Rat liver betaine–homocysteine S-methyltransferase equilibrium unfolding: insights into intermediate structure through tryptophan substitutions

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Equilibrium folding of rat liver BHMT (betaine–homocysteine methyltransferase), a TIM (triosephosphate isomerase)-barrel tetrameric protein, has been studied using urea as denaturant. A combination of activity measurements, tryptophan fluorescence, CD and sedimentation-velocity studies suggested a multiphasic process including two intermediates, a tetramer (I_4) and a monomer (J). Analysis of denaturation curves for single- and six-tryptophan mutants indicated that the main changes leading to the tetrameric intermediate are related to alterations in the helix $\alpha 4$ of the barrel, as well as in the dimerization arm. Further dissociation to intermediate J included changes in the loop connecting the C-terminal α -helix of contact between dimers, disruption of helix $\alpha 4$, and initial alterations in helix $\alpha 7$ of the barrel, as

well as in the dimerization arm. Evolution of the monomeric intermediate continued through additional perturbations in helix $\alpha 7$ of the barrel and the C-terminal loop. Our data highlight the essential role of the C-terminal helix in dimer–dimer binding through its contribution to the increased stability shown by BHMT as compared with other TIM barrel proteins. The results are discussed in the light of the high sequence conservation shown by betaine–homocysteine methyltransferases and the knowledge available for other TIM-barrel proteins.

Key words: betaine–homocysteine methyltransferase folding, monomeric intermediate, tetrameric intermediate, triosephosphate isomerase (TIM) barrel, tryptophan fluorescence.

INTRODUCTION

The increase in plasma levels of Hcy (homocysteine) has been established as an independent risk factor for cardiovascular [1] and Alzheimer diseases [2]. This fact prompted an increase in the interest in the enzymes that metabolize this amino acid, among others BHMT (betaine–homocysteine methyltransferase; EC 2.1.1.5) [3]. This enzyme uses betaine as the methyl donor to synthesize methionine, allowing recovery of one of the methyl groups used in the transmethylation reaction that led to choline synthesis. Its expression has been shown to be limited to liver and kidney in most species [4], but localization in rhesus-monkey (*Macaca mulatta*) lens has also been reported [5]. Changes in BHMT activity have been detected under several dietary conditions [6–8], during development [9] and in pathologies such as cirrhosis and hepatocellular carcinoma [10,11].

Rat liver BHMT is a 407-amino-acid cytosolic protein that is more than 90% identical at the amino acid level with its human and pig counterparts [12]. The enzyme contains zinc co-ordinated to three conserved cysteine residues [13,14] and assembles as a homotetramer [15–18]. Structurally the overall fold of the subunit consists of a $(\alpha/\beta)_8$ barrel [TIM (triosephosphate isomerase) barrel] that extends through the so-called ‘dimerization arm’ and a C-terminal α -helix extending towards the opposite dimer [18]. This last element confers additional strength to oligomer binding. BHMT possesses seven tryptophan residues per subunit located along the sequence (Figure 1). According to the crystal structure these residues are located in the following elements [18]: (a) loop 1, comprising residues 38–52, contains W44 (tryptophan-44 in the one-letter amino acid notation), which is involved

in substrate binding; (b) residues W169 and W279 appear on helices $\alpha 4$ and $\alpha 7$ of the barrel respectively; (c) W331, W342 and W352 are included in loops comprising accessory secondary elements; and (d), W373 is located in the loop connecting the C-terminal α -helix that extends to the opposite dimer. Moreover, the dimerization arm includes three of these amino acids, W331, W342 and W352 [17,18]. All these residues occupy the same structural elements in rat and human enzymes, a fact that can be extended to the rest of BHMT proteins identified by BlastP, except W169, which is not conserved in all of them.

Canonical TIM barrels are constituted by eight parallel β -strands surrounded by eight α -helices and are one of the most frequent and regular domain structures of globular proteins [19,20]. It has been calculated that, in Nature, one out of every ten proteins adopts this type of fold and, in most cases, with scarce sequence similarity. Folding of this standard structure has been the subject of study for many years, and the models proposed always include several intermediates [21,22]. Lately, the proposal of the existence of subdomains for folding and the discrepancies in the number and the elements that compose them has complicated this picture [22–26]. Here we present BHMT as a special case for this type of study, owing to the larger size of its subunit, as well as to the presence of a dimerization arm and an extra C-terminal α -helix. Distribution of tryptophan residues in different structural elements of the protein permits a deep insight into the mechanism of folding of this enzyme by analysis of the corresponding mutants, results that can be extrapolated to the whole BHMT family. Moreover, besides the study of the folding problem, the stability of this enzyme may be a key property for its functions under denaturant conditions such as those found in renal tubules.

Abbreviations used: ANS, 8-anilino-1-naphthalene-sulphonic acid; BHMT, betaine–homocysteine methyltransferase; DTT, dithiothreitol; Hcy, homocysteine; IPTG, isopropyl β -D-thiogalactopyranoside; MetS, methionine synthase; TIM, triosephosphate isomerase; TS, tryptophan synthase.

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Figure 1 Structure of rat BHMT

(A) Shows the oligomer containing the four subunits. The molecular surface of one of the subunits is shown to highlight the interface among the different molecules within the tetramer. The tryptophan residues are marked in one of the subunits. The region covered by the rectangle corresponds to the interface between the dimerization arm and the C-terminal helix from the upper and lower dimers respectively, and is fully labelled in (B). (B) Displays the protomer, showing the overall fold of the polypeptide chain: an $(\alpha/\beta)_8$ barrel (with corresponding helices labelled), including residues 10–318, an extended arm created by residues 319–380, and a long C-terminal α -helix built by the segment 381–406. The tryptophan residues are represented as stick models and are labelled.

