Cell. Mol. Life Sci. 63 (2006) 2792–2803
1420-682X/06/232792-12
DOI 10.1007/s00018-006-6249-6

Cellular and Molecular Life Sciences

Review

Betaine homocysteine S-methyltransferase: just a regulator of homocysteine metabolism?

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Received 26 May 2006; received after revision 3 July 2006; accepted 24 August 2006
Online First 3 November 2006

Abstract. Betaine homocysteine methyltransferase (BHMT), a Zn^{2+}-dependent thiolmethyltransferase, contributes to the regulation of homocysteine levels, increases in which are considered a risk factor for cardiovascular diseases. Most plasma homocysteine is generated through the liver methionine cycle, in which BHMT metabolizes approximately 25% of this non-protein amino acid. This process allows recovery of one of the three methylation equivalents used in phosphatidylcholine synthesis through transmethylation, a major homocysteine-producing pathway. Although BHMT has been known for over 40 years, the difficulties encountered in its isolation precluded detailed studies until very recently. Thus, the last 10 years, since the sequence became available, have yielded extensive structural and functional data. Moreover, recent findings offer clues for potential new functions for BHMT. The purpose of this review is to provide an integrated view of the knowledge available on BHMT, and to analyze its putative roles in other processes through interactions uncover to date.

Keywords. Betaine homocysteine methyltransferase, methionine, homocysteine, betaine, structure/function relationship, regulation, distribution, protein interaction.

Introduction

In recent years, homocysteine (Hcy) has become a subject of great interest, due to its implication as a risk factor for cardiovascular diseases. However, increases in plasma levels (tHcy) have also been detected in disorders such as psoriasis, renal insufficiency, spina bifida, Alzheimer, cognitive impairment in the elderly and adverse pregnancy outcome [1–4]. Elevated levels of tHcy are very common, and in fact can be detected in 10–20% of the normal population as a result of genetic [4] and non-genetic factors [3, 5]. The present interest in Hcy metabolism has brought to the front page the knowledge accumulated for over 50 years about this branch point of the methionine cycle and its relationship to a number of key cellular processes (Fig. 1) [6]. Most of the available information has been obtained in the liver, which processes 48% of the ingested methionine in humans [6]. Hence, the role of the liver as the main regulator of tHcy levels has been suggested [7]. Hcy, a non-protein thiol amino acid is generated through a reaction catalyzed by S-adenosylhomocysteine hydrolase (SAHH) that breaks the S-adenosylhomocysteine (SAH) produced through S-adenosylmethionine (SAM)-dependent methylations into Hcy and adenosine. This reaction is reversible and thermodynamically favors the synthesis of SAH, a potent inhibitor of many methylation reactions. To keep the levels of this inhibitor under control, elimination of the reaction products has to be coordinated with their synthesis. Thus, adenosine is metabolized by adenosine deaminase, whose K_m in the low micromolar range favors elimination under all conditions, whereas Hcy can be metabolized through several routes that differ

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in their K_m values, hence favoring one or another according to cell needs (Fig. 1): (i) synthesis of cystathionine, and thus entrance to the trans-sulfuration pathway; (ii) methylation to methionine, which can take place through the action of either methionine synthase (MS) or betaine homocysteine methyltransferase (BHMT) and (iii) export to the plasma. The trans-sulfuration pathway includes two reactions that depend on pyridoxal phosphate: those catalyzed by cystathionine β synthase (CBS) and by cystathionase or cysthathionine γ-lyase (CγL) that lead to the synthesis of cystathionine and cysteine, respectively (Fig. 1) [6]. These reactions yield cysteine for protein and glutathione synthesis, provided there is a sufficient intake of methionine to the mammalian cell. On the other hand, the methylation reactions involved in methionine synthesis link Hcy with the folate cycle and phosphatidylcholine (PC) metabolism. These are two of the few methylation reactions known to use methyl donors other than SAM. MS uses methyl tetrahydrofolate (MTHF) in a reaction that needs cobalamine in mammals, whereas BHMT utilizes betaine. Despite broad knowledge of the methionine cycle, the difficulties encountered in the purification of BHMT have long precluded a thorough investigation of this enzyme, until 1996 when Garrow [8] cloned the human protein. Since then, a large amount of structural and functional data about various mammalian BHMTs have emerged, making pertinent a review attempting to integrate this knowledge, in order to offer a general view of the current status of this field.

