad \text{(Eq. 5)}$$

From each curve-fit for the equilibrium unfolding-refolding studies, the value of $\Delta G^\circ$ for unfolding, extrapolated to zero urea concentration, was calculated using the relationships described in Matthews and Crisanti (15), Pace (16), and Nath and Uda gaonkar (17).

**Kinetic Refolding Properties of PyP-eSHMT—**Unfolding of eSHMT was performed by diluting 5-fold a concentrated enzyme solution (7.5 mg/ml) into 10 M urea. Refolding experiments were initiated by diluting the unfolded enzyme 10-fold into Tris buffer (3, 4). Native controls were made in 0.8 M urea with Tris buffer, and the final protein concentration was exactly the same as the samples used in these refolding experiments. The unfolded control was made in the same solution as the native control except that it contained 8 M urea. The unfolded and native controls were used to define the 0 and 100% fractions folded, respectively.

**Limited Proteolysis—**Subtilisin digestion of apo- and PyP-eSHMT during refolding was the same as described previously (4), except that the protease concentration was reduced to 0.5 mg/ml. Subtilisin was added to each refolding sample for 2 min at 4 °C after various periods of refolding. The digestion was stopped by the addition of phenylmethylsulfonyl fluoride before running on an SDS-PAGE gel.

**Urea Gradient and Native Gel Electrophoresis—**A 0–8 M linear urea polyacrylamide gel was made as described previously (4). Seventy-five $\mu$g of each form of eSHMT (3.3 mg/ml) in 20 mM potassium Pi, pH 7.3, were denatured by increasing temperature. Solid line, holo-eSHMT; dotted line, eSHMT-serine complex; dashed line, PyP-eSHMT.

**FIG. 1.** Thermograms obtained by differential scanning calorimetry of wild-type holo-eSHMT, holo-eSHMT-serine complex, and PyP-eSHMT. Solutions of the different forms of eSHMT (3.3 mg/ml) in 20 mM potassium Pi, pH 7.3, were denatured by increasing temperature. Solid line, holo-eSHMT; dotted line, eSHMT-serine complex; dashed line, PyP-eSHMT.

12.6 °C compared to the unreduced enzyme, resulting in an enzyme that is more thermally stable than the eSHMT-serine complex. This large increase in $T_m$ suggests that reduction of the internal aldimine may have resulted in a conformational change in the protein, which results in an altered folding pathway. Similar 12–13 °C increases in $T_m$ were observed for each of the single Trp mutant PyP-eSHMTs. Previously, Chaffotte and Goldberg had shown that the $\beta_I$ subunit of tryptophan synthase undergoes a conformational change upon reversion of the reduced eSHMT to pyruvate. The possibility that a conformational change results in an alternative folding pathway is addressed in the following experiments.

**Protease Digestion of Folding Intermediates—**Previous studies on the refolding of apo-eSHMT at 4 °C showed that subtilisin digested the enzyme into two large fragments during the initial stages of refolding. The 23-kDa domain covered residues 55–224, and the 17-kDa domain covered from residue 276 to the carboxyl terminus residue at 417 (a 20-kDa fragment was also present during digestion during the first 2 min of refolding). The native enzyme was completely resistant to subtilisin digestion under the conditions used during refolding. With apo-eSHMT, protease-resistant forms characteristic of native enzyme did not begin to appear until after 15 min. These protease digestion experiments were interpreted to show that the two domains of apo-eSHMT fold rapidly to protease resistant forms (4).

To test the refolding mechanism of the PyP-eSHMT, these studies with subtilisin were repeated. The protease digestion pattern, as analyzed by SDS-PAGE, is shown in Fig. 2. As observed before, if subtilisin is added to the initial refolding buffer and digestion allowed to proceed for 2 min, there are three bands that migrate at 23, 20, and 17 kDa, respectively (lane 1, apo-eSHMT; lane 2, PyP-eSHMT). Lanes 3, 4, 5, and 6 show the digestion pattern of PyP-eSHMT when subtilisin was added 0.25, 1.7, 17, and 60 min after initiation of refolding, respectively. Lane 7 shows the digestion pattern of PyP-eSHMT after refolding for 60 min at 4 °C and then for 10 min at 30 °C before addition of the protease. Lane 8 is the native PyP-eSHMT incubated with subtilisin for 2 min. Lane 9 is native PyP-eSHMT without subtilisin, and lane 10 is molecular mass standards.

