Different Unfolding Pathways for Mesophilic and Thermophilic Homologues of Serine Hydroxymethyltransferase[†,‡]

Anant Narayan Bhatt,[§] Koodathingal Prakash,[§] H. S. Subramanya, and Vinod Bhakuni*

Division of Molecular and Structural Biology, Central Drug Research Institute, Lucknow 226 001, India

Received May 15, 2002; Revised Manuscript Received July 9, 2002

ABSTRACT: To determine how much information can be transferred from folding and unfolding studies of one protein to another member of the same family or between the mesophilic and thermophilic homologues of a protein, we have characterized the equilibrium unfolding process of the dimeric enzyme serine hydroxymethyltransferase (SHMT) from two sources, *Bacillus subtilis* (bsSHMT) and *Bacillus stearothermophilus* (bstSHMT). Although the sequences of the two enzymes are highly identical (∼77%) and homologous (89%), bstSHMT shows a significantly higher stability against both thermal and urea denaturation than bsSHMT. The GdmCl-induced unfolding of bsSHMT was found to be a two-step process with dissociation of the native dimer, resulting in stabilization of a monomeric species, followed by the unfolding of the monomeric species. A similar unfolding pathway has been reported for *Escherichia coli* aspartate aminotransferase, a member of the type I fold family of PLP binding enzymes such as SHMT, the sequence of which is only slightly identical (∼14%) with that of SHMT. In contrast, for bstSHMT, a highly cooperative unfolding without stabilization of any monomeric intermediate was observed. These studies suggest that mesophilic proteins of the same structural family even sharing a low level of sequence identity may follow a common unfolding mechanism, whereas the mesophilic and thermophilic homologues of the same protein despite having a high degree of sequence identity may follow significantly different unfolding mechanisms.

There have been conflicting reports in the literature about the conservation of folding pathways of homologous proteins. A few studies have shown that homologous proteins with high levels of sequence identity or structural homology have similar folding mechanisms (*1*−*5*). However, studies on β-sheet proteins with nearly identical structures reported a markedly different folding mechanism for these proteins (*6*). On the basis of these reports, it was argued that the proteins having a high degree of sequence identity along with high α-helical content exhibit similar folding pathways (*6*). One of the ways to validate this suggestion is to compare the folding−unfolding mechanisms of mesophilic and thermophilic homologues of a protein having a high degree of sequence identity. There are a few comparisons of kinetics of folding and unfolding of mesophilic and thermophilic homologues of proteins (*2*, *7*−*9*). However, not much has been reported about the comparison of the equilibrium unfolding pathways of these proteins.

Pyridoxal 5′-phosphate (PLP)[1] is a versatile cofactor that is able to catalyze a spectrum of reactions on a variety of amino acid substrates in different structural protein contexts (*10*). Goldsmith and colleagues classified PLP-dependent enzymes into five different fold types on the basis of amino acid sequence comparison, predicted secondary structure elements, and available three-dimensional structure (*11*). Among these enzymes, the type I fold or aspartate aminotransferase family of proteins is the best structurally characterized fold type as of the 27 PLP-dependent enzymes with known three-dimensional structures, this family contains 18 of them (*12*). The enzymes in this class are catalytically active as homodimers but in some cases assemble into larger complexes. Each subunit folds into two domains: a large domain (the PLP binding domain) and a small domain (the C-terminal domain). The N-terminal part of the chain does not have a common fold within this class. Serine hydroxymethyltransferase (EC 2.1.2.1), a PLP-dependent enzyme which belongs to the type I fold family of PLP-dependent enzymes, is widely distributed in nature, and found both in prokaryotic and eukaryotic cells (*12*). SHMT from *Escherichia coli* as well as from several bacterial sources is dimeric, whereas the enzyme from mammalian sources is a homotetramer. The subunit molecular mass (M_r) of SHMT ranges from 45 to 53 kDa (*13*).

The main objective of our study was to compare the equilibrium unfolding pathways of thermophilic and mesophilic homologues of SHMT having a high degree of sequence identity. An extensive characterization of the structural and functional changes associated with GdmCl-induced unfolding of SHMT from *Bacillus subtilis* (bsSHMT) and *Bacillus stearothermophilus* (bstSHMT) has been

[†] A.N.B. and K.P. thank CSIR for financial assistance.
[‡] CDRI communication no. 6277.
* To whom correspondence should be addressed: Division of Molecular and Structural Biology, Central Drug Research Institute, Lucknow 226 001, India. Tel: 00−91−522−221411. Fax: 91-522-223405. E-mail: bhakuniv@rediffmail.com.
[§] These authors have contributed equally to this work.
[1] Abbreviations: SHMT, Serine hydroxymethyltransferase; PLP, Pyridoxal-5′-phosphate; GdmCl, Guanidine hydrochloride; ANS, 8-anilino-1-naphthalenesulphonic acid; DSC, differential Scanning calorimetry; AAT, aspartate aminotransferase.

10.1021/bi020356i CCC: $22.00
Published on Web 09/13/2002

carried out. Furthermore, the unfolding pathway of SHMT has also been compared with that reported for *E. coli* aspartate aminotransferase, a mesophilic protein which is a member of the type I fold family of PLP enzymes having a very low degree of sequence identity but a high degree of structural homology with SHMT.

EXPERIMENTAL PROCEDURES

Overexpression and Purification of bsSHMT. The expression plasmid containing the *B. subtilis* SHMT gene was transformed into the BL21(DE3) strain of *E. coli*. A single colony was inoculated into 10 mL of LB broth containing 100 μg/mL ampicillin and grown overnight at 37 °C. The cells were inoculated into 250 mL of LB medium containing 100 μg/mL ampicillin and grown for 3 h. For overexpression, the cells were induced with 0.4 mM isopropyl β-D-thiogalactopyranoside at an OD_{600} value of 0.6. After 3 h, the cells were harvested and the pellet was resuspended in 50 mM Tris buffer (pH 7.2) containing 1 mM EDTA and 2 mM β-mercaptoethanol. The cell suspension was kept in ice and sonicated until it was optically clear. It was then centrifuged at 24000*g* for 20 min, and the supernatant was directly loaded onto a Mono-Q column (Amersham Pharmacia Biotech, Uppsala, Sweden) pre-equilibrated with the same buffer. The column was washed with the 50 mM Tris buffer, 1 mM EDTA, and 1 mM β-mercaptoethanol containing 200 mM NaCl and the protein eluted using a linear gradient from 200 to 600 mM NaCl. The fractions containing SHMT were pooled, and ammonium sulfate was added to a final concentration of 1.1 M. The protein solution was loaded on a Phenyl Sepharose (high substituted) column pre-equilibrated with Tris buffer containing 1.1 M ammonium sulfate. After being thoroughly washed with the same buffer, the protein was eluted using a linear gradient from 1.1 M to 100 mM ammonium sulfate. The fractions containing SHMT activity were pooled and subjected to dialysis against 1 L of Tris buffer containing 0.1 mM PLP for 16 h with two changes. The purity of the enzyme sample that was obtained was checked using SDS−PAGE followed by silver staining and was found to be greater than 98% pure.

Overexpression and Purification of bstSHMT. The bstSHMT was cloned, overexpressed, and purified as described previously (*14*).

