Guanidine hydrochloride-induced reversible unfolding of sheep liver serine hydroxymethyltransferase

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Equilibrium unfolding studies of sheep liver tetrameric serine hydroxymethyltransferase (SHMT, EC 2.1.2.1) revealed that the enzyme assumed apparent random coil structure above 3 M guanidine hydrochloride (GdnHCl). In the presence of non-ionic detergent Brij-35 and polyethylene glycol, the 6 M GdnHCl unfolded enzyme could be completely (> 95%) refolded by a 40-fold dilution. The refolded enzyme was fully active and had kinetic constants similar to the native enzyme. The midpoint of inactivation (0·12 M GdnHCl) was well below the midpoint of unfolding (1·6 ± 0·1 M GdnHCl) as monitored by far UV CD at 222 nm. In the presence of PLP, the midpoint of inactivation shifted to a higher concentration of GdnHCl (0·6 M) showing that PLP stabilizes the quaternary structure of the enzyme. However, 50% release of pyridoxal-5′-phosphate (PLP) from the active site occurred at a concentration (0·6 M) higher than the midpoint of inactivation suggesting that GdnHCl may also act as a competitive inhibitor of the enzyme at low concentrations which was confirmed by activity measurements. PLP was not required for the initiation of refolding and inactive tetramers were the end products of refolding which could be converted to active tetramers upon the addition of PLP. Size exclusion chromatography of the apoenzyme showed that the tetramer unfolds via the intermediate formation of dimers. Low concentrations (0·3–0·6 M) of GdnHCl stabilized at least one intermediate which was in slow equilibrium with the dimer. The binding of ANS was maximum at 0·4–0·6 M GdnHCl suggesting that the unfolding intermediate that accumulates at this concentration is less compact than the native enzyme.

1. Introduction

Serine hydroxymethyltransferase (SHMT, EC 2.1.2.1), a pyridoxal-5′-phosphate (PLP) dependent enzyme, catalyzes the reversible interconversion of serine and glycine, a reaction which is the major source of one-carbon substituted folate cofactors that are essential for the synthesis of thymidylate, methionine and various other biomolecules. The urea-induced reversible unfolding of *Escherichia coli* SHMT (eSHMT), which is a dimer has been studied in detail. eSHMT, denatured with 8·3 M urea, could be refolded completely by a 10-fold dilution into 20 mM Tris buffer, pH 7·5, containing 5 mM of 2-mercaptoethanol (2-ME) and 1 mM ethylenediaminetetraacetic acid (EDTA). The enzyme initially refolds into an apodimer at 4°C, which binds PLP only when the temperature is raised to 30°C (Cai *et al* 1995). Further, the equilibrium unfolding and refolding studies with tryptophan mutants and protease sensitivity during refolding, revealed that the unfolded enzyme has two domains that rapidly fold to form monomers in which the N terminal 55 amino acids and a segment between residues 225–276 are disordered. This partially folded enzyme can form dimers and slowly undergo conformational change to the native state (Cai and Schirch 1996a). Fluorescence energy transfer and fluorescence anisotropy measurements on these tryptophan mutants of eSHMT show that the active site lysine 229 bound PLP

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remains more than 50 Å away from any tryptophan residue until the final rate-determining conformational change when it approaches each Trp residue at the same rate (Cai and Schirch 1996b).

SHMT isolated from sheep liver, on the other hand, is an oligomeric protein of four identical subunits (subunit M_r 53 kDa). The enzyme requires PLP for its catalytic activity. PLP present at the active site linked to lysine 256 of the enzyme via a Schiff's base gives a convenient visible spectral handle to monitor the changes at the active site. Also, the cofactor can be removed from the active site to get apoenzyme or alternatively the Schiff's base can be reduced with $NaBH_4$ to a non-hydrolyzable phosphopyridoxyl lysine adduct at the active site. These two forms of the enzyme can be used to determine the role of the cofactor in the protein folding process.

The sheep liver SHMT has 25% α-helical content as determined by UV CD spectroscopy (Manohar *et al* 1982; Bhaskar *et al* 1994; Brahatheeswaran *et al* 1996). Recently, the 3-dimensional structure of human liver tetrameric SHMT, which shares 90% identity with sheep liver SHMT, was determined (Renwick *et al* 1998). The monomer consists of three domains. The N-terminal small domain (residues 11–53) folds into 2α helices and one β strand. The middle large domain (residues 53–321) binds PLP and contains 9 helices and 7 stranded mixed β sheet. The C-terminal domain also contains an anti-parallel β sheet and 4 helices. The quaternary structure is a 'dimer of dimers' as suggested by us earlier (Jagath *et al* 1997a, b, c). PLP is at the interface of tight dimers and explains our earlier observations on its role in maintaining the oligomeric structure of the enzyme (Brahatheeswaran *et al* 1996; Jagath *et al* 1997a, b, c; Talwar *et al* 1997).