EXPERIMENTAL

Materials

DTT (dithiothreitol), betaine, homocysteine thiolactone, PMSF, pepstatin A, benzamidine, aprotinin, leupeptin, antipain, ampicillin, ZnCl_2 and ANS (8-anilino-1-naphthalene-sulphonic acid) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [$\text{methyl-}^{14}\text{C}$]Choline chloride (50–60 mCi/mmol) was a product from Amersham Pharmacia Biotech (Little Chalfont, Bucks., U.K.). IPTG (isopropyl β -D-thiogalactopyranoside) was obtained from Ambion (Austin, TX, U.S.A.). Optiphase HiSafe 3 scintillation fluid was supplied by E&G Wallac (Milton Keynes, U.K.). Protein-assay and electrophoresis reagents were purchased from Bio-Rad Laboratories (Richmond, CA, U.S.A.). PM-10 ultrafiltration membranes were products of Amicon Corp. (Beverly, MA, U.S.A.). Triton X-100, 2-mercaptoethanol and urea were purchased from Merck (Darmstadt, Germany). Chitin beads were obtained from New England Biolabs (Beverly, MA, U.S.A.). The rest of the buffers and reagents were of the best quality commercially available.

Site-directed mutagenesis

Replacement of tryptophan residues with phenylalanine in BHMT was carried out using the QuikChange[®] Site-Directed Mutagenesis Kit, following the manufacturer's (Stratagene) instructions. Single- and six-tryptophan mutants were constructed in the vector pBHMT-TYB12 using the following mutagenic oligonucleotides (only the sense sequence is included and the bases changed appear in bold): 5'-GGCTGGACCCT**TT**CACCCAGAGGCTG-3' (W44F); 5'-GTTGAAGAAGCCGTG**TT**CGCAGT-

CGAGGCC-3' (W169F); 5'-CAGAGTTGCCACCAGAT**TT**CG-ATATTCAAAAATACGCC-3' (W279F); 5'-GAAAAACATGGCAGCT**TT**CGGAAGTGGTTTGGACATG-3' (W331F); 5'-GCACACCAAACCC**TT**CATCAGGGCAAGGG-3' (W342F); 5'-GGGCCAGGAAAGAATACT**TT**CAGAAATCTTCGGATAG-3' (W352F); 5'-GCCCGATGCT**TT**TCGGAGTGACGAAAGGG-3' (W373F). Mutations were verified by automatic sequencing. Mutants conserving only one tryptophan residue (six-tryptophan mutants) were named according to the remaining residue as follows: W44, W169, W279, W331, W342, W352 and W373.

Expression of the fusion protein and purification of recombinant BHMT wild-type and mutants

Competent *Escherichia coli* BL21 (DE3) cells were transformed with the plasmid pBHMT-TYB12 [27], and 2.5-litre cultures were grown in Luria-Bertani medium containing 100 $\mu\text{g/ml}$ ampicillin. Induction was carried out for 16 h at 20 °C by the addition of 0.5 mM IPTG when the attenuation (D_{595}) of the culture was 0.6. Cells were harvested by centrifugation at 7500 g for 10 min, washed with water and stored at -70°C until use. Disruption of the cell pellets was carried out by sonication as previously described [27]. The soluble fraction was separated by centrifugation for 30 min at 13 000 g and loaded on chitin beads (10 ml) equilibrated in 20 mM Tris/HCl, pH 8.0, containing 500 mM NaCl, 0.1 mM EDTA and 0.1 % (v/v) Triton X-100, for purification. The column was washed as described previously, and incubated for 48 h at 25 °C for intein (protein-splicing element) cleavage before elution of either BHMT or its mutant [27]. The purified preparations were then dialysed and concentrated by ultrafiltration using PM-10 membranes, prior to addition of 50 %

glycerol and storage at -20°C . The purity of the samples was tested by SDS/PAGE and Coomassie Brilliant Blue staining.

Determination of the protein concentration

The protein concentration of the samples was measured using the Bio-Rad assay and BSA as a standard, or spectrophotometrically using the following calculated molar absorbance coefficients (ϵ_{280}): (a) $58\,220\text{ M}^{-1}\cdot\text{cm}^{-1}$ for the wild-type BHMT; (b) $52\,670\text{ M}^{-1}\cdot\text{cm}^{-1}$ for single-tryptophan mutants; and (c) $24\,920\text{ M}^{-1}\cdot\text{cm}^{-1}$ for six-tryptophan mutants in 6 M guanidinium chloride.

Activity measurements

BHMT activity measurements were carried out for 1 h at 37°C at saturating concentrations of betaine (6.5 mM) and Hcy (6.5 mM) using the radioassay previously described [28]. The protein concentration in the assays was 0.05 mg/ml, except for W352F, which needed 0.5 mg/ml for detection. Samples were incubated overnight at 10°C in the presence of urea (0–8 M) and the activity was measured. On the other hand, refolding of 8 M-urea-denatured samples was carried out: (a) by overnight dialysis at 4°C against 20 mM Tris/HCl (pH 8)/50 mM NaCl/10 mM DTT/500 μM ZnCl_2 (buffer C), including different urea concentrations (0–8 M); or (b) by dilution in buffer C. The final BHMT concentration in the refolding experiments was 0.05 mg/ml. Midpoints of urea-denaturation transitions were determined using the package GraphPad Prism (GraphPad Software Inc., San Diego, CA, U.S.A.). The kinetics exhibited by the enzyme with the substrates homocysteine and betaine were studied over the range of concentrations 6.5 mM–1 μM as previously described [27], using the same protein concentrations as those given above.

Gel-filtration chromatography

BHMT wild-type and mutants were loaded on a Superose 12 HR 10/30 gel-filtration column connected to an Advanced Protein Purification System (Waters, Millipore Corp., Milford, MA, U.S.A.) as previously described [27]. The presence of BHMT was detected by dot-blot analysis of the fractions using the anti-BHMT antiserum prepared in our laboratory, and these data were used to estimate the molecular masses of the proteins.