Differential Scanning Calorimetry. Differential scanning calorimetric experiments was performed on a Microcal VP-DSC machine using a protein concentration of 10 μM (*15*). The samples were scanned at a rate of 60 °C/h. Data reduction and analysis were performed using the Microcal Origin software. All the scans were found to be irreversible under the experimental conditions used for these studies.

SHMT Activity. The presence of the active enzyme was assessed by determining the extent of formation of an abortive complex formed between holo-SHMT, glycine, and 5-formyltetrahydrofolate (0.2 mM) as described previously (*16*). An enzyme concentration of 1 μM was used for the studies.

Guanidine Hydrochloride or Urea Denaturation of SHMT. Serine hydroxymethyltransferase (1 μM) was dissolved in 50 mM Tris buffer (pH 7.2) containing 1 mM EDTA and 2 mM β-mercaptoethanol in the presence and absence of increasing concentrations of GdmCl or urea and incubated overnight at 4 °C before the measurements were taken.

Circular Dichroism Measurements. CD measurements were taken with a Jasco J810 spectropolarimeter calibrated with ammonium (+)-10-camphorsulfonate. The results are expressed as relative ellipticity and plotted as percentage values. The CD spectra were recorded at enzyme concentrations of 1 and 10 μM for far- and near-UV measurements, respectively, with a 1 mm cell at 4 °C. The values that were obtained were normalized by subtracting the baseline recorded for the buffer having the same concentration of denaturant under similar conditions.

Synthesis of PyP-SHMT. The reduction of PLP aldimine was achieved according to the procedure of Cai and Schirch (*17*).

8-Anilino-1-naphthalenesulfonic Acid (ANS) Fluorescence Measurements. Fluorescence spectra were recorded with a Perkin-Elmer LS 50B spectrofluorometer in a 5 mm path length quartz cell at 4 °C. Aliquots of protein with a final concentration of 1 μM were equilibrated at the desired GdmCl concentration overnight at 4 °C. They were then mixed with a concentrated stock solution of ANS dissolved in the same buffer and incubated for 1 h. The excitation wavelength was 365 nm, and the emission was recorded from 400 to 560 nm. The final ANS concentration was 15 μM. Subtracting the baseline recording for the probe alone under similar conditions normalized the values.

Cross-Linking Using Glutaraldehyde. To native and GdmCl-treated SHMT (0.3 and 1 μM for bsSHMT and bstSHMT, respectively, or mentioned otherwise) was added an aliquot of 25% (w/v) glutaraldehyde to achieve a final concentration of 1% glutaraldehyde. This sample was incubated at 25 °C for 5 min followed by quenching the cross-linking reaction by addition of 200 mM sodium borohydride. After a 20 min incubation, 3 μL of 10% aqueous sodium deoxycholate was added. The pH of the reaction mixture was decreased to 2−2.5 by addition of orthophosphoric acid that resulted in precipitation of the cross-linked protein. After centrifugation (13237*g* and 4 °C), the obtained precipitate was redissolved in 0.1 M Tris-HCl (pH 8.0), 1% SDS, and 50 mM dithiothreitol and heated at 90−100 °C. SDS−PAGE was carried out on 10% gels.

Size Exclusion Chromatography. Gel filtration experiments were carried out on a Superdex 200HR 10/30 column (manufacturer's exclusion limit of 600 kDa for proteins) on an AKTA FPLC system (Amersham Pharmacia Biotech). The column was equilibrated and run on 50 mM Tris buffer containing 1 mM EDTA, 2 mM β-mercaptoethanol, and the desired GdmCl concentration at 4 °C. The SHMT solution (10 μM) was incubated at the desired GdmCl concentrations overnight at 4 °C. Two hundred microliters of this sample was loaded on the column and run at 4 °C with a flow rate of 0.3 mL/min.

RESULTS

Amino Acid Sequence and Structural Comparison of SHMT and Aspartate Aminotransferase (eAAT). The amino acid sequences of bsSHMT, bstSHMT, and aspartate aminotransferase (eAAT), members of the type I fold family of PLP enzymes, are compared in Figure 1. The sequences of bsSHMT and bstSHMT are ~77% identical and 89% homologous. The major variations in the sequences of the two enzymes were localized mainly in the C-terminus. The

```
Bstshmt     ----MNYLPQQDPQVFAAIEQERKRQ---HAKIELIASEN---FVSRAVMEAQGSVLTNK
Bsshmt      ----MKHLPAQDEQVFNAIKNERERQ---QTKIELIASEN---FVSEAVMEAQGSVLTNK
eAAT        MFENITAAPADPILGLADLFRADERPGKINLGIGVYKDETGKTPVLTSVKKAEQYLLENE

Bstshmt     YAEGYPGRRYYGGCEYVDIVEELARERAKQLFGAEHANVQPHSGAQANMAVYFTVLEHGD
Bsshmt      YAEGYPGKRYYGGCEHVDVVEDIARDRAKEIFGAEHVNVQPHSGAQANMAVYFTILEQGD
eAAT        TTKNYLG--IDGIPEFGRCTQELLFGKGSALINDKRARTAQTPGGTGALRVAADFLAKNT

Bstshmt     TVLGMNLSHGGHLTHGSPVNFSGVQYNFVA-GVP-ETHVIDYDDVRIKARFHRP--KLIV
Bsshmt      TVLGMNLSHGGHLTHGSPVNFSGVQYNFVEYGVDKETQYIDYDDVREKALAHKP--KLIV
eAAT        SVKRVWVSNPSWPNHKSVFNSAGLEVREYAY-YDAENHTLDFDALINSLNEAQAGDVVLF

Bstshmt     AGASAYPRIIDFAKFREIADEVGAYLMVDMAHIAGLVAAGLHPNPVPYAHFVTTT
Bsshmt      AGASAYPRTIDFKKFREIADEVGAYFMVDMAHIAGLVAAGLHPNPVPYADFVTTT
eAAT        HGCCHNPTGIDPTLEQWQTLAQLSVEKGWLPLFDFAYQG--FARGLEEDAEGLRAFAAMH

Bstshmt     THKTLRGPRGGLILCQEQFAKQIDKAIFQGIQGGPLMHVIAAKAVAFGEALQDDFKAYA
Bsshmt      THKTLRGPRGGMILCREFGKKIDKSIFPGIQGGPLMHVIAAKAVSFGEVLQDDFKTYA
eAAT        KELIVASSYSKNFGLYNERVGACTLVAADSETVDRAFSQMKAAIRANYSNPPAHGASVVA

Bstshmt     KRVVDNAKRLASALQNEGFTLVSGGTDNHLLLVDLRPQQLTGKTAEKVLDEVGITVNKNT
Bsshmt      QNVISNAKRLAEALTKEGIQLVSGGTDNHLILVDLRSLGLTGKVAEHVLDEIGITSNKNA
eAAT        TILSN--DALRAIWEQELTDMRQRIQRMRQLFVNTLQEKGANRDFSFIIKQNGMFSFSGL

Bstshmt     IPYDPESPFVTSGIR-IGTAAVTTRGFGLEEMDEIAAIIGLVLKNVGSEQALEEARQ---
Bsshmt      IPYDPEKPFVTSGIR-LGTAAVTSRGFDGDALEEVGAIIALALKNHEDEGKLEEARQRVA
eAAT        TKEQVLRLREEFGVYAVASGRVNVAGMTPDNMAPLCEAIVAVL----------------

Bstshmt     ----PSPL------
Bsshmt      ALTDKFPLYKELDY
eAAT        --------------
```

FIGURE 1: Amino acid sequences of bstSHMT, bsSHMT and eAAT. The sequence positions with identical residue are shown in green, and the positions that are homologous are shown in yellow.