General aspects of BHMT

BHMT catalyzes resynthesis of methionine using Hcy and the methyl donor betaine. It has been calculated that this reaction processes 25% of the cellular Hcy in an *in vitro* model of rat liver [9], also allowing recovery of part of the methylation equivalents (through betaine) used in PC synthesis, as explained below. This last point acquires more relevance because phospholipid methyltransferase (PEMT) and guanidinoacetate methyltransferase are responsible for most of the cellular Hcy production (Fig. 1) [4]. Moreover, BHMT is also involved in regulation of tonicity in the liver and kidney [10], since betaine exerts an important role as a cellular osmolyte to maintain cellular volume [11], and in the prevention of protein unfolding. Betaine is obtained from the diet, large amounts of this compound being found in seafood, wheat germ

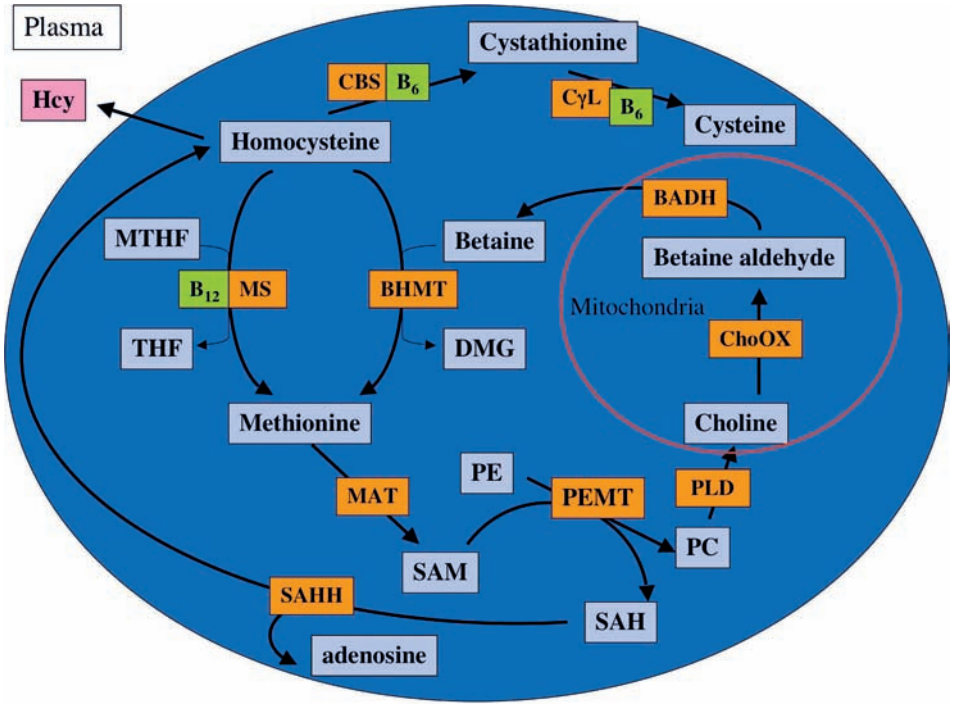

Figure 1. Homocysteine metabolism in the liver. The figure shows the reactions involved in Hcy metabolism in the liver. Enzymes are shown in orange, cofactors in green, cell metabolites in light blue and plasma metabolites in pink. Hcy, homocysteine; MTHF, methyl tetrahydrofolate; THF tetrahydrofolate; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; DMG, dimethylglycine; MS, methionine synthase (EC 2.1.1.13); BHMT, betaine homocysteine methyltransferase (EC 2.1.1.5); MAT, methionine adenosyltransferase (EC 2.5.1.6); SAHH, S-adenosylhomocysteine hydrolase (EC 3.3.1.1); PEMT, phosphatidylethanolamine N-methyltransferase (EC 2.1.1.17); PLD, phospholipase D (EC 3.1.4.4); ChoOX, choline oxidase (EC 1.1.3.17); BADH, betaine aldehyde dehydrogenase (EC 1.2.1.8); CBS, cystathionine β synthase (EC 4.2.1.22); CγL, cystathionine γ-lyase or cystathionase (EC 4.4.1.1).

or bran and spinach [10], whereas in mammalian cells it can be produced through choline oxidation that is mainly carried out in the liver and kidney (Fig. 1) [12]. This process occurs in the mitochondria and is catalyzed in two consecutive reactions by choline oxidase and betaine aldehyde dehydrogenase [13]. Choline is obtained either from the diet or from PC, one of the main phospholipids of membranes that can be synthesized by the Kennedy [14] and transmethylation pathways [15], the former being the most important in every cell type. On the other hand, transmethylation acquires more importance in the hepatocyte, where it can render about 40% of the PC synthesized by this cell [16]. In this process, phosphatidylethanolamine has to be methylated in three consecutive reactions using SAM as the methyl donor. PC degradation through phospholipase D renders phosphatidic acid and choline that enters the mitochondrial matrix from the cytosol using a specific transporter [17], whereas upon oxidation, betaine is extruded back to the cytosol by simple diffusion [13]. Thus, the overall result is that synthesis of choline by methylation consumes three SAM molecules, and methylation of Hcy by BHMT allows the recovery of one of these methylation equivalents to the methionine cycle, whereas the remaining four carbons of choline enter the one-carbon pool.