Sedimentation-velocity experiments

BHMT samples (0.1 and 0.2 mg/ml) at different urea concentrations (0–8 M) were loaded into an An50Ti rotor. Sedimentation-velocity experiments were performed at 188 000 g and 18°C in a XL-A analytical ultracentrifuge (Beckman–Coulter) using double-sector Epon/charcoal centrepieces. Absorbance scans (0.005 cm step size) were taken at 280 nm. Differential sedimentation-coefficient distributions, $c(s)$, were calculated by least-squares boundary modelling of sedimentation-velocity data using the program SEDFIT [29,30]. From this analysis, values obtained were corrected for solvent composition and temperature to obtain $s_{20,w}$ using the public domain software SEDNTERP, retrieved from the RASMB server [31]. Midpoints of unfolding transitions were calculated as for activity.

Intrinsic fluorescence and ANS-binding experiments

BHMT wild-type and mutants at different protein concentrations (50, 20 and 5 $\mu\text{g/ml}$) were incubated with urea (0–8 M) overnight at 10°C , whereas refolded samples were prepared from a concentrated protein stock denatured with 8 M urea overnight by either dilution or dialysis as for activity measurements. Samples

were excited at 295 nm for intrinsic-fluorescence determinations, using slit widths of 2.5 nm for the excitation and 5 nm for the emission. A minimum of ten fluorescence intensity spectra were recorded between 300 and 400 nm in a photon-counting SLM-8000 spectrofluorimeter at 23°C using $0.5\text{ cm} \times 0.5\text{ cm}$ cuvettes. The fluorescence was corrected by subtraction of the solvent signal. Unfolding curves were analysed as described for activity in order to obtain the transition midpoints.

ANS stock solutions were prepared in methanol and the concentration was determined using an ϵ_{370} value of $6800\text{ M}^{-1}\cdot\text{cm}^{-1}$. Pre-equilibrated unfolding reactions (0–8 M urea) containing 50 $\mu\text{g/ml}$ BHMT and controls containing no protein received different concentrations of the dye to obtain 0, 10, 20, 30 or 40 μM ; the final methanol concentration in the samples was 0.4 % (v/v). Mixtures were incubated for 1 h in the dark, and changes in the ANS emission upon protein binding were monitored at 470 nm from spectra recorded between 400 and 600 nm, using as excitation wavelength 380 nm. Data were corrected for baseline and instrumental factors.

CD measurements

Far- and near-UV CD spectra were recorded on a Jasco J-810 spectropolarimeter at 25°C [32]. Samples (0.25 mg/ml protein) and 0.1-cm-path-length cuvettes were used to obtain far-UV spectra, whereas 1 mg/ml protein samples and 1-cm-path-length cuvettes were required to obtain near-UV spectra of purified BHMT wild-type. After baseline subtraction the observed ellipticities were converted into mean residue ellipticities ($[\theta]_{\text{mrw}}$) on the basis of a mean molecular mass per residue of 110 Da. A minimum of five spectra was taken for each sample. Denaturation curves were prepared as for fluorescence experiments, and the transition midpoints were calculated as described for activity curves. Secondary-structure composition was calculated using Jasco software.

RESULTS AND DISCUSSION

BHMT is highly expressed in liver and kidney, two organs involved in osmoregulation of several species of animals. Adaptation to saline water up-regulates the urea cycle in liver, which in turn contributes to a dramatic increase in tissue urea levels. Similar effects can also be observed in kidney [4,33], where urea concentrations might reach molal levels [34], thus creating a denaturing environment for many enzymes. We therefore initiated the present study to gain an insight into BHMT behaviour in respect of urea denaturation. For this purpose we have monitored urea-induced unfolding of BHMT initially by activity measurements (Figure 2A). The profile showed plateaux between 0 and 2 M and 6 and 8 M denaturant that described the native and unfolded states. Moreover, the data could be fitted to the presence of two transitions with characteristic urea concentrations shown in Table 1. It is noteworthy that half of the enzyme activity was lost during each of these transitions. The reversibility of the process was studied either by dilution or by dialysis of the denatured samples in the presence or absence of ZnCl_2 . Full activity was only recovered by dialysis in the presence of the cation, demonstrating the reversibility of the process. Again, refolding showed two transitions characterized by denaturant concentrations of $1.47 \pm 0.08\text{ M}$ and $4.06 \pm 0.27\text{ M}$ (Figure 2A). These data are indicative of a hysteretic behaviour between unfolding and refolding that is more dramatic for the first transition of the curves.

The molecular dimensions of the different states detectable during equilibrium unfolding of BHMT were analysed by sedimentation velocity. Protein concentrations above the levels used

Figure 2 Monitoring unfolding and refolding by activity

Samples of BHMT wild-type and mutants were incubated in the presence of urea until equilibrium was reached. For refolding, samples denatured in 8 M urea were dialysed against different buffers containing variable concentrations of the denaturant in the presence of ZnCl₂. The denaturant effect was then evaluated measuring enzyme activity. (A) Shows the unfolding (■) and refolding (○) curves for wild-type BHMT (50 μg/ml); (B) the unfolding curve for W352F (0.5 mg/ml); (C) unfolding curves for W44F, W169F and W279F; and (D) unfolding curves for W331F, W342F and W373F.

Table 1 Parameters that define wild-type BHMT equilibrium unfolding

BHMT was incubated at different concentrations with 0–8 M urea and the equilibrium unfolding was followed by activity, sedimentation velocity, tryptophan fluorescence and CD. Values for the denaturant concentrations at midpoint of the transitions were calculated using equations for a two (1)-, three (2)- or four (3)-state mechanism using GraphPad Prism 3.0.