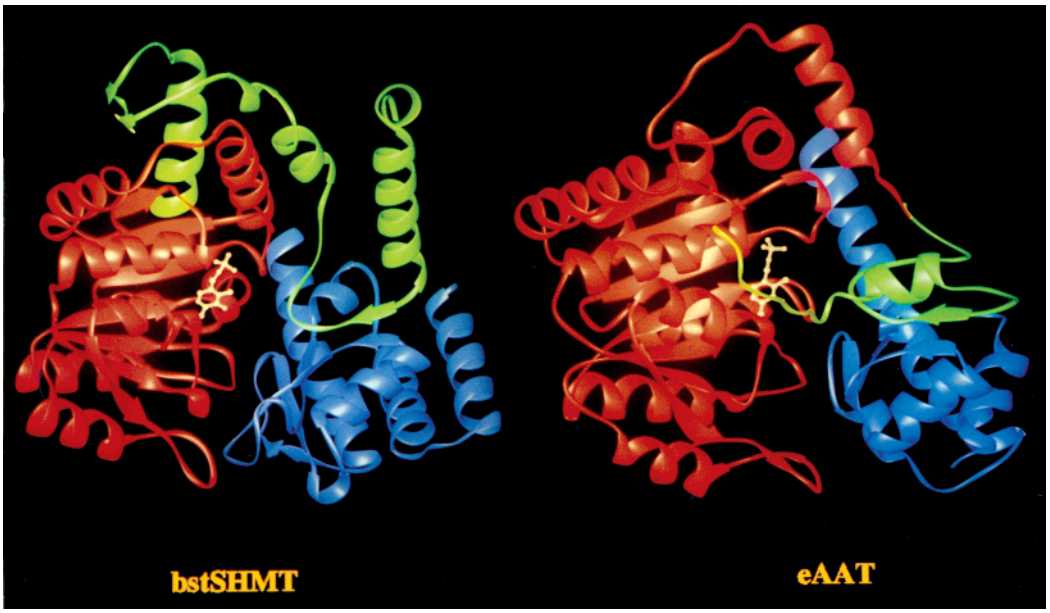

FIGURE 2: Schematic representation of the structure of *E. coli* aspartate aminotransferase and *B. stearothermophilus* SHMT. The diagram was produced using the program MolScript (*22*). Each subunit comprises three parts: the N-terminus (green), the PLP binding domain or P-domain (red), and the C-terminus (blue). The PLP molecule (white) is also shown.

sequence of *E. coli* aspartate aminotransferase is ~14% identical and ~43% homologous with that of SHMT, and the variations are dispersed throughout the sequence.

Figure 2 shows the comparison of the monomer structure of eAAT (*18*) and bstSHMT (*14*) as determined by X-ray crystallography. A high degree of structural homology exists between the two enzymes. The monomers of both the enzymes consist of three structural parts: the N-terminus, the PLP binding domain (P-domain), and the C-terminus (Figure 2). The three-dimensional structure of the enzyme shows the presence of two domains: a large P-domain and a smaller domain formed by the N- and C-terminal extensions. A very high degree of structural homology exists for the P-domain between the two enzymes. The C-termini also show a significant degree of homology; however, for bstSHMT, due to it having significantly more amino acid residues than eAAT (Figure 1) in the primary sequence, an extended structure is observed (*14*). The N-terminal regions

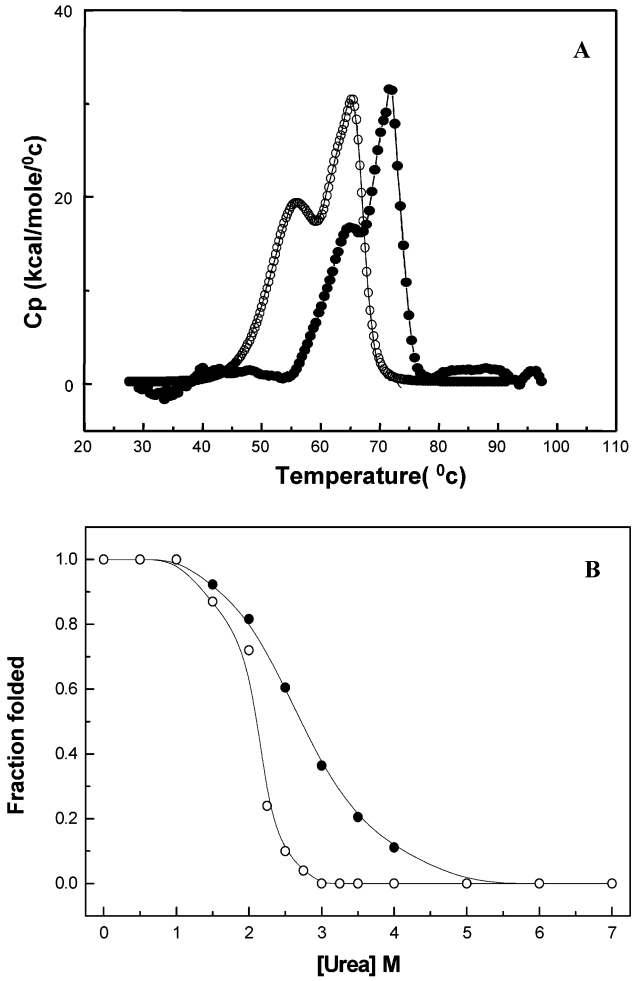

FIGURE 3: (A) Differential scanning calorimetric scans of native bsSHMT (○) and bstSHMT (●). The circles show the data points, and the line shows the fitting. (B) Urea-induced unfolding transition of native bsSHMT (○) and bstSHMT (●). A linear extrapolation of the baselines in the pre- and post-transitional regions was used to determine the fraction of enzyme within the transition region by assuming a two-state mechanism of unfolding.

of the enzymes show significant variation.

Enhanced Stability of bstSHMT. A differential scanning calorimetric study was carried out to compare the stability of bsSHMT and bstSHMT against thermal denaturation and is summarized in Figure 3A. For both enzymes, two distinct transitions were observed with increasing temperature. However, for bstSHMT, enhanced T_ms of 65 and 71 °C, respectively, as compared to values of 52 and 65 °C, respectively, for bsSHMT, were observed. Hence, bstSHMT is more stable than bsSHMT against thermal denaturation.

The two SHMTs exhibited similar CD spectra with maxima between 195 and 250 nm, suggesting that they have similar secondary structures. The urea denaturation of both the SHMTs was carried out, and the results are summarized in Figure 3B. For both enzymes, a sigmoidal decrease in the ellipticity value at 222 nm with increasing urea concentration was observed. However, the C_m, the concentration of urea required for half-denaturation of the enzyme, was found to be ~0.6 M higher for bstSHMT (2.8 M urea) than for bsSHMT (2.17 M urea), thus demonstrating that bstSHMT is more stable than bsSHMT against urea denaturation.

Unfolding Studies on SHMT. Several spectroscopic techniques and enzymatic activity measurements were used to study the GdmCl-induced unfolding of SHMT.