The similarity between protein bands on SDS-PAGE following digestion with subtilisin of PyP-eSHMT and those previously observed for apo-eSHMT provides support for a similar mechanism of domain folding being retained in the reduced enzyme. One difference is that a trace of the PyP-eSHMT that is resistant to subtilisin digestion starts to appear during the 1.7–3.7 min digestion period, which is sooner than observed...
with the apoenzyme, suggesting that in the PyP-eSHMT, the rate-determining step may be slightly faster than with apo-eSHMT.

**Spectral Properties of PyP-eSHMTs**—Treatment of wild-type holo-eSHMT with NaCNBH\(_3\) results in the reduction of the internal aldimine bond between PLP and the \(\epsilon\)-amino group of Lys\(_{229}\) to form a stable secondary amine that does not break the internal aldimine bond between PLP and the eSHMT. The solution was then analyzed by SDS-PAGE. Lanes 1 and 2, apo-eSHMT and PyP-eSHMT, respectively, with subtilisin added during the first 2 min of refolding; lanes 3–6, digestion pattern of PyP-eSHMT when subtilisin was added 0.25, 1.7, 17, and 60 min after initiation of refolding; lane 7, digestion pattern for PyP-eSHMT after refolding for 60 min at 4 °C and then for 10 min at 30 °C before addition of the protease; lane 8, native PyP-eSHMT incubated with subtilisin for 2 min; lane 9, native PyP-eSHMT without subtilisin; lane 10, molecular mass standards.

**Absorption spectra of holo- and PyP-eSHMT and fluorescent emission spectrum of apo-eSHMT.** Solid line, absorption spectrum of a 0.2 mg/ml solution of holo-eSHMT in 20 mM potassium Pi, pH 7.3; dashed line, absorption spectrum of the same enzyme solution after reduction with NaCNBH\(_3\) (PyP-eSHMT). Inset, dotted line, absorption spectrum of a 0.2 mg/ml solution of PyP-eSHMT; solid line, fluorescence emission spectrum of apo-eSHMT excited at 290 nm.

**FIG. 3.** Absorption spectra of holo- and PyP-eSHMT and fluorescent emission spectrum of apo-eSHMT. Solid line, absorption spectrum of a 0.2 mg/ml solution of holo-eSHMT in 20 mM potassium Pi, pH 7.3; dashed line, absorption spectrum of the same enzyme solution after reduction with NaCNBH\(_3\) (PyP-eSHMT). Inset, dotted line, absorption spectrum of a 0.2 mg/ml solution of PyP-eSHMT; solid line, fluorescence emission spectrum of apo-eSHMT excited at 290 nm.

**FIG. 2.** SDS-PAGE analysis of subtilisin digests during refolding of PyP-eSHMT. Aliquots of subtilisin were added at various times to refolding solutions of wild-type PyP-eSHMT at 4 °C and incubated for 2 min before stopping the reaction by the addition of an excess of phenylmethylsulfonyl fluoride. The solution was then analyzed by SDS-PAGE. Lanes 1 and 2, apo-eSHMT and PyP-eSHMT, respectively, with subtilisin added during the first 2 min of refolding; lanes 3–6, digestion pattern of PyP-eSHMT when subtilisin was added 0.25, 1.7, 17, and 60 min after initiation of refolding; lane 7, digestion pattern for PyP-eSHMT after refolding for 60 min at 4 °C and then for 10 min at 30 °C before addition of the protease; lane 8, native PyP-eSHMT incubated with subtilisin for 2 min; lane 9, native PyP-eSHMT without subtilisin; lane 10, molecular mass standards.