Parameter	BHMT (μg/ml)	D ¹ _{50%} (M)	D ² _{50%} (M)	D ³ _{50%} (M)
Activity	50 ²	2.79 ± 0.23	4.93 ± 0.34	–
Analytical	100 ¹	4.5 ± 0.1	–	–
ultracentrifugation	200 ¹	4.48 ± 0.06	–	–
Fluorescence	5 ²	2.47 ± 0.21	5.39 ± 0.05	–
	20 ²	2.95 ± 0.18	5.52 ± 0.2	–
	50 ²	3.11 ± 0.06	5.52 ± 0.04	–
CD	250 ²	3.53 ± 0.14	6.71 ± 0.15	–
	250 ³	2.26 ± 0.18	4.38 ± 0.11	6.77 ± 0.09

for activity measurements were needed for detection. The results indicated the presence of a single species at 0 M urea with an *s*_{20,w} of 9.0 ± 0.2 S, as expected for a globular tetramer

(Figure 3). Plateaux defining the native and unfolded states were observed over the same urea concentration ranges as those pertaining to the activity curves. The tetrameric component disappeared as the denaturant concentration increased, being approx. 50% at 4.5 M urea. In parallel, a new species with an *s*_{20,w} of 2.4 ± 0.1 S, as expected for a partially denatured monomer, became detectable. Moreover, the monomer evolved to render even smaller *s*_{20,w} values, becoming 1.8 ± 0.1 S at 8 M denaturant (Figure 3). Percentages of the different species showed a dependency on BHMT concentration that is more evident over the range at which half of the tetramer disappeared (1–4 M urea), and that included the initial 50% activity loss.

Rat BHMT contains seven tryptophan residues per monomer (at positions 44, 169, 279, 331, 342, 352 and 373) that, as intrinsic fluorophores, can serve as reporter groups for our study. Thus tryptophan fluorescence emission spectra of BHMT in the presence of 0–8 M urea have been recorded. The native protein has its emission maximum at 342 nm (Figure 4A), in contrast with the 334 nm *λ*_{max} showed by the human BHMT [35]. Both rat and human counterparts are 90% conserved at the amino acid level; even so, local differences in their crystal structures have been detected [17,18]. These small changes do not represent

Figure 3 Urea effect on BHMT oligomerization

Samples of wild-type BHMT were denatured in the presence of different concentrations of urea, and the effect on the oligomerization state of the enzyme was followed by sedimentation velocity. Sedimentation coefficients for the species generated were calculated as described in the Experimental section. (A) Shows $c(s)$ distributions derived from sedimentation-velocity profiles of BHMT in the presence of 0 M (—), 4.5 M (---) and 8 M urea (····) after correction to standard conditions ($s_{20,w}$). (B) Includes the percentage of tetramer (■, ○) and monomer (▲, △) upon unfolding for a typical experiment at 0.1 (open symbols) and 0.2 mg/ml (closed symbols).

significant alterations in enzyme specific activity, but reflect the tertiary structure around the tryptophan residues, as judged from their fluorescence emission spectra. This displacement of the maximum may be due to the presence of non-conserved charged residues around tryptophan residues or to their location in a more exposed environment in the rodent protein [36]. In both cases the experiments have been performed using recombinant BHMT expressed as a fusion protein to intein [27,35]. However, these constructions differed in the position of the intein fusion that for rat BHMT is N-terminally linked. These fusion proteins may show slight deviations in folding at their C-terminus, an area that includes four out of the seven tryptophan residues of the subunit, of special importance being the location of the C-terminal α -helix [18]. The spectrum of the unfolded BHMT is red-shifted to 355 nm, as expected for solvent-exposed tryptophan residues, a shift that was accompanied by a 35 % increase in fluorescence intensity. The maximum difference between native and unfolded states was observed at approx. 355 nm and better monitored by the 330/355 ratio (Figure 4B). Several protein concentrations were used to monitor BHMT equilibrium unfolding by fluorescence that again was stable until 2 M and above 6.5 M urea. Two transitions were observed in all the curves, the $D_{50\%}$ (denaturant concentrations at midpoint of the transition) values for the first transition being clearly dependent on the BHMT concentration (Table 1). Moreover, values of the fluorescence ratio between

3.5 and 4.5 M urea remained almost constant, suggesting the presence of a possible intermediate. BHMT refolding produced complete recovery of the fluorescence ratio but, again, hysteresis was observed among unfolding and refolding curves with $D_{50\%}^1$ values of 2.47 ± 0.21 and 2.67 ± 0.2 M respectively, the second transition being only detectable during refolding by dialysis. The change in fluorescence at 50 μ g/ml can be ascribed as for activity profiles: 50 % during each transition (Figure 4B). Comparison of the $D_{50\%}$ values obtained indicated a slight precedence of activity changes in the first transition, whereas dissociation took place earlier than the second transition either for activity or alterations in tertiary structure around tryptophan residues. ANS binding during urea unfolding of BHMT was also measured, the results indicating total absence of such binding to either of the enzyme states, thus suggesting that no increase in apolar surface exposure occurred during this process.

