Potential Pharmacological Chaperones for Cystathionine Beta-Synthase-Deficient Homocystinuria

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A. Ulloa-Aguirre, Y.-X. Tao (eds.), Targeting Trafficking in Drug Development,
Handbook of Experimental Pharmacology 245, DOI 10.1007/164_2017_72
Abstract
Classical homocystinuria (HCU) is the most common loss-of-function inborn error of sulfur amino acid metabolism. HCU is caused by a deficiency in enzymatic degradation of homocysteine, a toxic intermediate of methionine transformation to cysteine, chiefly due to missense mutations in the cystathionine beta-synthase (CBS) gene. As with many other inherited disorders, the pathogenic mutations do not target key catalytic residues, but rather introduce structural perturbations leading to an enhanced tendency of the mutant CBS to misfold and either to form nonfunctional aggregates or to undergo proteasome-dependent degradation. Correction of CBS misfolding would represent an alternative therapeutic approach for HCU. In this review, we summarize the complex nature of CBS, its multi-domain architecture, the interplay between the three cofactors required for CBS function [heme, pyridoxal-5′-phosphate (PLP), and S-adenosylmethionine (SAM)], as well as the intricate allosteric regulatory mechanism only recently understood, thanks to advances in CBS crystallography. While roughly half of the patients respond to treatment with a PLP precursor pyridoxine, many studies suggested usefulness of small chemicals, such as chemical and pharmacological chaperones or proteasome inhibitors, rescuing mutant CBS activity in cellular and animal models of HCU. Non-specific chemical chaperones and proteasome inhibitors assist in mutant CBS folding process and/or prevent its rapid degradation, thus resulting in increased steady-state levels of the enzyme and CBS activity. Recent interest in the field and available structural information will hopefully yield CBS-specific compounds, by using high-throughput screening and computational modeling of novel ligands, improving folding, stability, and activity of CBS mutants.

Keywords
- Heme
- High-throughput screening
- Homocysteine
- Protein misfolding
- Pyridoxal-5′-phosphate
- S-adenosylmethionine

Abbreviations
- AOAA: Aminooxyacetic acid
- AzMC: 7-Azido-4-methylcoumarine
- CBS: Cystathionine beta-synthase
- CGL: Cystathionine gamma-lyase
- Cth: Cystathionine
- Cys: Cysteine
- DMSO: Dimethyl sulfoxide
- HA: Hydroxylamine
- HCU: Classical homocystinuria
- Hcy: Homocysteine
- HTS: High-throughput screening
- Met: Methionine
Homocystinuria and Sulfur Amino Acid Metabolism

Homocystinurias are genetically determined disorders of sulfur amino acid metabolism characterized by the accumulation of homocysteine in tissues and blood, and its massive excretion in urine. The most common type of homocystinuria, the classical homocystinuria (HCU; OMIM# 236200), is an autosomal recessive inborn error resulting from the deficiency of cystathionine β-synthase (CBS), an enzyme expressed in several tissues including the liver, pancreas, kidney, and brain. Deficient activity of CBS impairs the reaction in which homocysteine is condensed with serine to produce cystathionine. HCU was first described in 1963 in two mentally retarded siblings in Northern Ireland with a rather characteristic clinical appearance and biochemically greatly elevated concentration of an amino acid reacting like cysteine to the cyanide nitroprusside test (Carson et al. 1963). This abnormal amino acid has been identified as homocysteine (Hcy; actually, it was Hcy disulfide homocystine), and authors suggested to name this defect as homocystinuria. Its incidence varies greatly with approximately 1:100,000 to 1:200,000 people worldwide, while expanded newborn screening suggests that this number is greatly underestimating the true rate of occurrence (Moorthie et al. 2014; Morris et al. 2017). In addition, there are countries in which the disorder appears more commonly, such as 1:65,000 in Ireland (Naughten et al. 1998) or the striking incidence of 1:1,800 in Qatar (Zschocke et al. 2009). Clinical consequences of CBS deficiency are variable with about equal proportion of two major forms of the disease. The most severe form manifests already in early childhood by affecting three systems: (a) central nervous system with cognitive impairment, epilepsy, and behavioral problems; (b) connective tissue with marfanoid features, osteoporosis, and progressive lenticular myopia resulting in lens dislocation; and (c) vascular system with thromboembolism. The milder form of the diseases may manifest in early to late adulthood by only thromboembolism without other clinical complications or may even remain asymptomatic (Skovby et al. 2010; Magner et al. 2011). In general, age of onset and clinical and biochemical severity correlate inversely with favorable response to treatment with pyridoxine, precursor of the CBS cofactor pyridoxal-5′-phosphate (PLP), which is the only treatment needed for the pyridoxine-responsive HCU patients. In the pyridoxine nonresponders, treatment includes low-protein diet with methionine-free amino acid supplements and
administration of betaine in some patients (Morris et al. 2017). The outcome of treatment is determined by the age of establishing diagnosis (only small proportion of patients worldwide are diagnosed by newborn screening), disease severity due to underlying genetic variants, and compliance with difficult-to-adhere dietary requirements. In general, in severe form of the disease, only early detection by newborn screening can prevent development of irreversible damage. Biochemically, HCU is characterized by grossly elevated levels of plasma total Hcy, methionine (Met), and S-adenosylhomocysteine (SAH) accompanied by significantly decreased plasma levels of cysteine (Cys) and cystathionine (Cth) (Stabler et al. 1993; Mudd et al. 2001; Orendac et al. 2003).

2 Cystathionine β-Synthase

CBS (EC 4.2.1.22) is a pivotal enzyme in the transsulfuration pathway, which resides at the junction where the metabolic fate of Hcy is decided (Fig. 1). CBS redirects the metabolic flux of Hcy from the competing methionine cycle, which converts Hcy back to Met, to the transsulfuration pathway, where Hcy is irreversibly removed from the cycle and transformed through two catalytic steps into Cys (Stipanuk 2004; Miles and Kraus 2004; Banerjee and Zou 2005). CBS catalyzes the first step by condensing Hcy with L-serine (Ser) to yield Cth, while the second enzyme of the transsulfuration pathway, cystathionine gamma-lyase (CGL), breaks down Cth into Cys, alpha-ketobutyrate, and ammonia. Both CBS and CGL enzyme require PLP cofactor, where either beta-replacement reaction catalyzed by CBS or gamma-elimination performed by CGL occurs. Cys is subsequently utilized by many processes within the cell, particularly in protein synthesis and generation of glutathione, the most important small molecule cellular antioxidant (Dickinson and Forman 2002). Recently, the relaxed substrate specificity of both transsulfuration enzymes, resulting in generation of hydrogen sulfide (H$_2$S), has attracted a lot of attention due to a multitude of effects exerted by this small gaseous molecule on many aspects of human physiology including cell signaling, vasorelaxation, angiogenesis, cytoprotection, inflammation, immunity, digestion, reproduction, and cancer (Szabo 2007; Predmore et al. 2012; Hellmich et al. 2015).

3 Modular Architecture of CBS

CBS is a unique PLP-dependent enzyme with a multi-domain architecture, complex structural and functional properties, and an intricate regulation, which are best illustrated on the extensively studied human enzyme (Miles and Kraus 2004; Banerjee and Zou 2005; Aitken et al. 2011; Majtan et al. 2014) (Fig. 2). The human CBS polypeptide consists of 551 amino acid residues yielding a subunit with a molecular size of around 63 kDa (Kraus et al. 1986). The enzyme assembles into native homotetramers, while each polypeptide is comprised of three functional and structural modules.
The N-terminal module encompassing the first ~70 residues binds the heme-b cofactor (protoporphyrin IX) (Fig. 3a) and lacks any significant structural elements.
The heme is thought to play a role in redox sensing (Banerjee et al. 2003) and/or enzyme folding (Majtan et al. 2008). Interestingly, CBSs from lower eukaryotes such as yeast lack heme entirely, thus strongly indicating that it is not essential for catalytic activity (Jhee et al. 2000; Maclean et al. 2000). The heme is axially coordinated by residues C52 and H65, relatively surface-exposed, and displaying a low spin and hexacoordinated state in both the ferrous (reduced) and ferric (oxidized) states (Green et al. 2001; Meier et al. 2001; Taoka et al. 2002). The presence of the heme gives the CBS its characteristic red color and is responsible for the unique spectral features of CBS, which have been extensively studied in an effort to shed light on its role and function (Taoka et al. 1998; Green et al. 2001; Pazicni et al. 2005; Cherney et al. 2007; Carballal et al. 2008; Kabil et al. 2011; Su et al. 2013). The purified ferric CBS displays a heme’s Soret peak at 428 nm with a broad αβ absorption band around 550 nm. In this oxidation status, heme is unreactive and inert to ligand exchange with exogenous molecules (Vadon-Le Goff et al. 2001). Upon reduction to the ferrous state, the Soret peak is red-shifted to 447 nm with a simultaneous resolution of αβ bands to 539 and 570 nm, while the enzyme retains its activity. However, the ferrous state is unstable and undergoes a ligand switch, where the heme axial ligand C52 (thiolate) is replaced by a neutral unknown ligand (Pazicni et al. 2005; Cherney et al. 2007). The ligand switch is irreversible, inactivates the enzyme, and is spectrally accompanied by a blueshift of the Soret peak from 447 to 424 nm. Ferrous CBS heme binds various small molecules, such as CO, NO, or cyanide, which results in inhibition of enzyme activity (Taoka and Banerjee 2001).
Due to the low CBS heme redox potential (-350 mV) (Singh et al. 2009a), the existence of a ferrous form of the enzyme in vivo and the feasibility of CO-based regulation under physiological conditions remain an open question. Recently, Kabil et al. (2011) have provided the first evidence of reversible inhibition of CBS by CO in the presence of a human flavoprotein and NADPH.