The BHMT gene

The BHMT gene contains eight exons and seven introns [18, 19] and in humans locates to chromosome 5q13.1–5q15 [20]. A BlastN search showed 61% identity between the human and mouse genes. 5′-RACE of human liver or HepG2 cell mRNA indicates a single species, whose open reading frame (ORF) encode a 406-amino-acid protein. Analysis of the 5′ end of the genomic DNA revealed a possible TATA box 26 bp and 28 bp upstream of the transcription initiation site for the human and mouse genes, respectively [18, 19]. In addition, 5′ of the human TATA box putative sites for several transcription factors are located, including Sp1 (four sites), activator protein 2 (one site), liver-specific or liver-enriched factors HNF-1, HNF-3 and CAAT-enhancer-binding protein, homeobox 4c, 4d and 4e and steroid receptors (glucocorticoids, progesterone, estrogens and androgens). This area of the mouse gene contains putative Sp1, AP2, AP4, NF1, GATA and CCAAT sites. 3′ to the TATA box, two more Sp1 sites have been identified in the human gene. Analysis of human promoter activity was performed in HepG2 cells [21], where only constructions including the TATA box showed transcriptional activity, the region –254/+1 containing all the essential elements for basal activity. Some negative regulatory elements are located in the region comprising –1793/–1063 with compensatory elements at –3175/–1793. Addition of methionine or ethionine to the HepG2 medium had no effect, whereas SAM reduced the activity. SAM-responsive elements have been located at –254/+1 [21]. However, to date, experimental data have only identified putative sites of regulation, and hence efforts to study the role of these regulatory elements in depth should be considered, especially in light of the hormonal effects described in early studies and that are discussed below.

In addition to these observations, screening of a mouse genomic library and a yeast two-hybrid study allowed identification of a BHMT pseudogene (mBHMTps) [19] and a transcript with 80% identity to BHMT called BHMT2 [2]. The sequence of the pseudogene showed 97% homology to mouse BHMT and contains a 1190-bp ORF [19]. The BHMT2 gene is located at chromosome 5q13.1–5q13.2; its ORF encodes a protein of 363 amino acids, of approximately 40.3 kDa and pI 5.61, and its origin seems to be a tandem duplication phenomenon [2]. At present, there is no information regarding the role of these new forms of BHMT. However, BHMT2 lacks approximately 40 residues of the C terminal, a fact that in light of the BHMT structural and folding data available may be crucial for its stability.

BHMT distribution

BHMT has been detected in eukaryotes and prokaryotes [22–24]. Most of the studies carried out to date have been done in mammals, where the protein can be detected after day 10 of gestation [25] and in adults [22]. Activity and expression measurements revealed the liver and kidney to be the main organs for BHMT and BHMT2 location [2, 8, 22, 26]. BHMT is very abundant in the liver in most species, representing 0.6–1.6% of the total protein [8, 22, 26], whereas in the kidney, the activity observed is lower for humans and pigs and very low for rats [22, 27]. In contrast, in sheep, the highest levels of activity correspond to the pancreas [28], such a distribution being attributed to the low oxidation of choline observed in these animals that could be an adaptation mechanism to the reduced levels of this metabolite in ruminant diets. BHMT has also been detected in the lens of Rhesus monkeys (*Macaca mulatta*), where it represents 0.5–10% of the total protein [29]. In this structure, BHMT appears concentrated in the oldest cells of the lens nucleus, which lack organelles such as nucleus and mitochondria as well as protein synthesis. Among cell lines, BHMT activity has been described in hepatoma cells [30], but not in skin fibroblasts or peripheral blood lymphocytes [31].

Subcellular fractionation of rat liver demonstrated BHMT activity only in the cytosol [22]. Immunohistochemistry confirmed these results, but also indicated that distribution in human and pig liver is homogeneous through the

lobule, whereas a gradient with high periportal expression is observed in the rat [32]. Western blot of human and pig kidney samples showed strong signals in the cortex [20], and immunohistochemistry revealed concentrated expression in the cytosol of the proximal tubules, in addition to a slight staining in distal tubules and collecting ducts [32]. Recently, analyses by SDS-PAGE, mass spectrometry and immunoblotting of liver membranes from C57BL/6J mice have detected BHMT in canalicular membranes and a low signal in basolateral membranes [33]. In summary, the fact that the liver and kidney are the main locations for BHMT expression confirms their importance for methionine metabolism and regulation of osmotic stress, processes both carried out mainly in these tissues. Moreover, the presence of the enzyme in renal cortex, the location of betaine production, rather than in medulla, where its role as osmolyte is exerted, speaks in favor of BHMT as a control point for betaine levels. In addition, the subcellular distribution of the enzyme is also in agreement with that of the other enzymes involved in methionine metabolism, but its presence in hepatocyte membranes and in the lens suggests additional roles for this protein.

Enzyme characterization and reaction mechanism.