Secondary Structural Changes. Figure 4A shows the change in mean residual ellipticity at 222 nm with increasing concentrations of GdmCl for the two SHMTs. For bsSHMT, the overall process was found to be biphasic, and an intermediate state appeared to be stable at ~0.65−1 M GdmCl. The transition midpoints defined by the horizontal baselines and the plateau were at 0.3 M GdmCl for the first phase and at 1.44 M GdmCl for the second phase. The far-UV CD spectra of the intermediate stabilized at 0.65−1 M GdmCl possessed ~64% of the native CD spectral amplitude (Figure 4A, inset I). These observations suggest that an ~36% loss of secondary structure of native dimeric bsSHMT is associated with the first transition and a complete loss of structure at high GdmCl concentrations.

For bstSHMT, a sigmoidal dependence of the decrease in mean residual ellipticity at 222 nm with increasing GdmCl concentrations was observed. Between 0 and 0.6 M GdmCl, a slight linear enhancement (from 100 to 106%) in the CD ellipticity of the native enzyme followed by a sharp decrease in CD ellipticity (from 106 to 25%) between 0.6 and 2.5 M GdmCl was observed. At >3.5 M GdmCl, a complete loss of the far-UV CD signal was observed (Figure 4A, inset II) which is an indication of the unfolding of the enzyme under these conditions. The C_m (the concentration of GdmCl required for half-denaturation) for GdmCl denaturation of bstSHMT was found to be ~1.29 M.

Tertiary Structural Changes and PyP Fluorescence. As no intrinsic fluorophore (tryptophan molecule) is present in bs- or bstSHMT, the reduction of bound PLP resulting in the formation of a pyridoxyl phosphate secondary amine, namely, PyP, that is fluorescent (*17*) was carried out. By this modification, a fluorescent probe is placed in the region of the enzyme between two domains, namely, the large domain and the C-terminal domain. Furthermore, the fluorophore remains attached in the protein even when the enzyme is completely unfolded and, hence, can provide information about the unfolding of the enzyme. For both enzymes, a significant difference between fluorescence emission wavelength maxima of PyP was observed for the native (389 nm) and denatured (398 nm) enzyme. Hence, a correlation between the GdmCl concentration and the fractional changes in emission λ_{max} was plotted for extracting information about stabilization of intermediates during GdmCl-induced unfolding of the enzyme (*19*) and is summarized in Figure 4B. For bsSHMT, two distinct transitions in the GdmCl concentration ranges of 0−0.65 and 0.7−1.8 M were observed with increasing GdmCl concentrations. However, for bstSHMT, a single transition between GdmCl concentrations of 0.6 and 2 M centered at ~1.25 M was observed.

Although similar profiles for GdmCl-induced unfolding of bsSHMT or bstSHMT were observed by far-UV CD and PyP fluorescence, a slightly higher C_m for GdmCl-induced unfolding was observed by PyP fluorescence. This is so because reduction of PLP to PyP in SHMT results in a slight stabilization of the enzyme (*17*).

Exposure of Buried Hydrophobic Clusters. The solvent exposure of nonpolar clusters during GdmCl-induced unfolding of bs- and bstSHMT was studied by the binding of ANS,

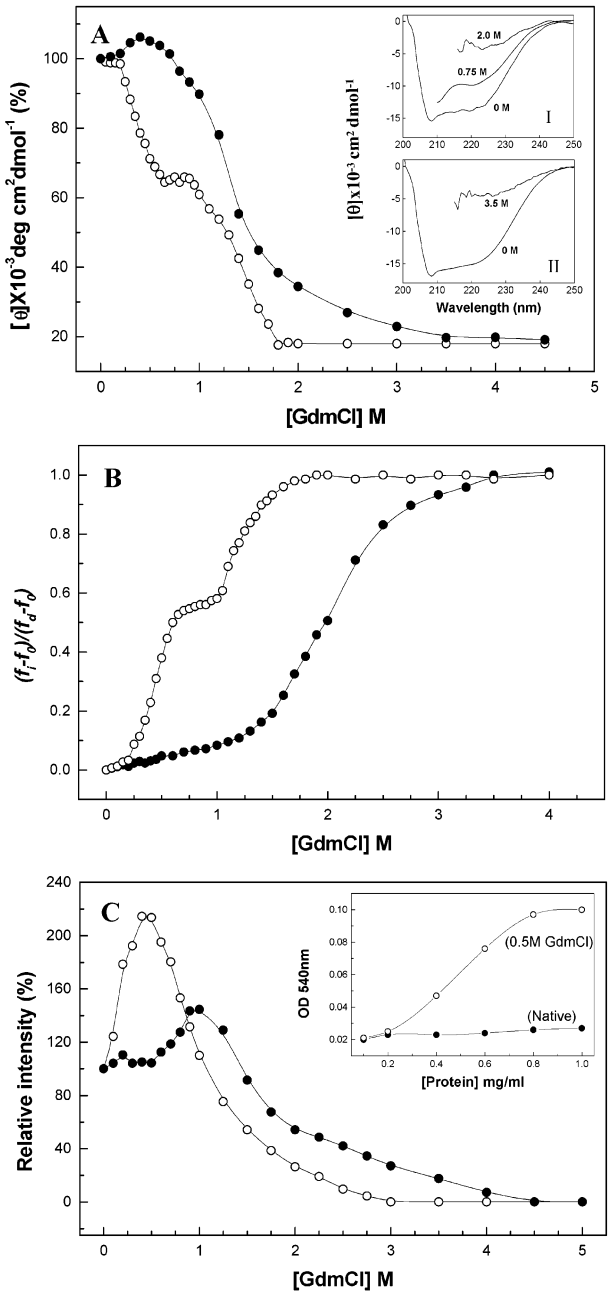

FIGURE 4: Equilibrium GdmCl-induced unfolding of bs- and bst-SHMT as studied by optical spectroscopic techniques. (A) GdmCl-dependent unfolding of bsSHMT (○) and bstSHMT (●) measured by far-UV CD spectroscopy monitored at 222 nm. The data are represented as a percentage of ellipticity at 222 nm taking the value for the native enzyme to be 100%. The insets show relative CD spectra of bsSHMT (inset I) and bstSHMT (inset II). The numbers in the inset represent the GdmCl concentration. (B) Plot of the fractional change in the wavelength of maximum fluorescence emission of PyP for bsSHMT and bstSHMT, $(f_i-f_o)/(f_d-f_o)$ vs GdmCl concentration. f_i is the wavelength for a particular sample, f_o is the wavelength in the absence of GdmCl, and f_d the wavelength at GdmCl concentration >3 M. The excitation wavelength was 335 nm, and the emission was recorded from 360 to 500 nm. The final enzyme concentration was 1 μM. The symbols are the same as in panel A. (C) GdmCl-induced unfolding of bsSHMT and bstSHMT monitored by following the changes in ANS fluorescence intensity at 460 nm. The data are represented as the percentage of fluorescence intensity at 460 nm, taking the value for the native enzyme to be 100%. The symbols are the same as in panel A. The inset shows the change in OD at 540 nm for native and 0.5 M GdmCl-stabilized bsSHMT at increasing protein concentrations.

a hydrophobic fluorescent dye (*20*), to the enzyme on GdmCl-induced denaturation. Figure 4C summarizes the changes in the fluorescence intensity of ANS on incubation with SHMT treated with increasing concentrations of GdmCl. For both bs- and bstSHMT, a single transition with respect to increasing GdmCl concentrations was observed. For bsSHMT, a transition between 0 and 2 M GdmCl, centered at 0.44 M, exhibited an enhancement of ~2.2 times in fluorescence intensity (at the maximum), indicating significant exposure of buried hydrophobic clusters in the enzyme at low denaturant concentrations. Furthermore, this transition overlaps with the first transition observed by far-UV CD studies (Figure 4A). These observations suggest that for bsSHMT the structural transition corresponding to low denaturant concentrations is associated with exposure of some buried hydrophobic clusters present in the native enzyme. Such a change in enzyme structure may lead to its aggregation due to hydrophobic interactions. This is indeed the case which was supported by the significant enhancement in OD at 540 nm (from 0.02 to 0.1) observed with increasing concentrations of enzyme for 0.5 M GdmCl-stabilized bsSHMT (Figure 4C, inset). For the native enzyme (control), no significant change in the OD value was observed, suggesting an absence of aggregation of the enzyme under similar conditions.

