The C-terminal domain of dimeric serine hydroxymethyltransferase plays a key role in stabilization of the quaternary structure and cooperative unfolding of protein: Domain swapping studies with enzymes having high sequence identity

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Abstract

The serine hydroxymethyltransferase from *Bacillus subtilis* (bsSHMT) and *B. stearothermophilus* (bstSHMT) are both homodimers and share ~77% sequence identity; however, they show very different thermal stabilities and unfolding pathways. For investigating the role of N- and C-terminal domains in stability and unfolding of dimeric SHMTs, we have swapped the structural domains between bs- and bstSHMT and generated the two novel chimeric proteins bsbstc and bstbsc, respectively. The chimeras had secondary structure, tyrosine, and pyridoxal-5′-phosphate microenvironment similar to that of the wild-type proteins. The chimeras showed enzymatic activity slightly higher than that of the wild-type proteins. Interestingly, the guanidium chloride (GdmCl)–induced unfolding showed that unlike the wild-type bsSHMT, which undergoes dissociation of native dimer into monomers at low guanidium chloride (GdmCl) concentration, resulting in a non-cooperative unfolding of enzyme, its chimera bsbstc, having the C-terminal domain of bstSHMT was resistant to low GdmCl concentration and showed a GdmCl-induced cooperative unfolding from native dimer to unfolded monomer. In contrast, the wild-type dimeric bstSHMT was resistant to low GdmCl concentration and showed a GdmCl-induced cooperative unfolding, whereas its chimera bstbsc, having the C- terminal domain of bsSHMT, showed dissociation of native dimer into monomer at low GdmCl concentration and a GdmCl-induced non-cooperative unfolding. These results clearly demonstrate that the C-terminal domain of dimeric SHMT plays a vital role in stabilization of the oligomeric structure of the native enzyme hence modulating its unfolding pathway.

Keywords: serine hydroxymethyltransferase; chimera; thermophilic; mesophilic; unfolding; guanidine hydrochloride; dissociation; cooperative unfolding

Serine hydroxymethyltransferase (SHMT), L-serine:tetrahydrofolate 5, 10-hydroxymethyltransferase is a pyridoxal-5′-phosphate (PLP)–dependent enzyme. SHMT reaction plays a major role in cell physiology as it is considered to be a key enzyme in the pathway for interconversion of folate coenzymes, which provides almost exclusively one carbon fragment for the biosynthesis of a variety of end products such as DNA, RNA, ubiquinone, and methionine (Schirch 1982; Shane 1989; Mc Neil et al. 1996). The physiological role of SHMT is the reversible interconversion of serine to glycine and irreversible hydrolysis of 5,10-CH^+-H_4PteGlu to 5-CHO-H_4PteGlu. SHMT shows a ubiquitous distribution

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Abbreviations: SHMT, serine hydroxymethyltransferase; PLP, pyridoxal-5′-phosphate; PyP, pyridoxamine-P; SEC, size exclusion chromatography; ESI-MS, electron spray ionization mass spectroscopy; Tm, midpoint of thermal denaturation; GdmCl, guanidium chloride.

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in nature, being found both in prokaryotes and eukaryotes (Venkatesha et al. 1998). SHMT from *Escherichia coli* and from several bacterial sources is dimeric, whereas enzyme from mammalian sources is a homotetramer; hence for SHMT, the dimer is the minimum structure necessary for the catalytic activity (Renwick et al. 1998; Venkatesha et al. 1998).

The subunit molecular weight (M_r) of SHMT ranges from 45 to 53 kDa (Chaturvedi and Bhakuni 2003). SHMTs from *E. coli* as well as from several other bacterial sources are dimeric and contain 2 mole of PLP per mole of enzyme; however, the *Mycobacterium tuberculosis* enzyme SHM1 is an exception as it has only one PLP per enzyme dimer (Chaturvedi and Bhakuni 2003). X-ray crystallographic study on bstSHMT has suggested that monomer of the enzyme is comprised of two major domains (Trivedi et al. 2002): the N-terminal domain (residues 1–279) and a C-terminal domain (residues 280–405). The N-terminal domain can be further divided into two subdomains, a small N-terminal domain (residues 1–80) and a large PLP binding domain (residues 81–279).

In pyridoxal-P–dependent enzymes, the N terminus has been shown to play an important role in inter-subunit interactions (Sandmeier and Christen 1980; Schirch et al. 1986). For sheep liver cytosolic SHMT, which is a tetrameric enzyme, N terminus deletion studies have demonstrated that in addition to the cofactor PLP, the N-terminal arm of enzyme plays an important role in stabilizing the tetrameric structure of SHMT (Jagath et al. 1997). This was further supported by the recently reported targeted mutagenesis studies (Jala et al. 2003), in which several amino acids of the N-terminal domain of enzyme were found to play an important role in stabilization of tetrameric configuration of the enzyme. However, no information on the role of N- or C-terminal domain in stabilization of dimeric configuration of SHMT is available.

SHMT from *Bacillus subtilis* (bsSHMT) and *B. stearothermophilus* (bstSHMT) are both homodimers and share a very high degree of sequence identity of ~77%. However, compared with bsSHMT, bstSHMT shows a significantly higher stability both against thermal and urea denaturation (Bhatt et al. 2002). Equilibrium unfolding studies (Bhatt et al. 2002) have shown that bsSHMT unfolding is a two-step process, with the initial step being the dissociation of the native dimer into monomer followed by the unfolding of the stabilized monomeric species. In contrast, the bstSHMT unfolding is a highly cooperative process in which the dissociation and unfolding of the native dimer occurs simultaneously without stabilization of any folded monomer.

In this article, we present comparative structural and denaturation studies on the two domain-swapped chimeric isoenzymes, bsbstc and bstbsc, and their wild-type counterpart bs- and bstSHMT. The analysis of the obtained results in light of the role of N- and C-terminal domains of the enzyme in stabilization of native dimeric configuration and modulation of cooperativity associated with denaturation of enzyme molecule has also been discussed.

Results and Discussion

In pyridoxal-P–dependent enzymes, limited proteolysis results in the formation of a core protein devoid of N terminus (Sandmeier and Christen 1980; Schirch et al. 1986). Studies using these fragmented proteins have provided significant information on the role of N-terminal domain of these enzymes on the stabilization of quaternary structure and also the catalytic activity (Sandmeier and Christen 1980; Kim and Churchich 1983; Schirch et al. 1986). Based on the information from the crystal structure of the tetrameric SHMTs, it was suggested that the N-terminal regions of the enzyme clasp across the "tight dimers," conferring stability on the tetramer of enzyme (Scarsdele et al. 2000). Studies on sheep liver cytosolic SHMT have shown that the apoenzyme lacking first 16 N-terminal residues is present predominantly in dimeric form (Jagath et al. 1997), suggesting that N-terminal arm of the enzyme along with the cofactor PLP stabilizes the quaternary structure of tetrameric SHMT (Scarsdele et al. 2000). The crystal structure of several dimeric SHMTs is reported (Trivedi et al. 2002). The dimeric SHMTs have also been extensively characterized with respect to its catalytic function, but no information on the role of N- or C-terminal domain in stabilization of the native conformations of these dimeric enzymes is available.