In contrast with the heme domain, the catalytic domain is well structured and includes thirteen α-helices and two β-sheets (Meier et al. 2001). The central module spanning the residues 70–386 represents the catalytic segment, where the catalytically active PLP cofactor binds via Schiff bond to the ε-amino group of the K119 residue (Kery et al. 1999). Based on sequential and structural similarities, CBS catalytic core belongs to a homogeneous β (or fold type II) family of PLP-dependent enzymes with O-acetyl-L-serine sulfhydrylase (sometimes referred to as cysteine synthase) being the most homologous with CBS (Christen and Mehta 2001; Meier et al. 2001). All members of the β family carry out α,β-replacement/elimination reactions which in itself warrants for a similar catalytic mechanism and to some extent relaxed substrate specificity. Indeed, CBS ping-pong catalytic mechanism initiates with a formation of an external aldimine of PLP with Ser followed by transformation to an aminoacrylate intermediate. The subsequent reaction of aminoacrylate with a second substrate, Hcy, represents the rate-limiting step and yields an external aldimine of PLP with Cth. The reaction is concluded by the release of Cth and restoration of the internal aldimine. Due to the spectral overlap of

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**Fig. 3** The binding clefts of the cofactors heme and PLP in the catalytic core. (a) The heme-binding domain is in green. The structural elements of the catalytic core that contribute to the cavity are represented in blue. The heme and PLP cofactors are in sticks. The iron of heme (in orange) is axially ligated with residues H65 and C52. (b) PLP is covalently bound to ε-amino group of lysine residue K119. Multiple residues of the catalytic core (shown in sticks) interact with and stabilize the PLP within the catalytic site.
the heme and PLP in human CBS, the spectral characteristics of the reaction intermediates and the catalytic mechanism were described in heme-independent CBS from yeast (Jhee et al. 2000). Some of these reaction intermediates were later confirmed in a crystal of human truncated CBS, from which the heme cofactor had been removed by CO (Bruno et al. 2001), as well as in the crystal structure of *Drosophila* CBS (Koutmos et al. 2010). Alternative CBS reactions, which result in production of H₂S (Chen et al. 2004), follow the same reaction mechanism even though the kinetic parameters for the individual alternative substrates are less favorable compared to the canonical condensation of Ser and Hcy (Singh et al. 2009b).

The C-terminal regulatory domain, also known as the Bateman module (Fig. 2), consists of two consecutive so-called CBS motifs (CBS1, residues 412–471; CBS2, residues 477–551) that reflect a characteristic α+β pattern observed in many other CBS domain containing proteins of unrelated function, where it usually fulfills a regulatory role and/or sensing function upon binding adenosine analogs (Bateman 1997; Shan et al. 2001; Scott et al. 2004; Ereno-Orbea et al. 2013a). The CBS motifs are structurally related by a twofold axis that runs parallel to their central β-sheets. This spatial arrangement generates two symmetrical cavities (named as sites S1 and S2), which represent potential binding sites for SAM. However, although similar in size, the two cavities are very different in physical-chemical properties, and only one of them (S2) hosts the CBS allosteric activator SAM (Ereno-Orbea et al. 2014; McCorvie et al. 2014). The sterical interference of the Bateman module with the catalytic core results in inhibition of the enzyme’s catalytic activity, while binding of SAM releases an intrasteric autoinhibitory block and activates the enzyme (Pey et al. 2013; Ereno-Orbea et al. 2014). The regulatory domain is connected to the catalytic core via a relatively long and flexible linker (residues 386–411), which enables a regulatory domain rearrangement leading to the activation of the enzyme upon binding of SAM (Majtan et al. 2014; Ereno-Orbea et al. 2014). A pair of interleaved CBS domains shares an identical fold despite having only 7% sequence identity: CBS1 spans the residues 412–471 having an ααββα-fold, while CBS2 covers residues 477–551 showing an αβαββα-fold (Ereno-Orbea et al. 2013b).

The Bateman module plays (at least) two essential roles. On the one hand, it is responsible for the tetramerization of the enzyme, while on the other hand, it determines the activation state of the enzyme. For several years it was known that the artificial removal of the Bateman module region disassembles the protein into homodimers, although it was not clear which specific residues were relevant in the association between subunits. We answered this question by showing that artificial removal of residues 516–525, within the quite unique extended loop (residues 513–529) connecting the two last β-strands (β15, β16) of the CBS2 motif, resulted in the irreversible disassembly of the tetrameric native protein into dimers (Ereno-Orbea et al. 2013b). Interestingly, the length of this loop is significantly shorter in dimeric CBS enzymes from organisms, such as fruit fly or honey bee (Koutmos et al. 2010; Oyenarte et al. 2012). Undoubtedly, the most important role of the Bateman module is to regulate the CBS activity. The recently obtained structural data
demonstrates that in the absence of SAM, the Bateman module is placed atop the entrance of the catalytic cavity (Ereno-Orbea et al. 2013b), thus hampering the access of substrates into the catalytic site (Fig. 4). In this conformation, the enzyme maintains a low activity (or basal) state. Binding of SAM to site S2 of the Bateman module causes a relative rotation of the two CBS motifs that weakens the network of interactions that maintains them anchored to the catalytic core (Ereno-Orbea et al. 2014). The direct consequence is a displacement of the regulatory domain that activates the enzyme by allowing for unrestricted access of substrates to the catalytic center.

Taking into account the crucial role of CBS in metabolism of sulfur amino acids, it is surprising that the architectural, structural, and particularly regulatory features are not as conserved across phyla as one would anticipate. The presence of the heme-binding domain in CBS enzymes is unique within the family of PLP-dependent enzymes, and, moreover, heme is present only in a subset of CBS enzymes (Majtan et al. 2014). More importantly, SAM-mediated regulatory mechanism is not universal for CBS enzymes. For example, CBS enzymes from parasitic protozoans or nematodes entirely lack both the N-terminal heme-binding domain and the C-terminal regulatory SAM-binding module suggesting that the central catalytic module represents a self-sustainable and fully catalytically competent unit (Nozaki et al. 2001; Williams et al. 2009; Vozdek et al. 2012). The lack of regulatory domain is understandably accompanied by insensitivity of the CBS enzyme to SAM-mediated activation. However, despite possessing the C-terminal CBS domains, CBS enzymes from insect or yeast are not regulated by SAM (Maclean et al. 2000; Koutmos et al. 2010). Interestingly, while both enzymes are highly active and do not respond to SAM activation, yeast CBS, but not the one from fruit fly, binds SAM (Majtan et al. 2014).

### 4 Posttranslational Regulation of CBS

As mentioned above CBS lies at a point of significant metabolic control and regulation. As Cys is a precursor for the biosynthesis of glutathione, regulation of CBS in response to various reactive oxygen species (ROS) has been considerably explored. In addition to heme-based redox regulation, whose relevance and feasibility still remains unclear, a subset of CBS enzymes including human CBS contains another putative redox switch of nuclear function, the CXXC oxidoreductase motif (Meier et al. 2001; Taoka et al. 2002). Tumor necrosis factor alpha (TNFα), which enhances ROS levels, induces a 50–60% increase in CBS activity by yielding a truncated form of the enzyme (Zou and Banerjee 2003). CBS is a target of SUMOylation on the residue K211, which inhibits the CBS activity by 28% in the absence or by 70% in the presence of human polycomb protein 2 (Agrawal and Banerjee 2008). While CBS has been long thought to be located solely in the cytoplasm, SUMOylated CBS has been found in the nucleus (Kabil et al. 2006). However, the significance of CBS SUMOylation and its role in the nuclear compartment remain unknown. CBS has also been found in mitochondria, where it accumulates during ischemia/hypoxia and...
leads to inhibition of ROS production and stimulation of cellular bioenergetics most likely via increased mitochondrial H₂S production (Teng et al. 2013; Szabo et al. 2013); however, the mechanism how CBS gets into mitochondria is unclear since it lacks any mitochondrial targeting sequence. Recently, CBS was found to be activated over twofold by S-glutathionylation at the residue C346, which needs first to be oxidized in order to be modified by GSH efficiently (Niu et al. 2015).