**FIG. 4.** Fluorescence emission spectra of the native and unfolded wild-type and single Trp mutant forms of PyP-eSHMT. Each panel records the fluorescence emission spectrum of the unfolded PyP-eSHMTs (dotted line) and the native PyP-eSHMTs (solid line). Enzyme concentrations were all 0.15 mg/ml in Tris buffer, pH 7.5. The number, location, and approximate distances (with respect to PyP) of the Trp residues are shown in the middle three panels for the single Trp forms of eSHMT. The left panel shows the results for the wild-type PyP-eSHMT, and the far right panel shows the Trp\(^{-}\) mutant eSHMT, which contains no Trp residues. All excitations were at 290 nm.

**TABLE I**

| Trp \(^{16}\) | Trp \(^{343}\) | Trp \(^{385}\) |
|---|---|---|
| \(E\) (\%) | 0.30 | 0.89 | 0.13 |
| \(R_{\text{min}}\) (Å) | 19.0 | 19.5 | 19.2 |
| \(R_{\text{2/3}}\) (Å) | 23.9 | 24.2 | 24.0 |
| \(R_{\text{max}}\) (Å) | 30.1 | 30.4 | 30.2 |
| \(R_{\text{0}}\) (Å) | 21.9 | 13.8 | 26.4 |
| \(R_{\text{0}}\) (Å) | 27.5 | 17.1 | 32.9 |
| \(R_{\text{0}}\) (Å) | 34.7 | 21.4 | 41.5 |

**Energy transfer between Lys\(^{229}\)-bound PyP and individual Trp residues in PyP-eSHMT**
eSHMT migrated faster than the homodimer of wild-type apo-eSHMT because of the extra negative charge on the PLP phosphate group. In the refolded mixture of apo-eSHMT and PyP-eSHMT, a band that ran intermediate to the two homodimers was observed in native gel electrophoresis, showing that hybrid dimers had formed (data not shown). No FRET was observed in the solution containing hybrid dimers. This suggests that all FRET observed in the samples recorded in Fig. 4 occurs between the Trp and PyP residues in the same subunit.

The distance between each Trp residue and the active site PyP can be determined from both fluorescent lifetime measurements of Trp residues and by donor quenching (5–8). The fluorescent lifetimes for each single Trp mutant eSHMT were determined in the apoenzyme form (4). The fluorescence by the bound PyP, making a distance measurement difficult. Therefore, this method was not used for this mutant. Table I records the distance calculations only by the donor quenching method. The results show that Trp183 PyP-eSHMT is the closest to the active site PyP and Trp385 is the most distant. These distances (RQDD) have an error due to the lack of knowledge about the orientations of Trp and PyP in the native enzymes. The results of anisotropy measurement were used to calculate the maximum and minimum distances between donor (Trp) and receptor (PyP) to account for this uncertainty of the orientation factor (13). Table I records the maximum and minimum distance between each Trp residue and the PyP. However, the accuracy of the absolute distance is not so important in this study.

**Equilibrium Unfolding-Refolding Studies**—Previous studies monitored the environment of each Trp residue for the apo-eSHMTs under equilibrium unfolding-refolding conditions (4). It was observed that Trp16 underwent local unfolding at a lower concentration of urea than either Trp183 or Trp385, which unfolded at similar urea concentrations (shown as dotted lines in Fig. 5). Evidence was obtained for a three state model (Equation 5) for both Trp16 and wild-type apo-eSHMTs, in which the intermediate Ie was the dominant species at 2.1 M urea (4).