For obtaining a core protein devoid of N-terminal domain, limited proteolysis of both dimeric bs- and bstSHMT using α-chymotrypsin was carried out. Figure 1 shows the SDS-PAGE profile of native bs- and bstSHMT on proteolysis with α-chymotrypsin. For bsSHMT, two major protein bands, corresponding to the native protein (Band I) and to the proteolysed fragment (Band II) of the bsSHMT along with several minor low-molecular-weight protein bands were observed on treatment of enzyme with α-chymotrypsin. The N-terminal sequencing of the protein corresponding to the major fragmented band (Band II) showed that it had the N-terminal amino acid sequence TNKYA (data not shown). This suggests that proteolysis of bstSHMT with α-chymotrypsin led to removal of first 47 amino acid residues from the N-terminal sequence of protein. However, for bstSHMT no effect of α-chymotrypsin on the enzyme was observed, as only a single protein band corresponding to the native protein was observed, suggesting that protein is resistant to the protease. These results demonstrated that the core protein devoid of N terminus could not be obtained by limited proteolysis of bstSHMT. Hence, for getting the information on the role of N- and C-terminal domain of dimeric SHMT in protein stability and folding, we took a domain swapping approach in which we swapped the C-terminal domains of bs- and bstSHMT and investigated the

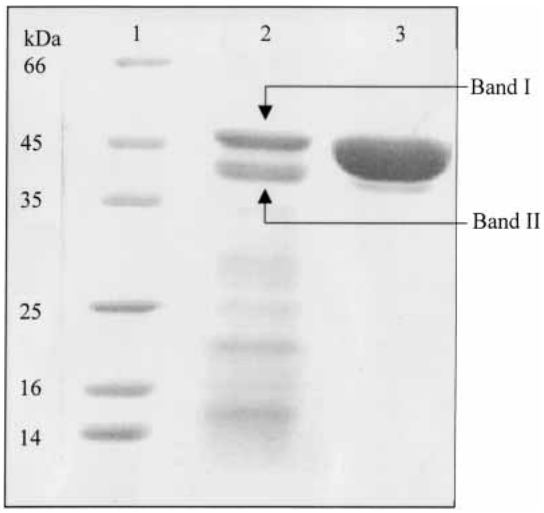

Figure 1. SDS-PAGE analysis of bst- and bsSHMT on limited proteolysis with α-chymotrypsin. The protein-to-protease ratio was 100:1. Lanes *1* to *3* represent molecular weight markers, α-chymotrypsin–treated bsSHMT, and α-chymotrypsin–treated bstSHMT, respectively. The protease and protein samples were incubated for 1 h at 4°C. The reaction was stopped by using protease inhibitor cocktail (Sigma). The samples were analyzed by SDS-PAGE.

resultant chimeric proteins for alterations in the structural and functional properties and folding pathway compared with their wild-type counterparts.

Structural and functional characterization of the chimeras

The fragments encoding the N- and C-terminal domains of bs- and bstSHMT were amplified independently and ligated to exchange C-terminal domains between their conserved Glu and Phe residues, toward the end of the N-terminal domain, encoded by the EcoRI site (Fig. 2A). The resulting chimeric proteins were called bsbstc (N-terminal domain of bsSHMT with the swapped C-terminal domain of bstSHMT) and bstbsc (N-terminal domain of bstSHMT with the swapped C-terminal domain of bsSHMT), having 402 and 413 residues, respectively (Fig. 2B). The proteins were expressed in *E. coli* and purified to homogeneity (Fig. 3A). The observed molecular weights of the chimeric proteins bsbstc and bstbsc were 45.12 and 46.53kDa, respectively. The obtained chimeras showed a purity of >98% as determined by electron spray ionization mass spectroscopy (ESI-MS[1]; data not shown). The effect of the exchange of C-terminal domain on the molecular dimension and quaternary structure of bs- and bstSHMT was analyzed by carrying out size-exclusion chromatography (SEC) and glutaraldehyde cross-linking studies on the wild-type proteins and the chimeras. Figure 3B summarizes the SEC[1] profile of the bs- and bstSHMT and the chimeras bsbstc and bstbsc on an S-200 Superdex column. The native bsSHMT has a retention volume of 13.6 mL, but for its C-terminal exchanged chimera bsbstc, a significantly higher retention volume of 14.3 mL was observed under identical conditions. Similarly, the native bstSHMT has a retention volume of 13.8 mL, but its C-terminal exchanged chimera bstbsc has a significantly higher retention volume of 14.6 mL under identical conditions (Fig. 3B). As the wild-type enzymes and the chimeras are of almost similar molecular weight of ~45kDa, the higher retention volume for chimeras on SEC suggests a significantly reduced molecular dimension for them compared with their wild-type counterparts. Under identical conditions, the decrease in the molecular dimension of the chimeras compared with the wild-type enzymes is possible only either if in the native conformation they are more compact than the wild-type protein or if they are present as monomer rather than dimer that the native wild-type proteins are. To dissect between these two possibilities, glutaraldehyde cross-linking studies on bsbstc and bstbsc were carried out. Figure 3B, inset, shows the SDS-PAGE profile of the glutaraldehyde cross-linked bsbstc and bstbsc. For both the cross-linked chimeras, a single protein band of ~90 kDa was observed on SDS-PAGE. As the molecular weight of ~45 kDa was obtained for the chimeras from ESI-MS, the molecular weight of 90 kDa obtained from cross-linking studies for them suggests that the two chimeras exist as dimers in native state under physiological conditions. Hence, the reduction in molecular dimension for the chimeras compared with their wild-type proteins is due to the compaction of native conformation of enzyme as a result of swapping of the C-terminal domain in these proteins.

The effect of domain swapping on the structural features of the enzyme was studied by carrying out comparative far-UV, near-UV, and visible CD studies on the two chimeric proteins and their wild-type counterparts. Figure 4A summarizes the comparison of the secondary structure present in the chimeras bsbstc and bstbsc and their wild-type proteins bs- and bstSHMT. A very similar far-UV CD spectrum with ellipticity maxima at 208 and 222 nm, typical of proteins with high α-helical content, was observed for the wild-type and chimeric proteins. These observations demonstrate that no significant alteration occurs in secondary structure of the enzymes on swapping of C-terminal domain.

The native SHMT shows a strong near-UV and visible CD signal due to the presence of buried tyrosine residues and the bound PLP in the enzyme, respectively (Strickland 1972). Figure 4B summarizes the comparison of the near-UV and visible CD spectra of the chimeras bsbstc and bstbsc and their wild-type proteins bs- and bstSHMT. For both the wild-type enzymes and the chimeras, a strong near-UV and visible CD signal was observed, suggesting the presence of buried tyrosine residues and bound PLP in all the proteins studied. Furthermore, compared with the wild-type