Fig. 4 Basal and activated states of human CBS. Surface-ribbon (a) and schematic (b) representation of the successfully crystalized human CBSΔ516-525 dimers in the basal (left) and the SAM-bound activated state (right). The two complementary subunits are represented in orange and cyan, respectively. The two main protein structural blocks (catalytic core and Bateman module) are indicated. The position of PLP within the catalytic cavity is marked with a black asterisk. In the basal state (left), the Bateman module is placed on top of the catalytic cavity of the complementary monomer where it impairs the access of substrates into the cleft (the loops defining the entrance to the catalytic site are represented with a red line in panel b). Binding of SAM to site S2 of the Bateman module (green asterisk in panel a) makes the enzyme progress toward its activated state (right). In this state, the entrance to the catalytic site (indicated with a green arrow in panel a) is freely accessible for the substrates. Of note, the site S1 of the regulatory domain (represented with a red asterisk) is occluded in the basal state, and it becomes exposed only upon activation of the enzyme.
4.1 Binding of SAM by CBS: Stabilization of the Enzyme

Recent ultrasensitive calorimetric titrations of human CBS with SAM have supported that both types of sites (S1 and S2) in the full-length enzyme are operational, and furthermore, they can independently regulate CBS activity and stability in vitro (Pey et al. 2013). Strikingly, a total binding capacity of six SAM molecules per CBS tetramer was found, each tetramer binding two SAMs with high affinity (with a Kd ~10 nM) and four SAMs with low affinity (Kd ~370 nM). Binding isotherms were consistent with both types of sites binding independently (noncooperatively) the ligand, and attempts to alternatively use a model with a total binding capacity of four dependent sites with negative cooperativity yielded poor results (Pey et al. 2013, 2016b).

Despite the inherent complexity of SAM binding to tetrameric CBS [with up to 15 different ligation states, many of them degenerate, and their population strongly dependent on SAM concentration (Pey et al. 2013)], thermodynamic analyses of SAM binding combined with functional assays support different and independent functions for both types of sites. In Fig. 5a, we display for sake of simplicity the five most representative ligation states in functional terms. The SAM concentration required for half-activating CBS was experimentally found to be 3.2 μM (Pey et al. 2013), in excellent agreement with the SAM concentration required to half-saturate the low-affinity sites predicted by our equilibrium analyses (about 4 μM; state (2,2); Fig. 5b, c). Logically, the high-affinity sites become significantly occupied at much lower concentrations (Fig. 5b). Since SAM binding to the two different types of sites is independent, a plausible hypothesis is that the different types of sites exert different functions. SAM binding is known to stabilize CBS in vitro toward chemical denaturation and, importantly, inside cells (Prudova et al. 2006), and therefore, a possible role for the high-affinity sites might be modulation of CBS stability. Analysis of CBS thermal stability by differential scanning calorimetry (DSC) revealed a denaturation mechanism through two main and independent transitions, one corresponding to the regulatory domains (RDs) at lower temperatures and the other one manifesting denaturation of catalytic domains (CDs) at high temperatures [Fig. 6a; (Pey et al. 2013)]. Denaturation enthalpies for these transitions indicate an almost complete loss of tertiary structure upon denaturation, but the domains retain significant amounts of residual secondary structure (Fig. 6a). Importantly, denaturation of RDs and CDs behaves as independent processes and conforms to simple kinetic models in which the unfolded domains U (in equilibrium with the native domains, N) are not significantly populated (Fig. 6b; Schemes 1 and 2), and thus, denaturation of each type of domain phenomenologically follows a simple two-state mechanism (Fig. 6b, Scheme 2). This result has an important implication to understand CBS stability in vitro and possibly in vivo: the widely different thermal stabilities of RDs and CDs reflect different time scales for irreversible denaturation, with the former denaturing on a scale of hours-days while the latter denaturing in a time scale of months (Fig. 6c). Therefore, CBS may transiently exist in vivo in a situation in which fully activated forms of CBS (due to irreversible denaturation of...
Fig. 5  Equilibrium binding of SAM and its analogs to human CBS. (a) Five representative ligation states depicting different intermediate states with functional relevance: the (0,0) state has no SAM bound; states (1,0) and (2,0) have one or two high-affinity sites occupied (thus, high kinetic stability); states (2,2) and (2,4) have high-affinity sites saturated and activating sites half or fully occupied, respectively; (b) dependence of the degree of binding for SAM (solid line = stabilizing sites; dashed line = activating sites) indicating relevant ligation states for stabilization and activation; (c) dependence of the degree of binding for SAM, SAH, and S-adenosylornithine (SAO) (solid lines = stabilizing sites; dashed line = activating sites) indicating relevant ligation species for stabilization and activation. The degree of binding is normalized using the number of sites for each type. Data are from simulations performed using 1 μM CBS tetramer and the binding affinities and models described elsewhere (Pey et al. 2013, 2016a; Majtan et al. 2016) using a binding polynomial formalism.
Fig. 6 Conformational stability of human CBS is modulated by SAM binding. (a) Thermal denaturation (at rate of 2 K min\(^{-1}\)) for human CBS (5 μM in protein monomer) by far-UV CD spectroscopy (at 222 nM; upper panel) or by DSC (lower panel). The transitions corresponding to denaturation of RDs and CDs are indicated by arrows. (b) Different models used to discuss the kinetic stability of human CBS. In Scheme 1, a Lumry-Eyring model in which native (N) and unfolded (U) domains exist in equilibrium, while the unfolded domains undergo an irreversible denaturation step to a final state (F). Assuming that the population of U is always comparatively low \([i.e., X_U < \langle X_N + X_F \rangle]\), this model is phenomenologically described by a two-state kinetic model (Scheme 2). In Scheme 3, we extend this model to the independent and irreversible denaturation of RDs and CDs, considering their widely different kinetic stabilities (see panel c). (c) Decay of the fraction of native RDs and CDs according to the DSC studies reported previously (Pey et al. 2013). It must be noted that in a time scale of a few days, most of the RDs are denatured while most of the CDs are active, showing a large population of the RD\(_d\),CD\(_n\) intermediate in Scheme 3. (d) Kinetic models and mechanism used to support the role of high-affinity sites on the kinetic stabilization of RDs.
RDs) may be significantly populated several hours after its synthesis and folding to the native state, but no longer regulated by SAM (Fig. 6b, Scheme 3).

4.2 Binding of SAM by CBS: Activation of the Enzyme

Structural insight into SAM-mediated regulation has been hindered for decades by the inability to obtain diffracting crystals of a full-length CBS. Recently, Ereno-Orbea et al. identified a flexible loop spanning residues 516–525 within CBS2 domain of the regulatory module, whose deletion yielded an enzyme biochemically indistinguishable from a native WT CBS in terms of catalytic activity and response to SAM, except for its dimeric oligomeric status (Ereno-Orbea et al. 2013b). The construct was successfully crystallized and yielded the crystal structure of human CBS in the basal, SAM-free conformation (Figs. 2 and 4) (Ereno-Orbea et al. 2013b). The crystal structure of CBS in its basal conformation and the identification of critical residues involved in the autoinhibition process allowed us to devise an artificial, constitutively activated E201S CBS mutant. This mutant has been successfully co-crystallized with SAM and yielded the activated conformation of CBS with bound SAM (Figs. 2 and 4) (Ereno-Orbea et al. 2014). The structure of CBS in its basal conformation suggested the presence of two plausible SAM binding sites within the Bateman module of each monomer (designated as S1 and S2), where S1 was occluded by structural elements from the catalytic core and several bulky hydrophobic residues, while S2 was exposed and thus could represent the primary binding site for SAM. Indeed, the structure of the activated state revealed one SAM ligand per monomer nested only within S2 site, despite the S1 site being solvent accessible (Ereno-Orbea et al. 2014). The availability of structural information on CBS resulted in the proposal of a molecular mechanism of CBS allosteric regulation (Fig. 7). In the absence of SAM, CBS rests in its basal state with the Bateman module placed atop the entrance of the catalytic cavity, thus pushing the flexible loops delineating the entrance to the catalytic site of the protein core toward its closed conformation (state 0). Upon binding of SAM to site S2 of the Bateman module, the two CBS motifs rotate with respect to each other, thus weakening their interaction with the protein core. This facilitates the migration of the Bateman module from above the catalytic cavity and eliminates the occlusive effect formerly imposed (steps 1 and 2). As a consequence of this conformational change, the loops delineating the entrance to the catalytic core can progress toward an open conformation. The two Bateman modules from complementary monomers associate through their α-helices forming an antiparallel CBS module, a disk-shaped complex of two Bateman modules (step 3). At this point, the enzyme becomes fully activated. The substrates access the catalytic cavity and promote the closure (collapse) of the flexible loops around the catalytic site (step 4). Once the catalytic reaction has occurred, the products abandon the cavity, thus promoting opening of the loops (step 5). SAM is released from the Bateman module, thus causing an inverse rotation of the two CBS motifs toward their basal orientation and the subsequent disassembly of the CBS module (steps 7 and 8). The Bateman module
migrates back toward its initial position atop the catalytic cavity of the complementary monomer, and the activation cycle is concluded (Ereno-Orbea et al. 2014).

