ACTIVE-SITE MUTAGENESIS STUDY OF RAT LIVER BETAINE HOMOCYSTEINE S-METHYLTRANSFERASE.

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Short page heading: Betaine homocysteine methyltransferase active site.
SYNOPSIS

A site-directed mutagenesis study of putative active-site residues in rat liver betaine homocysteine S-methyltransferase has been carried out. Identification of these amino acids was based on data derived from a structural model of the enzyme. No alterations in the circular dichroism spectra or the gel filtration chromatography elution pattern were observed in the mutants, thus suggesting no modifications in the secondary structure content or the association state of the proteins. All the mutants obtained showed a reduction of the enzyme activity, the most dramatic case being that of E159, followed by Y77 and D26. Changes in affinity for either of the substrates, homocysteine or betaine, were detected when substitutions were carried out on E21, D26, F74 and C186. Interestingly, D26 postulated to be involved in homocysteine binding, has a strong effect on betaine affinity. The relevance of these results is discussed in the light of very recent structural data obtained for the human enzyme.

Keywords: structural modelling, site-directed mutagenesis, methionine metabolism, homocysteine metabolism, kinetic study.

Abbreviations: BHMT, betaine homocysteine methyltransferase; Hcy, homocysteine, AdoMet, S-adenosylmethionine; AdoHcy, S-adenosylhomocysteine; ADA, adenosine deaminase; CBS, cystathione β-synthase; MS, methionine synthase; CB-Hcy, S(δ-carboxybutyl)-L-homocysteine; SS, secondary structure.
INTRODUCTION

Homocysteine (Hcy) represents a key branch point in methionine metabolism [1]. Its synthesis is catalysed by S-adenosylhomocysteine hydrolase (EC 3.3.1.1) in a reaction that is thermodynamically shifted to the production of S-adenosylhomocysteine (AdoHcy). This compound is a potent inhibitor of the transmethylation reactions dependent on S-adenosylmethionine (AdoMet) [2], therefore elimination of Hcy has to be carried out coupled with its synthesis to maintain the methylation ratio, AdoMet/AdoHcy. Three are the enzymes that use Hcy, cystathionine β synthase (CBS, EC 4.2.1.22), methionine synthase (MS, EC 2.1.1.13) and betaine homocysteine S-methyltransferase (BHMT, EC 2.1.1.5). CBS initiates the transulfuration pathway leading to cystathionine, which is further used for the synthesis of cysteine [3]. The other two enzymes remethylate Hcy to render methionine in reactions that use different methyl donors, methyltetrahydrofolate and betaine. These methyl donors connect the pathway with folate metabolism and choline oxidation allowing the recovery of one of the methylation equivalents used in choline synthesis, leading to the incorporation of 4 of its carbon units in the one-carbon pool [1]. This picture is completed by the fact that AdoMet, vitamin B₆ and vitamin B₁₂ control directly or indirectly the activity of these three enzymes. Alterations in the Hcy levels reflect changes in any of the reactions mentioned above. Thus, homocystinuria has been related to deficiencies in CBS and methylene tetrahydrofolate reductase (EC 1.7.99.5). Moreover, elevated plasma levels of Hcy (hyperhomocysteinemia) have also been detected in patients with cardiovascular disease, this increase thus being considered an independent risk factor for the development of this type of illness [4,5].

BHMT is the enzyme of Hcy metabolism least studied. As the majority of these enzymes is an oligomer, composed of four identical subunits [6-8], that in rat liver contains 407 amino acids (accession number AF038870). Its localisation is cytosolic and restricted to the liver and kidney in most of the species studied [9,10], but its apparition in lens has also been reported in Rhesus monkey [11]. Conservation among
mammalian enzymes is very high, being more than 80% identical at the nucleotide level, and more than 90% at the amino acid level for the rat, human, mouse and pig liver proteins [12]. This enzyme contains zinc coordinated to three conserved cysteine residues (217, 299 and 300 according to the human sequence)[13,14]. One of the mutations detected so far in patients with moderate hyperhomocysteinemia (G199S) has been suggested to play an important role in the secondary structure of the enzyme [15]. Alterations of betaine levels and decreases in BHMT activity have been observed in choline deficiencies [16], whereas in methionine deficiencies the enzyme maintains the levels of this essential amino acid for mammalian growth and development [17]. Using vitamin B$_{12}$ deficient diets a decrease in enzyme activity has been observed [18], whereas increases have been detected in low protein diets combined with alcohol, alcohol liquid diets, high protein diets and methionine supplemented diets [19]. Studies in developing rats have shown that fetal liver activity is lower than in the adult, a peak being observed in the first 10 days of the neonate [20]. Expression of BHMT mRNA is decreased in cirrhotic livers and hepatocellular carcinoma [21,22], and increased in samples of animals with a methionine deficient diet [23]. Moreover, hydrocortisone and thyroxine treatments also affect BHMT activity [20]. Recently, it has been shown that BHMT regulates the expression of apolipoprotein B, leading to an increase in related lipoproteins [12], and it has been located bound to tubulin in liver extracts [24]. On the other hand, betaine has been proposed to play a role in the protection of proteins against denaturation in kidney, due to the high level of urea accumulated in the cells [25], a similar role being ascribed to the lens BHMT [11].