Variations in the secondary-structure content of BHMT during urea unfolding were monitored using CD in the absorption band of the peptide bond (200–230 nm). For sensitivity reasons, protein concentrations used for CD experiments were higher than those for fluorescence and activity measurements. Minima at 222 and 210 nm were features of the native BHMT spectrum (Figure 5), the major secondary structure elements being α -helix and β -sheet, in agreement with the crystallographic data [17,18]. Ellipticity plots at 220 nm, as a function of urea concentration, showed a pretransition baseline until 1.5 M denaturant, followed by a non-linear loss of helicity until 7.5 M (Figure 6). This profile could be fitted to the presence of either three or four states, this last case rendering the best fit. The characteristic parameters that define the transitions detected are shown in Table 1. The first transition occurred at 1.5–3 M denaturant, representing a 20 % change in ellipticity. This change preceded a 50 % reduction in activity and total tryptophan fluorescence, the helicity changes occurring earlier than dissociation (compare curves obtained by CD and sedimentation velocity respectively), thus suggesting an intermediate of the type I_4 . The urea concentration range at which the second transition took place was 3.5–5 M, and this represented an additional 25 % change in ellipticity. It occurred in coincidence with the disappearance of the remaining activity and comprising the midpoint for tetramer $\leftrightarrow$ monomer transition observed in sedimentation-velocity experiments, thus leading to monomeric intermediate J. In contrast, this interval coincided with the plateau and the early steps of the remaining transition observed by tryptophan fluorescence. Moreover, the third transition on CD curves, accounting for the main ellipticity decrease, appeared at 6–7.5 M denaturant, a range at which no further changes in either activity or fluorescence were observed and where only monomers that decrease their $s_{20,w}$ were detectable. Refolding profiles showed similar behaviour, thus confirming the reversibility of the process. Addition of 0.4 M Na_2SO_4 did not improve the stability of the possible intermediates, but displaced the curve to slightly higher denaturant concentrations (results not shown). Near-UV spectra showed high complexity and complete loss of the features of native tertiary structure above 6 M denaturant, thus confirming the results previously shown by fluorescence spectroscopy (Figure 6C). A four-state mechanism of the type $N \rightarrow I_4 \rightarrow J \rightarrow U$ has been described for other TIM barrel proteins, such as the α -subunit of tryptophan synthase (TS), and both intermediates were identified also at 3 and at 5–6 M urea [21]. However, this is not a general mechanism, the number and organization level of the intermediates varying from one TIM barrel to another [22,37]. Larger percentages of the native signal were preserved in BHMT intermediate states as compared with TS (80 versus 40 % for I_4 and 55 % versus unfolded for J) [21]. These differences might arise from the characteristics of the oligomeric assemblies under

Figure 4 Tryptophan fluorescence of BHMT and urea denaturation curves

Samples of wild-type BHMT and mutants were used to record fluorescence intensity after excitation at 295 nm. (A) Shows the BHMT spectrum at 50 μ g/ml in the absence or presence of 8 M denaturant. (B) Includes data of urea unfolding at equilibrium. Symbols indicate unfolding at 5 μ g/ml (■) and 50 μ g/ml (▲). (C), (D), (E) and (F) show the urea denaturation curves of the BHMT mutants included in the present study at 50 μ g/ml. The graphs show the ratio of emission intensities at 330 and 355 nm for a typical experiment, the continuous line being the fitting of the data to a mechanism with a monomeric intermediate. A.U., arbitrary units.

study, among them the absence of the partner subunit in TS and the presence of the C-terminal helix of contact with the lower dimer in BHMT.

As deduced from the wild-type-enzyme data, secondary-structure changes seemed to precede alterations in tertiary structure; however, further information derived from similar analyses carried out on tryptophan mutants will clarify this point. For this purpose, single- and six-tryptophan mutants were prepared by replacement of tryptophan by phenylalanine. Expression and purification of the mutants were performed as described for the wild-type protein without noticeable variations in yield or purity for the single substitutions. However, six-tryptophan mutants showed a reduced level of expression; that of W44, W342 and W352 was negligible. Moreover, purification of W169, W279, W331 and W373 rendered 15% of the control yield, as expected from its low level of expression. No modifications in the secondary-structure composition of the mutants were detectable by far-UV CD as compared with the wild-type (Figures 5A and

5B). In addition, gel-filtration chromatography and sedimentation velocity showed identical behaviour for wild-type and mutants, indicating preservation of the oligomeric state. Exception to this rule was W352F, which was eluted mostly as expected for a dimer and showed a calculated $s_{20,w}$ of 6.0 ± 0.1 S. All the six-tryptophan mutants contained the W352F substitution, but preserved their tetrameric association. This unexpected result may indicate compensating effects of other mutations around the dimerization arm, precluding dissociation of the tetramer. As for specific activity, only W169F, W279F and W331F showed values comparable with that of the wild-type protein, whereas W44F and W342F showed a 35% decrease, W373F a 60% reduction and W352F only 7% of the control activity. In addition, none of the six-tryptophan mutants showed detectable BHMT activity (Table 2). Kinetics for homocysteine and betaine of the mutants with reduced activity revealed decreases in affinity for both substrates (Table 3). Exception to this rule was W352F, which showed the same parameters as the wild-type protein, thus

Figure 5 Far-UV CD and fluorescence spectra of BHMT mutants

Samples of BHMT proteins were used to record their far-UV CD (250 $\mu\text{g/ml}$) and fluorescence emission (20 $\mu\text{g/ml}$) spectra. For fluorescence recording an excitation wavelength of 295 nm with excitation and emission slits of 2.5 and 5 nm respectively were used. A minimum of five (CD) or ten (fluorescence) spectra were recorded in each case. (A) and (B) include the CD spectra for single- and six-tryptophan mutants respectively. (C) and (D) show the fluorescence emission spectra for the single- and six-tryptophan mutants respectively. All panels include the spectrum of BHMT wild-type (wt) for comparison. A.U., arbitrary units.

indicating an effect at the V_{max} level (Table 3). These data probably reflect the presence of W352F mostly as a low-activity dimer, but the existence of a small amount of tetramer could also account for the reduced activity observed. Surprisingly, despite the high conservation in the amino acid sequence, our results do not completely match those previously published for analogous single-tryptophan mutants prepared in human BHMT [35,38]. In fact, W44F and W352F showed trends similar to those obtained for human W44A and W352A, but not to human W352F [38].

Single-tryptophan mutants were used to obtain urea denaturation curves by activity measurements (Figures 2C and 2D). As for wild-type BHMT, the inactivation profiles showed two transitions with the characteristic denaturant concentrations shown in Table 2, except for W352F, which denatured through a single transition (Figure 2B). Comparison of the values revealed important differences for the first transition shown by W44F, a residue involved in betaine binding [17], its $D^1_{50\%}$ being lower than that for the wild-type, and thus reflecting an effect on active-site stability. The characteristic $D_{50\%}$ for W352F was also lower than that shown by the first transition of the control protein. However, in this case this higher susceptibility to urea cannot be separated from the fact that the dimer may be a less stable assembly and

thus easier to inactivate. As for the second transition, only W342F showed a clear decrease in its $D^2_{50\%}$, whereas the rest of the mutants behaved in approximately the same way as the control.