This intermediate, Trp16 was largely solvent-exposed, whereas Trp183 and Trp385 were still mostly buried. The transitions for wild-type apo-eSHMT of N to Ie and Ie to U had similar ΔG° values of 4.1 and 4.4 kcal/mol, respectively (4). Trp183 and Trp385 eSHMTs had equilibrium profiles that were fit by a two-state model since both of these Trp residues had similar fluorescent properties in the intermediate and the native states. A urea gradient gel of apo-eSHMT showed that the unfolded enzyme in 8 M urea migrated more slowly than the native dimer. Between 1.5 and 3 M urea, a second band that moved considerably more slowly was observed where Ie was most stable. The urea gradient gel and size exclusion chromatography results were interpreted to show that in Ie, the enzyme was still a partially unfolded dimer with an increased Stokes radius. No evidence for a compact monomeric form of apo-eSHMT could be seen.

In this study, both the equilibrium experiments and the urea gradient gel were repeated with the PyP-eSHMTs. Both the environment of each Trp residue and its proximity to the PyP group can be monitored by observing the fluorescence emission at 335 (Trp) and 380 (PyP) nm, respectively. As shown in Fig. 5, Trp16 PyP-eSHMT undergoes equilibrium unfolding-refolding with the changes in fluorescence at 335 and 380 nm parallel each other and a midpoint in the 3–3.2 M range of urea. This is compared to a midpoint at about 1.6 M for the Trp16 apoenzyme (dotted line, Fig. 5) (4). The transitions for the fluorescence changes at both 335 and 380 nm fit a two-state model as described by the solid lines. The values for ΔG° calculated from the two-state fit for the 335 and 380 nm curves gave 3.3 and 3.7 kcal/mol for unfolding, respectively (Table II). Likewise, the fluorescence changes at 335 and 380 nm for Trp385 PyP-eSHMT parallel each other and fit a two-state transition model with a midpoint at 3.5 M urea. The ΔG° values are slightly higher than for Trp16 PyP-eSHMT, with values of 4.2 and 4.0 kcal/mol for the transitions monitored at 335 and 380 nm, respectively (Table II). These results suggest that in PyP-eSHMT, Trp16 and Trp385 become solvent-exposed (fluorescence at 335 nm) under the same conditions as they are...
separated by distance from the active site PyP (fluorescence at 380 nm).

The same equilibrium experiments with wild-type and Trp183 PyP-eSHMTs gave different results. First, for Trp183 PyP-eSHMT, the reduced PyP quenches the fluorescence of Trp183, resulting in no increase of fluorescence in the unfolded state compared to the native state at its native fluorescence maximum of 323 nm (Fig. 4). Even at 335 nm, the difference in fluorescence emission between the unfolded and native state is small, with the unfolded state having the greater value. This small change in signal between the unfolded and native states makes it difficult to calculate the fraction folded from the fluorescence at 335 nm. Even at 335 nm, the unfolded state has a slightly greater fluorescence at 335 nm than the native enzyme. This is the result of two opposing factors. Unfolding results in a decrease in the Trp fluorescence as the buried Trp residue is exposed to a hydrophilic solvent. In opposition, unfolding increases fluorescence by decreasing its quenching as the PyP group becomes more distant. There is essentially no change in either the fluorescence quenching of Trp183 or exposure to solvent during the transition of N to Ie (from 0 to 4.5 M urea). Both the decrease in quenching and increased exposure to a hydrophilic environment occur with the same magnitude during the Ie to U transition at 323 nm (4.5–8 M urea) (data not shown).

The equilibrium unfolding-refolding curve, monitored by both FRET at 380 nm and Trp fluorescence at 335 nm (Fig. 5), was performed between 0.6 and 6 M enzyme. No concentration effect was observed in these curves for any of the mutant enzymes or the wild-type enzyme (data not shown). As shown by a large increase in mobility of PyP-eSHMT in a urea gradient gel (upper panel in Fig. 6), it is likely that a monomer-dimer equilibrium exists in the 3–5 M urea range. The fact that no concentration effect was observed in either Trp fluorescence or FRET in the equilibrium unfolding studies suggests that FRET is the same in both the monomer and dimer forms of the enzyme. If some FRET had occurred between subunits, then one should observe increased FRET with increasing enzyme concentration. This is further support for the conclusion that all FRET occurs between the three Trp residues and PyP in the same subunit.