Finally, structural data on this enzyme are very limited, and hence, the objective of this paper is to get insight into the role of active-site residues. For this purpose, we have built a structural model of the rat liver protein and the role of the proposed active-site amino acids has been tested by site-directed mutagenesis. The validity of the identification of catalytically important residues derived from this model has been confirmed with the report of the first structure of a human BHMT complex [8] while
this manuscript was in preparation. Therefore, our results are discussed in the light of the data derived from that X-ray structure.
EXPERIMENTAL

Materials.

Betaine, homocysteine thiolactone, PMSF, pepstatin A, aprotinin, leupeptin, antipain, DTT, ampicillin, Freund's adjuvant and the molecular mass standards for gel filtration chromatography were products from Sigma Chemical Co. (St. Louis, MO, USA). [methyl-14C] choline chloride (50-60 mCi/mmole) was supplied by Amersham International (Little Chalfont, Bucks., UK). IPTG was a product of AMBION (Austin, TX, USA). Superose 12 HR 10/30 was purchased from Amersham Pharmacia (Uppsala, Sweden). Optiphase HiSafe 3 scintillation fluid was obtained from E & G Wallac (Milton Keynes, UK). Goat ant-rabbit IgG-horseradish peroxidase, Bio-Rad protein assay kit I and the electrophoresis reagents were from Bio-Rad (Richmond, CA, USA). YM-30 ultrafiltration membranes were purchased from AMICON Corp. (Beverly, MA, USA). Chemiluminescence Renaissance reagents was obtained from DuPont New England Nuclear (Boston, MA, USA). Triton X-100 was purchased from Merck (Darmstadt, Germany). QuikChange Site-directed mutagenesis kit and the Impact-CN system were supplied by Stratagene (La Jolla, CA, USA) and New England Biolabs (Beverly, MA, USA), respectively. The rest of the buffers and reagents were of the best quality commercially available.

Sequence alignments, secondary structure prediction and template searching.

Sequence of rat liver BHMT (SwissProt O09171) was used to search homologous enzymes and predict its secondary structure. Homologous proteins have been searched using Psi-BLAST server [26]. Alignments between BHMT and other enzyme sequences were made with the CLUSTAL X program [27] and were corrected manually when necessary. For secondary structure (SS) prediction, three different methods were used: PHD [28], PSIPRED [29] and JPRED [30]. We considered as reliable secondary elements those with a score over six (nine being the maximum). All these three methods provided very similar results.
On initiating this study no structure for the BHMT family of enzymes was known as revealed by a Psi-BLAST search. Thus, to find a template for BHMT model building, different strategies were tried. First, some enzymes with known structure and functionally related to BHMT, as Zn$^{2+}$-dependent transferases or methyltransferases, were tested as possible templates with no success. Therefore, protein-fold recognition methods were applied using 3D-PSSM [31], THREADER [32] and BIOINBGU [33] threading algorithms. Enzymes with good scores by at least two methods were tested as templates for BHMT homology modelling, and were used to build a preliminary model when the following criteria were fulfilled: 1) their secondary structure correlated with BHMT SS prediction, and 2) some relevant residue conservation was observed.

**Homology modelling of the BHMT protein structure.**

A three-dimensional structure for BHMT has been modelled by comparative protein modelling methods using the MODELLER package [34]. This program uses spatial restraints from the input templates and the model to be built has to satisfy the restraints, as much as possible. Preliminary models were built from an alignment based on SS correlation between the templates and the predicted BHMT SS. To consider definitively a protein as a good template, the cysteines involved in Zn$^{2+}$ binding had to be oriented properly in the preliminary models. One of the proteins tested, adenosine deaminase (ADA), fulfilled all the selection criteria, and hence, used as a template (pdb code: 1a4l) [35]. Initially, fifteen BHMT models were built, but only that with the fewest restraints violation and lowest energy was evaluated, and several cycles of realignment and model building were repeated until no further optimisation of the model was obtained. The final model was evaluated with VERIFY3D [36] and PROCHECK [37]. JOY [38] and COMPARER [39] programs were used to generate structure-based alignments between the target and the template in every cycle of the model building process.