In an earlier study, it was established that the urea-induced unfolding of tetrameric SHMT was reversible only in the presence of a non-ionic detergent, Brij-35, and polyethylene glycol (PEG-3350). The refolding was dependent on the protein concentration and temperature. Maximum refolding was achieved only when the final protein concentration was less than 25 µg/ml and the refolding temperature was 30°C. The tetramer was found to unfold via a 'pre-denaturation transition' characterized by the dissociation of the tetramer into dimers followed by the formation of a putative unfolding intermediate as monitored by the far UV CD and 8-anilinonaphthalene sulfonic acid (ANS) binding properties of the protein at intermediate concentrations of urea (1–2 M). The cofactor, apart from its role in catalysis, served to stabilize the quaternary structure of the enzyme and also afforded considerable protection against urea-induced unfolding, in the case of reduced holoSHMT (Venkatesha *et al* 1998).

In the present paper, we describe the reversible dissociation and unfolding pathway of sheep liver SHMT in the presence of GdnHCl and show the presence of at least one detectable unfolding intermediate which is stabilized at low concentration of GdnHCl.

2. Materials and methods

The following chemicals were obtained from Sigma Chemicals Co., St. Louis, MO, USA: 2-ME, EDTA, DL – dithiothreitol (DTT), PLP, guanidine hydrochloride (GdnHCl), D-alanine, polyethylene glycol-3350 (PEG-3350), polyoxyethyelene-23-lauryl ether (Brij-35, 30% w/v); L-[3-^{14}C]serine (55 mCi/mmol) was from Amersham International, Bucks, England; 5,6,7,8-tetrahydrofolate (H_4-folate) was prepared by the method of Hatefi *et al* (1959). All other chemicals used in the study were of the highest purity available.

Large scale purification of SHMT from sheep liver was achieved by the procedure described by Baskaran *et al* (1989). The enzyme was dialyzed against 50 mM potassium phosphate buffer, pH 7·3, containing 1 mM 2-ME, 1 mM EDTA with four changes to remove excess PLP and such an enzyme free of excess PLP was used in all the experiments. The apoenzyme was prepared from the holoenzyme using D-alanine as the substrate (Schirch *et al* 1993). Protein concentration was determined by the method of Lowry *et al* (1951) using bovine serum albumin as the standard. Enzyme activity was determined as described by Manohar *et al* (1982) using L-[3-^{14}C]serine as substrate. Activity of the apoenzyme was determined after a short pre-incubation (3 min) with 50 µM PLP. The reduced holoenzyme was prepared by reducing the Schiff's base linkage with sodium borohydride (200 µmol) for 1 h at 4°C in the dark (Schirch *et al* 1993).

2.1 *Equilibrium unfolding studies*

The buffer used in the following studies consisted of 50 mM potassium phosphate buffer, pH 7·3, 1 mM DTT, 2 mg/ml PEG-3350, 0·02% Brij-35 (buffer A), ± 50 µM PLP and the specified concentration of GdnHCl.

2.1a *Guanidine hydrochloride-induced inactivation*: The holoenzyme (25 µg/ml) was incubated with various concentrations of GdnHCl at either room temperature (30 ± 2°C) or at 4°C for 4 h. At the end of incubation, an aliquot of the mixture was assayed. Corresponding concentrations of GdnHCl were included in the assay buffer also to prevent refolding during the assay. In the experiment where the apoenzyme was treated with GdnHCl, 50 µM PLP was added 3 min before the assay and enzyme activity determined.

2.1b *Circular dichroism measurements*: Far UV circular dichroism measurements were made at 25°C with

a Jasco J720 spectropolarimeter using a bandwidth of 1 nm and scan speed of 20 nm/min. The protein concentration was 25 μg/ml. Each sample was scanned 10 times and an average ellipticity at 222 nm after correcting for the buffer reading was used to calculate the mean residue ellipticity (θ_{MRE}) using the equation

$$\theta_{MRE} = \theta \times MRW/10 \times c \times d, \quad (1)$$

where θ = observed ellipticity, MRW = mean residue molecular weight (110·4 Daltons), c = concentration of protein in mg/ml, d = path length in cm.

2.1c *Fluorescence spectroscopy*: (i) *Intrinsic fluorescence*: Intrinsic fluorescence measurements were made at 25°C using a Spex fluorolog spectrofluorimeter. Protein concentration was 25 μg/ml. The excitation wavelength was 280 nm and the bandwidth was 3·55 nm. Fluorescence intensities at 329 nm for the holo-, reduced holo- and the apoenzyme, where the difference between native and 6 M GdnHCl unfolded protein was maximum, were converted to relative fluorescence and plotted against GdnHCl concentration. (ii) *Data analysis:* The fluorescence and CD data were converted to plots of f_{app} versus denaturant concentration according to Nath and Udgaonkar (1995). (iii) *ANS fluorescence*: The proteins (50 μg/ml) were incubated with various concentrations of GdnHCl for 4 h at 25°C. An aliquot of the stock ANS was added at the end of incubation such that the final concentration of ANS was 20 μM. The fluorescence was monitored at 489 nm after exciting at 373 nm using a bandwidth of 3·55 nm. Each data point is an average of ten such scans after subtracting for the buffer reading. The ANS fluorescence at 0 M GdnHCl was taken as 100%.