The urea gradient gel for PyP-eSHMT (Fig. 6, upper panel) is very different from the one previously shown for apo-eSHMT (4). In Fig. 6, the gel shows that in the region where N is starting to form Ie, the enzyme migrates significantly faster than the native state. This suggests that the enzyme has dissociated into a monomer but is still compact. As the amino acid sequence of the mutants is the same, it is unlikely that the difference is attributable to changes in the amino acid sequence. Instead, it could be that the mutant enzymes adopt a different conformation and are more compact at low urea concentrations. This would result in a decrease in the mobility of the enzyme and a faster migration in the gel. The fact that the wild-type enzyme does not migrate as fast as the mutant enzymes could be due to differences in the secondary or tertiary structure of the enzymes. It is possible that the wild-type enzyme adopts a more extended conformation, which results in a slower migration in the gel.

Using the equilibrium unfolding pattern of fluorescence at 380 nm for Trp183 PyP-eSHMT, a graph of how the relative concentration of N, Ie, and U vary with urea concentration was constructed (Fig. 6). The results show that at 5.5 M urea Ie is almost the only species present. In Ie, both Trp165 and Trp285 are solvent-exposed and separated by more than 50 Å from the PyP, but domain 1 containing Trp165 remains folded and in close contact with the Lys229-PyP group. The closeness of Trp183 and the PyP in Ie is also supported by the changes in fluorescence at 335 nm for Trp183 PyP-eSHMT with increasing urea. As shown in Fig. 4 for Trp183, the unfolded state has slightly greater fluorescence at 335 nm than the native enzyme. This is the result of two opposing factors. Unfolding results in a decrease in the Trp fluorescence as the buried Trp residue is exposed to a hydrophilic solvent. In opposition, unfolding increases fluorescence by decreasing its quenching as the PyP group becomes more distant. There is essentially no change in either the fluorescence quenching of Trp183 or exposure to solvent during the transition of N to Ie (from 0 to 4.5 M urea). Both the decrease in quenching and increased exposure to a hydrophilic environment occur with the same magnitude during the Ie to U transition at 323 nm (4.5–8 M urea) (data not shown).
The rate of change in Trp fluorescence was monitored by FRET. The excitation was at 335 nm, and emission was monitored at 380 nm. The anisotropy of the PyP of wild-type PyP-eSHMT was monitored every 30 s during refolding. The arrows are the values as defined in the figure. The excitation was at 335 nm, and emission was monitored at 380 nm. The solid line is a fit to the data to two parallel first order reactions with rate constants of 0.0024 and 0.017 s⁻¹. They had relative amplitudes of 0.064 and 0.050, respectively.

A different pattern was observed for Trp16 PyP-eSHMT. At 4°C, there was a rapid blue shift after initiation of refolding but not a large increase in fluorescence (data not shown). The fluorescence intensity returned more slowly and did not reach 50% of its native value until about 1600 s. This was also observed in studies on the rate of refolding of Trp16 apo-eSHMT (4). These results show that the amino-terminal residues are not in their native environment in the first stable kinetic intermediate. A model of this first kinetic intermediate is referred to as Iₖ in Scheme 1.

The measurement of FRET at 380 nm during refolding was repeated for each single Trp mutant and wild-type PyP-eSHMT at 30°C. The appearance of FRET at 380 nm is plotted versus time in Fig. 8, as is the decrease in fluorescence for Trp183 PyP-eSHMT at 323 nm (quenching). Each curve is fit by a single exponential rate (solid line), and the first order rate constants are given on each curve. The results show that all three Trp residues approach the active site PyP at the same rate. The first order nature of each curve suggests that there is a single structure for the kinetic intermediate (Iₖ) that goes to the native state (as defined by FRET) by a single first order process. This two-state conversion of Iₖ to the native state is also supported by the isosbestic point at 355 nm, as recorded in the spectra in Fig. 7.