**Expression of the fusion protein and purification of rat liver BHMT.**
The sequence of rat liver BHMT included in the Ndel/BamHI sites of the pET11a expression vector [7] was cloned in pBluescript M13 (+) KS, and later in the Ndel/Not I restriction sites of pTYB12 to obtain an intein/chitin-binding-BHMT fusion protein. Competent *E. coli* BL21(DE3) cells were transformed with the plasmid pBHMT-TYB12 and 1l cultures were grown in LB medium containing 100 µg/ml ampicillin. Induction was carried out for 16 hours at 20ºC by adding 0.5 mM IPTG at A₅₉₅ 0.6. Cells were harvested by centrifugation, washed with water and stored at -70ºC until use. Disruption of the cell pellets was carried out by sonication at 4ºC in a Branson 250 sonifier (30 pulses of 30s at 30s intervals, output power level 20) in 10 volumes of 20 mM Tris/HCl pH 8.0, containing 0.5 M NaCl, 0.1 mM EDTA, 0.1% (v/v) Triton X-100 and protease inhibitors (2 µg/ml aprotinin, 1 µg/ml pepstatin A, 0.5 µg/ml leupeptin, 2.5 µg/ml antipain, 0.1 mM benzamidine, 0.1 mM PMSF). The soluble fraction was separated by centrifugation for 30 min at 13000 g and loaded on Chitin beads (10 ml) for purification. The column was washed first with 30 bed volumes of the buffer, followed by 10 volumes of 20 mM Tris/HCl pH 8.0, containing 50 mM NaCl, 0.1 mM EDTA (buffer A) and 3 volumes of buffer A containing 30 mM 2-mercaptoethanol (buffer B). The column was incubated for 48 hours at room temperature to allow intein cleavage and eluted with 3 volumes of buffer B. The purified preparation was then dialysed against buffer A (3 l) in order to eliminate a small peptide originated during cleavage of the fusion protein. The final purification yield was 4 mg/g of bacteria. For storage purified BHMT was made 50% glycerol and kept at -20ºC.

**Site-directed mutagenesis.**

Residues of interest were mutated using the QuikChange Site-directed mutagenesis method, following manufacturer’s instructions. Mutants were constructed in the vector pBHMT-TYB12 using the mutagenic oligonucleotides included in table 1.
Mutations were verified by sequencing the entire cDNA, using the dideoxy termination method [40].

**Activity and kinetic determinations.**

BHMT activity measurements were carried out for 1 hour at 37°C using the radioassay previously described [3]. Kinetics for homocysteine and betaine were performed in a concentration range between 1 µM and 6.5 mM at a saturating concentration of either betaine (6.5 mM) or homocysteine (6.5 mM).

**Determination of secondary structure.**

Far-UV circular dichroism spectra of purified BHMT and mutants were recorded on a Jasco J-810 spectropolarimeter at 25°C [41], using samples of 0.1-0.2 mg/ml protein concentration and 0.1 cm pathlength cuvettes. After baseline subtraction the observed ellipticities were converted to mean residue ellipticities ($\theta_{\text{mrw}}$) on the basis of a mean molecular mass per residue of 110 Da. Secondary structure composition was calculated using the Jasco software. A minimum of five spectra was taken for each sample.

**Gel filtration chromatography.**

BHMT and mutant samples of 100 µl (50 µg) were injected on a Superose 12 HR 10/30 gel filtration column connected to an Advanced Protein Purification System (Waters). Equilibration and elution were performed using 20 mM Tris/HCl pH 8.0, containing 200 mM NaCl and 0.1 mM EDTA at a flow rate of 0.3 ml/min at 4 ºC. Absorption at 280 nm was recorded and 210 µl fractions collected, to detect the presence of BHMT by Dot-blot. The protein standards used and their elution volumes were: Dextran Blue (2000 kDa) 8.69 ml, apoferritin (443 kDa) 9.87 ml, β-amylase (200 kDa) 11.34 ml, alcohol dehydrogenase (150 kDa) 12.07 ml, carbonic anhydrase (29 kDa) 15.12 ml, and ATP (551 Da) 19.26 ml.
Production of a polyclonal anti-BHMT antiserum.