2.1d *Size exclusion chromatography*: A Superose 6 HR 10/30 analytical gel filtration column was used on a Pharmacia FPLC system for monitoring the changes in the protein elution pattern as a function of GdnHCl concentration. The protein concentration was 62·5 μg/ml and was incubated in buffer A containing 0·1 M KCl and the desired concentration of GdnHCl. The column was calibrated with standard markers of known M_r.

2.2 *Refolding of SHMT unfolded in 6 M GdnHCl*

Refolding of SHMT from 6 M GdnHCl was initiated by a 40-fold dilution of unfolded protein into the refolding buffer (buffer A) in the presence and absence of 50 μM PLP such that the final protein and GdnHCl concentrations were 12·5 μg/ml and 0·15 M, respectively. Native protein incubated with 0·15 M GdnHCl under identical conditions served as the control. The apoenzyme was also refolded in a similar way by dilution of GdnHCl. The refolded enzymes were assayed either immediately or after 1 h incubation at 30°C.

2.2a *Size-exclusion chromatography of the refolding intermediates*: A Superose 6 HR 10/30 analytical size-exclusion chromatography column was used on a Pharmacia FPLC instrument to separate the refolding intermediates. The column was equilibrated with buffer A containing 0·15 M GdnHCl and 0·1 M KCl. The column was calibrated with standard proteins of known M_r. Aliquots (2·5 μg/0·2 ml) were injected at various time intervals. The absorbance of the eluate was monitored at 280 nm.

3. Results

3.1 *GdnHCl-induced unfolding of SHMT*

3.1a *Activity studies*: The enzymes, either holo or apoenzyme (25 μg/ml) were incubated with various concentrations of GdnHCl at room temperature and residual activity was assayed at the corresponding GdnHCl concentration, after 2 h. A plot of residual activity against GdnHCl is given in figure 1. It can be seen that the activity progressively decreased with increasing concentration of GdnHCl and almost complete activity was lost in the case of apo- and holoSHMT at 0·5 M GdnHCl. The midpoints of inactivation for the apo- and holoSHMT were 0·25 and 0·12 M GdnHCl, respectively. It should be mentioned that the apoenzyme was reconstituted with 50 μM PLP before the assay, whereas the holoenzyme was assayed in the absence of any added PLP. When the inactivation was carried out in the presence of excess PLP (50 μM) the denaturation curve shifted to the right and complete inactivation was observed at 0·9 M GdnHCl. The shift in the concentration required for 50% inactivation (0·6 M) upon the addition of PLP suggested that PLP stabilizes the native enzyme to some extent. In order to monitor the loss of PLP from the active site, the holoenzyme (125 μg/ml; in the absence of any added PLP) was incubated at concentrations of GdnHCl indicated in figure 1 and dialyzed against buffer containing the corresponding concentration of GdnHCl. The absorbance at 425 nm was monitored and as shown in figure 1 (open triangles) the loss of PLP from the active site occurred at higher concentrations of GdnHCl (0·6 M, midpoint) compared to the midpoint of inactivation (0·12 M). This and the earlier observation from this laboratory, which had suggested that aminoguanidine compounds function as inhibitors of SHMT activity (Acharya and Appaji Rao 1992), indicated that the extreme sensitivity of SHMT to inhibition by GdnHCl at low concentration could be due to inhibition by interaction at the active site. In order to examine this further, the holoenzyme (25 μg/ml) was incubated at 0, 0·075 and 0·15 M GdnHCl for 2 h. The activity of this enzyme was assayed at varying concentrations of L-serine in the range 0–4 mM. The enzyme, in the absence of GdnHCl, served as the control. It can be seen from the