Fig. 7 Schematic representation of the allosteric activation of human CBS by SAM. The two complementary monomers of the CBS dimer are represented in orange and blue, respectively. SAM is in green. Substrates (serine in pink and homocysteine in turquoise) and products (cystathionine in yellow and water in purple) are also shown. The flexible loops delineating the entrance into the catalytic site are represented by a red line above the catalytic cleft. The relative position of heme and PLP are depicted based on available structural information.
Of note, the comparable activation to the one achieved by binding of SAM can also be achieved by partial thermal denaturation of the enzyme or by the presence of an activating missense mutation, such as the artificial E201S or the pathogenic S466L (Majtan et al. 2010; Ereno-Orbea et al. 2014). However, such activated conformations are relatively unstable in the absence of SAM. The presence of SAM in the S2 site of each subunit triggers formation of a disk-shaped CBS module (Ereno-Orbea et al. 2013b). Such conformation of activated CBS with bound SAM is stable and remarkably resembles the crystal structure of the SAM-insensitive insect CBS (Koutmos et al. 2010; Ereno-Orbea et al. 2014). Identification of the molecular mechanism of CBS activation by SAM allows to propose mechanisms of how the pathogenic missense mutations impair the regulation and activation of the enzyme.

5 CBS-Deficient Homocystinuria Is a Misfolding Disease

HCU is caused mostly by point mutations within the CBS gene sequence (Kraus et al. 1999; Mudd et al. 2001). Of the 164 mutations reported so far, the overwhelming majority (85%) are missense substitutions (http://medschool.ucdenver.edu/krauslab). Mutations have been detected in all functional domains of the CBS enzyme. Although the most frequent mutations, such as I278T, T191M, G307S, or R336C, affect the catalytic domain, the majority of disease-causing CBS mutations do not target critical residues involved in catalysis. Thus, their pathogenicity probably originates from a different mechanism. In general, missense mutation can interfere with proper folding of the enzyme into its native form, its stability, and conformational flexibility or cause other structural perturbations all leading to a misfolded protein (Pey et al. 2013). Such misfolded protein molecules may provoke a loss-of-function phenotype due to nontoxic protein aggregation with accelerated degradation. Accelerated degradation of misfolded enzymes is characteristic of many inherited metabolic disorders, such as phenylketonuria and Gaucher disease as well as HCU (Muntau et al. 2014).

The first evidence that HCU is associated with protein misfolding came from a study on a group of Slavic CBS-deficient patients from the former Czechoslovakia (Janosik et al. 2001). Western blot analysis of fibroblast extracts showed normally assembled tetrameric CBS only in WT control fibroblasts, while in patient samples, the CBS antigen, if any, was only detected as a high molecular weight aggregate devoid of heme and correlated with little or no CBS activity. Additional studies involving heterologous expression of 27 frequent CBS mutants in E. coli and in Chinese hamster ovary cells followed by Western blot detection confirmed their aggregation tendencies and the lack of CBS activity (Kozich et al. 2010; Melenovska et al. 2015). A recent immunofluorescence microscopy study in transiently transfected HEK-293 cells provided an in situ evidence of CBS mutant aggregation as well as its correlation with mutant residual activity (Casique et al. 2013). In summary, these indications strongly suggest that many cases of CBS deficiency are caused by misfolding of mutant CBS and allow to consider HCU as a conformational disease.
6 Use of Chaperones in CBS-Deficient Homocystinuria

As discussed above the evidence strongly indicate that CBS deficiency can be considered a conformational disorder. Knowledge of this pathogenetic mechanism offers novel therapeutic options devised for the group of misfolding diseases. In general, there are three different small molecule treatment strategies currently available to rescue misfolded mutant proteins and to restore their homeostasis (Muntau et al. 2014), namely, chemical chaperones, pharmacological chaperones, and proteostasis modulators. All these therapeutics are often called chaperones as they help the mutated protein to adopt native active conformation similarly to cellular molecular chaperones, the proteins of cellular quality control and repair machinery responsible for proper folding and assembly of cellular proteins (Hartl and Hayer-Hartl 2009). Chemical chaperones are diverse low molecular weight compounds that do not directly and/or specifically interact with mutated misfolded proteins. Typically, their mode of action relies on altering solvent conditions to stabilize the native state (Street et al. 2006; Bolen and Rose 2008). In addition, chemical chaperones may also induce expression of molecular chaperones or otherwise enhance their activity (Singh and Kruger 2009; Majtan et al. 2010). The pharmacological chaperones, which often resemble natural ligands or cofactors of the target protein, can specifically bind and stabilize the native conformation of mutant proteins, thus preventing the fast degradation and/or aggregation (Bernier et al. 2004; Pey et al. 2008; Parenti et al. 2014). Thus, the pharmacological chaperones increase the steady-state levels of the mutant proteins and stimulate their residual enzymatic activity. The last group of small molecule chaperones, the proteostasis modulators, influences the generic function and steady-state levels of molecular chaperones and other components of cellular protein quality control apparatus (Balch et al. 2008).

6.1 CBS Cofactors and Their Precursors

As described above, CBS needs the assistance of three cofactors: heme, PLP, and SAM. Although only PLP is necessary for the catalytic cycle, heme has been shown to be important for proper folding and optimal activity, while SAM activates and kinetically stabilizes the enzyme.

6.1.1 Pyridoxine

PLP is a cofactor in more than 160 distinct enzymatic activities, especially in the metabolism of amino acids, and its possible involvement in correct folding of several enzymes has been reviewed elsewhere (Cellini et al. 2014). The idea of supplementation with pyridoxine (vitamin B6) as a precursor of PLP in inborn errors affecting PLP-dependent enzymes can be traced back to 1963 (Greengard and Gordon 1963). In 1967, Drs. Barber and Spaeth reported that three homocystinuric patients normalized their homocystine levels in urine after administration of pharmacological doses of pyridoxine (250–500 mg per day for a period of 2–4 weeks) (Barber and
Spaeth 1967). However, not all patients responded to such treatment and two forms of HCU were described: pyridoxine responders or nonresponders (Brenton and Cusworth 1971). It was evident that pyridoxine responsiveness was not due to correction of vitamin B$_6$ deficiency. Subsequent studies suggested that pyridoxine-responsive patients exhibit higher residual CBS activity in their cultured fibroblasts (typically 1–9%) or in plasma (typically 4–22%) (Fowler et al. 1978; Alcaide et al. 2015). The seminal work of Dr. Harvey Mudd showed that approximately half of patients can benefit from vitamin B$_6$ treatment (Mudd et al. 1985), although recent data indicate that this type of HCU may be more frequent with substantially milder phenotype (Orendac et al. 2003) or even clinically asymptomatic (Skovby et al. 2010).