Rat liver BHMT purified as described by González et al [7] was used to immunise New Zealand rabbits. Preimmune serum was obtained before injection of the antigen. The immunisation protocol used was that previously described by our laboratory [42]. The specificity of the response was tested by immunoblotting (figure 1), and the best results were obtained using a 1:50000 dilution (v/v) of the antiserum.

SDS-PAGE and Western blot.

Denaturing gel electrophoresis of the samples was carried out on 10% PAGE-SDS gels. Staining was performed using Coomassie blue R-250. When needed a western blot of the gel was performed using the buffer system described by Mingorance et al [42], and revealed using the anti-BHMT antiserum mentioned above.

Dot-blot.

Samples of the column fractions (maximum volume 50 µl) were spotted on nitrocellulose membranes. After denaturation using 6 M guanidinium chloride (50 µl) the membrane was washed twice with TTBS, before the blocking step using low-fat dry milk (3% w/v). The membrane was washed again with TTBS and incubated with a 1:50000 (v/v) solution of the anti-BHMT polyclonal antiserum prepared in our laboratory. Under these conditions the only band detected corresponds to BHMT as judged by SDS-PAGE gels (figure 1). Membranes were revealed using Renaissance, the exposed films subjected to densitometric scanning and the data used to elaborate the elution profile.

Determination of the protein concentration.

Protein concentration of the samples was measured using the Bio Rad kit I, and BSA as a standard, or spectrophotometrically using a calculated molar absorbance
coefficient ($\varepsilon$) at 280 nm of 58020 M$^{-1}$cm$^{-1}$ in 6M guanidinium chloride. This coefficient calculated from the sequence was corrected for the mutants as appropriate.
RESULTS AND DISCUSSION

BHMT belongs to the family of thiol/selenol methyltransferases. Sequence homology among these enzymes is not high, but a strong conservation of two motifs, which include the three cysteine residues involved in zinc binding, is observed. In addition, secondary structure prediction renders very similar results for all these proteins (figure 2). Thus, it is believed that all of them may share a common folding. At the start of this study no structure for this family of proteins was available, except for two domains of the vitamin B₁₂-dependent MS that have no homology with BHMT [43,44]. Consequently, it was necessary to search for templates among possible structural homologues in order to build a model for BHMT structure. Thus, searches were carried out based on either function similarity or fold-recognition methods, success being achieved only by the use of the latter. Most of the suggested templates presented the typical TIM barrel fold, but only those proposed by at least two of the methods employed were used to build preliminary models. Further selection was carried out based on the following criteria: a) good correlation between their SS and that predicted for BHMT; b) conservation of catalytically relevant residues in key areas of the protein; and c) correct positioning of the cysteines involved in zinc binding according to the previous data for BHMT [14]. Only mouse ADA fulfilled these criteria, and hence was considered for further analysis.

Mouse ADA belongs to the family of metal-dependent hydrolases and catalyses the hydrolysis of adenine to render inosine, a reaction linked to methionine metabolism since, among other pathways, adenine can be produced by AdoHcy hydrolysis in the same reaction that leads to Hcy [45]. Moreover, ADA is a zinc-dependent metalloenzyme in which the metal assumes the same role postulated in BHMT, activating a water molecule for a further nucleophilic attack [35,46,47]. Sequence similarity between BHMT and ADA is 52%, being only 13% identical. Their predicted SS correlate well, and in addition some of the zinc ligands are conserved (C217 of BHMT aligns with H214 of ADA, and both C299 and C300 with D297) (figure 3). The
structural model constructed does not include the 47 N-terminal amino acids. This segment is absent in many of the members of the thiol/selenol family, suggesting a non-essential role of this area for the overall folding. The best model obtained is shown on figure 4. The proposed BHMT structure is a \((\alpha/\beta)_8\) barrel, in which the three cysteines identified as zinc ligands (C217, C299 and C300) are pointing to the centre of the barrel. One of the eight \((\alpha/\beta)\) blocks comprising the core of the barrel was not predicted for the thiol/selenol methyltransferases, and was introduced in the model on the basis of the alignment with ADA. The final model presents only 2.6% of the residues located in disallowed regions according to a Ramachandran plot, an acceptable value for models constructed using this approach. Evaluation of the model with verify 3D produces all positive scores.