For bstSHMT, a transition, however, with a very small enhancement (from 100 to 140 fluorescence units) in ANS fluorescence was observed between 0.4 and 1 M GdmCl, indicating only a slight alteration in the exposure of buried hydrophobic clusters of bstSHMT at low GdmCl concentrations.

Dissociation of PLP from the Enzyme. For SHMT, a significant difference in the visible CD signal of the native and apo or denatured enzyme has been reported due to the loss of PLP bound to the enzyme (*16*). The native SHMTs exhibit a unique peak at 420 nm due to bound PLP that disappears with the loss of PLP from the enzyme either on unfolding or in the apoenzyme. For both enzymes, a sigmoidal dependence of the loss of the CD signal at 420 nm with increasing GdmCl concentrations was observed (Figure 5A). For bsSHMT, the transition was observed between 0.2 and 0.8 M GdmCl which corresponds to the first transition observed by far-UV CD (Figure 4A) for this enzyme. However, for bstSHMT, it was observed at higher GdmCl concentrations, between 0.4 and 1.3 M GdmCl.

Figure 5B shows that enzyme activity of both bs- and bstSHMT was lost via a strong cooperative transition between 0.1 to 0.8 M and between 0.5 to 1.3 M GdmCl, respectively. For both bs- and bstSHMT, the transitions observed by near-UV CD coincide with that observed by enzymatic activity measurements, thus suggesting that the loss of PLP from the enzyme results in the loss of enzymatic activity.

The spectroscopic studies demonstrate that unfolding of bsSHMT by GdmCl is a noncooperative process involving two discrete steps probably of dissociation of the native dimer into the monomeric intermediate followed by unfolding of the monomeric intermediate (discussed later). In contrast, for bstSHMT, only a single transition at a similar GdmCl concentration was observed with all the spectroscopic techniques, suggesting that the GdmCl-induced unfolding of bstSHMT occurs in a cooperative manner with dissociation

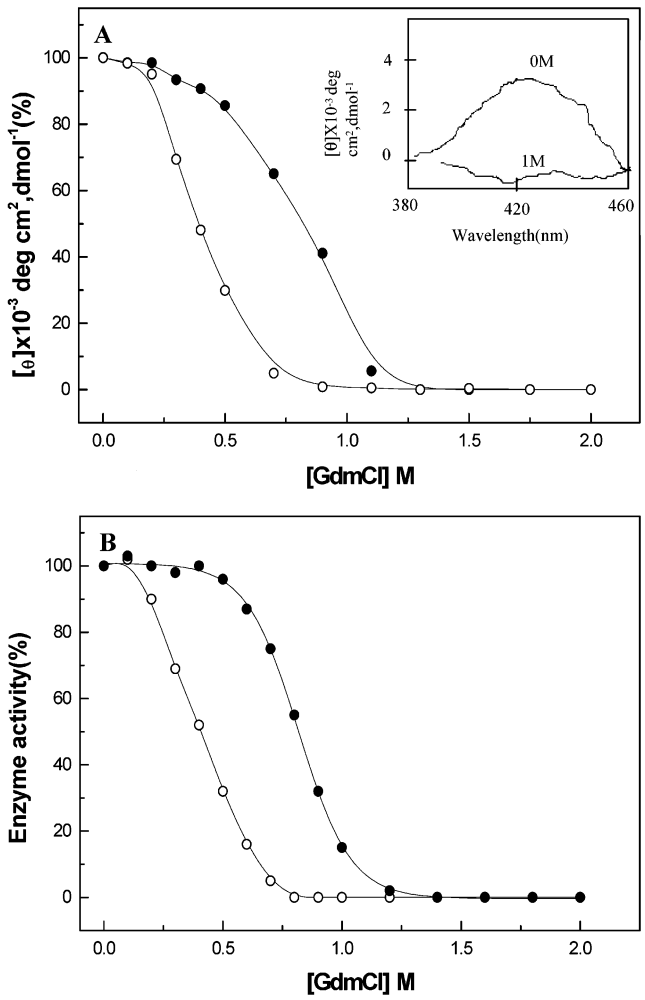

FIGURE 5: (A) GdmCl-dependent unfolding of bsSHMT (○) and bstSHMT (●) measured by near-UV CD spectroscopy monitored at 422 nm. The data are represented as the percentage of ellipticity at 422 nm, taking the value for the native enzyme to be 100%. The inset shows the visible CD spectra of native and 1 M GdmCl-stabilized SHMT. (B) Changes in enzymatic activity of bsSHMT and bstSHMT with increasing concentrations of GdmCl. The data are represented as the percentage of enzymatic activity, taking the value for the native enzyme to be 100%. The symbols are the same as in panel A.

and unfolding of the enzyme occurring in a concerted manner.

Effect of GdmCl on the Subunit Assembly and Molecular Dimension of bs- and bstSHMT. (1) Cross-Linking Studies. Glutaraldehyde cross-linking studies were carried out to study the effect of GdmCl treatment on the subunit configuration of bs- and bstSHMT and are summarized in panels A and B of Figure 6, respectively. For glutaraldehyde-cross-linked native bsSHMT, a single protein species corresponding to the dimer was observed. However, for the 0.5 M GdmCl-treated enzyme, two species corresponding to the dimer and the monomer were observed (Figure 6A) with the monomer was being the major species and a very small population of the dimer (comparison based on the relative intensity of the two protein bands). At 1 M GdmCl, only protein species corresponding to monomers were observed.

For bstSHMT, up to a GdmCl concentration of 1 M, only a protein band corresponding to the dimer was observed (Figure 6B). This demonstrates that unlike bsSHMT for which significant dissociation of the native dimer into monomers was observed at even 0.5 M GdmCl, no effect on the subunit structure of native bstSHMT is observed up to even 1 M GdmCl. For 1.5 M GdmCl, both the dimer and monomer were observed; however, the monomer was the major component. At 2 M GdmCl, only protein species corresponding to monomers were observed.