Attempts to purify BHMT have been carried out using rat, pig and human liver samples [8, 30, 34, 35]. SDS-PAGE and gel filtration chromatographies of partially purified preparations indicated calculated molecular weights of 45 and 270 kDa, respectively, thus leading to the proposal that BHMT is a hexamer of identical subunits. However, crystallization data and sedimentation velocity experiments demonstrated later that a tetramer is the association arrangement that better defines recombinant BHMT [36–38]. These discrepancies between proteins of animal and recombinant origin cannot exclude other arrangements such as those observed in morpheeins that may be preferred i.e. after post-translational modifications of the polypeptide chain.

BHMT uses L-Hcy and betaine for the synthesis of methionine and dimethylglycine (DMG) following an ordered Bi-Bi mechanism [34, 39]. Hcy is the first substrate to be bound and DMG the first product to leave, whereas no binding of betaine or DMG is observed in the absence of Hcy. Kinetic characterization of the enzyme rendered K_m values for betaine and L-Hcy in the micromolar range. The values for betaine were calculated to be 100 μM for the human protein, but the latest report elevates this value to 2.4 mM [8, 30, 35]. L-Hcy K_m values have also been determined, being 120 μM for the human liver BHMT, but the non-quantitative hydrolysis procedure used to obtain Hcy from Hcy thiolactone makes these data variable between assays. Recombinant BHMTs showed slight differences in the affinity for the substrates as compared with the animal protein data [40, 41]. D-Hcy is not a substrate, whereas dimethylacetothetin can substitute for betaine as the methyl donor, and in this case an increase of 47-fold in V_{max} has been observed [8]. The search for inhibitors of the enzyme included many metabolites, reducing agents and analogs of the substrates [30, 34, 35, 42–46]. The potency and effect of all these compounds were tested with proteins of different origins and in some cases differences in behavior among BHMTs were observed (Table 1).

BHMT structure

Mammalian BHMT proteins are highly conserved, in fact, human and pig enzymes are 88% identical at the nucleotide level and 94% at the amino acid level [8]. BlastP revealed homology to regions involved in SAM binding for B_{12}-dependent MS. The motif G(ILV)NCX(20,100)(ALV)X(2)-(LV)GGCCX(3)PX(2)I, typical for the structural family of Zn^{2+}-dependent thiol and selenol methyltransferases, was also identified. This motif contains three cysteines (217, 299 and 300) essential for catalysis as they bind Zn^{2+} to activate Hcy. This process led to thiolate formation and liberation of a proton for nucleophilic attack to the methyl group of betaine [40]. Mutants in these cysteines showed no activity and a low Zn^{2+} content, thus confirming their role in cation binding [48]. The fourth ligand for Zn^{2+} binding was identified as Y160 [49]. Moreover, double mutants at positions 214/215 and 297/298 showed very low activity, highlighting their relevance [50]. This same study showed that none or only small changes are allowed at these positions or at 301. Moreover, the need for glycines in locations contiguous to the cation-binding cysteines giving flexibility to this environment was also stated.

Crystal structures for the human and rat liver proteins have been obtained [38, 49]. The human protein used was a mutant in six residues (five non-active-site cysteines and a proline), and its free and Zn^{2+}-ligated structures, as well as its complex with CB-Hcy, were solved by multiple anomalous diffraction (MAD). The oxidized form of the protein showed a disulfide bond linking C217 and C299 [38]. The main feature of the subunit is a $(\alpha/\beta)_8$ barrel composed by residues 11–318 of the sequence (Fig. 2). Several unordered areas are detected in the absence of the cation, while its presence allowed solution of the 38–52 loop. Residues downstream of 318 form an extended structure called the dimerization arm (DA) that interacts with L7 of the opposite monomer. Moreover, a loop comprising residues 362–365 (the hook) in this DA wraps the equivalent loop of the other monomer. Superposition of the hooks provides a flat surface that allows interaction with another dimer generating a tetramer of 222-point symmetry. Therefore, the BHMT structure obtained is a

Table 1. Inhibitors of BHMT: compounds exerting the strongest effects on BHMT activity are shown, along with their IC_{50} and the source of the protein used in the assay.

Inhibitor	IC_{50}	Type of inhibition	BHMT source	Reference
L-methionine	2 mM	competitive against betaine, non-competitive against L-Hcy	rat, human	34, 46
DMG	10 µM	non-competitive	rat, human	30, 34, 46
D- and L-cystine			rat	34
SAM			rat, human	44, 46
SAH		competitive against L-Hcy, non-competititve against betaine	rat	42
3,3-Dimethylbutirate	450 µM	competitive against betaine	human	30
Isovalerate	300 µM	competitive against betaine	human	30
Butyrate	1 mM	competitive against betaine	human	30
CB-Hcy	6.5×10^{-6}M	competitive against betaine	human	30, 43
TAMB	45 µM	competitive against betaine	rat	35
Val-Phe-ψ[PO_2^--CH_2]-Leu-His-NH_2	1 µM		rat	45
(R,S)-5-(3-amino-3-carboxy-propylsulfanyl)-pentanoic acid	87 nM	competitive against betaine	human recombinant	47
(R,S)-6-(3-amino-3-carboxy-propylsulfanyl)-hexanoic acid	200 nM		human recombinant	47
(R,S)-2-amino-4-(2-carboxymethylsulfanyl-ethylsulfanyl)-butyric acid	96 nM		human recombinant	47