The typical \((\alpha/\beta)_8\) barrel fold is one of the largest and most regular structures described to date. Several features are repeated in all the proteins folded this way, one of them being the location of the active-site at the centre of the barrel's cavity. As shown in figure 2, \(\beta\)-strands and \(\alpha\)-helixes are more conserved and form the barrel's core, whereas more variability is present at the loops. Residues involved in catalysis are located in the strands surrounding the cavity, and the loops following the C-ends of these strands provide the amino acids related to substrate specificity. In our model the highly conserved motifs I (G(LIV)NC\(^{217}\)) and II (GGCC\(^{300}\)) [14], containing the zinc-binding cysteines, are located in loops at the C-ends of two \(\beta\)-strands in the barrel. Moreover, due to the fact that this family of enzymes binds zinc and all use Hcy, it is possible to postulate that the most conserved loops may be related to their binding. Following the same criteria, those areas less preserved, but common among enzymes using the same methyl donor, could be related to betaine binding. Thus, several interesting residues have been identified in our model: E21, D26, T73, F74, Y77, A119, E159, T184 and C186. Some of them have been selected due to their proper location at the C-ends of \(\beta\)-strands in the model. Besides, they are conserved in all the enzymes of the family (A119, E159 and T184), align with relevant residues of ADA (E21 and D26).
or were proposed to be next to the Zn$^{2+}$-binding site (C186) (figure 3). The rest of the selected residues (T73, F74 and Y77) are present in a conserved motif, $^{73}$Tx(ST)(YF), that contains more aromatic amino acids directed to the barrel's cavity, as required for betaine binding [48]. All these facts make these residues ideal candidates for their further study by site-directed mutagenesis.

In order to clearly establish the role of the residues identified in the structural model several mutants were prepared (table 1). For this purpose, the IMPACT CN system was used, since it allowed a faster purification to homogeneity of the proteins under study. In our case, the expression vector of choice was pTYB12, in order to protect the BHMT N-terminal from bacterial aminopeptidases. Cleavage was achieved as described by Breksa et al [14], leading to a BHMT protein including three extra amino acids at its N-terminal (AGH). Addition of these extra residues did not interfere with either activity or SS, since the levels of methionine synthesis achieved and the CD spectra were similar to those obtained from a liver-purified (18.7 nmol/min/mg) and a pETT11a overexpressed BHMT (18.5 nmol/min/mg). Total and cytosolic expression levels were similar for wild type and mutants, as judged by Coomassie blue staining of SDS-PAGE gels (data not shown). The purification yield was similar (approximately 4 mg/g bacteria), and in addition an anti-BHMT antiserum was able to recognise all the mutants with similar specificity. Moreover, the purification procedure was the same for all the proteins, suggesting that BHMT conformation was not significantly altered in the mutants. Far-UV CD spectroscopy showed no significant differences among spectra (figure 5). The content in main secondary structure elements has been calculated and appears in table 2. The composition obtained compares favourably to the values from prediction methods, and those derived from the X-ray structure of human BHMT at the $\alpha$-helix content, whereas the $\beta$-sheet percentage resulting from this analysis is overestimated. This observation concurs with the difficulties in the accurate assignation of $\beta$-sheet structural elements from CD spectra, often being rigorously resolved using complementary spectroscopies [41,49,50]. In addition, the oligomeric state of the
mutants was also evaluated by gel filtration chromatography. No significant changes in
the elution pattern were observed, the elution volume corresponding to that of a
globular protein with an estimated Mr of 187000. Therefore, it can be inferred that none
of the residues studied plays a significant role in the association of the subunits.

All the mutant proteins exhibited a reduced activity (table 3). Four groups can be
identified, those with a 70% of activity (E21K, E21A, C186S, C186A, F74A, A119G,
T73G, T184G), those with an intermediate level (D26A), others with a 15-10% of
activity (Y77A, D26I), and finally those with less than a 2% of the original level
(E159G, E159K). To get insight the significance of these changes, kinetics for all the
mutants within the first three groups were carried out (table 3). This type of analysis
was not performed in the rest of the mutants due to the low sensitivity of the assay, on
the other hand the most accurate for BHMT activity determinations [51]. $K_m$ values
obtained for the recombinant wild type protein differed from those previously published
for the rat liver-purified BHMT [52,53], being 9- and 3-fold higher for Hcy and betaine,
respectively. In fact, the $K_m$ obtained for Hcy is closer to that published for the human
liver-purified enzyme [54] than to that for the recombinant human BHMT [13].
Regarding betaine $K_m$, the values differed strongly from those published for the human
liver enzyme, that are approximately 2.3 mM [13]. Mutant proteins E21A, T73G, F74A,
C186A and T184G showed increases in affinity for Hcy. Changes of either sign were
also detectable in betaine affinity. Slight increases, approximately 30%, were observed
for E21A, E21K, C186S and C186A. In contrast, decreases (more than 3-fold) were
patent for D26A and F74A, the $K_m$ values reaching the millimolar range as described for
the human liver BHMT [13]. Thus, based on these results it can be inferred that D26
and F74 play a role in betaine binding, since the mutants generated for these residues
show a diminished activity and a dramatic change in affinity for this substrate. With
respect to Hcy binding, the strongest effect, a 4- to 5-fold increase in affinity, was
shown by E21A and C186A. However, the hydroxide-dependent conversion of Hcy
thiolactone to Hcy is not quantitative [55], and hence has a clear effect on the real
concentration of the amino acid in each assay, a fact that can hardly be estimated. Therefore, it could be suggested that E21 and C186 may play a role in Hcy binding, but its importance cannot be directly quantitated.