(2) Size Exclusion Chromatography. For studying the effect of GdmCl on the molecular dimensions of bs- and bstSHMT, size exclusion chromatographic studies on the S-200 Superdex column in the absence and presence of increasing concentrations of GdmCl were carried out and are summarized in panels C and D of Figure 6. For the native bsSHMT dimer, a single peak with a retention volume of 12.6 mL was observed (Figure 6C). However, for the 0.5 M GdmCl-stabilized enzyme, two peaks having retention volumes of 8.7 and 13.9 mL (designated as A and B, respectively) were observed (Figure 6C, inset). Peak A appeared at a dramatically reduced retention volume of 8.7 mL, which is the void volume (8 mL) of the column (exclusion limit of 600 kDa), indicative of vastly increased hydrodynamic radii for the enzyme under these conditions (i.e., an aggregated species). Peak B observed at a significantly higher retention volume of 13.9 mL compared to that of the native dimeric enzyme (12.6 mL) is suggestive of highly reduced hydrodynamic radii for the enzyme under these conditions. The cross-linking studies (Figure 6A) showed a stabilization of the bsSHMT monomer under these conditions which indeed will have a significantly reduced hydrodynamic radius compared to that of the native dimeric enzyme; hence, peak B corresponds to the bsSHMT monomer. The presence of both the monomer and aggregates under these conditions indicates that they exist in equilibrium under conditions at which the column was run. The structural studies (Figure 4A) showed a significant loss of secondary structure for 0.5 M GdmCl-stabilized bsSHMT; hence, the monomer stabilized under these conditions is probably a partially unfolded monomer of bsSHMT. As the bsSHMT species stabilized at 0.5 M GdmCl has a tendency to aggregate (Figure 4C), enzyme concentration-dependent size exclusion chromatographic studies were carried for studying the aggregation properties of these monomers. At an enzyme concentration of 2 μM under these conditions, only the enzyme species corresponding to the monomer was observed (retention volume of 13.9 mL corresponding to peak B). These observations demonstrate that 0.5 M GdmCl-stabilized monomers of bsSHMT undergo concentration-dependent aggregation. At a GdmCl concentration of 3 M, a single peak having a highly reduced retention volume of 11.0 mL was observed, indicating a significantly larger hydrodynamic radius for the enzyme under these conditions which is possible only when the enzyme is in an unfolded conformation. The stabilization of the unfolded monomer of the enzyme at 3 M GdmCl is supported by collective information from the spectroscopic (Figure 4) and cross-linking studies (Figure 6A) reported above.

For native bstSHMT, a single peak at a retention volume of 12.6 mL was observed which is similar to that observed for bsSHMT, suggesting that in the native conformation the two enzymes have relatively similar hydrodynamic radii (Figure 6D). For 0.2−1 M GdmCl-stabilized bstSHMT, a single peak, however, at an enhanced retention volume of 14.2 mL was observed. These observations suggest that

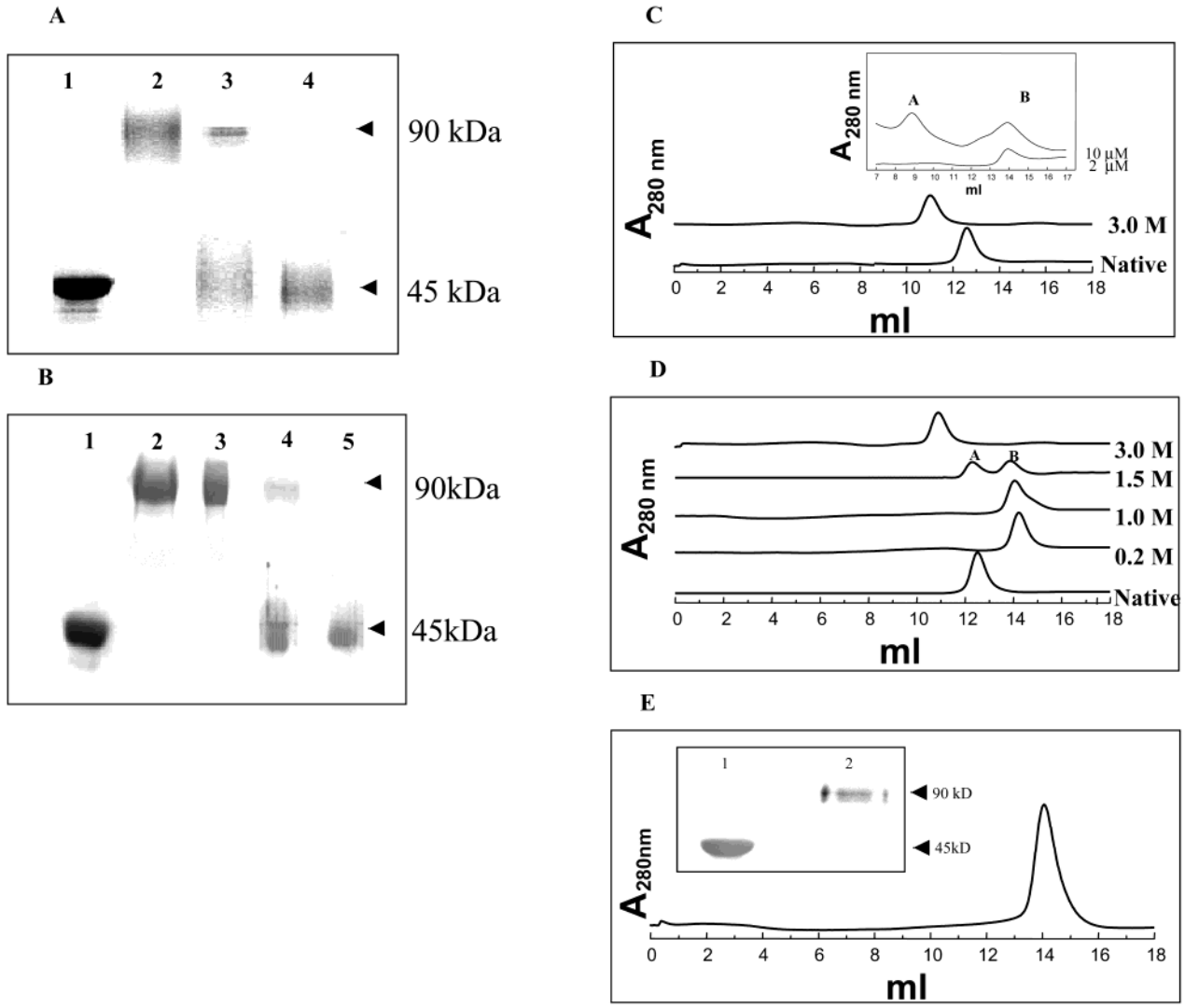

FIGURE 6: GdmCl-induced changes in molecular dimension and subunit configuration during enzyme unfolding. The SDS−PAGE profiles of glutaraldehyde cross-linked GdmCl-treated bsSHMT and bstSHMT are presented in panels A and B, respectively. In panel A, lanes 1 and 2 represent the native and cross-linked native enzyme, respectively. Lanes 3 and 4 represent cross-linked samples of 0.5 and 1 M GdmCl-stabilized bsSHMT, respectively. In panel B, lanes 1 and 2 are the same as in panel A. Lanes 3−5 represent cross-linked samples of 0.5, 1.5, and 2 M GdmCl-stabilized enzymes, respectively. Chromatographic profiles of bsSHMT (C) and bstSHMT (D) on the Superdex 200HR 10/30 column on the AKTA FPLC system at increasing GdmCl concentrations. In panel C, the inset shows the profile for 0.5 M GdmCl-stabilized bsSHMT at 10 and 2 μM enzyme. The curves have been displaced on the *Y*-axis for display purposes. Panel E represents the chromatographic profiles of 0.5 M NaCl-stabilized bstSHMT on then Superdex 200HR 10/30 column on AKTA FPLC system. The inset shows the SDS−PAGE profiles of gluteraldehyde cross-linked 0.5 M NaCl-treated bstSHMT. The lanes 1 and 2 represent un-cross-linked and cross-linked samples, respectively.