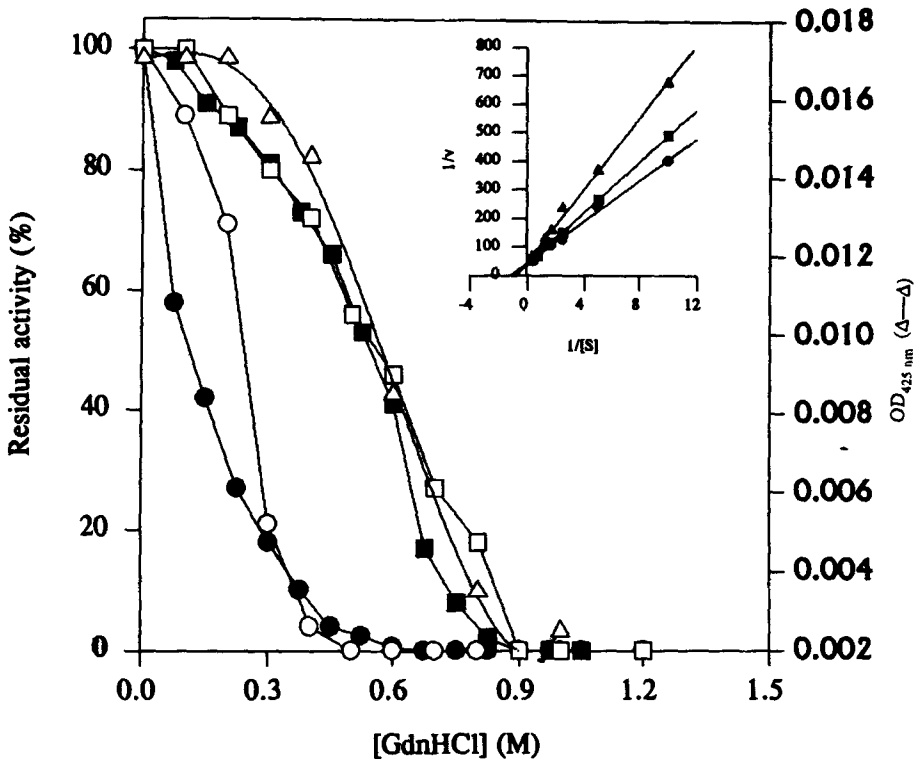

Figure 1. Effect of GdnHCl on SHMT activity and PLP binding. The protein concentrations used for monitoring the activity and loss of PLP were 25 and 125 μg/ml, respectively. (●, ○), Activity changes of holo- and apoSHMT, respectively. (■, □), Activity changes of the holoenzyme and apoenzyme, respectively in the presence of 50 μM PLP. (Δ), Absorbance changes. *Inset*: Lineweaver-Burk plot of 1/v vs 1/[Ser] concentrations at varying concentrations of GdnHCl.

double reciprocal plot (figure 1, inset) that the lines intersect at a common point on the Y-axis indicating that GdnHCl may be functioning as a competitive inhibitor of the enzyme at low concentrations. The K_i value calculated from this data was 40 mM.

3.1b *Spectral studies*: GdnHCl-induced unfolding of holo-, apo- and reduced holoenzyme was monitored by using far UV CD and fluorescence spectroscopy. Figure 2a represents the secondary structural changes of all the three forms of the enzyme upon incubation with GdnHCl. Though reduced holoSHMT was marginally more stable compared to the holo- and apoSHMT at low denaturant concentration, unfolding midpoints of all the 3 forms of the enzyme are similar (1·6 ± 0·1 M GdnHCl). Above 3 M GdnHCl all the proteins assume apparent random coil structures. Fluorescence intensity, which indicates the environment around the tryptophan side chains, remains unchanged up to 0·8 M GdnHCl in all the 3 forms of the enzyme. Above 3 M the tryptophan side chains become completely exposed to the solvent. The unfolding midpoints as monitored by fluorescence for the holo/apoenzyme and reduced holoenzymes are 2·0 and 2·2 M GdnHCl respectively (figure 2b). The observation that the midpoints of denaturation of reduced holoSHMT and other two forms of SHMT are similar, indicates that the PLP present at the active site as a stable phosphopyridoxyl lysine residue does not afford any significant protection against GdnHCl-induced unfolding unlike in the case of urea-induced unfolding (Venkatesha *et al* 1998). The noncoincidence of denaturation curves also suggests that the unfolding of SHMT may not be occurring by a two state mechanism.

3.2 *Size exclusion chromatography*

The changes in the hydrodynamic volume of apo-, holo- and reduced holoenzymes as a function of GdnHCl were monitored using size exclusion chromatography (figure 3a, b, c). In the absence of any denaturant, holo and reduced holoenzymes eluted as a single symmetrical peak (peak 1) whose elution volume corresponded to 210 kDa when compared to the elution profiles of the standard M_r markers processed under identical conditions.