Very recently the structures of human BHMT, Zn\(^{2+}\)-depleted and Zn\(^{2+}\)-repleted in complex with a transition-state analogue, S(\(\delta\)-carboxybutyl)-L-homocysteine (CB-Hcy), have been published [8]. The overall fold described for the monomer is a \((\alpha,\beta)_8\) barrel as that proposed in our structural model. Moreover, the complex with CB-Hcy highlighted the role of some amino acids in the active-site, among them D26, E159, and Y77 (numbered according to the human BHMT sequence). Such residues are the same that show important effects on activity in our mutagenesis study (table 3). The postulated roles for these residues are related to Hcy binding (D26), establishing stereospecificity for L-Hcy (E159), and interacting with the carboxyl group of the methyl donor (Y77)[8]. Activity measurements of the corresponding mutants revealed strong reductions in methionine synthesis in all the cases, except for D26A, where an important diminishment in affinity for betaine was detectable, an effect also shown by F74A. Substitutions carried out in D26 are related to changes in the charge and the volume of the side chain (slight decrease in D26A and increase in D26I). It is relevant to note that D26A affects the affinity for betaine, in contrast to its role in Hcy binding proposed and deduced from its high level of conservation among the thiol/selenol methyltransferase family [8]. In the human BHMT crystal structure, D26 interacts with the backbone amide of Q72 from \(\beta_2\)-strand. This interaction brings apart \(\beta_1\) and \(\beta_2\)-strands, creating a cleft between them, which has been proposed to be important in Hcy accommodation. Nevertheless, our results suggest another role for D26 related to betaine binding. Accordingly, D26 seems to be involved in shaping the active-site, and this role could also be reflected by changes in activity and the affinity for betaine. An increase in the \(K_m\) values for betaine was also patent in F74A, where a reduction in the length and volume of the side chain is produced, thus indicating a role for the aromatic ring in betaine binding. F74 precedes F76 and Y77, which are involved in betaine
binding, as suggested in the CB-Hcy complex with human BHMT. In addition, F74 is packed against other non-polar residues, and substitution of phenylalanine by alanine would break this packing, resulting in a disruption of the local conformation, and therefore affecting betaine binding.

The results obtained with E159 mutants confirmed the crucial importance of its side chain in BHMT activity, since either of the substitutions performed (elimination of the side chain or change of charge in the ε-group) abolished methionine production. Moreover, the carboxybutyl moiety of CB-Hcy served to suggest the binding site for glycinebetaine through its carboxyl group, a role ascribed to Y77 of human BHMT [8]. Again our model suggested an essential position for this residue, and in fact its substitution by alanine produced a strong reduction of the activity, thus highlighting the importance of its side chain.

Other residues located close to the Hcy binding site also show interesting features when their substitution is carried out. A conservative change performed on C186, as that produced by the inclusion of serine, exerted no effect on substrate affinity. However, in C186A when the amino acid chain is shortened and the -SH or -OH groups disappear an increase in Hcy affinity is detectable, suggesting a role for this position in substrate binding. Such a role could not be ascribed in the human crystal structure, since this is one of the five cysteine residues, considered non-essential, that were mutated to avoid aggregation [8]. In addition, mutation of T184, just two residues downstream in the sequence, also affected Hcy affinity. T184 interacts through its side chain with N216, which is in motif I (GVNC) involved in Zn\(^{2+}\) binding. Elimination of the side chain by substitution for glycine suppressed this interaction, and hence could have an indirect effect on ion binding, and therefore in Hcy affinity. Mutations carried out on human N216 showed decreases in activity, that in the case of N216A were comparable to that produced by T184G, but effects on affinity were not explored [14].