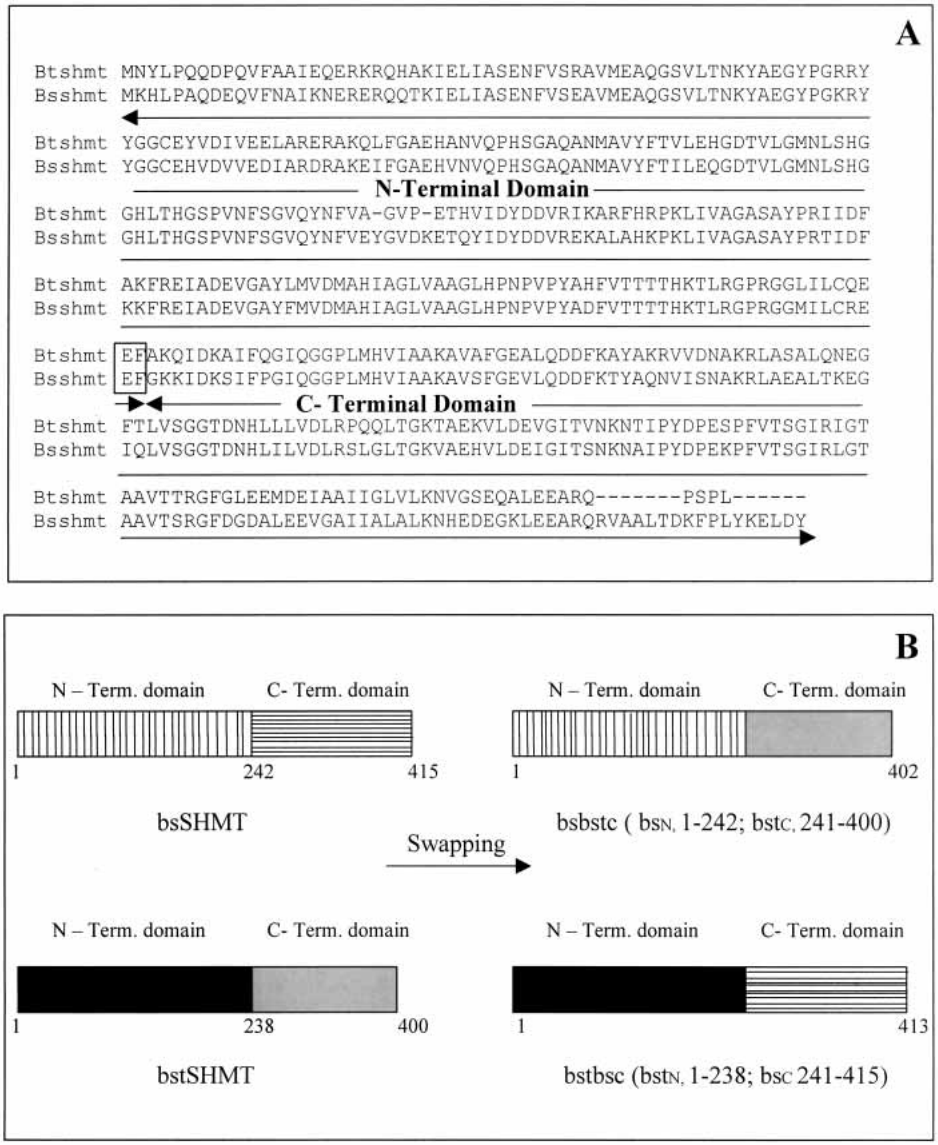

Figure 2. (*A*) Sequence alignment of bst- and bsSHMT performed by using the program clustarW showed the N- and C-terminal domains of SHMT. The open box shows the position of conserved E and F residues at the end of N-terminal domain. (*B*) Schematic diagram showing the swapping of the C-terminal domains between bst- and bsSHMT performed for this study. The numbers denote the place of amino acid in the primary sequence of the protein.

proteins, a significantly higher visible CD signal at 420 nm was observed for the chimeric proteins. This observation suggests that the protein bound PLP in the wild-type proteins is present in a less asymmetric environment compared with that in the chimeric proteins.

The presence of exposed hydrophobic clusters in the native conformation of the four proteins was studied by using ANS fluorescence (Stryer 1965) and is summarized in Figure 4C. For the wild-type proteins bsSHMT and bstSHMT, ANS fluorescence having emission wavelength maxima at 465 nm was observed, suggesting the binding of ANS molecules to the exposed hydrophobic patches present in the native conformation of both these enzymes. However, at the same protein concentration, the ANS fluorescence intensity observed for bsSHMT was significantly higher than that for the bstSHMT. This suggests a significantly higher exposure of hydrophobic surfaces in native bsSHMT compared with bstSHMT. For the two chimeras bsbstc and bstbsc, ANS fluorescence maxima at 520 nm with fluorescence intensity similar to that for the free ANS was observed that suggests that ANS molecules did not bind to the native conformation of the two chimeras (Stryer 1965). This is possible only when there are no significantly exposed hydrophobic clusters present in the native conformation of these two chime-

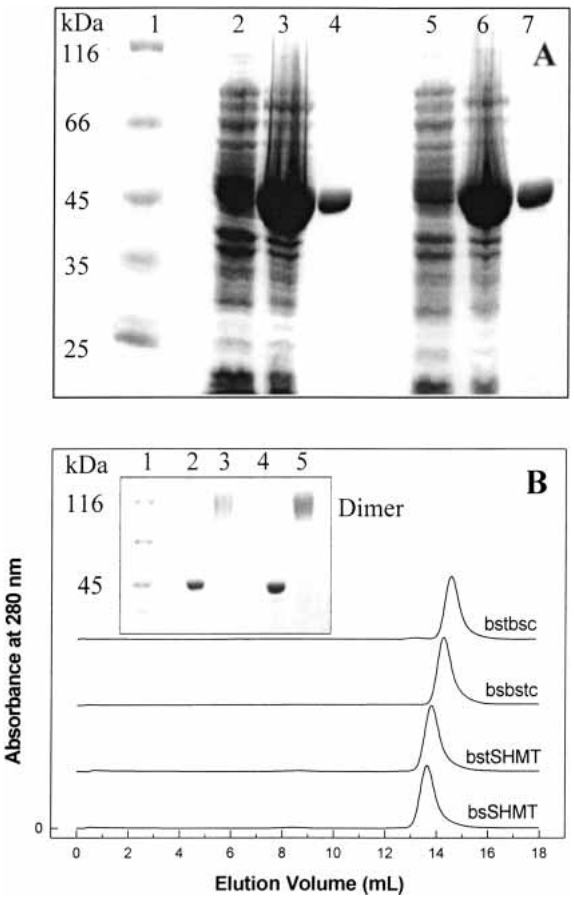

Figure 3. (*A*) SDS-PAGE analysis of *E. coli* lysate overexpressing the chimeras bsbstc and bstbsc. (Lane *1*) Molecular weight markers. (Lanes *2–4*) The cell lysates of uninduced and induced cultures and the purified protein, respectively, for bsbstc. (Lanes *5–7*) Cell lysates of uninduced and induced cultures and the purified protein, respectively, for bstbsc. (*B*) Size-exclusion chromatographic profiles for bsSHMT, bstSHMT, bsbstc, and bstbsc on Superdex 200HR column at pH 7.5 and 25°C. (*Inset*) Profile of glutaraldehyde cross-linked chimeras. (Lane *1*) Molecular weight markers. (Lanes *2*,*3*) Un–cross-linked and cross-linked samples of bsbstc at pH 7.5. (Lanes *4*,*5*) Un–cross-linked and cross-linked samples of bstbsc in the same condition.

ras. These observations indicate that probably the chimeras have a compact conformation with the hydrophobic portions buried in the core of the protein, whereas the wild-type proteins due to the presence of exposed hydrophobic portions in native conformation have an open conformation. This possibility is also supported by the results of SEC, in which the wild-type proteins show greater hydrodynamic radii compared with the chimeras (Fig. 3B), demonstrating an open conformation for the wild-type enzymes compared with the chimeras.