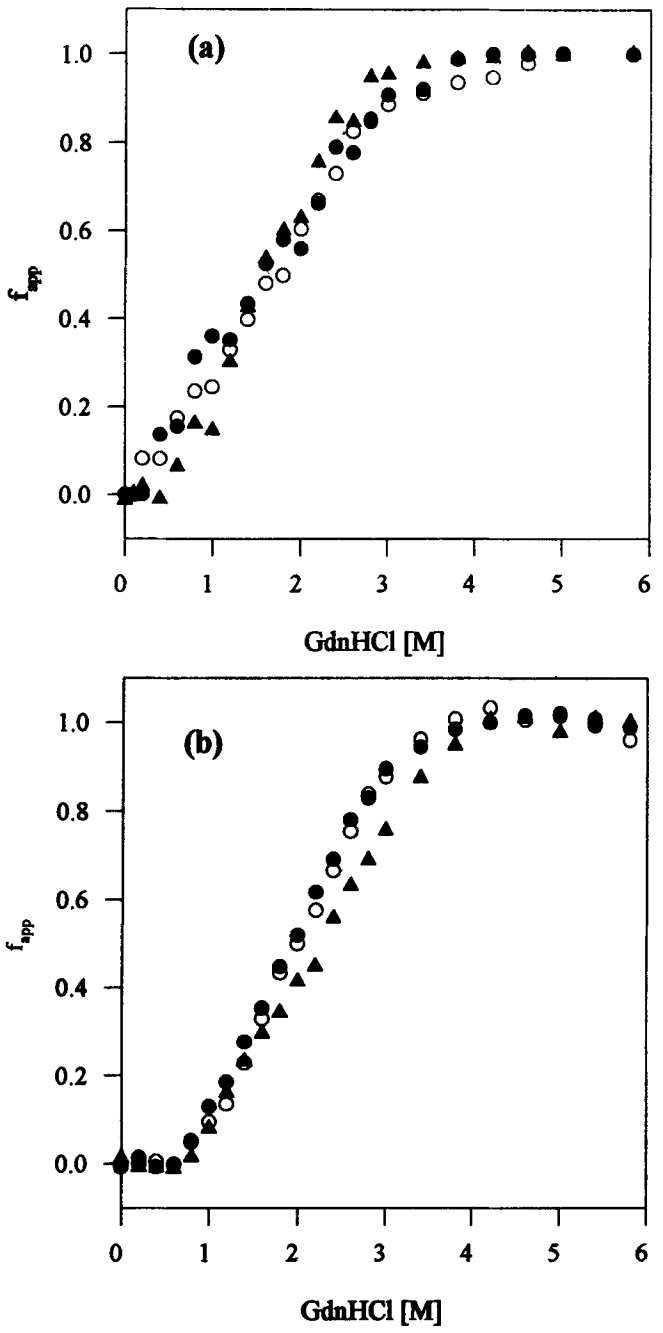

Figure 2. GdnHCl-induced structural changes of holo, apo and reduced holoSHMT. (**a, b**), Far UV CD and fluorescence spectra, respectively. The proteins (25 μg/ml) were separately incubated at specified concentrations of GdnHCl for 2 h at room temperature. (●, ○), Holo- and apoSHMTs, respectively. (▲), Reduced holoSHMT.

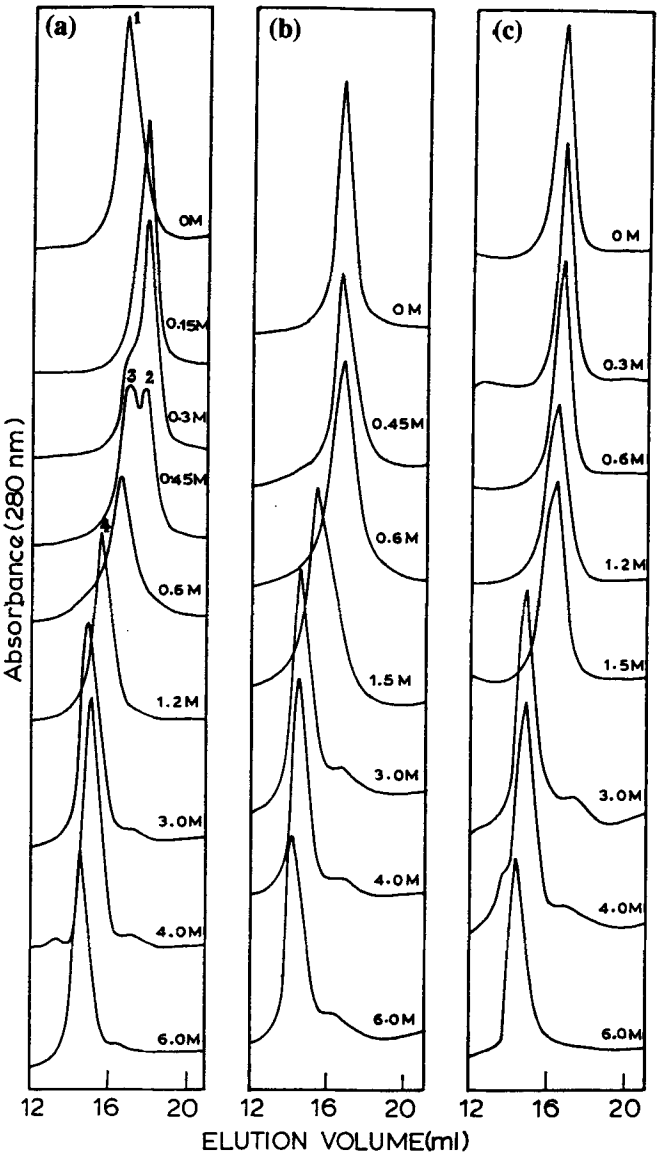

Figure 3. Size exclusion chromatography of the GdnHCl treated SHMTs. Proteins (125 μg/ml) incubated at the specified concentrations of GdnHCl were chromatographed on a FPLC Superose 6 HR 10/30 column equilibrated with the same concentration of GdnHCl in buffer C. The flow rate was 0·4 ml/min. (**a, b** and **c**), Elution profiles obtained with apo-, holo- and reduced holoSHMT, respectively. The column was calibrated with cytochrome C (12,900) carbonic anhydrase (29,000), BSA (66,000), alcohol dehydrogenase (150,000), β-amylase (200,000) and apoferritin (443,000).