Finally, all our data indicate that the structural model presented here shows the main features of the BHMT monomer, and allows the identification of key residues for
enzyme activity. Their importance has been confirmed by site-directed mutagenesis and kinetic studies, allowing the ascription of a further role for D26 in the active-site. The results obtained are consistent with those derived from X-ray diffraction, thus suggesting that these types of structural models can be useful tools for other families of enzymes with unknown structure, allowing the identification of key residues involved in catalysis.
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REFERENCES


**TABLE 1**

**Mutagenic oligonucleotides.** The primers synthesised for site-directed mutagenesis are included. Only the sense strand is shown, and the substitution performed appears underlined.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Mutagenic oligonucleotide</th>
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<tr>
<td>E21A</td>
<td>5'-GCTTAAATGCTGGCGCAGTCGTGATCGG-3'</td>
</tr>
<tr>
<td>E21K</td>
<td>5'-CGCTTAAATGCTGGCAAAGTCGTGATCGG-3'</td>
</tr>
<tr>
<td>D26A</td>
<td>5'-GTCGTGATCGGAGCGTGGGGGATTTGTC-3'</td>
</tr>
<tr>
<td>D26I</td>
<td>5'-GTCGTGATCGGAATTGGGGGATTTGTC-3'</td>
</tr>
<tr>
<td>T73G</td>
<td>5'-CGAACGTCATGCAGGGCTTCACCTTTCTATGC-3'</td>
</tr>
<tr>
<td>F74A</td>
<td>5'-CGAACGTCATGCAGACCGCCACTTTCTATGC-3'</td>
</tr>
<tr>
<td>Y77A</td>
<td>5'-GCAGACCTTCTACTTGCTGCAAGGTGGAC-3'</td>
</tr>
<tr>
<td>A119G</td>
<td>5'-GGATGCATTGGTGGAAGGATGTTGAG-3'</td>
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<td>E159G</td>
<td>5'-GGACTTCCTCATTGCAAGGTATTTTGAAATG-3'</td>
</tr>
<tr>
<td>E159K</td>
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<tr>
<td>C186A</td>
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</table>
TABLE 2

Comparison of the content in secondary structure elements. Data of the content in the main secondary structure elements obtained from circular dichroism spectra are compared with those derived from prediction methods and X-ray diffraction studies on human BHMT. CD calculation is within the 10% error due to protein concentration measurements. Predicted and X-ray diffraction values are based on 360 amino acids that are those shown in the solved structure.

<table>
<thead>
<tr>
<th></th>
<th>CD spectra (%)</th>
<th>Predicted (%)</th>
<th>X-ray diffraction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-helix</td>
<td>28.6</td>
<td>34</td>
<td>38</td>
</tr>
<tr>
<td>β-sheet</td>
<td>31.4</td>
<td>7</td>
<td>11</td>
</tr>
<tr>
<td>turns</td>
<td>10.1</td>
<td></td>
<td>20</td>
</tr>
<tr>
<td>random coil</td>
<td>20.9</td>
<td></td>
<td>31</td>
</tr>
</tbody>
</table>
### TABLE 3

**Kinetic parameters for betaine homocysteine S-methyltransferase.** BHMT activity was measured as described under Experimental for 1 h at 37ºC. Kinetics were performed in the 1 µM to 6.5 mM range for both substrates, Hcy or betaine. Affinities for the substrates in mutants with less than 15% of the *wild type* activity were not determined (n.d.).