Urea denaturation of the mutants was also monitored by fluorescence spectroscopy. For this purpose, spectra for all the mutants were obtained in the absence of urea in order to establish the wavelengths of maximal emission in each case (Figures 5C and 5D, and Table 4). Deviations from control value were observed in some mutants, corresponding to red-shifts of 4, 2 and 3 nm for W169F, W331 and W373 respectively and a 2 nm blue-shift for W279F. Again, as for activity measurements, single-tryptophan mutants did not behave as previously described for its human homologues [35]. Only W169F showed the 4 nm red-shift described for the equivalent human BHMT mutant, whereas W352F behaved as the control and W279F showed a blue-shift. Fluorescence intensity is moderately increased for all the single-tryptophan mutants as compared with the wild-type protein. Deviations from this behaviour were again shown by W169F, which showed a fluorescence intensity similar to that of the control, and W279F, which showed a 300% increment in this parameter (Figure 5C). These results indicated that, in the presence of W44, W279, W331, W342, W352 and W373, the

Figure 6 Folding of BHMT followed by CD

BHMT wild-type samples were incubated with urea and used to record their CD spectra in the far- (0.25 mg/ml) and near-UV (1 mg/ml). (A) and (B) include the corrected signal of CD at 220 nm for a typical experiment. (A) Shows the data for the complete unfolding profile, the continuous line being the fitting of the data to the equations for an unfolding mechanism with a monomeric intermediate. (B) Shows an enlargement of the lower scale of urea concentrations comparing fits for a three- (—) and four-state (· · · ·) mechanisms. (C) Includes the near-UV spectra of BHMT at several denaturant concentrations.

Table 2 Activity determinations in BHMT single-tryptophan mutants

BHMT mutants were produced and purified as the wild-type protein and assayed for methionine synthesis. In addition, their unfolding by urea was monitored by activity and the data were fitted to a three-state model. Denaturant concentrations that characterize the process were calculated. The data show the values obtained for a typical experiment carried out in triplicate (mean ± S.D.).

Enzyme	BHMT activity (nmol/min per mg)	Urea denaturation	
		$D^1_{50\%}$ (M)	$D^2_{50\%}$ (M)
Wild-type	17.52 ± 2.38	2.79 ± 0.23	4.93 ± 0.34
W44F	11.2 ± 0.49	1.08 ± 0.39	4.63 ± 0.1
W169F	16.27 ± 0.69	2.7 ± 0.31	5.31 ± 0.24
W279F	16.01 ± 0.62	2.32 ± 0.27	4.85 ± 0.15
W331F	14.77 ± 0.79	2.74 ± 0.45	4.66 ± 0.14
W342F	11.27 ± 0.19	2.44 ± 0.35	4.24 ± 0.2
W352F*	1.23 ± 0.12	0.73 ± 0.15	—
W373F	6.91 ± 0.42	3.16 ± 0.47	5.24 ± 0.49

* Performed at 0.5 mg/ml, owing to low activity, and fitted to a two-state model.

Table 3 Kinetic data of single-tryptophan mutants

Single-tryptophan mutants showing deviations from wild-type behaviour were used to determine their kinetics against homocysteine and betaine. The values included in this Table are the means ± S.D. from a typical experiment carried out in triplicate.

Enzyme	K_m^{Hcy} (μM)	K_m^{betaine} (μM)	k_{cat} (s ⁻¹)
Wild-type	106.4 ± 11.3*	333.3 ± 25.3*	0.013
W342F	500.4 ± 52.3	1610 ± 110	0.0084
W352F	51.6 ± 7.9	331.4 ± 39.8	0.00092
W373F	256.2 ± 29.6	980.9 ± 56.6	0.00518

* Data from [27].

Table 4 Parameters that define tryptophan fluorescence of BHMT mutants

Spectra for BHMT tryptophan mutants were obtained and their characteristic λ_{max} was established. In addition, urea denaturation curves were performed and adjusted to a three-state model for the calculation of the denaturant concentrations that characterize the midpoints of the transitions. The values shown are from a typical experiment performed in triplicate (means ± S.D.).

Enzyme	λ_{max} (nm)	Urea denaturation	
		$D^1_{50\%}$ (M)	$D^2_{50\%}$ (M)
Wild-type	342	2.95 ± 0.18	5.52 ± 0.2
W44F	342	2.69 ± 0.2	5.05 ± 0.12
W169F	346	3.22 ± 0.3	5.54 ± 0.17
W279F	340	2.53 ± 0.27	5.08 ± 0.09
W331F*	342	3.71 ± 0.08	—
W342F	342	2.80 ± 0.11	4.64 ± 0.05
W352F†	342	1.25 ± 0.16	2.77 ± 0.16
W373F	341	3.07 ± 0.09	4.89 ± 0.04
W169	342	1.89 ± 0.2	3.66 ± 0.15
W279	342	3.08 ± 0.19	4.91 ± 0.05
W331	344	2.07 ± 0.22	4.02 ± 0.3
W373	345	2.62 ± 0.29	5.17 ± 0.2

* Fits better to a two-state model.