The rate of appearance of FRET at 380 nm was studied with Trp183 PyP-eSHMT at temperatures between 4 and 30°C. The In of the first order rate constant is plotted against the reciprocal temperature (Arrhenius plot) in degrees Kelvin in Fig. 9. The slope of the straight line was used to calculate a ΔH of 22.5 kcal/mol. At 25°C, TₐS is 2 kcal/mol.

Fluorescence Anisotropy of Trp Residues and PyP during Refolding—The kinetic studies on the rate of appearance of Trp fluorescence at 335 nm suggests that a kinetic intermediate (Iₖ)
is formed in which both Trp$^{183}$ and Trp$^{385}$ are buried in a hydrophobic environment. Trp$^{16}$ is also in a relatively hydrophobic environment, but the intensity suggests that it is not in its native state. The results of kinetic refolding monitored by FRET (Figs. 7 and 8) also show that all three Trp residues are more than 50 Å from the PyP attached to Lys$^{229}$ after the initial hydrophobic collapse. This suggests that the peptide chain between domains 1 and 2 that contains Lys$^{229}$-PyP may still be free and disordered in solvent. To determine the nature of the environment of the PyP in I$_k$, we measured its anisotropy every 30 s during refolding at 4 °C (Fig. 10). The anisotropy of the PyP in the wild-type PyP-eSHMT in 8 M urea is 0.351, and in the unfolded state in 8 M urea it is 0.064. This change in values between the native and unfolded states represents the change in rotational freedom of the Lys$^{229}$-PyP and not a solvent effect since free Lys-PyP has an anisotropy value of 0.031 and 0.036 in 0.8 and 8 M urea, respectively. The anisotropy results show that after initiating refolding by the 10-fold dilution of unfolded enzyme in 8 M urea, about 70% of the difference in unfolded and folded state anisotropy has been regained in the 15 s it takes to record the first value (Fig. 10). During the next 1000 s, more than 95% of the anisotropy of the native state has been regained. The regain of the last 30% of anisotropy is not first order or second order, but is best fit by the equation for two parallel first order reactions (solid line) with rate constants of 0.017 and 0.0024 s$^{-1}$ and relative amplitudes of 0.050 and 0.064, respectively. This suggests that there are at least two forms of the enzyme involved in this kinetic process.

During the initial few min after initiating folding, little FRET has occurred (Fig. 7), but more than 70% of the native anisotropy of PyP has been regained (Fig. 10). These results show that during the formation of the kinetic intermediate I$_k$, the PyP attached to Lys$^{229}$ has become partially immobilized, but is still not in its native environment, since the PyP remains distant from any of the Trp residues.

Fluorescence anisotropy was also determined for each Trp residue in the single Trp apo-eSHMTs and single Trp PyP-eSHMTs. The anisotropy values changed from 0.03–0.04 in the unfolded enzymes to 0.11–0.14 in their native states. For each Trp, including Trp$^{16}$, anisotropy had returned to the native state value within 30 s after initiation of refolding (data not shown).

**DISCUSSION**

**Kinetic Properties of Refolding of PyP-eSHMT**—A model of a kinetic folding intermediate M (Equation 1) from previous studies on apo-eSHMT (3, 4) is shown in Scheme 1. This model showed that domains 1 and 2 fold in less than 10 s at 4 °C, but the amino-terminal 55 residues and residues between 225 and 276 remain disordered and sensitive to digestion with subtilisin. The previous studies also suggested that M could form a dimer (D') that cannot bind PLP. Because there was no fluorescent probe in the section between residues 225 and 276, the only available evidence as to its structure in M and D' was its sensitivity to digestion with subtilisin. This segment of the amino acid sequence is important because it contains not only the active site Lys$^{229}$, which binds PLP, but several other residues that have been shown to be involved in catalysis (20, 21). Reducing the enzyme-bound PLP with NaCNBH$_3$ forms a stable secondary amine that absorbs at 335 nm. This mild procedure now places a fluorescent probe in the critical active site region and serves as a fluorescent acceptor to receive energy transferred from Trp residues in FRET experiments. Unexpectedly, the reduction of the PLP aldimine resulted in a significant increase in stability of the enzyme, suggesting that the PyP-eSHMT may have a different conformation. A similar conclusion was reached with the β$_s$ subunit of E. coli tryptophan synthase, where the reduction of the coenzyme resulted in a significant decrease in immunoreactivity (19).