The effect of domain swapping on the functional properties of the enzymes was studied by monitoring the enzyme activity of the bs- and bstSHMT and their chimeric proteins bsbstc and bstbsc and is summarized in Figure 4D. The bsSHMT showed significantly lesser enzymatic activity than did bstSHMT. However, the chimeras, bsbstc and bstbsc, showed significantly higher enzymatic activities compared with the wild-type proteins. The chimera bsbstc showed the maximum enzymatic activity among the four proteins studied.

The results of the enzymatic activity studies in combination with the ANS binding, SEC, and visible CD studies on wild-type and chimeric proteins of bs- and bstSHMT, as reported above, suggest that the swapping of C-terminal domain between the bs- and bstSHMT results in burial of the exposed hydrophobic clusters present in the native conformation of the wild-type proteins. This will lead to a difference in ratio of exposed nonpolar to polar surface area in native conformation of wild-type and chimeric proteins, and probably as a result of this, the chimeric proteins have a more compact structure compared with wild-type proteins. In SHMT the protein-bound PLP has to be in an asymmetric environment for elucidating enzymatic activity (Cai et al. 1995). The studies presented above demonstrate that the protein-bound PLP in chimeric proteins is in a higher asymmetric environment than that in the wild-type protein, and this might be the reason for the higher enzymatic activity observed for the chimeric proteins compared with wild-type counterparts.

GdmCl-induced equilibrium unfolding

For understanding the role of structural domains of SHMT in modulating the unfolding behavior of the enzyme, comparative GdmCl-induced unfolding studies on the chimeras and their wild-type counterparts were carried out.

The alterations in the secondary structure of wild-type and chimeric proteins during GdmCl-induced denaturation were studied by monitoring the changes in the CD ellipticity at 222 nm. As no intrinsic fluorophore (tryptophan molecule) is present in bs- or bstSHMT, the reduction of bound PLP resulting in the formation of pyridoxyl-P secondary amine, namely, pyridoxamine-P (PyP) that is fluorescent (Cai and Schirch 1996), was carried out. By this modification, a fluorescent probe is placed in the region of the enzyme between two domains, namely, the large N-terminal 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 on the unfolding of the enzyme. For all the four proteins studied, a significant difference between fluorescence emission wavelength maxima of PyP was observed for native (~389 nm) and denatured (~398 nm) enzymes. Hence, correlation between the GdmCl concentration and the fractional changes in emission wavelength maxima was plotted for extracting information on stabilization of intermediates during GdmCl-induced unfolding of enzymes (Prakash et al. 2002).

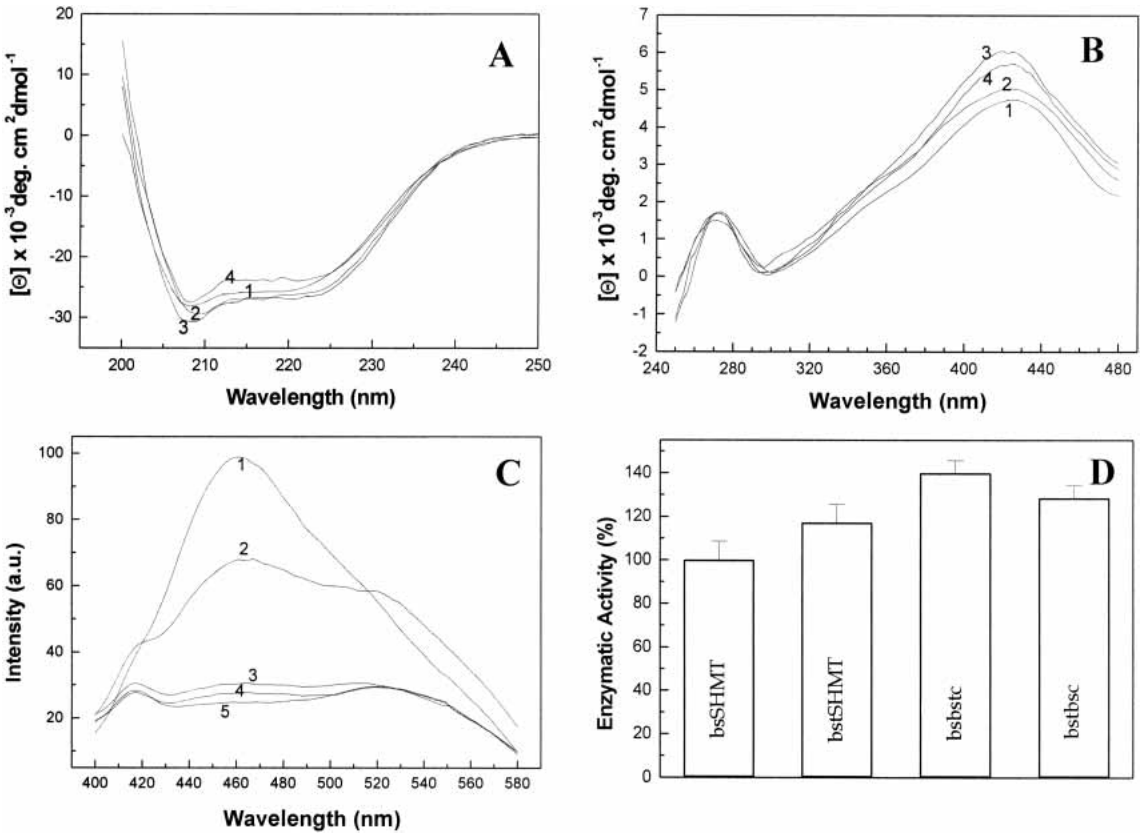

Figure 4. Structural properties of bsSHMT, bstSHMT, bsbstc, and bstbsc at pH 7.5 and 25°C. (*A*) Near-UV CD spectra of native bsSHMT (curve *1*), bstSHMT (curve *2*), bsbstc (curve 3), and bstbsc (curve *4*). (*B*) Visible and near-UV CD spectra of native bsSHMT, bstSHMT, bsbstc, and bstbsc. The numbering is the same as in *A*. (*C*) Changes in ANS fluorescence spectra on incubation with wild-type and chimeric proteins. The curves *1* to *5* represent samples of bsSHMT, bstSHMT, bsbstc, bstbsc, and free ANS, respectively. (*D*) Relative enzymatic activity of bsSHMT, bstSHMT, bsbstc, and bstbsc. The data have been presented as percentages with the value obtained for bsSHMT taken as 100%.