dimer of dimers with a small tetramerization interphase (780 $Å^2$ are buried) [38]. Electron density maps of the transition state complex revealed the features and the residues involved in L-Hcy (Zn^{2+}, D26 and E159) and betaine (W44, F76, Y77, Y160, F261 and F267) binding [38]. A parallel mutagenesis study carried out on the rat BHMT was in good agreement with the role proposed for these residues in the human enzyme [41]. Changes at positions E159 and Y77 showed the largest decreases in activity, but D26 and F74 seem to have a role in betaine binding, whereas E21 and C186 also influence L-Hcy binding [41]. Solution of the rat BHMT crystal showed an α helix formed by residues 381–407 extending from one monomer to the opposite to bind the dimers, contributing additional interactions to tetramer stabilization [49]. Electron maps contributed new data such as the N-terminal (1–9) and the L2 (82–90) loop. This last loop shows high mobility and plays a role in Hcy binding. The presence of CB-Hcy provides protection against trypsin proteolysis at loop L2, as well as against oxidation [51]. No effect on oligomerization is exerted by the oxidant methylmethane thiosulfonate (MMTS), but reversible effects on activity and cation binding were observed. These results and the fact that mutations of non-active-site cysteines did not abolish the inhibitory effects of MMTS indicate that oxidation occurs on active-site cysteines.

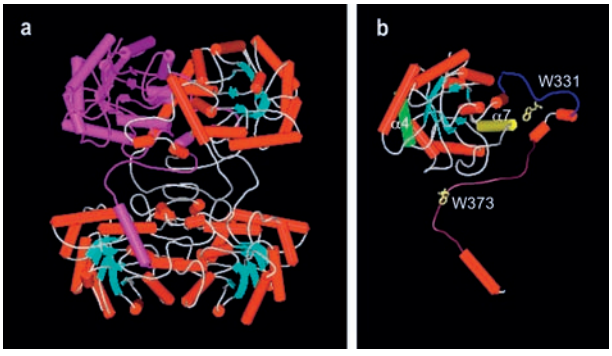

Figure 2. BHMT structure. (*a*) A schematic representation of the structure of the rat BHMT tetramer, with one of the monomers highlighted in magenta. (*b*) The details of the monomer are depicted. Secondary-structure elements involved in folding are shown: helix α4 of the barrel (green); helix α7 of the barrel (yellow); dimerization arm around W331 (blue); and C-terminal loop around W373 (magenta).

Mutations at other positions relevant for the rest of the Pfam 02574 family of Zn^{2+}-dependent thiol and selenol methyltransferases have also been studied [52]. These include stop codons at positions equivalent to the last residue in other members of the family and in BHMT2, deletions of the C-terminal helix and at the DA. These mutants showed either very low activity and expression or could not be purified. Moreover, since BHMT is the only oligomer of the family, analysis of the residues at the DA was also carried out. Several mutants, R346A, W352A and Y363A, showed alterations in the association state. Elimination of the DA destabilized BHMT

leading to its rapid degradation in *Escherichia coli*. Some of the DA residues may also be involved in catalysis or substrate binding, as is the case for H338. Structural information about BHMT2 is limited to hydrophobic plots of its sequence. Such plots show no evidence of transmembrane segments, and thus exclude BHMT2 as an integral membrane protein. Using human and mouse sequences, Prosite identified sites for putative post-translational modifications [2]. The sequence GGCC-GTXPXHI of the zinc-binding domain is conserved, but no significant methylation activity was observed in the initial report by Chadwick et al. [2]. Future advances in BHMT2 structure may shed light on the association level of this protein, as well as on its structure and the differences with BHMT.

Insights into BHMT folding

Barrels of the $(\alpha/\beta)_8$ type are the most common fold among proteins of known structure. In fact, 10% of protein structures have been calculated to belong to this group. Most of the members of this family whose folding has been studied to date are monomers. Thus, the study of oligomers should provide additional knowledge about the contribution of association features to stability. Among these oligomers, BHMT is of special interest due to the presence of the C-terminal helix as an extension that keeps the tetramer tighly packed. To date, only equilibrium unfolding data for recombinant rat BHMT have been published [53]. However, the high level of identity shown by mammalian BHMTs makes these results extrapolatable. Urea has been used as the perturbing agent and its effects monitored by several techniques that showed a number of transitions. Sedimentation velocity allowed detection of only tetramers and monomers during the unfolding process. Analysis of the results indicated a four-step mechanism involving two intermediates, a tetramer and a monomer, described as N_4-I_4-4J-4U.