Reduction of the coenzyme did not significantly change the basic folding mechanism as determined by the kinetic properties during refolding of PyP-eSHMT. The protease digestion pattern remains the same (Fig. 2). Trp$^{16}$ and Trp$^{385}$ are rapidly buried in a hydrophobic environment, and Trp$^{183}$ does not reach its native state of fluorescence until the final step in the folding pathway. After the first few seconds of refolding, the intermediate I$_k$ of PyP-eSHMT has the same structural properties as the kinetic intermediate proposed for apo-eSHMT (Scheme 1) (4). The evidence supporting this view is summarized in Table III, where the kinetic properties (time to reach 50% of the value between unfolded and native state) were monitored by Trp fluorescence, FRET between Trp residues and Lys$^{229}$-bound PyP, anisotropy, and regain of the protease-resistant form during the refolding of PyP-eSHMTs.

This study thus adds to our understanding of the structure of M and D' (Equation 1). Fluorescence anisotropy measurements show that Trp$^{16}$ is partially immobilized but not completely buried in a native-like environment during the first few seconds of refolding. Also, the anisotropy studies showed that the PyP group attached to Lys$^{229}$ is mostly immobilized in the first few seconds and approaches the rotational freedom of the native state at 4 °C in a 20-min period. Fully folded enzyme is not present after this 20 min period as indicated by only a small amount of protease-resistant form of the enzyme (Fig. 2) and low FRET at 380 nm (Fig. 7).

Most importantly, the studies by FRET at 380 nm show that in M, and probably also in D', all three Trp residues are more than 50 Å from the Lys$^{229}$-PyP moiety. This provides direct evidence that the rate-determining step of folding brings a loop region into proximity of all three Trp residues, as found in the native state. In the previous study, we used size exclusion chromatography to determine the oligomeric state during refolding at 4 °C (3). A brief study was done with the PyP-
Serine Hydroxymethyltransferase

Intermediate (Le) and the PyP is still close to it. These results suggest that the active site region remains compact and native-like. An additional support for this view is that the quenching of Trp183 PyP-eSHMT, has increased from about 2.5 M urea for Trp183 apo-eSHMT to about 6 M urea for the PyP-eSHMT. The ΔG° for unfolding of domain 1 and the active site PyP is about 14 kcal/mol compared to about 5 kcal/mol for domain 1 in the apoenzyme (Table II in this paper and Table II in Ref. 4). Apparently the reduced PLP has strong interactions with residues in domain 1. In our previous study it was concluded that in the equilibrium denaturation of the holo-enzyme, the first event was the loss of the PLP to form apoenzyme between 1 and 2 M urea (3). By reduction of the PLP, we have lost this first step and the PyP remains bound at the active site. This provides a possible explanation of why domain 1 was stabilized in PyP-eSHMT.

In the study of apo-eSHMT, it was concluded that Ie is a dimer. This was supported not only by size exclusion chromatography experiments but by a urea gradient gel that showed decreased mobility for the apoenzyme in the 2–3 M urea range that was interpreted as being caused by a partially unfolded dimer (4). The urea gradient gel for PyP-eSHMT shows very different results (Fig. 6) compared to the urea gradient gel from the apoenzyme studies. It appears that Ie in the 4 M urea range has significantly greater mobility than the native enzyme, even though both the amino-terminal region and domain 2 have been partially unfolded. This suggests that the enzyme is becoming a monomer and that at 5.5 M urea the Ie of PyP-eSHMT is a monomer. The Ie of PyP-eSHMT is not observed on the kinetic folding pathway. Since PLP adds to the refolding enzyme only after it has passed through the rate-determining step, it is unlikely that the structure shown in Scheme 2 is on the kinetic pathway for folding of this enzyme. Herold et al. (24) have characterized a monomeric fragment of aspartate aminotransferase that folds independently and binds PLP. This fragment has some sequence identity and is of similar size to domain 1 plus the active site region of eSHMT, shown as Ie in Scheme 2 (25, 26). It will be of interest to determine whether the domain shown as Ie in Scheme 2 can also fold independently and bind PLP as a monomer as observed with aspartate aminotransferase.