The exact biochemical basis for pyridoxine responsiveness in HCU remains unknown, particularly due to the discrepancies among data obtained from in vitro, bacterial, and eukaryotic systems, animal models of HCU on one side and homocystinuric patients on the other side. Mutation analysis in HCU patients revealed that some mutant CBS enzymes, such as the P49L, A114V, I278T, R266K, or R336H, confer vitamin B$_6$ responsiveness, while patients carrying other missense CBS mutants, e.g., R125Q, E176K, T191M, T262M, or G307S, do not respond to such treatment. The most obvious hypothesis to explain pyridoxine responsiveness in HCU is the possibility of an increased $K_m$ for PLP of the mutant protein, which would require higher concentrations of this cofactor to achieve the full activity. A study on cultured fibroblasts from several B$_6$-responsive and B$_6$-nonresponsive patients showed that the concentration of PLP needed to achieve maximal saturation of CBS apoenzymes is directly proportional to the observed phenotype (Lipson et al. 1980). This study concluded that the B$_6$ nonresponsiveness is due to either lack of any residual CBS activity or highly reduced affinity of mutant CBS for PLP, which cannot be rescued by therapeutically safe doses of pyridoxine. Similarly, it is plausible that the PLP-dependent proteins are not fully loaded with the cofactor in vivo. PLP is highly reactive, so its free concentration must be very low inside cells. Increasing the availability of PLP precursor could increase the saturation of mutant CBS enzymes, thus increasing their kinetic stability without apparent cofactor affinity issue (Oppici et al. 2016). Although many HCU patients clearly benefit biochemically and clinically from pyridoxine administration, the mice carrying the most common pyridoxine-responsive mutation I278T do not exhibit pyridoxine responsiveness (Chen et al. 2006). Moreover, several mutant enzymes known to respond to vitamin B$_6$ in vivo do not exhibit such behavior in vitro following expression in different systems. In summary, the clinically well-established and widely used phenomenon of pyridoxine responsiveness in HCU remains mechanistically largely unknown, and, similarly to other studied enzymes, a possibility that PLP functions as a chaperone seems plausible (Cellini et al. 2014).

### 6.1.2 Protoporphyrin IX (Heme)

Binding of heme to human CBS was described more than two decades ago (Kery et al. 1994). Although some earlier studies suggested that heme moiety in CBS is functioning as a redox sensor that regulates the catalytic activity (Taoka et al. 1998;
Taoka et al. 2001), additional work indicates that heme is not directly involved in catalysis (Bruno et al. 2001). In contrast, its importance for the proper folding of human CBS has been well documented. Expression studies in heme biosynthesis-deficient strains of *E. coli* and *S. cerevisiae* demonstrated substantial CBS misfolding and aggregation in the absence of heme or protoporphyrin supplementation (Majtan et al. 2008, 2011). In combination with other studies showing a correlation between the heme content and aggregation propensity of CBS mutants (Janosik et al. 2001), these data suggest that heme incorporation (possibly co-translational) is crucial for proper CBS folding.

Indeed, heme precursors or heme analogs were shown to improve folding of a number of mutants expressed in prokaryotic and eukaryotic systems. About half of 27 mutants expressed in *E. coli* in the presence of heme precursor 5-aminolevulinate (500 μM) showed increased formation of native tetramers with a rescue of CBS activity (Kopecka et al. 2011). Mutations responsive to treatment with the heme precursor were located in all domains of the enzyme. Interestingly, 5-aminolevulinate had substantially different effect on two mutations located in the heme-binding pocket. Whereas tetramerization and activity of the R266K CBS mutant was significantly improved, the H65R mutant affecting the histidine residue critical for heme axial ligation failed to show any changes in response to the treatment. Five selected pathogenic CBS mutants were recently expressed in CHO-K1 cells in the presence of 77 μM heme arginate in order to directly supply the CBS cofactor (Melenovska et al. 2015). The results essentially verified the findings from the *E. coli* expression system. One of the mutations (R125Q) exhibited an outstanding heme sensitivity, and addition of heme arginate to a fibroblast culture obtained from a patient homozygous for this mutation rescued also the activity in these human cells and increased production of cystathionine. All these data suggest that there may be a limited set of CBS mutants, which could increase their residual activity in the presence of heme or its analogs, and that heme pocket might represent a potential therapeutic target in HCU.

### 6.1.3 S-Adenosylmethionine

As Fig. 1 illustrates, the methyl group of Met becomes activated by ATP with the addition of adenosine to the sulfur of methionine, thus forming SAM. SAM is an important biological sulfonium compound and the second most often used substrate in enzymatic reactions after ATP (Cantoni et al. 1975). A majority of methylation reactions occurring in the cell are catalyzed by methyltransferases, which utilize SAM as the methyl donor forming methylated product and SAH. In turn, SAH is a potent inhibitor of methylation reactions catalyzed by methyltransferases (Glick et al. 1975). In addition, SAH hydrolase catalyzes the formation of SAH in the excess of Hcy. Therefore, SAM activation of CBS represents a critical mechanism for maintaining the balanced methylation as well as redox potential. This fine balance disturbed by, e.g., the lack of CBS activity, leads to an increased formation of Hcy and SAH, inhibition of methylation reactions, and normal or increased SAM plasma concentrations in HCU patients (Orendac et al. 2003).
Kozich et al. have explored the possibility of SAM or SAH stimulating the residual CBS activity in a large set of missense mutants using *E. coli* expression system (Kozich et al. 2010). Mutant proteins were distributed into three groups based on the observed effect of the tested ligand: clear activation similar to the WT, clear inhibition, or absence of activation. In many instances, inhibition by SAH was also observed (Kozich et al. 2010). The study suggested that SAH hydrolase inhibitors could decrease SAH concentration and thus alleviate the inhibitory effect on some CBS mutants. However, such treatment would automatically lead to an increase of Hcy concentration. Another possibility suggested by the authors was an administration of SAM to stimulate residual activity of several mutants. However, reactive properties, intrinsic instability, and charged character of SAM make such an approach likely unsuccessful.

### 6.2 Chemical Chaperones

Chemical chaperones represent a group of small organic molecules that are not specific for any particular protein. Many of the chemical chaperones are osmolytes or sugars often being accumulated intracellularly in response to environmental stress (Leandro and Gomes 2008; Nascimento et al. 2008). The most accepted mechanism for their positive effect on protein stability is destabilization of unfolded states leading to a thermodynamic stabilization of their native state (Street et al. 2006; Bolen and Rose 2008). Moreover, they were found to provide an additional stabilization to improperly folded proteins, to reduce protein aggregation, to prevent non-specific and/or undesired interactions with other proteins, and to alter the expression and activity of cellular molecular chaperones (Nascimento et al. 2008). Some of the most widely used chemical chaperones are, for example, glycerol, dimethyl sulfoxide (DMSO), trimethylamine-N-oxide (TMAO), 4-phenylbutyric acid (PBA), sorbitol, or betaine.

First report on the use of chemical chaperones for rescuing activity of several CBS mutants came from the group of Dr. Warren Kruger (Singh et al. 2007). Earlier he developed a yeast complementation assay, where yeast lacking endogenous CBS gene (cys4) regained its ability to grow on a cysteine-free medium only when functional mutant CBS was expressed from a plasmid, thus linking residual activity and growth (Kruger and Cox 1994; Kruger et al. 2003). By using five chemical chaperones (DMSO, glycerol, proline, TMAO, and sorbitol), these authors classified eight pathogenic missense CBS mutants into three groups based on their rescued CBS activity (Singh et al. 2007). Surprisingly, prediction of solvent accessible surface area suggested that the rescuable CBS mutants are the ones that are predicted to cause a decrease in the solvent-exposed area. Interestingly, the mixture of several chemical compounds was found to be more effective than the individual chaperones in rescuing an I278T CBS mutant. Chemical chaperones were found to enhance a formation of native tetramers as well as CBS-specific activity. This effect seemed to be associated with an improvement of the folding efficiency or stability of the folded CBS proteins. The I278T CBS mutant was also found in a follow-up work rescuable
by an increasing concentration of ethanol up to 6% in a concentration-dependent manner, while 10% ethanol resulted in a total yeast growth inhibition (Singh and Kruger 2009). These studies therefore concluded that chemical chaperones present during CBS mutant expression could rescue folding and enzymatic activity and thus could represent a possible pathway toward treatment of HCU.

A remarkably different effect of chemical chaperones and cosolvents was described by Majtan et al. (2010). By systematic screening of different concentrations of three chemical chaperones using *E. coli* expression system, they identified conditions that remarkably increased the recovery of tetrameric and fully active CBS mutants, while their response to SAM and thermal activation varied significantly. The lack of response to both activating stimuli of R125Q and E176K indicated that their improved folding and newly adopted conformation were unable to reach the activated state. Increased levels of molecular chaperones, particularly DnaJ, in *E. coli* soluble extracts suggested a rather indirect effect of the chemical chaperones on folding of CBS mutants.

To assess the number of patients that may benefit from chemical chaperone therapy, Kopecka et al. studied the effect of three osmolytes (glycerol, betaine, and taurine) on assembly and activity of a large set of 27 CBS mutants expressed in *E. coli* representing about 70% of known CBS alleles (Kopecka et al. 2011). Betaine was able to improve tetramer formation and CBS activity in a third of the mutants, while glycerol was found to be even more effective rescuing about half of the studied mutants. Taurine did not show any effect at all. The study also indicated that the topology of the mutation may determine the ability of the chemical chaperone to improve folding as 11 out of 14 solvent-exposed mutations were substantially more responsive to the chaperone treatment compared to 3 out of 13 buried missense mutations. The authors estimated that, considering the frequency of examined patient-derived mutations amenable to chemical chaperone treatment, approximately one tenth of HCU patients might benefit from such a therapeutic approach.