It is evident from figure 3a that the apoenzyme dissociated at lower concentrations of GdnHCl (0·15–0·3 M) as indicated by the appearance of a protein peak (peak 2) whose hydrodynamic volume was less than that of the tetrameric form. The apparent M_r of the dissociated tetramer was determined using standard M_r markers developed at 0·15 M GdnHCl and was found to be 120 kDa which approximately corresponded to the dimeric form of SHMT. With increasing denaturant concentration, the dimer began to unfold and eluted earlier upon gel filtration. Interestingly at 0·45 M GdnHCl, two peaks with elution volume 16·2 ml (peak 2) and 15·2 ml (peak 3)

were observed. The peak 2 corresponded to the dimeric form and peak 3, although eluting at the same position as the native tetramer, does not represent the folded tetramer as the native tetramer was almost completely dissociated by 0·2 M GdnHCl. Therefore, it might represent an unfolding intermediate which is less compact than the dimer and more compact than the completely unfolded form. The equilibrium between the dissociated tetramer and this intermediate is slow when compared to the time of chromatography, hence they can be separated on the column. The holo (figure 3b) and reduced holo enzymes (figure 3c), on the other hand, do not show the presence of dimer at low concentrations of GdnHCl. This is probably because dissociation and unfolding are simultaneous in this case. Beyond 0·6 M GdnHCl, the elution profiles of both holo- and reduced holoSHMT shift to the left indicating an increase in Stoke's radius as the proteins begin to unfold. The presence of only a single peak beyond 0·6 M GdnHCl concentration indicated that the equilibrium between completely folded and other forms (unfolded or unfolding intermediate, if any) was very fast when compared to the time for chromatography. Hence, these peaks represent the weighted average of all the forms present at equilibrium. Above 3 M GdnHCl, there was no significant change in the elution volume up to 6 M indicating that the proteins unfold almost completely by 3 M GdnHCl.

3.3 *ANS binding studies*

The binding of the hydrophobic fluorescent dye, ANS, can be used as a very sensitive tool to monitor the exposure of hydrophobic regions of a protein. Both holo- and apoSHMTs show increased ANS binding even at very low concentrations of GdnHCl. The ANS binding continues to increase up to 0·6 M GdnHCl and then decreases before reaching a minimum at 2 M GdnHCl (figure 4). A comparison of ANS binding curves obtained using the holo- and apo- forms of the enzyme indicates that they are essentially similar. This suggests that the holoenzyme may also follow a similar unfolding pathway as that of the apoenzyme, although the holoenzyme does not show any apparent dissociation and the formation of an unfolding intermediate.