For deeper insight into the changes taking place during urea unfolding, tryptophan mutants were prepared and analyzed by activity measurements and tryptophan fluorescence [53]. Only W352F showed a different behavior that corresponds to a low-activity dimer, as previously described for the analogous human mutant [39, 52]. Differences in fluorescence parameters among equivalent mutants for recombinant human and rat enzymes exist and have already been discussed in the light of their relative positions to a number of non-conserved residues around them [38, 49, 53, 54]. Unfolding profiles indicate that changes around W331 allow for differentiation between dissociation and intermediate denaturation. Comparison of the results for wild-type and mutants allowed identification of the structures modified as urea concentration increases (Fig. 2). First, changes around helix α4 of the barrel (W169) and DA (W331) take place before detection of I_4. Dissociation of I_4 to intermediate J occurs through alterations in the loop that connects the C-terminal helix (W373), in the α7 helix (W279) and the final modifications in helix α4 of the barrel (W169) and DA (W331). Unfolding of intermediate J involves the remaining changes in the α7 helix of the barrel (W279) and in the loop that connects the C-terminal helix (W373), as well as the last modifications in secondary structure. These results cannot exclude the presence of dimeric intermediates in this process since, as judged from W352F data, those would be highly susceptible to urea, and hence difficult to detect. Comparison of BHMT unfolding data to those of other $(\alpha/\beta)_8$ barrels indicates the importance of the additional elements of difference (DA and C-terminal helix) to the stability of the subunit and the oligomer.

BHMT regulation

Dietary effects. Ever since its discovery, important efforts have been made to elucidate the effects of different dietary compositions on BHMT, the preferred model being the rat. The number of studies combining alterations in the dietary content of sulfur amino acids and lipotropes is large, and they have not always yielded consistent results. Increases in BHMT activity, protein and mRNA levels have been reported with supplements of methionine, choline, betaine and cystine, but some of these effects are only obtained when combined with restrictions in another of these components [18, 55–59]. Feed restriction also increases BHMT activity with small elevations in expression, this last effect being ascribed to increased blood glucocorticoids [18]. On the other hand, decreases in BHMT activity have been observed with selenium-deficient diets, these reductions being more pronounced when combining selenium and folate deficiencies [60, 61]. Most of these dietary conditions also affect related enzymes such as choline oxidase, thus leading to alterations in betaine levels or changes in the thiol redox status of the cell, inducing oxidative processes. Thus, BHMT activity can be modified by the levels of its methyl donor or by oxidation of its active site. Other interesting models for studying dietary supplements are sheep and pigs. Ruminants fed wheaten diets exhibit elevations of hepatic BHMT activity that are not due to methionine deficiency since supplementation with this amino acid produces additional increases, while reducing methionine adenosyltransferase (MAT) activity and, thus, leading to plasmatic hypermethioninemia [62]. Pigs show low choline oxidase activity, as in humans, and deficiencies in methionine do not affect hepatic BHMT, but increase its activity in the kidney. Elevations in the liver activity were detected with addition of choline and betaine, whereas choline has no effect in the renal enzyme [63].

The high incidence of alcoholic cirrhosis in developed countries has elicited a deep interest in the study of ethanol effects. These have been explored in combination with different types of diets due to the influence of alcohol on the intestinal absorption of methionine and folic acid [64, 65]. Increases in hepatic BHMT activity were detected in rats fed diets including 3.5% casein or liquid diets including ethanol [65, 66], and further elevations were observed by addition of betaine to the liquid diets [67]. Micropigs fed folate-deficient diets showed increased BHMT activity, which was partially blocked by ethanol [68]. In other animal models, such as Syrian hamsters, increases in BHMT activity induced by ethanol were only observed when 4-methylpyrazole, an inhibitor of alcohol dehydrogenase, was included in the treatment [69]. Altogether these results indicate an interplay between ethanol and not only the diet composition, but also its presentation. Moreover, the effects of alcohol will also depend on the animal model of choice, especially if its alcohol dehydrogenase levels are high.

Hormonal treatments. As described above, the BHMT promoter contains regulatory elements for several hormones, and future studies will take advantage of this knowledge. However, to date, most of the data available on BHMT regulation come from early studies on the effects exerted in the whole methionine cycle by several hormonal treatments. Increases in BHMT activity and mRNA levels were observed by the action of hydrocortisone, cortisol and triamcinolone, whereas growth hormone only induced BHMT mRNA [18, 34, 65, 70–76]. On the other hand, reductions in activity were described for thyroxine and renal hypertrophia induced by testosterone [18, 34, 65, 70, 71, 75, 77, 78]; in this last case, methotrexate administration further reduced renal activity. Depletion of choline and betaine from liver produced by testosterone could be responsible for its effects, but the increased renal methionine uptake can also contribute. Insulin was effective in counteracting the stimulation of BHMT activity and mRNA levels produced by triamcinolone in rat hepatoma H4IIE cells [74]. These hormones affect the transcription rate in a manner parallel to their effects on mRNA levels, indicating their role in regulation of BHMT transcription. During pregnancy, tHcy also increases with inverse correlation to betaine after week 16. This effect could be due to the high levels of estrogens and cortisol that characterize this process [75]. Altogether, these results are in agreement with the presence of regulatory elements for some of these hormones in the BHMT promoter, but as pointed out earlier, deeper studies on their behavior are needed.