Figure 5, A and B, shows the changes in mean residual ellipticity at 222 nm and fractional changes in emission wavelength maxima of PyP fluorescence, respectively, with increasing concentration of GdmCl for the bsSHMT and chimera bsbstc. For bsSHMT, between 0 and 4 M GdmCl, a biphasic dependence of loss of CD signal at 222 nm (Fig. 5A), and changes in PyP fluorescence (Fig. 5B) were observed with increasing GdmCl concentration, suggesting the stabilization of an intermediate at low GdmCl concentration during the unfolding process. The stabilization of an intermediate during GdmCl-induced unfolding of bsSHMT was further confirmed by ANS fluorescence studies. The ANS molecules do not bind to the fully folded or fully unfolded proteins but show strong binding to partially unfolded intermediates due to the presence of exposed hydrophobic residues in them (Stryer 1965; Prakash et al. 2002). Figure 5C shows the changes in ANS fluorescence at 465 nm on incubation with bsSHMT treated with increasing concentration of GdmCl. A single peak centered at 0.5 M GdmCl was observed for changes in ANS fluorescence for bsSHMT incubated at increasing GdmCl concentration, suggesting that an intermediate of bsSHMT with significantly exposed hydrophobic clusters is stabilized at 0.5 M GdmCl. These results suggest that the bsSHMT undergoes a GdmCl-induced non-cooperative unfolding. Earlier reported studies have demonstrated that low GdmCl concentration induces dissociation of the native enzyme dimer of bsSHMT, resulting in stabilization of a folded monomer of enzyme (Bhatt et al. 2002). So during GdmCl-induced unfolding, first the native dimer of bsSHMT dissociates into monomer, followed by the unfolding of the stabilized monomer. In contrast, for the chimera bsbstc, a sigmoidal loss of CD ellipticity at 222 nm with increasing concentration of GdmCl was observed between 0 and 3 M GdmCl (Fig. 5A). Furthermore, an almost complete loss of CD signal at 222 nm was observed at 3 M GdmCl, suggesting the presence of an unfolded protein at the end of the observed transition. For fractional changes in emission wavelength maxima of PyP fluorescence with increasing GdmCl concentration also, a sigmoidal dependence of change in population of native

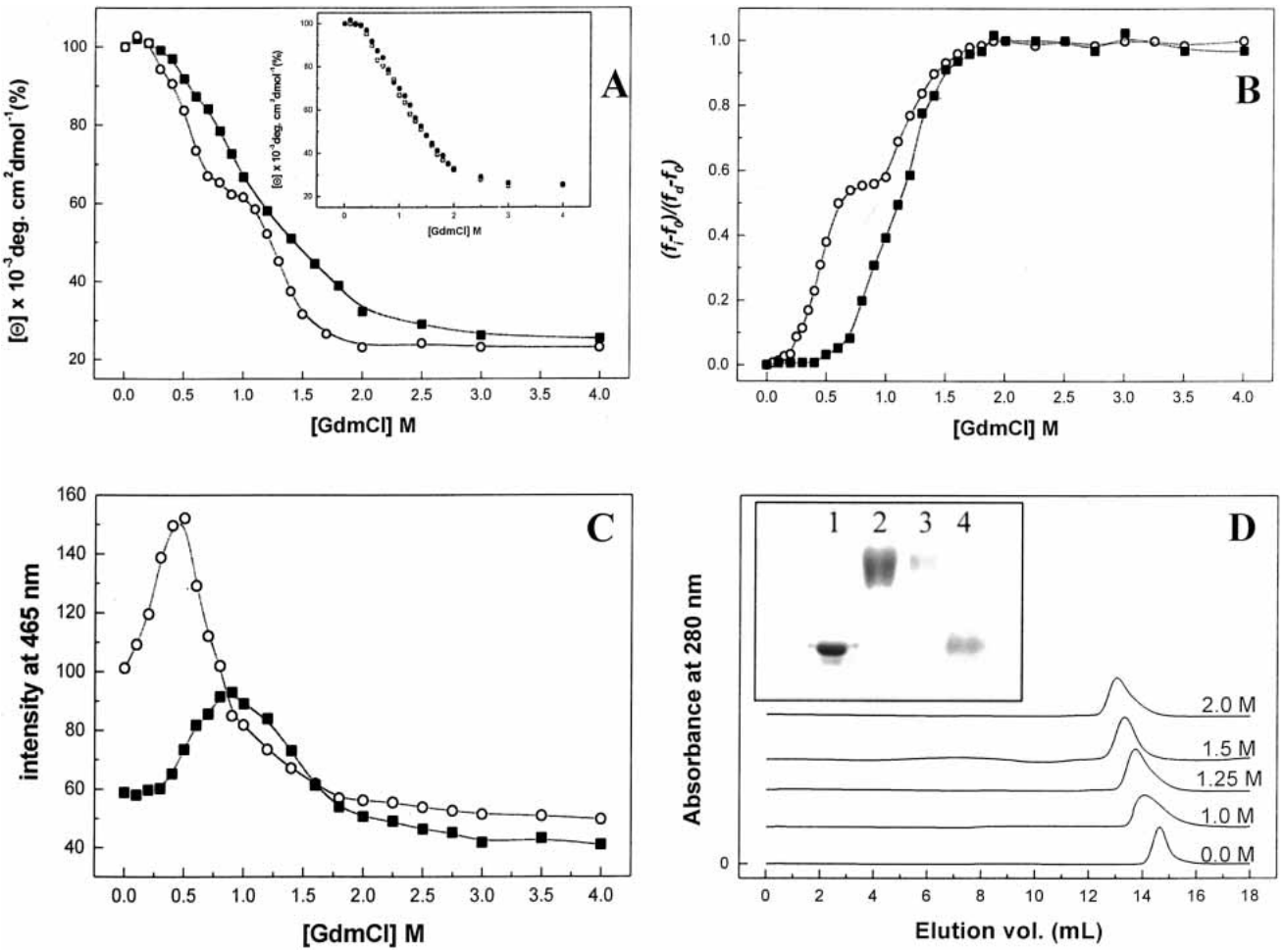

Figure 5. Equilibrium GdmCl-induced unfolding of bsSHMT and the chimera bsbstc, as studied by optical spectroscopic techniques and size-exclusion chromatography. (*A*) GdmCl-dependent unfolding of bsSHMT (circles) and bsbstc (squares) measured by far-UV CD spectroscopy monitored at 222 nm. The data are represented as percentage of ellipticity at 222 nm, taking the value for the native enzyme to be 100%. (*Inset*) The protein concentration dependent changes in CD ellipticity at 222 nm for bsbstc. The symbols in the inset represent 1 μM (open symbols) and 5 μM (filled symbols) protein concentration data. (*B*) Plot of fractional change in the wavelength of maximum fluorescence emission of PyP for bsSHMT and bsbstc, $(f_i - f_o)/(f_d - f_o)$ 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 is the wavelength at GdmCl concentration >3 M. The excitation wavelength was 335 nm, and the emission was recorded from 350 to 450 nm. The final enzyme concentration was 1 μM. The symbols are the same as in *A*. (*C*) GdmCl-induced unfolding of bsSHMT (circles) and bstbsc (squares) monitored by following the changes in ANS fluorescence at 465 nm. (*D*) Size-exclusion chromatographic profiles for GdmCl-treated bsbstc on Superdex 200HR column at pH 7.5 and 25°C. The numbers in the figure represent concentrations of GdmCl at which the samples were incubated and run on the column. (*Inset*) The profile of glutaraldehyde cross-linked GdmCl-treated bsbstc samples. (Lane *1*) Uncross-linked bsbstc native protein. (Lanes *2–4*) Glutaraldehyde cross-linked samples of 0, 1, and 2 M GdmCl-treated bsbstc samples.