More recently, the effect of PBA was examined on a set of 27 CBS mutants under folding-permissive conditions of mammalian cells (Melenovska et al. 2015). PBA is an FDA-approved drug for treatment of urea cycle disorders; however, many studies reported its positive effect on other diseases as well (Kolb et al. 2015). While in urea cycle disorders PBA conjugates with glutamine and thus serves as an ammonia detoxifying agent, the mode of action of PBA in misfolding diseases remains elusive. Most evidence suggests that PBA downregulates the endoplasmic reticulum stress as well as acts as a chemical chaperone (Kolb et al. 2015). However, PBA only mildly increased the specific activity in a small set of CBS mutants (Melenovska et al. 2015). This result suggests that a generic effect of PBA does not have any noticeable impact on CBS mutants in contrast to other misfolded proteins.
6.3 Molecular Chaperones and Proteasome Inhibitors

Many chemical chaperones were also found to function indirectly by inducing expression and to promote the function of endogenous molecular chaperones. Molecular chaperones belong to the proteostasis network, which encompasses pathways that control protein synthesis, folding, trafficking, aggregation, disaggregation, and degradation (Powers et al. 2009). A mutated protein may represent a challenge to the proteostasis network, and thus additional assistance from molecular chaperones is needed to cope with the stress induced from misfolded and aggregated protein. In general, such assistance to increase steady-state levels of mutated protein can be achieved by either upregulation of mechanisms leading to refolding of misfolded polypeptide or downregulation of degradation pathways increasing the probability for the protein to adopt its proper conformation. While the first approach relies on induction of molecular chaperones such as HSP70, HSP60, or HSP40, the latter seeks inhibition of proteasome function.

The role of molecular chaperones in the rescue of CBS mutants was first explored using the most common I278T CBS mutant (Singh and Kruger 2009). Manipulation of the cellular chaperone environment resulted in a dramatically restored enzyme stability and activity. Involvement of molecular chaperones was suggested by the initial studies, where either ethanol or a mild heat shock resulted in better growth of yeast expressing I278T CBS mutant accompanied by an increased steady-state levels of CBS protein. Ethanol treatment upregulated the HSP70, while levels of HSP104 remained largely unchanged. Interestingly, levels of the small co-chaperone HSP26 were significantly decreased for the mutant, but not for the WT. The ability of ethanol to restore function was found to be linked with the function of a cytosolic HSP70 in I278T CBS mutant folding. On the contrary, HSP26 seemed to allow misfolded I278T to be presented and rapidly degraded via the ubiquitin/proteasome pathway. Consistently, the use of bortezomib, a proteasome inhibitor, resulted in the rescue of this CBS mutant. These approaches were later successfully extended to a larger set of CBS mutants (Singh et al. 2010). Remarkably, its efficacy was confirmed in a patient-derived fibroblasts and homocystinuric mice expressing the I278T mutant CBS. Taken together, the work suggested that manipulation of the molecular chaperone levels, particularly an induction of HSP70 by proteasome inhibitor or other agents, might represent a useful novel approach for treatment of HCU.

The efficacy of proteasome inhibitors to correct homocystinuric phenotype was subsequently explored in two HCU mouse models (Gupta et al. 2013). Mice lacking endogenous CBS, but expressing either I278T or S466L human CBS mutant, were treated with ONX-0912, an oral proteasome inhibitor currently in clinical trials as an anticancer drug (Zhou et al. 2009), and/or bortezomib, a parenteral proteasome inhibitor studied previously and approved by FDA in 2003 for treatment of multiple myeloma. While either treatment-induced expression of multiple molecular chaperones in the liver, such as HSP70, HSP40, and HSP27, increased steady-state levels and activity of the mutant CBS enzyme and resulted in lowering Hcy levels to within a normal range, the response rates varied between the studied
mouse models. Mice carrying the S466L CBS mutant responded positively much more frequently and consistently than the mice expressing the I278T CBS. Interestingly, microarray analysis on livers harvested from the I278T mice responsive or unresponsive to these treatments revealed significant downregulation of several genes in steroid hormone metabolism in responding versus non-responding animals. These data provide strong preclinical evidence that proteasome inhibitors should be considered as potentially useful in treatment of misfolding diseases caused by a missense mutation, such as HCU.

7 Rational Approach in Search for CBS Pharmacological Chaperone

Except for the use of high doses of vitamin B₆ in pyridoxine-responsive HCU patients, therapeutic applicability of the studied chemical or pharmacological chaperones and proteostasis modulators so far is low mainly due to their non-specificity and a risk of significant off-site effects and a requirement of high doses, which are often toxic. Clearly, much more focused and targeted approach is needed in order to develop a small molecule treatment for HCU. In addition to an obvious unmet need of HCU patients, two main factors can contribute to such efforts. First, CBS has been recently recognized as an enzyme responsible for H₂S biosynthesis. While the physiological relevance of CBS alternative reactivity leading to an in vivo generation of H₂S remains to be answered (Kožich et al. 2016; Majtan et al. 2017), a multitude of pathological and physiological effects of H₂S has attracted a lot of attention in recent years and has lead to a development of many H₂S probes and CBS activity assays employing alternative substrates suitable for high-throughput screening and a search for CBS-specific activity modulators (Holt et al. 2009; Asimakopoulou et al. 2013; Lin et al. 2014). Second, high-resolution crystal structures of human full-length CBS have recently been solved in both the basal (Ereno-Orbea et al. 2013b; McCorvie et al. 2014) and the activated SAM-bound conformations (Ereno-Orbea et al. 2014). Structural information about the PLP-containing catalytic center as well as the SAM-binding allosteric site in the C-terminal regulatory domain of CBS are crucial for structure-guided, computer-aided drug design. Therefore, these two factors can propel new avenues of research in the field toward rational design of small molecules targeting CBS folding, stability, or activity.

7.1 High-Throughput Screening for CBS Inhibitor

At first, the idea of using specific inhibitors for restoring mutant enzyme activity may sound counterintuitive. However, there is a precedent for it from the lysosomal storage disorders. Miglustat (N-butyl-1-deosynojirimycin; NB-DNJ) is a substrate reduction therapy for Gaucher disease type 1 patients, who showed anaphylactic reactions to the available enzyme replacement therapies (Bennett and Mohan 2013). In addition, miglustat acts as an active site inhibitor and chaperone-like compound
preventing misfolding and rapid degradation (Alfonso et al. 2005). The term active site-specific chaperones was coined for a group of small molecule inhibitors of enzymes, which shift the folding equilibrium of a mutated enzyme in favor of a proper, native-like folding, thus preventing rapid degradation and improving subsequent processing and trafficking of the mutants (Fan 2008). Once the mutant enzyme folding has been rescued by the action of a specific competitive inhibitor, the inhibitor can be displaced by a highly concentrated substrate to allow the function of the enzyme. Thus, the ideal properties of such chaperoning inhibitors are (1) high affinity to the active site of the enzyme, where the inhibitor can serve as a scaffold for folding and/or stabilizer of the domain; (2) high cellular permeability and subcellular distribution, which is particularly relevant for posttranslationally modified enzymes, such as those responsible for lysosomal storage disorders; and (3) smooth dissociation of the inhibitor from the enzyme’s catalytic center, so it can be replaced by a natural substrate (Fan 2008). Screening for identification of such chaperoning inhibitors generally includes three stages. First, an in vitro activity assay, preferably suitable for high-throughput screening of chemical libraries, is employed to estimate the binding affinity between a compound and an enzyme and to determine the IC₅₀ values for best hits. Second, cell-based chaperone enhancement assay is used for evaluation of hits (typically with IC₅₀ lower than 10 μM). Third, successful leads from cell-based evaluation are assessed for in vivo efficacy in an animal model expressing the misfolded mutant enzyme and showing clear clinical symptoms of enzyme deficiency.

CBS currently represents one of many therapeutically attractive PLP-dependent enzymes, which have not yet been successfully targeted. Currently, there are only two widely used CBS inhibitors, aminooxyacetic acid (AOAA) and hydroxylamine (HA) (Whiteman et al. 2011). However, in addition to their poor potency, both compounds are insufficiently selective due to their targeting of the PLP cofactor in other PLP-dependent enzymes. AOAA has been recently tested for its chaperoning effect on seven human CBS mutants expressed in mammalian cells (Melenovska et al. 2015). The inhibitor only marginally affected the residual activity of the studied CBS mutants suggesting that AOAA does not induce proper folding, rescue activity, or stabilize the native conformation of CBS mutants. Selectivity of the commonly used pharmacological inhibitors of CBS and CGL have been recently evaluated showing that, while there are several CGL selective inhibitors available, there are none that are CBS-specific (Asimakopoulou et al. 2013). Moreover, both AOAA and HA were significantly more potent inhibiting CGL over CBS.