† Fits to a three-state model of the type N₂–2J–2U.

fluorescence signal is quenched to different extent, an effect especially important for W279. On the other hand, suppression of W169 did not have an important influence in the intensity. As expected, six-tryptophan mutants showed a decrease in fluorescence intensity that reached 50 % of the control value for W169, W279 and W331, whereas this reduction represented 65 % for W373. At equivalent protein concentrations the fluorescence emission spectra of six-tryptophan mutants do not sum up to that of wild-type BHMT. The complexity of the effects on the spectral features of the mutants, which differed from simple additive phenomena, converged to indicate that the global tryptophan fluorescence emission of BHMT is highly affected by local alterations (microenvironment) often considered to be structural quenching [39]. The large increase in fluorescence intensity showed by W279F corresponded to that observed for the corresponding human mutant [35]. However, the rest of the single-tryptophan mutants deviated from the previously described behaviour, W169F being the only one exhibiting a fluorescence intensity similar to the wild-type BHMT, whereas its human equivalent showed a decrease in this parameter [35]. In addition, W44F, W331F, W342F, W352F and W373F moderately increased their intensity of emission, whereas no change compared with the control was detected in their human counterparts. All these discrepancies could be explained in the light of the relative positions of tryptophan residues in human and rat BHMT structures [17,18], as well as by the presence of some of the nonconserved residues

around them. Of special interest are the changes N257G and E353Q observed in the rat sequence [12], both representing important alterations in the environment around these residues, the main effects being related to the size and charge of the replaced amino acids. Changes of charges around tryptophan residues, as well as the presence of metal ions, are among the causes that have been described to quench fluorescence of these residues [39]. Thus mutation N257G in the proximity of W342 (subunit A) and W373 (subunit B) of rat BHMT, as well as that of E353Q in the neighbourhood of W279, W331 and W352, might have an influence on the conformation around these areas and, therefore, on the fluorescence properties of these residues. Moreover, we cannot ignore the influence that the C-terminal α -helix might have in respect of solvent accessibility to this area, an aspect unknown for human BHMT [17].

The $\lambda_{\max}$ values for the single-tryptophan mutants at urea concentrations where the intermediate was maximally populated indicated that only for W44F, W169F, W342F and W373F was a red-shift observed in this parameter. Thus a higher exposure to the polar solvent for the remaining tryptophan residues is obtained in these cases. As for the six-tryptophan mutants, W373 presented a 5 nm red-shift, thus suggesting a conformational change at the loop connecting the C-terminal α -helix, which resulted in a higher exposure of this residue to the polar solvent. However, this change in $\lambda_{\max}$ was observed at higher urea concentrations than the moderate red-shifts (1–2 nm) for W169 and W331, thus suggesting that a slight change in solvent exposure around these two tryptophan residues preceded a larger alteration around W373. Total exposure to the solvent indicated by the presence of the maximum wavelength of emission at 355 nm was obtained at 8 M urea in all the cases.

Denaturant profiles for all the mutants showed two transitions, except for W331F, which shows a profile fitting better to a two-state model (Figure 4). As for the wild-type BHMT, dependence on the protein concentration was observed only for the first of these changes, as expected for a dissociation phenomenon. Urea concentrations at midpoints of the transitions showed deviations from control values, especially for W352F and W331F (Table 4). In fact, $D^{250\%}$ values for W352F approached those of $D^{150\%}$ of the wild-type, thus indicating a lower stability for the dimer. Values for single-tryptophan mutants related to changes in the environments of the remaining tryptophan residues, effects that can be ascribed either to individual alterations or to composite variations in several of these residues. Considering the first transition, W44F and W279F showed higher susceptibilities to denaturant concentrations, thus indicating changes in the tertiary structure around any of the remaining tryptophan residues (W169, W331, W342, W352 and W373). As for the second transition, different behaviours can be observed. First, deviations from control $D^{250\%}$ values were observed for W44F, W279F, W342F and W373F. In fact, these values for W342F and W373F were lower than those calculated for W44F and W279F. Thus the observed changes can be related in the first place to residues 44, 169, 279, 331 and 352 and later to the environments surrounding amino acids 169, 331, 342, 352 and 373. Secondly, W169F behaved as the wild-type for this second transition, indicating a global change in tertiary structure around all the tryptophan residues but W169 in intermediate denaturation. As mentioned above, a special case is that of W331F, which showed only one transition with a characteristic denaturant concentration between $D^{150\%}$ and $D^{250\%}$ values for the control. This behaviour indicated that changes around W331 allowed differentiation between dissociation and intermediate denaturation, as could be expected from its localization at the dimerization arm [17,18]. This picture can be clarified by analysis of the results for six-tryptophan mutants,

Figure 7 Schematic representation of the order of events taking place during BHMT unfolding

Native and unfolded states as well as the intermediates detected during BHMT unfolding appear as boxes. Tertiary structure alterations as detected by the use of tryptophan mutants are also included according to their $D_{50\%}$. Abbreviations are: $\alpha 4$ and $\alpha 7$ represent these same helices of BHMT barrel; DA, the dimerization arm; CL, the loop connecting the C-terminal helix; and SS, the last change in secondary structure that takes place during monomer unfolding.

which revealed deviations to lower denaturant concentrations for W169, W331 and W373 as compared with the native protein (Table 4). Thus BHMT denaturation was initiated by changes in the tertiary structure involving the environment of W169, W331 and W373, leading to an intermediate (Figure 7). Denaturation of this species, the second transition, started again by alterations in the tertiary structure around W169, followed by changes around W331, continued through alterations surrounding W279 and W373, and finished with the production of the denatured monomer. Looking at the secondary-structure level, the alterations detected by fluorescence corresponded in a first step to changes in the $\alpha 4$ helix of the barrel (W169) and in the dimerization arm (W331). Denaturation then continued by effects on the loop connecting the C-terminal α -helix (W373), and changes in areas around tryptophan residues other than W342 and W373, as confirmed by the results with six-tryptophan mutants W169, W279 and W331. These additional alterations in the $\alpha 4$ and $\alpha 7$ helices of the barrel and the dimerization arm occurred earlier than the final loss of activity and led to the monomeric intermediate detected by fluorescence spectroscopy. Evolution of the monomeric intermediate is then observed, starting with changes around tryptophan residues other than W342 and W373, as confirmed by data of the six-tryptophan mutant W279. These alterations thus referred to the $\alpha 7$ helix of the barrel (W279), and are concomitant with complete inactivation. In a second step, tryptophan residues distinct from W44 and W279 were affected, and only changes around the loop connecting the C-terminal helix can be observed (W373).