Other agents. Administration of nitrous oxide to rats oxidized vitamin B_{12} and reduced MS activity in liver, leading to an increase in BHMT activity [79], a compensatory effect that was not observed in sheep [80]. Hypertonicity studies carried out in guinea pigs induced decreases in BHMT mRNA and protein levels in liver and kidney [81], thus preserving betaine to protect against the osmotic stress in the organs involved in such a regulatory system, while no effect was exerted on the high pancreatic activity. Other systems studied include valproate-induced neural tube defects [82] and microarray analysis of arsenic and TPA-treated mouse livers [83].

Knockout models. To date there is no knockout mouse for BHMT. However, there are several descriptions of knockout mice for other enzymes where BHMT function has been studied and that show the common feature of changes in tHcy levels. BHMT activity is increased in MTHFR (–/–) [84] and CTα (–/–) mice [85], whereas no change is observed in PEMT-null mice [3] and CBS (+/–) [86]. Dietary betaine supplements induce BHMT activity, preventing severe hepatic steatosis in null MTHFR mice, while reducing Hcy levels around 40%. On the other hand, such a supplement in the drinking water reduces BHMT activity in CBS (+/–) mice and has no effect in PEMT (–/–). Such effects can be expected from the interrelationships between the eliminated activities and remethylation through BHMT (Fig. 1). Thus, reduction in the flux through the folate cycle due to MTHFR elimination down-regulates MS, whereas partial suppression of CTα increases PC synthesis through transmethylation, leading to a higher production of Hcy and an elevated consumption of SAM. Thus, increasing the use of betaine by BHMT will help in recovering methylation equivalents. Such a need disappears in PEMT (–/–) mice, and hence no effect on BHMT is observed, these results highlighting the importance of PEMT in Hcy production. In another model, the TNFR-1 knockout mouse, increased pHcy was also observed after intragastric ethanol infusion, and this elevation was reduced by betaine [87]. In this case, although a decrease in BHMT mRNA was detected, no change in enzyme activity was observed.

Interactions of BHMT

The extraordinary abundance of BHMT in liver, as well as its presence in the lens, where betaine is not produced, suggests its potential implication in processes other than methionine synthesis. To date, BHMT has been identified during the search for protein-protein interactions in several systems, although the significance of these observations remains unclear. These studies include screening of cDNA expression and yeast two-hybrid libraries, two-dimensional gel electrophoresis and immunoprecipitation. Among the interacting partners identified are apoB mRNA [54], tubulin [88], hepatitis B virus (HBV) [89] and transglutaminases [90]. In addition, BHMT2 and BHMT frag-

ments were found in a prion protein (PrP) screening [2] and in autolysosomal and lysosomal membranes [91], respectively. The most promising results, providing a direct interaction, were those of tubulin, transglutaminase and lysosomal membranes. Fragments of BHMT were found in autolysosomal and lysosomal membranes [92], asssociation with microtubules was observed by colocalization using confocal microscopy, and coassembly of BHMT with tubulin dimers was also obtained [88]. In addition, transglutaminases modify BHMT on its C-terminal end, or produce cross-linked 260- and 520-kDa multimers [90]. All these data suggest new roles for BHMT, since the fragments lacking part of the N terminal may adopt different folding patterns, and hence could play another function. Such new foldings may also arise from association with tubulin or could derive from post-translational modifications. On the other hand, the rest of the studies mentioned failed to demonstrate a direct association to BHMTs. In fact, no direct binding of recombinant BHMT to apoB mRNA [93] or effects in its editing process [94] were observed, even when BHMT overexpression induces apoB mRNA, and both mRNAs are elevated in dietary treatments. Thus, as for apoB mRNA, these data are suggestive of a role for BHMT in the process that could be indirect, not involving an interaction with these components. No immunoprecipitation of BHMT2 linked to PrP was reported [2], and its presence in membranes, in contrast to the cytosolic distribution of BHMT, was taken as indicative of the non-functional character of this interaction. However, this idea may have to be revised in the light of new data that found BHMT in hepatocyte membranes [33]. Immunoprecipitation also failed to prove interaction with HBV, but differential gel electrophoresis (DIGE) and mass spectrometry data from HBV hepatocellular carcinoma showed underexpression of BHMT in well-differentiated tumors [95].