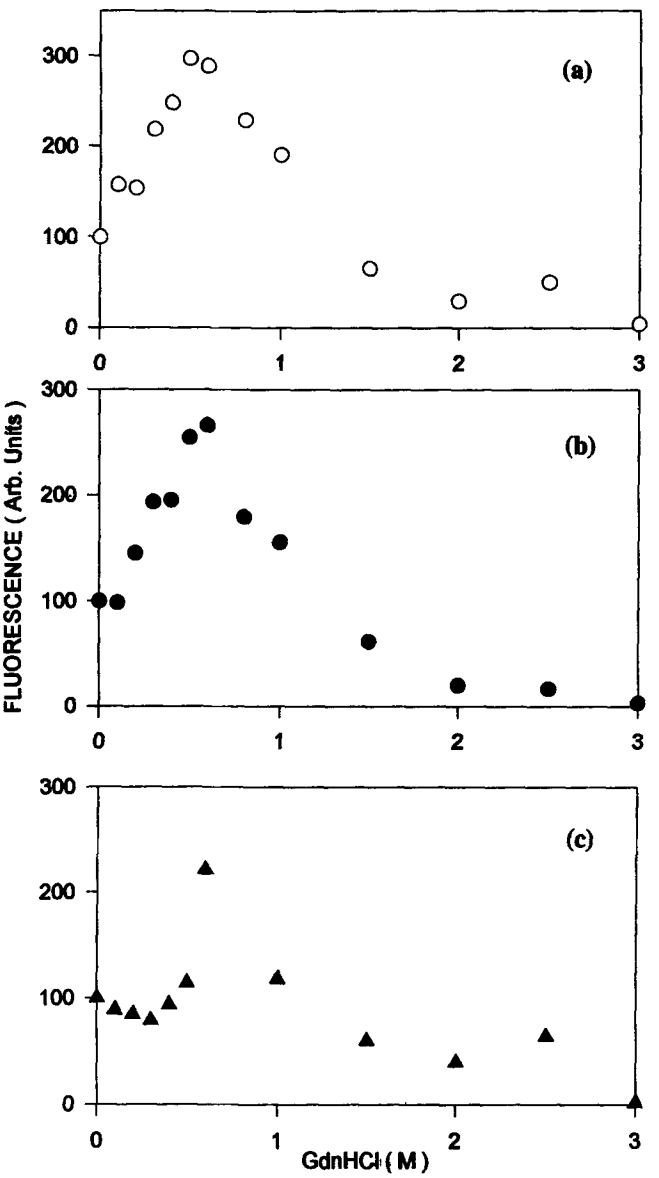

Figure 4. ANS binding properties of SHMT. The apo- (**a**), holo- (**b**) and reduced holoSHMT (**c**) (50 μg/ml) were incubated with various concentrations of GdnHCl. ANS (10 μM) was added and the fluorescence intensity at 489 nm was monitored after exciting at 372 nm.

3.4 *Reversibility of unfolding*

SHMT incubated in the presence of 6 M GdnHCl had an apparent random coil structure with its tryptophan side chains completely exposed to solvents as monitored by far UV CD and fluorescence, respectively. Such an enzyme could be refolded to recover >95% activity, by 40-fold dilution into refolding buffer. The recovery of enzyme activity was maximum when the final protein concentration was 12·5 μg/ml and the refolding temperature was 30°C. The kinetic and spectral properties of the control and refolded SHMTs were comparable (table 1). The absence or presence of PLP during the initial stages of refolding did not affect the final recovery of activity. PLP added just before the assay was able to restore activity, suggesting that the coenzyme may not be required for the initiation of refolding. Oligomeric status of the protein during refolding was monitored by size exclusion chromatography as described in § 2. The profiles are represented in figure 5. The protein

Table 1. Kinetic and spectral properties of the control and refolded SHMT.

	Control enzyme	Refolded enzyme
Specific activity	6·1	5·9
L-Ser K_m	0·9 mM	1·1 mM
H_4-folate K_m	0·8 mM	0·9 mM
Mean residue ellipticity ($deg.cm^2.dmol^{-1}$)	16,200	16,050
Fluorescence (relative to 1 for the native SHMT)	1	>0·95

when refolded either in absence or presence of PLP eluted as a single peak with a retention volume of 15·2 ml which corresponds to the elution volume of the native tetramer under similar conditions. But the enzyme refolded in the absence of the coenzyme is inactive and can be converted to the active form by the addition of PLP.

4. Discussion

It is becoming increasingly evident that intermediary structures precede the final folded conformation (Matthews 1993; Ptitsyn *et al* 1995). Hence it is of utmost importance to stabilize these intermediates under equilibrium conditions. Folding intermediates become populated when the native structure is sufficiently destabilized by perturbing a strong tertiary interaction between two units of secondary structure, incubating the apoproteins under mildly denaturing conditions (Kirby and Steiner 1970; Kuwajima 1989) or by varying the nature of the denaturant. In an earlier study, urea was used to denature sheep liver SHMT. In the present study we have characterized the pathway of GdnHCl-induced reversible unfolding of sheep liver SHMT which shows that the change in the denaturant does affect the equilibrium distribution of intermediates. If a protein requires a coenzyme/cofactor for its stability and activity, the removal of the coenzyme/cofactor renders the apoproteins less stable than the corresponding holoproteins. This results in the accumulation of unfolding intermediates under mildly denaturing conditions. The results presented here on the GdnHCl-induced unfolding of the holo- and apoSHMTs show that some of the intermediates are indeed stabilized upon removal of the cofactor, PLP.

4.1 *Mechanism of unfolding*

Scheme 1 depicts the minimum model proposed to explain the GdnHCl-induced unfolding/refolding of sheep liver SHMT.

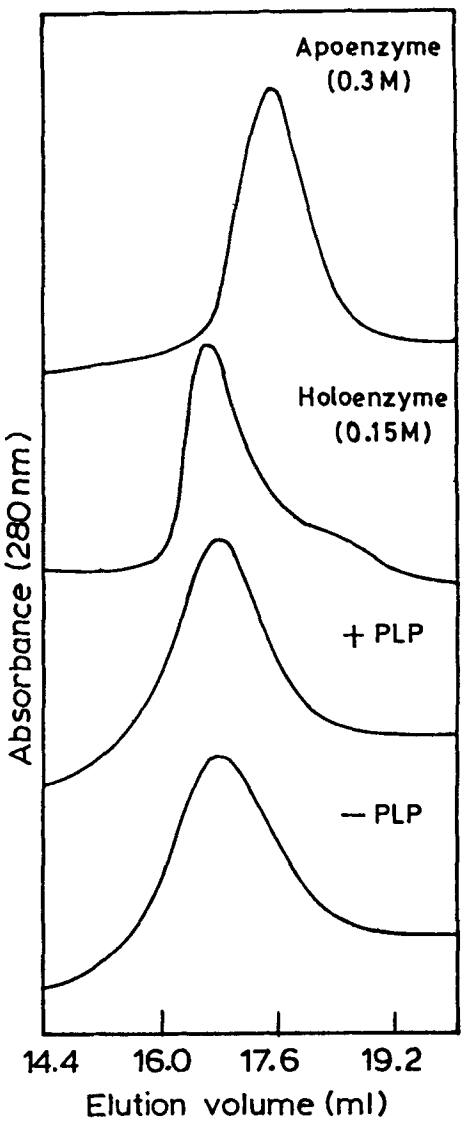

Figure 5. Size exclusion chromatography of refolding intermediates. A Superose 6 HR 10/30 column was used to separate refolding intermediates as described in § 2. The eluting proteins was monitored at 280 nm. The column was calibrated with the same M_r markers as in figure 3. The PLP concentration was 20 μM.