Tryptophan fluorescence allowed identification of just one of the intermediates in BHMT unfolding. Thus, in order to infer the changes that led to tetrameric-intermediate production, comparison with far-UV CD data was needed. Out of the several transitions, one occurring at 2–3.5 M urea involved a tetramer-to-monomer dissociation exhibiting a dependency on the protein concentration. Thus, according to the Mass Action Law, this transition should result in higher $D_{50\%}$ values when evaluated by CD than the corresponding fluorescence indices. Hence, changes detected by tryptophan fluorescence that took place at much lower denaturant concentrations than I_4 accumulation (3–3.5 M) could be ascribed to events preceding its production. This was the case for the first transitions corresponding to W169 and W331 (Figure 7), and hence the tetrameric intermediate should be a product of alterations in helix $\alpha 4$ and the dimerization arm. In this line, transitions occurring at the interval where I_4 accumulates (Figure 6B) and detected at lower protein concentrations might be related to tetrameric intermediate evolution, as is the case for the first transitions of W373 and W279 mutants. In addition, the second transition detected by fluorescence in W169 and W331 mutants also took place at denaturant concentrations between

tetrameric (far-UV CD) and monomeric (tryptophan fluorescence) intermediate accumulation, but at urea concentrations larger than W373 and W279 initial transitions. Thus these data suggested that I₄ transition to the monomeric intermediate occurs initially through changes in the loop connecting the C-terminal α -helix and alterations in helix α 7, followed by the last modifications in helix α 4 and the dimerization arm. As a last step, comparison between fluorescence and CD data should also give an idea of the monomeric intermediate evolution. Unfolding of the monomeric intermediate, as deduced from fluorescence experiments, starts with changes around W279 (helix α 7) and continued through alterations around W373 (C-terminal loop), these changes being followed by the last effects in secondary structure that are detected at even higher denaturant concentrations. Unfortunately, following this final transition detected by CD spectroscopy no further changes are observed by either of the techniques used in the present study, and hence no further events could be ascribed.

The proposed model includes only tetrameric and monomeric intermediates (Figure 7). However, fluorescence and activity data derived from W352F, a dimeric mutant, indicated the high susceptibility of the BHMT dimer to urea denaturation. Taking into account the concentrations of denaturant at which tetramer dissociated, it was possible that failure to detect dimers was due to their low stability that precluded their accumulation. Moreover, it cannot be excluded that the low-stability dimers dissociated through sedimentation-velocity experiments, as has been described to take place for other oligomers [40]. Thus the presence of dimeric intermediates during BHMT unfolding cannot be completely excluded.

A point currently being discussed is the number of subdomains through which TIM-barrel folding takes place [22–26]. On the basis of the location of tryptophan residues in BHMT and our results it is not possible to shed much more light on to the answer to this question. However, our data indicated independent events for unfolding of two α -helices of the barrel, and thus suggested the presence of at least two subdomains for folding, one including helix α 7 and another, the least stable, comprising helix α 4. The ascription of these helices to subdomains of different stability as those indicated by Silvermann et al. [22] for TIM may be a reflection of the influence of the dimerization arm and the extra C-terminal helix on the whole oligomer. As shown in the rat BHMT structure, this helix is stacked against the dimerization arm and helix α 7 of the barrel, protecting these structural elements from the solvent [18]. Thus perturbation of the C-terminal helix is needed for dissociation and disruption of helix α 7. Previous reports attempting to produce deletion mutants of this C-terminal at G372 were unsuccessful, suggesting a strong contribution of this area to oligomer stability [38], a fact demonstrated by our results.

According to our data, BHMT did not suffer strong inactivation or global tertiary structural changes until the concentration of urea reached 2–2.5 M, indicating that most of the enzyme properties are preserved over this range of denaturant concentrations. Physiologically our data could have some relevance for the role of BHMT in liver and kidney, especially since the presence of this enzyme should account for a large need of betaine, a known cellular osmoprotectant [41]. Compensatory effects have been recently demonstrated in hypertonicity models, where a decrease in BHMT expression, and hence in activity, in those organs was produced [33]. These compensatory effects could be extended as to increase remethylation of Hcy through methionine synthase (MetS), another member of the Pfam 02574 family, as has been observed in chronic ethanol feeding for the maintenance of vital tissue levels of S-adenosylmethionine [42]. For this purpose, MetS should be as stable as BHMT under increased osmolarity. Comparison of the available data that is restricted to the crystal

structures revealed (α/β)₈ barrels as the main features in both proteins [17,18,43]. These data, unfortunately, shed no light on MetS's stability as compared with that of BHMT. Moreover, the relative abundance of BHMT and MetS is quite different, the former being one of the most abundant proteins in liver [4,5]. This high level of BHMT in liver and kidney, along with its stability at moderate urea concentrations, could also be related to other functions of this enzyme, among them interaction with tubulin [44] or its role as a ψ -crystallin [5]. These structural functions are not linked to betaine consumption, at least in rhesus-monkey lens, as no choline oxidation can occur, owing to the lack of mitochondria in those cells [5].

In conclusion, the mechanism of BHMT unfolding included two intermediate states, a tetramer and a monomer. Analysis of the fluorescence properties of the tryptophan mutants indicated that dissociation was concomitant with alterations in the dimerization arm and the loop connecting the C-terminal helix, and intermediate conformations originated from perturbations in these structures, as well as in the barrel. Moreover, the contribution of the extra C-terminal helix to the barrel stability indicated an essential role for additional elements of difference among TIM-barrel proteins.

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