dimer of protein to unfolded monomer was observed. For bsbstc, the sigmoidal dependence of the monitored signals (both CD and fluorescence) with the increasing concentration of GdmCl suggests that the protein undergoes a cooperative unfolding of the native dimer without stabilization of any monomeric intermediate during this process. This is also supported by the lack of protein concentration dependence of the denaturation profile of bsbstc as observed by far-UV CD studies (Fig. 5A, inset). Furthermore, the ANS fluorescence studies (Fig. 5C) did not show very significant enhancement in fluorescence intensity on incubation of enzyme with increasing GdmCl concentration, thus supporting the absence of stabilization of any intermediate during GdmCl-induced unfolding of bstSHMT. The absence of any monomeric intermediate during GdmCl-induced unfolding of bsbstc is further confirmed by the SEC in conjunction with glutaraldehyde cross-linking studies on bsbstc at increasing concentration of GdmCl as summarized in Figure 5, D and inset. For all the samples studied, only a single peak on SEC was observed, suggesting the presence of a single species of protein under these conditions. However, there was a progressive decrease in retention volume from

14.3 mL for the native protein to 13.05 mL for 2 M GdmCl-treated bsbstc samples treated with increasing concentration of GdmCl. This observation suggests a progressive enhancement in the hydrodynamic radii of the bsbstc samples on treatment with increasing concentration of GdmCl. Such a situation can occur only when the dimeric enzyme undergoes progressive unfolding without dissociation into monomers. This is also supported by the glutaraldehyde cross-linking studies (Fig. 5D, inset), in which protein dimer is observed even at 1 M GdmCl. However, at high GdmCl concentration, ~2 M, in which the unfolding transition is over and the protein is unfolded, protein monomeric species were observed.

Figure 6, A and B, shows the changes in mean residual ellipticity at 222 nm and fractional changes in emission wavelength maxima of PyP fluorescence respectively, with increasing concentration of GdmCl for bstSHMT and chimera bstbsc. For bstSHMT, a sigmoidal dependence of loss of CD signal at 222 nm (Fig. 6A) with increasing GdmCl concentration was observed. Furthermore, an almost complete loss of CD signal at 222 nm was observed at the end of the transition, suggesting a complete unfolding of enzyme associated with the transition. The changes in PyP fluorescence of bstSHMT with increasing GdmCl also showed a sigmoidal dependence (Fig. 6B). The ANS fluorescence studies also showed no significant changes in the fluorescence intensity on incubation of the protein with increasing GdmCl concentration (Fig. 6C). These observations collectively suggest a GdmCl-induced cooperative unfolding of the native bstSHMT dimer to the unfolded monomer.

However, for the chimera bstbsc, a biphasic dependence of loss of CD signal at 222 nm with increasing concentration of GdmCl was observed and an intermediate state appeared to be stabilized between 0.95 and 1.2 M GdmCl. The transition midpoints defined by the horizontal base lines and the plateau were at 0.8 M for the first phase and at 1.4 M GdmCl for the second phase. About 40% loss of CD signal at 222 nm was found to be associated with the first transition and a complete loss of secondary structure associated with the second transition (Fig. 6A). Furthermore, the position of the first transition observed at low GdmCl concentration was dependent on the protein concentration, whereas no such dependence of protein concentration was observed for the second transition (Fig. 6A, inset). These observations suggest that the first transition observed at low GdmCl concentration probably corresponds to dissociation of native dimer of enzyme. The stabilization of an intermediate during GdmCl-induced unfolding of bstbsc was also supported by the PyP fluorescence studies (Fig. 6B). For bstbsc, a biphasic dependence of fractional changes in emission wavelength maxima of PyP fluorescence with increasing concentration of GdmCl was observed, and an intermediate state appeared to be stable between 1 and 1.2 M GdmCl. This observation is further supported by the results from ANS fluorescence studies in which a single peak centered at ~1 M GdmCl is observed (Fig. 6C), thus confirming the stabilization of an intermediate of bstbsc at low GdmCl concentration.

The subunit configuration of the intermediate of bstbsc stabilized at low GdmCl concentration was studied by SEC and glutaraldehyde cross-linking and is summarized in Figure 6, D and inset, respectively. The SDS-PAGE of the glutaraldehyde cross-linked sample of 0.9 M GdmCl-treated bstbsc showed a protein band corresponding to monomer of the enzyme (Fig. 6D, inset i), suggesting the dissociation of the native protein dimer resulted in stabilization of a monomer under these conditions. Further characterization of the monomer stabilized at 0.9 M GdmCl was carried out by SEC studies (Fig. 6D, inset ii). For 0.9 M GdmCl-treated bstbsc, three peaks (a, b, and c) with retention volumes of 10.2, 14.5 and 15.1 mL, respectively, were observed. The peak a was observed at a significantly decreased retention volume of 10.2 mL, which is close to the void volume of the column, thus indicating that the protein species under this peak corresponds to a very high-molecular-weight species, probably an aggregated species. The peak b had slightly lesser retention volume than the native protein dimer and hence may correspond to the partially unfolded dimer. The peak c, having higher retention volume, was found to be corresponding to the folded monomer as obtained by the retention volume for the markers on the same column (Fig. 6E). The presence of three peaks suggests that these three species are in equilibrium under the conditions in which the experiment was performed. However, studies using low protein concentration under similar conditions showed only two peaks: one corresponding to monomer of enzyme and another to the aggregated species (Fig. 6D, inset). These results collectively suggest that treatment of bstbsc with low concentration of GdmCl results in dissociation of enzyme. Furthermore, the 0.9 M GdmCl-stabilized monomer of bstbsc was found to undergo aggregation, as demonstrated by the protein concentration-dependent SEC results (Fig. 6D, inset). The reason for observing both the dimeric and monomeric species of enzyme on SEC and not in the glutaraldehyde cross-linking experiment is that a significantly higher protein concentration was used for SEC studies compared with cross-linking studies.

The comparative results of the GdmCl-induced unfolding of the wild-type and the chimeras, as reported above, demonstrate that swapping of C-terminal domain of bsSHMT with that of bstSHMT results in enhancement in cooperativity in the enzyme molecule, and the resultant chimera bsbstc undergoes cooperative unfolding from dimer to unfolded monomer rather than a non-cooperative unfolding with stabilization of folded monomer, as is the case for its wild-type protein bsSHMT. In contrast, the swapping of C-terminal domain of bstSHMT with that of bsSHMT de-