Acknowledgment—This work was performed in part at the Center for Fluorescence Spectroscopy, University of Maryland at Baltimore, School of Medicine. We thank Dr. Henryk Malak for his generous help and useful suggestions.

REFERENCES
1. Plamann, M., Stauffer, L., Urbanowski, M., and Stauffer, G. (1983) Nucleic Acids Res. 11, 2065-2075
2. Schirch, V., Hopkins, S., Villar, E., and Angelaccio, S. (1985) J. Bacteriol. 163, 1-7
3. Cai, K., Schirch, D., and Schirch, V. (1995) J. Biol. Chem. 270, 19294–19299
4. Cai, K., and Schirch, V. (1996) J. Biol. Chem. 271, 2987–2994
5. Stryer, L. (1978) Annu. Rev. Biochem. 47, 819–846
6. Wu, P. G., and Brand, L. (1984) Anal. Biochem. 128, 1-13
7. Selvin, P. R. (1995) Methods Enzymol. 246, 300–334
8. Förster, T. (1965) in Modern Quantum Chemistry (Sinanoglu, O., ed) Vol III, pp. 93–157, Academic Press, New York.
9. Blond, S., and Goldberg, M. E. (1986) Proteins 1, 247–255
10. Schirch, L. G., and Mason, M. (1963) J. Biol. Chem. 238, 1032–1037
11. Lakowics, J. R., Gryczynski, I., Lazzio, G., Wicak, W., and Johnson, M. L. (1994) Protein Sci. 3, 625-637
12. Chen, R. F. (1967) Anal. Lett. 1, 35–42
13. Davis, R. F., Eisinger, J., and Blumberg, W. E. (1979) Biophys. J. 26, 161–194
14. Copo, J. F., and Pace, C. N. (1983) Biochemistry 22, 2654–2658
15. Matthews, C. R., and Crisanti, M. M. (1981) Biochemistry 20, 784–792
16. Pace, C. N. (1975) Crit. Rev. Biochem. 3, 11–20
17. Nath, U., and Udagawa, J. B. (1995) Biochemistry 34, 1702–1713
Serine Hydroxymethyltransferase

18. Schirch, V., Shostak, K., Zamora, M., and Gautam-Basak, M. (1991) J. Biol. Chem. 266, 759–764
19. Chaffotte, A. F., and Goldberg, M. E. (1983) Biochemistry 22, 2708–2714
20. Stover, P., Zamora, M., Shostak, K., Gautam-Basak, M., and Schirch, V. (1992) J. Biol. Chem. 267, 17679–17687
21. Angelaccio, S., Pascarella, S., Fattori, E., Bossa, F., Strong, W., and Schirch, V. (1992) Biochemistry 31, 155–162
22. Schmid, X. F. (1992) in Protein Folding (Creighton, T. E., ed) pp. 203–218, Freeman, New York
23. Leistler, B., Herold, M., and Kirschner, K. (1992) Eur. J. Biochem. 205, 603–611
24. Herold, M., Leistler, B., Hage, A., Luger, K., and Kirschner, K. (1991) Biochemistry 30, 3612–3620
25. Alexander, F. W., Sandmeier, E., Mehta, P. K., and Christin, P. (1994) Eur. J. Biochem. 219, 953–960
26. Pascarella, S., Schirch, V., and Bossa, F. (1993) FEBS Lett. 331, 145–149