Pathological implications of BHMT

Changes in tHcy levels have been observed in important pathologies, but not always correlating to alterations in BHMT expression and activity levels. Moreover, when effects on this enzyme were detected they were not directly related to mutations in the protein sequence. In fact, the results suggest that BHMT alterations are a secondary event in the development of the pathologies studied, a role that may acquire more importance once some of the interactions mentioned above are studied in depth. Elevations of tHcy appear in cirrhosis [96], coronary artery disease [31] and renal disease [97], whereas decreases were observed in streptozotocin-induced diabetes [72–74]. Decreased BHMT mRNA and protein levels were observed in cirrhotic patients and animal models, the strongest effect being observed upon HCV [98, 99]. Implication of BHMT defects in renal disease is rather indirect and based on the presence of high plasma DMG levels and increased betaine excretion, thus suggesting that decreased enzyme activity is an important factor for the detected hyperhomocysteinemia. The changes observed in BHMT in diabetes depend strongly on the agent used for induction of this condition in the animal models, and hence while alloxan produces reductions in BHMT activity [100], streptozotocin-treated [72–74] and Zucker diabetic fatty rats show increased activity and mRNA levels [101]. Diabetes as well as alcoholism are known to increase losses of zinc, an effect that deserves further studies due to its putative consequences in BHMT structure and oxidation state.

Hyperhomocysteinemia may appear as a consequence of defects in CBS or of inborn errors of remethylation due to either alterations in cobalamine metabolism and MS activity or in MTHFR function [102]. As mentioned before, elevated levels of tHcy are considered a risk factor for cardiovascular disease, and hence several studies have been devoted to finding treatments to correct this problem. Folate fortification carried out in the USA and Canada proved to reduce stroke-related mortality, and additional vitamin B_6 and B_{12} supplements appeared to benefit certain subgroups among this population [103, 104]. At present, treatment of patients with homocystinuria includes pyridoxine in combination with folic acid and vitamin B_{12} or methionine-restricted cystine-supplemented diets, and recently, betaine has been included as an adjunct to these treatments [105, 106]. However, betaine therapy, besides reducing tHcy, increases methionine levels and some cases of cerebral edema have been reported [102]. Benefits of betaine therapy result from increases in the flux through BHMT, although its activity has not been reported to be reduced in coronary artery disease [31]. Screening for BHMT mutations in vascular patients with hyperhomocysteinemia or subjected to coronary angiography identified six mutations [107, 108]. Among them, G199S was found in conserved regions of the sequence, and a Chou/Fasman analysis suggests a turn to coil rearrangement for this mutant protein [107]. Another mutation, R239Q, was also present in the ORF, but no significant association with the severity and extent of the disease was observed [108]. In fact, this human polymorphism is the normal allele in rat and mouse. The incidence of this mutation in neural tube defects has also been explored, leading to the conclusion that the Q allele may reduce the risk for development of this problem [109]. Linkage analysis of thoracic aortic aneurysm, familial abdominal aortic aneurysm, Wagner syndrome (vitreoretinopathy) and spinal muscular atrophy mapped to regions in chromosome 5 close to BHMT gene location, thus making it a putative candidate of study in these diseases [20, 110]. The presence of BHMT in lens [29] could be related to its role in betaine removal when

osmotic stress disappears, a key process, since osmotic swelling is a major factor in the development of some types of cataracts [11, 111]. Other studies of gene expression profiling have also shown upregulation of BHMT in Barret's esophagous patients [112].

Future directions

The fact that BHMT is one of the most abundant proteins of mammalian liver, as well as its association with proteins such as tubulin suggest roles other than just remethylation of Hcy for this protein. At present no clues about these other functions exist, and the interactions reported deserve deeper studies. Its presence in the lens, an organ where the cells lack mitochondria, and hence choline oxidation, also deserves further study, as does its binding to membranes. Protein moonlighting among metabolic enzymes is quite common, and hence it is a possibility that should not be excluded for BHMT. Another aspect that remains in doubt is the association state of the protein. It is clear that recombinant BHMTs appear as tetramers, but data from purified proteins indicate higher oligomerization levels. Such discrepancies could be derived either from other oligomerization arrangements of the protein, as occurs in morpheeins, or from association with other proteins. In fact, interaction with transglutaminases produces higher-order association arrangements. Little information exists regarding post-translational modifications or other levels of regulation. Finally, the role, structure and regulation of BHMT2 are almost unknown. Altogether, the data available to date are centered on the traditional role of BHMT in methylation, but new possibilities are being opened that seem to link this enzyme to key processes that remain unexplored.

Acknowledgements. The work in the authors' laboratories was supported by grants from the Ministerio de Educación y Ciencia (PM 97/0064, BMC 2002-0243 and BFU 2005-00050 to M. A. P. and SAF 2003-03713 to D. P.-S.) and Fondo de Investigación Sanitaria (FIS 01/1077 and RCMN C03/08 to M. A. P.). We wish to thank B. Ashley Morris for style and grammatical corrections.

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