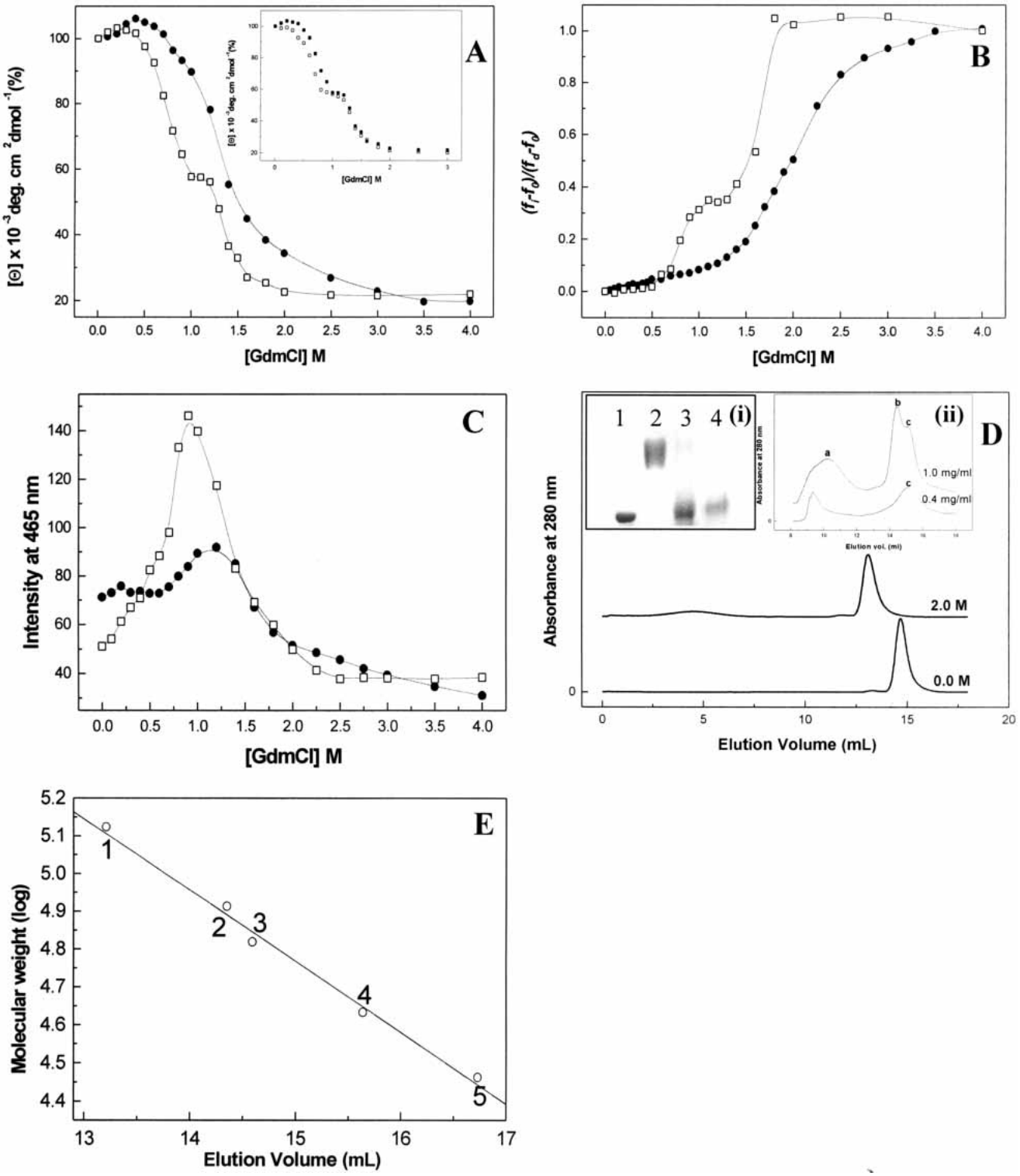

Figure 6. Equilibrium GdmCl-induced unfolding of bstSHMT and the chimera bstbsc as studied by optical spectroscopic techniques and size-exclusion chromatography. (*A*) GdmCl-dependent unfolding of bstSHMT (circles) and bstbsc (squares) measured by far-UV CD spectroscopy monitored at 222 nm. The data are represented as percentages of ellipticity at 222 nm, taking the value for the native enzyme to be 100%. (*Inset*) Protein concentration dependent changes in CD ellipticity at 222 nm for bsbstc. The symbols represent 1 μM (open symbols) and 5 μM (filled symbols) protein concentration data. (*B*) Plot of fractional change in the wavelength of maximum fluorescence emission of PyP for bstSHMT and bstbsc, $(f_i - f_o)/(f_d - f_o)$ 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 is 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 *A*. (*C*) GdmCl-induced unfolding of bstSHMT (*circles*) and bsbstc (*squares*) monitored by following the changes in ANS fluorescence at 465 nm. (*D*) Size-exclusion chromatographic profiles for GdmCl-treated bstbsc on Superdex 200HR column at pH 7.5 and 25°C. (*Inset i*) The profile of glutaraldehyde cross-linked GdmCl-treated bstbsc samples. (Lane *1*) Un–cross-linked bstbsc native protein. (Lanes *2–4*) represent glutaraldehyde cross-linked samples of 0, 0.9, and 2 M GdmCl-treated bstbsc samples. (*Inset ii*) shows the size-exclusion chromatography (SEC) profiles of 0.9 M GdmCl-treated bstbsc samples at protein concentrations of 0.4 and 1 mg/mL. (*E*) Plot of elution volume vs. molecular weight (log) of standard marker proteins on SEC. The molecular weight markers (given as numbers) are *1*, 133 kDa (*E. coli* MTHFR; *2*, 82 kDa (*Streptococcus* pn. Hyluronate lyase); *3*, 66 kDa (BSA); *4*, 43 kDa (ovalbumin); and *5*, 29 kDa (carbonic anhydrase). The buffer used was 50 mM Tris.Cl (pH 7.5) containing 100 mM NaCl.

creases the cooperativity within the enzyme, and the resultant chimera bstbsc undergoes non-cooperative unfolding with stabilization of a folded monomer in contrast to the native enzyme, which shows cooperative unfolding from dimer to unfolded monomer.

The results of the comparative studies on the GdmCl-induced denaturation of the wild-type and the chimeric proteins, as reported in this article and summarized in Figure 7, demonstrate that the wild-type protein bsSHMT and the chimera bstbsc—both having C-terminal domain of bsSHMT but N-terminal domain of bsSHMT and bstSHMT, respectively—undergo dissociation into monomers at low GdmCl treatment. However, the wild-type protein bstSHMT and the chimera bsbstc, both of which have C-terminal domain of bstSHMT but different N-terminal domains of bstSHMT and bsSHMT, respectively, were resistant to low GdmCl. These results demonstrate that the C-terminal domain of the dimeric enzymes bsSHMT and bstSHMT plays an important role in stabilization of native dimer of SHMT, thus regulating the cooperativity associated with the unfolding processes of these enzymes.

Materials and methods

Designing of chimeras

Clones of bsSHMT and bstSHMT (Bhatt et al. 2002) were used as templates to amplify the fragments necessary for the designing of chimeras. For the chimera bsbstc, different oligonucleotides used for the amplification of the N-terminal domain of bsSHMT (720 bp; 1 to 240 amino acids) were upstream 5′ggAATTCCATAT gAAACATTTACCTgCgCAAgAC 3′ and downstream 5′GGA ATTCTTCACGGCAAAGGATCATACC 3′. Reaction was carried out for 30 cycles in a total volume of 50 μL with 1 U Taq polymerase, and the thermocycler condition was 1 min at 95°C, 1 min at 52°C, and 2 min at 72°C. For the amplification of C-terminal domain of bstSHMT (480 bp; 241 to 400 amino acids), the different oligonucleotides used were upstream 5′ ggAATTC gCCAAACAgATCgACAAAgCCATTTTC 3′ and downstream 5′ CCgCTCgAGTTACgTCAAAgCAgCAACgCgTTg 3′. The amplification condition was the same as for the N-terminal domain except the annealing temperature was 56°C. To construct the gene encoding chimeric protein bsbstc, the N-terminal domain of bsSHMT was initially cloned between the NdeI and EcoRI sites of pET-22b (+) followed by the addition of C-terminal domain of bstSHMT between the EcoRI and XhoI sites. For designing bstbsc chimera, the same strategy as mentioned above was followed. The different oligonucleotides used for N-terminal domain of bstSHMT (714 bp, 1 to 238 amino acids) were upstream 5′ ggAATTCCATATgAAATACTTgCCACAACAAgAT3′, and downstream 5′ggAATTCCAgggAAAACggCTTTgTCgA 3′. For C-terminal domain of bsSHMT, the primers were upstream 5′ggAATTCAAggCggCCCTC3′, and downstream 5′ CCgCTC gAgTTAATAATCTAATTCTTTATATAA 3′. All the PCR conditions were the same as for bsbstc except the annealing temperatures, which were 52°C for the former reaction and 47°C for the later one. The proper construction of the chimeras was confirmed by the sequencing of DNA. The resultant constructs were transformed into BL21 (DE3).