bstSHMT at low GdmCl concentrations has a significantly reduced hydrodynamic radius compared to that of the native dimeric enzyme; however, the cross-linking studies (Figure 6B) showed the presence of a dimeric enzyme species under these conditions. These observations collectively suggest that a low concentration of GdmCl induces compaction of the native conformation of bstSHMT, resulting in stabilization of a compact dimer of the enzyme. A similar compaction of the native conformation of the dimeric enzyme glucose oxidase has recently been reported by us (*15*). For 1.5 M GdmCl-stabilized bstSHMT, two peaks (designated A and B) having retention volumes of 12.3 and 13.9 mL, respectively, corresponding to partially unfolded compact dimeric and monomeric species, respectively (based on the observation from cross-linking, Figure 6B, and spectroscopic studies, Figure 4), were observed. The presence of both a partially unfolded compact dimer and monomers under these conditions indicates that these two species exist in equilibrium under conditions at which column was run. The equilibrium between the partially unfolded compact dimer and monomer at 1.5 M GdmCl is also supported by the structural studies (Figure 4A), where a significant denaturation was observed under these conditions. For the 3 M GdmCl-stabilized enzyme, a single peak having a retention volume of ~10.9 mL was observed, suggesting the presence of an unfolded enzyme under these conditions (as discussed for bsSHMT).

The results of glutaraldehyde cross-linking along with the results of size exclusion chromatography reported above suggest that treatment of bsSHMT with a low concentration of GdmCl results in dissociation of the native dimeric enzyme and the monomers are stabilized. At higher GdmCl concentrations, only unfolded monomeric species were observed. In contrast for bstSHMT, no stabilization of monomers was observed at low concentrations of GdmCl; however, under

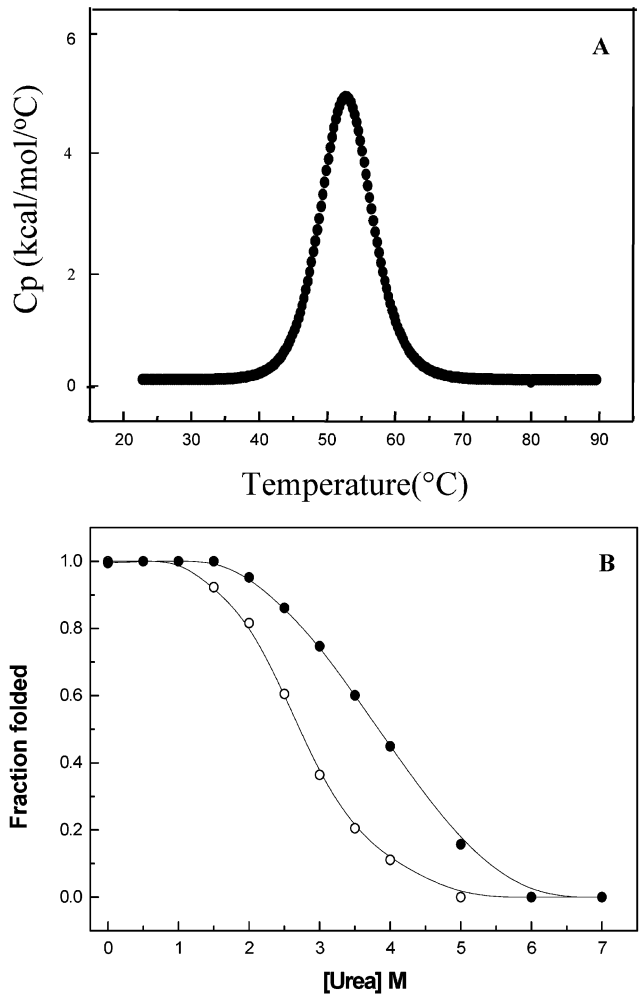

FIGURE 7: (A) Differential scaning calorimetric scan of 1 M GdmCl-stabilized bsSHMT. (B) Urea-induced unfolding transition of native (○) and 0.5 M GdmCl-stabilized bstSHMT (●). A linear extrapolation of the baselines in the pre- and post-transitional regions was used to determine the fraction of enzyme within the transition region by assuming a two-state mechanism of unfolding.

these conditions, the enzyme was found to undergo compaction of its conformation (Figure 6D), resulting in stabilization of a compact dimeric species. At higher GdmCl concentrations, unfolded bstSHMT was observed.

Properties of a GdmCl-Stabilized Compact Dimer of bstSHMT. The GdmCl-stabilized compact dimer of bstSHMT exhibited enzymatic activity similar to that of the native dimer (Figure 5). Furthermore, the structural properties of the compact dimer were also very similar to those of the native dimeric enzyme (Figure 4). However, a significant difference in the stability of the two dimers was observed. Figure 7B summarizes the urea denaturation profiles of 0.5 M GdmCl-stabilized bstSHMT as monitored by changes in CD ellipticity at 222 nm. An enhancement in C_m of urea by ~1.3 M urea was observed for the 0.5 M GdmCl-stabilized enzyme as compared to that of the native enzyme, thus demonstrating that GdmCl-induced compaction of the native conformation of bstSHMT results in enhanced enzyme stability.

Mechanism of GdmCl-Induced Stabilization of the Compact Dimer of bstSHMT. GdmCl is an electrolyte with a pK_a of ~11, which means that at a pH below this the GdmCl molecule will be present in fully dissociated form, i.e., as

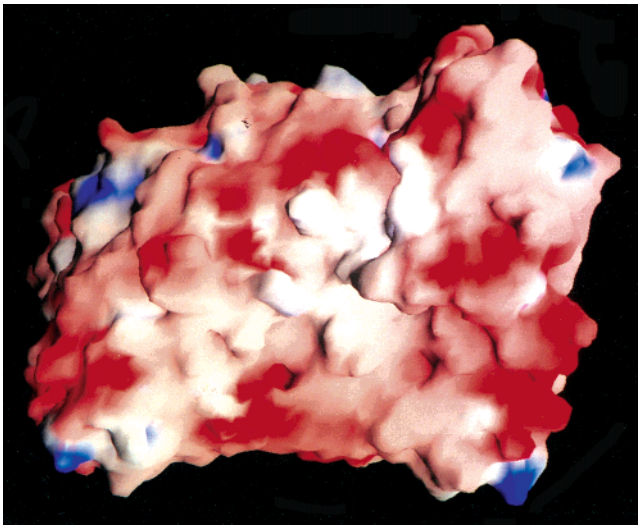

FIGURE 8: Molecular surface of the bstSHMT dimer colored according to electrostatic potential (blue for negative and red for positive potential). The molecular surface was displayed using GRASP.