To identify novel, specific, and potent new inhibitors for CBS, a CBS activity assay compatible with high-throughput screening (HTS) is necessary. This type of high sensitive HTS assay for CBS has been described using label-free mass spectrometry to quantify the unlabeled product of the canonical CBS reaction (Holt et al. 2009). Screening of a proprietary chemical library of over 25,000 compounds using this assay identified 22 compounds as activators. Unfortunately, the authors did not reveal the identity of the CBS activators. However, a follow-up study explored the effect of S-adenosylethionine, a close analog of SAM, on Hcy
levels and H$_2$S production in mice (Jensen et al. 2011); thus, one could assume that the structures of the identified CBS activators were closely related to SAM.

The growing interest in H$_2$S metabolism and its contribution to human health and disease has resulted in the development of reaction-based fluorescent probes offering a versatile and sensitive set of screening tools for H$_2$S detection (Lin et al. 2014). An HTS-capable assay using 7-azido-4-methylcoumarine (AzMC) as a novel H$_2$S probe producing robust fluorescent signal has been recently developed (Thorson et al. 2013) and used to identify a set of 12 substances, mostly related to flavonoids, as good inhibitors and, in some cases, with a remarkable selectivity for CBS over CGL. More recently, a similar approach applied to a library of marine natural products and their synthetic derivatives has allowed to identify polyandrocarpamine derivatives as scaffolds to develop new CBS inhibitors (Thorson et al. 2015). Taken together, flavonoid and polyandrocarpamine scaffolds may serve as useful starting points for the development of potent and selective CBS inhibitors capable to correct CBS mutant misfolding and thus to rescue residual CBS activity.

Several additional screens for CBS modulators (typically, inhibitors) have been conducted over recent years. Some of this activity was facilitated by discoveries showing that the upregulation of CBS in certain types of cancer acts as a cancer cell-derived pro-proliferative, pro-angiogenic, and bioenergetics stimulatory factor (Szabo et al. 2013; Szczesny et al. 2016; Hellmich and Szabo 2015; Szabo 2016). Zhou and colleagues used a high-throughput tandem microwell assay using Ellman’s reagent (5,5'-dithiobis-(2-nitrobenzoic acid), DTNB) to trap and detect H$_2$S. The screened library contained 21,599 unique substances, including 2,697 compounds obtained from the National Cancer Institute, 1,563 FDA- or foreign-approved drugs from the Johns Hopkins Clinical Compound Library, 14,400 compounds from Maybridge HitFinder collection, as well as smaller in-house collections, and identified 35 hit compounds belonging to nine different structural classes. After potency and CBS/CGL selectivity analyses, the authors focused on three polycyclic ketone-based selective inhibitors of human CBS (NSC111041, NSC67078, and SP14311008) and characterized the inhibition as a mixed-type inhibition. Using docking analysis, authors concluded that these compounds appear to bind to CBS at sites other than the PLP binding site. The NSC67078, also known in the literature as toxoflavin, xanthothricin, or PKF118-310, demonstrated antiproliferative effects on cancer cells, although this effect was attributed to independent pharmacological action of this compound, including the inhibition of sirtuin 1/2 and of the beta-catenin pathway (Leow et al. 2010; Choi et al. 2013; Antony et al. 2014).

The most recent CBS screen was published in 2016 (Druzhyna et al. 2016). This screen used an AzMC-based detection method and included 8,866 clinically used drugs and well-annotated pharmacological compounds, a composite collection of 11 commercially available libraries and a small custom library assembled from known PLP-dependent enzyme inhibitor compounds. The compounds that emerged from the screen as CBS inhibitors with potency comparable to AOAA were hexachlorophene, tannic acid, aurintricarboxylic acid, and benserazide. These four compounds were further characterized, along with the principal reference
compound AOAA and the secondary reference compound NSC67078, in cell-based assays. Unexpectedly, the second reference compound (NSC67078), in addition to inhibiting CBS-induced AzMC fluorescence with an IC$_{50}$ of $\sim$1 $\mu$M, also inhibited AzMC fluorescence induced by the H$_2$S donor GYY6137 with an IC$_{50}$ of $\sim$6 $\mu$M. These findings suggested that the observed inhibitory effects of this compound were due to a combination of direct CBS inhibition as well as H$_2$S scavenging and/or interference with the assay. Considering the four hit compounds with respect to the results of previous screens, hexachlorophene and aurintricarboxylic acid have not been previously identified as a CBS inhibitor. The identification of tannic acid as a CBS inhibitor is also novel, although Thorson and colleagues have previously identified several different polyphenolic compounds (including rutin) as CBS inhibitors (Thorson et al. 2013). Benzerazide (Fig. 8a), which was found to have an IC$_{50}$ of $\sim$30 $\mu$M, was previously detected in the screen as a CBS inhibitor with lower potency [IC$_{25}$ = 125 $\mu$M, (Thorson et al. 2013)]. The potency difference can be attributed to the fact that this compound is extremely labile and oxidation-prone, and its CBS inhibitory potency markedly decreases with storage in solution (Druzhyna et al. 2016). As expected from prior studies with CBS silencing and with AOAA, which inhibit cancer cell proliferation (Szabo et al. 2013), all of the identified hit compounds inhibited the proliferation of HCT116 colon cancer cells. Further work was, in turn, conducted to characterize the effects of benzerazide. It was demonstrated that not only benzerazide but also its active metabolite 2,3,4-trihydroxybenzylhydrazine (THBH, also known as Ro 04-5127, Fig. 8a) exerted comparable CBS inhibitory potency and antiproliferative effects in cancer cells. To explore the putative mode of the compound’s CBS inhibitory effect, docking calculations were conducted with two distinct PLP-benserazide derivatives that could be potentially formed (Fig. 8b, c): compound 1 (a derivative of the coupling between the free amine of the unmodified benzerazide with the formyl group of PLP) and compound 2 (a derivative obtained by reaction of THBH with the respective moiety of PLP). While both molecules adopted a highly similar geometry with respect to each other and the crystallographic-free PLP cofactor, compound 1 demonstrated higher docking scores due to the fact that interaction geometry permitted the formation of additional hydrophobic contacts with the protein environment as well as extensive hydrogen-bonding interactions between the trihydroxybenzene ring and polar residues located at the periphery of the cavity such as H203, Y223, and Y308.

Taken together, recent, renewed activity in the field identified a number of scaffolds, which may serve as useful starting points for the development of potent and selective CBS modulators.

### 7.2 Dawn of Rational Design of CBS Ligands

Alternatively, structure-guided rational design can be used to identify novel CBS activity modulators and to improve those found by HTS approaches. However, structure-guided methods depend on the availability of high-resolution 3D (X-ray
or NMR) structures of a given drug target, e.g., enzyme or receptor, bound to its natural substrate(s) and/or allosteric ligand(s), thus allowing for virtual in silico screening of large collections of chemical compounds.

### 7.2.1 Inhibitors Targeting CBS Catalytic Site

The currently available structural information on human CBS makes it feasible to embark on a path toward CBS drug discovery and development via rational design. Although the crystal structure of a human CBS catalytic core has been solved more than a decade ago (Meier et al. 2001; Taoka et al. 2002), it did not reveal the PLP-bound reaction intermediates and thus did not provide an insight into residues that may be important for substrate binding and catalysis. However, both crystal structures of the truncated human CBS (PDB IDs 1JBQ and 1M54) showed an overall fold of the catalytic core of the enzyme, binding site for heme cofactor and catalytic center with bound PLP. Insight into reaction intermediates came from the crystal structures of *Drosophila melanogaster* CBS as apoenzyme (PDB ID 3PC2),