Overexpression and purification of chimeras

The recombinant bsSHMT and bstSHMT were overexpressed and purified as described earlier (Bhatt et al. 2002). A single colony of the desired chimera bsbstc was inoculated into 10 mL LB broth containing 100μg/mL ampicillin and grown overnight at 37°C with vigorous shaking. The cells were then subcultured into 250 mL LB broth containing the same antibiotic and grown till OD 0.6, followed by the induction with 0.5 mM-isopropyl β-D-thiogalactopyranoside. The cells were harvested after 3 h of induction, and the pellet was resuspended in 50 mM Tris buffer (pH 7.5) containing 1 mM EDTA and 2 mM β-mercaptoethanol and stored at −80°C in the presence of 10% glycerol. Frozen cells were thawed

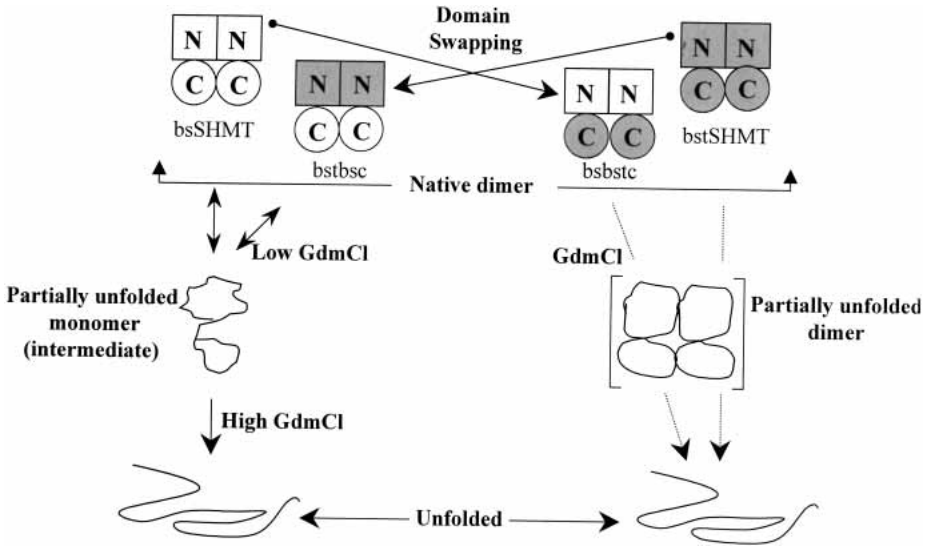

Figure 7. Schematic representation of GdmCl-induced unfolding of wild-type bsSHMT, bstSHMT, and chimeras bsbstc and bstbsc.

on ice and sonicated until the solution was optically clear. The cell lysate was then centrifuged at 24,000*g* for 20 min, and the supernatant was directly loaded onto a Mono-Q column (Amersham Pharmacia Biotech) pre-equilibrated with the same buffer except glycerol. The column was washed with the similar buffer containing 200 mM NaCl, and the protein was eluted by using a linear gradient of buffer containing 200 to 600 mM NaCl. During the gradient the desired protein eluted ~400 mM of NaCl. The obtained protein was ~90% pure. This partially purified protein was then salted out with 65% ammonium sulfate, redissolved, and loaded onto the Superdex 200 HR 10/30 column equilibrated with 50 mM Tris buffer (pH 7.5; containing 1 mM EDTA, 2 mM β-mercaptoethanol, and 50 mM NaCl). The fractions containing the desired protein were pooled and subjected to dialysis against 1 L Tris buffer containing 0.1 mM PLP for 16 h with two changes. The overexpression and purification of the other chimera bstbsc was the same as described for bsbstc except for that it was eluted from Mono Q at ~300 mM NaCl.

SHMT activity

The activity of the enzyme was assayed with threonine as the substrate. The product acetaldehyde was continuously measured by observing the decrease in absorbance at 340 nm upon reduction of NADH in the presence of alcohol dehydrogenase (Schirch and Gross 1968).

GdmCl denaturation of SHMT

SHMT (5 μM) was dissolved in Tris buffer (50 mM at pH 7.5 containing 1 mM EDTA and β-mercaptoethanol) in the presence and absence of increasing concentrations of GdmCl and incubated for overnight at 25°C before taking the measurements.

CD measurements

CD measurements were made 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 measured at an enzyme concentration of 5 μM and 20 μM for far- and near-UV or visible CD measurements, respectively, with a 1-mm cell at 25°C. The values obtained were normalized by subtracting the baseline recorded for the buffer having 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 (1996).

Fluorescence measurements

Fluorescence spectra were recorded with Perkin-Elmer LS 50B spectrofluorometer in a 5-mm path-length quartz cell at 25°C. An aliquot of protein with final concentration of 1 μM was equilibrated at the desired GdmCl concentration overnight at 25°C. The excitation wavelength for PyP fluorescence measurements was 335 nm, and the emission was recorded from 350 to 450 nm.

For ANS fluorescence measurements, 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 10 μM.

Cross-linking using glutaraldehyde

To native and GdmCl-treated wild-type and chimeric SHMTs (100 μg/mL), an aliquot of 25% (w/v) glutaraldehyde was added so as to make a final concentration of 1% glutaraldehyde. This sample was incubated for 10 min at 4°C followed by quenching the cross-linking reaction by adding 200 mM sodium borohydride. After 20-min incubation, 3 μL 10% aqueous sodium deoxycholate was added. The pH of the reaction mixture was lowered to 2 to 2.5 by addition of orthophosphoric acid, which resulted in precipitation of the cross-linked protein. After centrifugation (24,000 *g*, 4°C) the obtained precipitate was redissolved in 0.1 M Tris/HCl (pH 8.0), 1% SDS, and 50 mM β-merceptoethanol; heated at 90°C to 100°C for 2 min; and finally monitored on 8% SDS PAGE.

Size-exclusion chromatography

Gel filtration experiments were carried out on a Superdex 200HR 10/30 column (manufacturer's exclusion limit 600 kDa for proteins) on AKTA FPLC (Amersham Pharmacia Biotech). The column was equilibrated and run with Tris buffer (50 mM containing 1 mM EDTA and 2 mM β-mercaptoethanol) containing the desired GdmCl concentration at 25°C. The SHMT solution (10 μM) was incubated at the desired GdmCl concentrations for overnight at 25°C; 200 μL of this sample was loaded on the column and run at 25°C with a flow rate of 0.3 mL/min.

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