<table>
<thead>
<tr>
<th></th>
<th>$V_{\text{max}}$ (nmol/min/mg)</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$K_m$ (Hcy) (µM)</th>
<th>$K_m$ (betaine) (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>18.67 ± 0.14</td>
<td>0.014</td>
<td>106.4 ± 11.3</td>
<td>333.3 ± 25.3</td>
</tr>
<tr>
<td>E21A</td>
<td>8.39 ± 0.25</td>
<td>0.006</td>
<td>17.47 ± 3.2</td>
<td>138.5 ± 13.2</td>
</tr>
<tr>
<td>E21K</td>
<td>12.82 ± 0.34</td>
<td>0.009</td>
<td>72.66 ± 8.9</td>
<td>195.1 ± 16.87</td>
</tr>
<tr>
<td>D26A</td>
<td>6.81 ± 0.17</td>
<td>0.0051</td>
<td>62.93 ± 2.6</td>
<td>1174 ± 102.6</td>
</tr>
<tr>
<td>D26I</td>
<td>2.86 ± 0.67</td>
<td>0.0021</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>T73G</td>
<td>6.09 ± 0.2</td>
<td>0.0045</td>
<td>49.25 ± 7.9</td>
<td>258.4 ± 17.1</td>
</tr>
<tr>
<td>F74A</td>
<td>9.69 ± 0.26</td>
<td>0.0072</td>
<td>57.44 ± 7.8</td>
<td>968.6 ± 99.5</td>
</tr>
<tr>
<td>Y77A</td>
<td>1.36 ± 0.01</td>
<td>0.001</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>A119G</td>
<td>10.05 ± 0.3</td>
<td>0.0075</td>
<td>67.98 ± 9.46</td>
<td>305.6 ± 29.6</td>
</tr>
<tr>
<td>E159G</td>
<td>0.305 ± 0.02</td>
<td>0.00023</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>E159K</td>
<td>0.11 ± 0.025</td>
<td>0.000082</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>T184G</td>
<td>7.77 ± 0.6</td>
<td>0.0058</td>
<td>34.46 ± 13.2</td>
<td>548.1 ± 55.4</td>
</tr>
<tr>
<td>C186S</td>
<td>8.01 ± 0.26</td>
<td>0.006</td>
<td>74.45 ± 11.1</td>
<td>206.5 ± 19.9</td>
</tr>
<tr>
<td>C186A</td>
<td>10.12 ± 0.41</td>
<td>0.0076</td>
<td>24.73 ± 5.64</td>
<td>185.2 ± 11.4</td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

**Figure 1. Specificity of the anti-BHMT antiserum.** BHMT was purified and used for rabbit immunisation as described under Experimental. Immune serum was collected and used to test for specificity in western blot. For this purpose, 10 µg of either purified protein (lane 1) or rat liver cytosol (lane 2) were loaded on SDS-PAGE gels and transferred to nitrocellulose membranes. The molecular weight of the markers is stated on the right.

**Figure 2. Alignment of enzyme sequences belonging to the thiol/selenol methyltransferase family.** The alignment includes data from the thiol/selenol methyltransferase family. The highly conserved secondary structure prediction among these proteins is presented as grey-shadowed regions (α-helix with white characters and β-strands with black characters). The α-helices and β-strands comprising the barrel in human BHMT crystal structure are highlighted below the sequences. As it can be observed, only β2 was not predicted. The conserved residues are marked with an asterisk below the sequence alignment, whereas the residues mutated in this work are indicated by their sequence number. Motifs I and II comprising the Zn$^{2+}$-binding residues (C217, C299 and C300) appear included in squares, as well as the conserved motif Tx(ST)(YF)x. Proteins aligned are: rat liver BHMT, *E. coli* B$_{12}$-dependent MS, S-methylmethionine Hcy methyltransferase from *Arabidopsis thaliana* and S-methylmethionine selenocysteine methyltransferase from *Astragabus bisulcatus*.

**Figure 3. Alignment of mouse ADA and rat liver BHMT used in model’s construction.** Secondary structure elements, α-helices and β-strands (grey-shadowed regions with white and black characters, respectively) of ADA and those built for BHMT model are shown. The secondary structure elements comprising the barrel in the human BHMT crystal structure are schematically represented below the sequence.
alignment. As can be observed, seven out of eight \( \beta/\alpha \) blocks were assigned properly in the BHMT model. Zn\(^{2+}\)-ligands identified for both enzymes are included in squares.

**Figure 4. Structural model for rat liver BHMT.** The structural model of BHMT built using mouse ADA as a template revealed a \((\alpha/\beta)_8\) barrel as the monomer fold. The main secondary structure elements appear in grey (\(\alpha\)-helices) and black (\(\beta\)-strands). The three cysteine residues involved in Zn\(^{2+}\)-binding are represented as ball and sticks, and the zinc atom was modelled between them.

**Figure 5. Circular dichroism spectra of wild type and mutant BHMT.** Only CD spectra of wild type and the most relevant mutants, whose activity is less than 15% of the original BHMT, are shown for graphical purposes. Symbols indicate: wild type (solid line), D26I (bold dashed line), Y77A (dotted line), E159G (squares) and E159K (thin solid line). Spectra are the average of five scans.
Figure 1
Figure 2
Figure 3
\[ \Theta_{\text{max}}, 10^3 \text{ (degree-cm}^2\text{-dmol}^{-1}) \]

\[ \lambda \text{ (nm)} \]