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**Fig. 8** Docking of PLP inhibitor benserazide to human CBS. (a) The two potential derivatives of the reaction between PLP and either the unmodified benserazide (1) or the benserazide’s active metabolite THBH (2). (b, c) Proposed binding mode of compound 1 (b) and compound 2 (c) (shown as balls and sticks) in the PLP binding cavity of CBS shown in a ribbon representation and an electrostatic potential colored surface. A number of residues involved in binding are depicted in sticks, while hydrogen bonds with residues of the PLP cavity are shown as yellow dashed lines (Druzhyna et al. 2016)
in aminoacylate intermediate (PDB ID 3PC3), and in complex with serine (PDB ID 3PC4) (Koutmos et al. 2010). Substrate binding to the active center PLP induced a general collapse of the active site pocket, particularly of a loop containing residue S116. This residue corresponds to an S147 in human CBS located on loop L145-148 (Fig. 9). While the conformational flexibility in this loop was later found relevant for accessibility of the catalytic center in human enzyme as well, three other loops, namely, L171-174, L191-202, and L295-316 (Fig. 9), have been found crucial for the formation of an entrance to the catalytic cavity in the full-length human CBS (Ereno-Orbea et al. 2013b, 2014; McCorvie et al. 2014). These loops were found collapsed only in the case of a substrate present in the catalytic cavity (Koutmos et al. 2010). In addition, the conformation and flexibility of these loops was found to be substantially impacted by the presence of a regulatory domain, thus explaining its autoinhibitory function. Particularly, the loops L171-174 and L191-202 were found compressed into the catalytic cavity and rigid by the presence of the regulatory domain from the complementary subunit (Ereno-Orbea et al. 2013b). The SAM-mediated activation and formation of the CBS module in the regulatory region of the enzyme resulted in relaxation of the loops, thus increased accessibility of the catalytic site, and observed higher enzymatic activity (Ereno-Orbea et al. 2014). Taken together, the available structural information allowed us (1) to understand the formation and binding of CBS reaction intermediates, (2) to identify important residues and overall structure of the catalytic cavity, and (3) to recognize the importance of conformational flexibility of the loops defining the entrance to the catalytic site.

![Fig. 9](image-url) Loops delineating the entrance to the CBS catalytic cavity. Structure of the basal (gray, PDB ID 4LOD) and the activated (pink, PDB ID 4PCU) conformation of the catalytic core of human CBS. In the basal state, the loops delineating entrance to the PLP cavity (L145-148, L141-174, L191-202, and L295-316; shown by arrows) remain in a “closed” conformation due to the presence of the Bateman module (not shown) above the catalytic cavity. Upon binding of SAM, the Bateman module migrates from atop the catalytic cavity, thus allowing for relaxation of the loops and subsequent “opening” of the catalytic site (the shift is indicated with a blue arrow). Artificial removal of the regulatory domain has a similar effect.
Currently there is no specific inhibitor for CBS. It is our belief that increased interest in H₂S biogenesis and its modulation with the availability of the structural determinants will propel the advances in pursuit of a CBS inhibitor. In addition to the search for a CBS-specific compound disrupting the reaction mechanism, importance of flexibility versus rigidity of the loops delineating the entrance to the catalytic cavity could be exploited as a new potential site for ligand binding. Indeed, the docking analysis of CBS inhibitor NSC111041 mapped the compound to a site different from the PLP binding site (Zhou et al. 2013). NSC111041 was found inserted into a small pocket with its 2-hydroxy, 5-keto, and 8-imine groups forming four hydrogen bonds with T146, S147, and Y223. Interestingly, residues T146 and S147 are located on the loop L145-148 identified earlier to be important for conformation of the catalytic site. It is plausible that ligands such as NSC111041 would stabilize the region enough to allow a CBS missense mutant to refold and/or to fold into a native-like conformation, thus functioning as a pharmacological chaperone.

7.2.2 Activators or Kinetic Stabilizers Targeting CBS Regulatory Domain

The relevance of the CBS regulatory domain as a drug target has been recently highlighted, thanks to its potential connection with intracellular CBS turnover. While activation of CBS by SAM has been known for a long time (Finkelstein et al. 1975) and its molecular mechanism has been recently uncovered (Eren-Osbea et al. 2014; McCorvie et al. 2014), the role of the regulatory domain in kinetic stabilization of the enzyme has been just recently discovered (Pey et al. 2013). Differential scanning calorimetry analyses of WT and several pathogenic CBS mutants have shown that denaturation of the regulatory and the catalytic domains are independent and kinetically controlled processes (Fig. 6). Therefore, their stabilities in vivo could be linked to their half-lives toward irreversible denaturation at 37°C (i.e., their kinetic stabilities). The stability of the regulatory domain is significantly decreased among pathogenic mutants compared to the WT. Surprisingly, pathogenic mutations located in the catalytic domain impaired the stability of the regulatory domain as well, supporting the notion of communication between the regulatory and the catalytic domains in the native structure and thus underlining the importance of stabilization of the regulatory domain in CBS-deficient homocystinuria (Pey et al. 2013).

More importantly, current knowledge implies that specific ligands targeting CBS allosteric sites (Fig. 10) could be found or designed in order to independently modulate CBS activity and kinetic stability. Ligand-induced kinetic stabilization of the regulatory domain (Pey et al. 2013; Majtan et al. 2016; Pey et al. 2016b) of a missense CBS mutant would result in increased intracellular levels of the protein. For example, Pey et al. found out that the half-life for irreversible denaturation of the regulatory domain in CBS mutants is as much as 200-fold lower compared to the WT, thus making it extremely kinetically unstable (Pey et al. 2013). We hypothesize that ligand-induced stabilization of its regulatory domain would remedy its abnormal susceptibility toward denaturation, thus rescuing the CBS activity...
in vivo. On the other hand, ligand-induced activation similar to a natural CBS ligand SAM would increase the residual activity or ameliorate an impaired regulation in certain mutants. The majority of CBS pathogenic mutations do not prevent SAM binding, but rather interfere with the molecular mechanism of the regulatory domain rearrangement and formation of the CBS module (Pey et al. 2013; Ereno-Orbea et al. 2014). As an example, binding affinity of SAM to D444N CBS mutant is significantly lower, thus increasing the $K_{act}$ for SAM ~100 times (Evande et al. 2002) and, at the same time, partially increasing the enzyme’s activity twofold (Ereno-Orbea et al. 2013b). Furthermore, the D444 residue was found to be an important residue involved in SAM binding and its accommodation within the allosteric binding site (Ereno-Orbea et al. 2014; McCorvie et al. 2014). We hypothesize that there may be identified and/or designed a ligand with higher affinity for the D444N mutant than SAM, thus rescuing the physiological regulation and activation of the enzyme.

8 Future Prospects

Misfolding due to the presence of a missense mutation represents an increasingly better understood pathogenic mechanism in HCU. CBS mutations often display difficulties to fold to the native/active state and show low kinetic stability of this active state. Therefore, we can envision several ways to at least partially correct the effect of a missense mutation on CBS folding and stability. While treatment with pyridoxine as a precursor of catalytically active cofactor PLP works in roughly half of HCU patients, novel treatments need to be devised to address an unmet need of
the remaining affected individuals. Other alternatives, such as enzyme replacement therapy (Bubil et al. 2016) or gene therapy (Jacobs et al. 2011; Muthuramu et al. 2015), have been explored and/or are currently in development. Studies using various CBS cofactors or their analogs, chemical chaperones, or proteostasis regulators suggest that development of a small molecule treatment for HCU is a possible, but very challenging task. The presence of multiple missense mutations among HCU patients and their different impact on CBS properties represent a major challenge. It is likely that the individualized patient-tailored therapeutic approach would need to be developed in order to address impact of each mutation or a very small subset of mutations independently. In order to effectively screen for a promising chaperone or ligand, development of assays compatible with HTS reporting not just an effect on activity of a purified mutant enzyme is needed. Such screening tools should detect the effect of a tested compound on folding of CBS mutant in its natural environment, thus pointing out an approach to the development of cell-based assays. With a recent progress in CBS structure determination, alternative strategy through virtual computer-aided structure-guided screening is equally viable. Targeting catalytic site cavity in search for inhibitor or SAM binding site in search of kinetic stability regulator and/or catalytic activator bears a lot of potential to yield novel scaffolds for detailed biochemical characterization and further optimization.

Acknowledgment  This study was supported by the American Heart Association Scientist Development Grant 16SDG3004000 (to TM), Junta de Andalucia (P11-CTS-07187 to ALP), Basque Foundation for Health and Research (BIOEF and EITB Maratoia 2015 to LAMC) by grants from the Spanish Ministry of Economy, Industry and Competitiveness (BFU2010-17857 and BFU2013-47531-R to LAMC) and from the Czech Health Research Council (16-30384A to VK). TM and JPK would like to thank Orphan Technologies Ltd. for support of their research. ALP would also like to acknowledge support from Prof. Jose Manuel Sanchez-Ruiz (University of Granada) through the Spanish Ministry of Economy, Industry and Competitiveness grant (BIO2015-66426-R) and FEDER funds. LAMC would also like to thank the Spanish Ministry of Economy, Industry and Competitiveness for the Severo Ochoa Excellence Accreditation to CIC bioGUNE (SEV-2016-0644). In addition, VK received an institutional support from projects RVO-VFN 64165 and Progres Q26.

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