Scheme 1: $M_4 \leftrightarrow M_2 \leftrightarrow (I)_n \leftrightarrow 4U$,

where M_4: tetramer; M_2: dimer; $(I)_n$: one or more intermediates; U: completely unfolded protein.

Physical evidence for the presence of intermediates in the unfolding of SHMT was obtained using size exclusion chromatography (figure 3a). In case of the apoenzyme, the removal of PLP results in the dissociation of the tetramer as well as in the stabilization of one of the unfolding intermediates (figure 3a). Initially, the population of this intermediate (peak 3) increased with time at the expense of the dimer (peak 2). After 2–3 h of

Table 2. Comparative analysis of the urea and GdnHCl-induced unfolding pathway of tetrameric SHMT.

	Urea	GdnHCl
Inactivation	Loss of PLP	Competitive inhibition
Dissociation and unfolding	Distinct events	Concerted
Predenaturation transition	Present (holoSHMT)	Not evident
Protection by PLP against unfolding	Present	Absent
Oligomeric status during refolding	Dimer (– PLP)	Inactive tetramer/ intermediate (– PLP)
	Tetramer (+ PLP)	Active tetramer (+ PLP)

incubation another species appeared at 14·6 ml (peak 4) which was less compact than the first intermediate and more compact than the completely unfolded protein (figure 3a). The area under this peak increased with time at the expense of the first intermediate before reaching an equilibrium value. This species with a retention volume of 14·6 ml, might represent a second intermediate or could be an oligomeric product of the first intermediate. To check for the latter possibility, the effect of protein concentration on the accumulation of these intermediates was monitored. In the range of protein concentrations employed (62·5–187·5 μg/ml), when the system reached equilibrium, the relative area under these peaks at all protein concentrations were comparable (data not shown). Higher concentrations of protein could not be used because of the problem of aggregation. The aggregated product which appeared above 250 μg/ml eluted at 9·2 ml (data not shown). Whether the holo- and the reduced holoenzymes also show similar intermediate(s) could not be clearly established because the unfolding intermediate eluted at the same elution volume as the folded tetramer and at 0·3–0·4 M GdnHCl, substantial amounts of native tetramer was still present. At higher GdnHCl, the intermediates are in fast exchange with the unfolded state resulting in a single peak which represents the weighted average of both the forms. But the results of ANS binding studies (figure 4) indicated that all the three forms of the enzyme unfold via a similar pathway. The maximum binding observed at 0·5 M GdnHCl suggests that the unfolding intermediate that accumulates at this concentration is less compact. A comparison of the urea/GdnHCl-induced unfolding of tetrameric SHMT is presented in table 2.

The inactivation in the case of urea-induced denaturation was found to be due to the loss of PLP from the active site, whereas, in case of GdnHCl, the inactivation is due to competitive inhibition rather than the direct removal of PLP from the active site (figure 1). The predenaturation transition, defined as the event leading to dissociation and the formation of an intermediate dimer, which was very distinct during the urea-induced unfolding of holoSHMT is not evident in case of the GdnHCl-induced unfolding. The apoenzyme, in both the cases, dissociates to dimers prior to unfolding. In the case of GdnHCl, probably because of its stronger denaturing capacity than urea (Greene and Pace 1974), the dimer is not a very stable intermediate. During both unfolding pathways, the dimer, probably directly without further dissociation, unfolded into an intermediate which showed increased affinity towards ANS. In the case of GdnHCl-induced unfolding, this intermediate is stabilized at low GdnHCl concentration (0·45 M) and can be captured in size exclusion chromatography.

Another interesting comparison is the role of the cofactor during urea and GdnHCl-induced unfolding. In the case of urea-induced unfolding, the phosphopyridoxyl lysine residue present at the active site was able to afford considerable protection against unfolding. In the case of GdnHCl, the unfolding midpoints of all the three forms of the enzyme are comparable. Similarly, during the refolding pathway, in case of urea, the PLP was found to affect the dimer/tetramer equilibrium and shifted the equilibrium towards the formation of active tetramers. During the refolding of GdnHCl denatured SHMT, in the absence of PLP, the protein elutes with an elution volume corresponding to that of the tetramer and can be converted to the active form upon addition of PLP. The results presented in this paper demonstrate that change of denaturant affects the equilibrium distribution of the unfolding intermediates.

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