Gdm^+ and Cl^-. The presence of these ions would influence the stabilizing properties of proteins and/or enzymes. Compaction of the native conformation and/or enhanced thermostability of the protein due to Gdm^+ (cation) binding to negatively charged moieties of protein has been reported for multimeric (*15*) and monomeric proteins (*23*). The electrostatic surface potential of bstSHMT from its crystal structure as obtained by GRASP (*24*) is shown in Figure 8. The enzyme has a predominant negative charge with clusters of negative charges localized on the surface of the enzyme molecule. Due to localization of these negative charge clusters, the enzyme molecule will experience repulsive electrostatic interaction, resulting in stabilization of an open structure of the enzyme at neutral pH [as explained previously (*25*)]. The interaction of Gdm^+ cations with negatively charged moieties present in the enzyme would result in weakening of the repulsive electrostatic force, thus permitting forces favoring compaction of the enzyme's conformation. The cation (Gdm^+)-induced compaction of the native conformation of bstSHMT is further supported by the studies using NaCl. Incubation of bstSHMT with 0.5 M NaCl results in stabilization of the compact dimer of the enzyme, which is similar to that stabilized by 0.5 M GdmCl, as observed by the reduction in the hydrodynamic radii of the enzyme by size exclusion chromatography and glutaraldehyde cross-linking (Figure 6E).

DISCUSSION

The simplest mechanism that accounts for all experimental observations on GdmCl-induced unfolding of bsSHMT is

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

where D is native dimer, M* a partially unfolded monomer and U the unfolded enzyme. The partially unfolded monomeric intermediate M* that is thermodynamically stable at about 0.65 to 1MGdmCl retained about 64% of the α-helical structure (Figure 4A), but was enzymatically inactive (Figure 5B) and had significant exposure of buried hydrophobic clusters due to which it has tendency to aggregate (Figure

4C). Furthermore, this intermediate M* showed a significant enthalpy associated with its thermal denaturation (Figure 7A). At higher concentrations of GdmCl the unfolded enzyme was observed.

For eAAT, an unfolding pathway very similar to that observed for bsSHMT has been reported (*21*). Low concentrations of GdmCl (between 0.9 and 1.1M) induce dissociation of native dimer, resulting in stabilization of a monomeric intermediate. The dissociation of native dimer is associated with loss of about 50% α-helical structure present in the native enzyme, exposure of buried hydrophobic clusters, loss of enzymatic activity and dissociation of PLP from the enzyme. All these properties are similar to that observed for GdmCl-induced dissociation of the native dimer of bsSHMT under similar conditions. At higher concentrations of denaturant, the enzyme monomer was reported to be unfolded. These observations suggest that bsSHMT and eAAT follow a similar unfolding pathway.

For bstSHMT, a significantly different pathway of GdmCl-induced unfolding was observed which on the basis of the results obtained could be summarized as follows:

$$D \Leftrightarrow D^* \Leftrightarrow U$$

where D is native dimer, D* a compact dimer and U the unfolded enzyme.

Unlike in bsSHMT, no discrete step of dissociation of the native dimer resulting in stabilization of any monomeric species was observed for bstSHMT. However, at low concentrations of GdmCl (up to 0.5M) compaction of the native dimer of enzyme (species D*) was observed by examination of hydrodynamic radii using size exclusion chromatography. Furthermore, this compact dimer showed a higher stability against urea denaturation as compared to native enzyme (Figure 7B) suggesting that compaction of the native conformation of bstSHMT leads to enhanced stability of the enzyme. At higher GdmCl concentration unfolding of the enzyme with dissociation and denaturation occurring in a concerted manner was observed.

The bstSHMT shares a very high degree of sequence identity with bsSHMT and a low degree of sequence identity but high degree of structural homology with eAAT. However, the unfolding pathway for bstSHMT was found to be very different from that of bsSHMT or eAAT, which have a similar unfolding pathway. The bstSHMT undergoes a highly cooperative unfolding with dissociation and unfolding of native dimer occurring in a single step whereas bsSHMT and eAAT undergo a non cooperative unfolding with stabilization of intermediates during the unfolding process. These observations suggest that for the family of fold type I of PLP binding proteins the thermophilc proteins unfold more cooperatively than their mesophilic counterparts.

ACKNOWLEDGMENT

We thank Prof. Appaji Rao and Prof. HS Savithri for providing the clone of bstSHMT. The DST supported IRHP DSC facility is thanked for DSC experiments. Dr. C. M. Gupta is thanked for his constant support and helpful discussions.

REFERENCES

1. Clarke, J., Cota, E., Flower, S. B., and Hamill, S. J. (1999) *Structure 7*, 1145−1153.
2. Perl, D., Welker, C., Schindler, T., Schroder, K., Marahiel, M. A., Jaenicke, R., and Schimd, F. X. (1998) *Nat. Struct. Biol. 256*, 229−235.
3. Kargelund, B. B., Hojrup, P, Jensen, M. S., Schjerling, C. K., Jull, E., Knurdsen, J., and Poulsen, F. M. (1996) *J. Mol. Biol. 256*, 187−200.
4. Hooke, S. D., Radford, S. E., and Dobson, C. M. (1994) *Biochemistry 33*, 5867−5876.
5. Stackhouse, T. M., Onuffer, J. J., Matthews, C. R., Ahmed, S. A., and Miles, E. W. (1988) *Biochemistry 27*, 824−832.
6. Dalessio, P. M., and Ropson, I. J. (2000) *Biochemistry 39*, 860−871.
7. Hollien, J., and Marqusee, S. (2002) *J. Mol. Biol. 316*, 327−340.
8. Dams, T., and Jaenicke, R. (1999) *Biochemistry 38*, 9169−9178.
9. Ogasahara, K., Nakamura, M., Nakura, S., Tsunasawa, S., Kato, I., Yoshimoto, T., and Yutani, K. (1998) *Biochemistry 37*, 17527−17544.
10. Pascarella, S., Schirich, V., and Bossa, F. (1993) *FEBS Lett. 331*, 145−149.
11. Grishin, N. V., Phillips, M. A., and Goldsmith, E. J. (1995) *Protein Sci. 4*, 1291−1304.
12. Schneider, G., Kack, H., and Lindqvist, Y. (2000) *Structure 8*, R1−R6.
13. Rao, N. A., Talwar, R., and Savithri, H. S. (2000) *Int. J. Biochem. Cell Biol. 32*, 405−416.
14. Trivedi, V., Gupta, A., Jala, V. R., Saravanan, P., Rao, J., Rao, N. A., Savithri, H. S., and Subramanya, H. S. (2002) *J. Biol. Chem.* 277, 17161−17169.
15. Akhtar, Md. S., Ahmed, A., and Bhakuni, V. (2002) *Biochemistry 41*, 3819−3827.
16. Cai, K., Schirch, D., and Schirch, V. (1995) *J. Biol. Chem. 270*, 19294−19299.
17. Cai, K., and Schirch, V. (1996*) J. Biol. Chem. 271*, 27311−27320.
18. Jager, J., Moser, M., Sauder, U., and Jonsonius, JN.(1994) *J. Mol. Biol. 239*, 285−305.
19. Prakash, K, Prajapati, S, Ahmad, A, Jain, S. K., and Bhakuni, V.(2002) *Protein Sci. 11*, 46−57.
20. Stryer, L. (1965) *J. Mol. Biol. 13*, 482−485.
21. Herold, M., and Kirschner, K. (1990) *Biochemistry 29*, 1907−1913.
22. Kraulis, P. J. (1991) *J. Appl. Crystallog. 24*, 946−950.
23. Mayers, L. M., and Schmid, F. X. (1993) *Biochemistry 32*, 7994−7998.
24. Nicholls, A. (1992) *GRASP, Graphical representation and analysis of surface potential properties, Columbia University, New York.*
25. Ahmad, A., Akhtar, S. Md., and Bhakuni, V. (2001) *Biochemistry 40*, 1945−1955.

BI020356I