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REVIEW ARTICLE

Targeting Cystathionine Beta-Synthase Misfolding in Homocystinuria by Small Ligands: State of the Art and Future Directions

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Abstract: Classical homocystinuria (HCU) is the most common loss-of-function inborn error of sulfur amino acids 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 non-functional aggregates or to undergo proteasome-dependent degradation. Thus correction of CBS misfolding represents 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-adenosyl-L-methionine) as well as the intricate allosteric regulatory mechanism only recently explained thanks to advances in CBS crystallography. While roughly half of the patients responds 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 increased 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.

Keywords: Classical homocystinuria, cystathionine beta-synthase, high-throughput screening, homocysteine, pharmacological chaperones, protein misfolding, heme, pyridoxal-5'-phosphate, S-adenosylmethionine.

1. INTRODUCTION

Classical homocystinuria (HCU; OMIM# 236200) is an autosomal recessive inborn error of sulfur amino acid metabolism. It is characterized by highly elevated levels of L-homocysteine (Hcy) in body fluids and tissues [1]. 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 [2]. This abnormal amino acid has been identified as Hcy and the authors suggested to name this defect homocystinuria. HCU is the most common disorder of sulfur amino acid metabolism. Its incidence varies greatly with approximately 1:200,000 to 1:335,000 people worldwide, while expanded newborn screening suggests that this number is greatly underestimating the true rate of occurrence [1].

In addition, there are countries in which the disorder appears more commonly, such as 1:65,000 in Ireland [3], 1:6,400 in Norway [4] or the striking incidence of 1:1,800 in Qatar [5]. If left untreated, HCU is accompanied by dislocated optic lenses with or without severe myopia, skeletal and connective tissue abnormalities, osteoporosis, cognitive impairment up to mental retardation and significantly increased cardiovascular complications, such as atherosclerosis, thromboembolism and stroke [1]. Biochemically, the HCU is characterized by grossly elevated levels of total plasma Hcy accompanied with significantly diminished plasma levels of cysteine (Cys) and cystathionine (Cth) and normal or elevated plasma methionine (Met) levels [1, 6]. The available therapeutic approaches primarily rely on the reduction of total Hcy accumulation (reviewed in [7]). Since Met is a precursor of Hcy, a Met-restricted diet is the cornerstone of the treatment. Though such approach is quite effective in normalizing the biochemical profile and prevents the onset, progression or exacerbation of clinical symptoms,

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compliance is generally poor, particularly for teen-aged patients. In such cases, administration of betaine, which serves as a methyl donor for remethylation of Hcy back to Met, was found beneficial to maintain low levels of total plasma Hcy. In a specific subset of affected individuals, the so called pyridoxine-responsive patients, supplementation of vitamin B_6 significantly helps to maintain low Hcy plasma levels by increasing the residual activity of cystathionine beta-synthase (CBS).

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 [8-10]. 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. These two enzymes require an active form of pyridoxine (vitamin B_6), pyridoxal-5'-phosphate (PLP), as a 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 [11]. Recently, the relaxed substrate specificity of both transsulfuration enzymes, resulting in generation of hydrogen sulfide (H_2S), 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 [12-14].

1.1. CBS Modular Architecture

CBS is a unique PLP-dependent enzyme with a multidomain architecture, complex structural and functional properties and an intricate regulation, which are best illustrated on the extensively studied human enzyme [9, 10, 15, 16] (Fig. **2**). The human CBS polypeptide consists of 551 amino acid residues yielding a subunit with a molecular size of around 63 kDa [17]. 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), which is thought to play a role in redox sensing [18] and/or enzyme folding [19]. 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 [20-22]. 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 [20, 23-28]. The purified ferric CBS displays a heme's Soret peak at 428 nm with a broad αβ adsorption band around 550 nm. In this oxidation status, heme is unreactive and inert to ligand exchange with exogenous molecules. 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 enzymes 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 [24, 25]. The ligand switch is irreversible, inactivates the enzyme and is spectrally accompanied by a blue-shift of the Soret peak from 447 nm to 424 nm. Ferrous CBS heme binds various small molecules, such as CO, NO or cyanide, which results in inhibition of enzyme activity [29]. Due to the low CBS heme redox potential (-350 mV) [30], the existence of a ferrous form of the enzyme *in vivo* and the feasibility of CO-based regulation under physiological conditions has been an open question. Recently, Kabil *et al.* [27] have provided the first evidence of reversible inhibition of CBS by CO in the presence of a human flavoprotein and NADPH.

The central module spanning the residues 70 to 386 represents the catalytic segment, where the catalytically active PLP cofactor binds *via* Schiff bond to the ε-amino group of the K119 residue [31]. 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 being the most homologous with CBS [21, 32]. 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 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 the heme and PLP in human CBS, the spectral characteristics of the reaction intermediates and the catalytic mechanism were described in a heme-independent CBS from yeast [33]. Some of these reaction intermediates were later confirmed in the crystal of human truncated CBS, from which the heme cofactor had been removed by CO [34] as well as in the crystal structure of *Drosophila* CBS [35]. Alternative CBS reactions, which result in production of H_2S [36], 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 [37].

The C-terminal module of the enzyme houses a tandem repeat of CBS domains (also referred to as the Bateman module), a conserved structural motif named after CBS. This motif is found in diverse and functionally unrelated proteins, where it usually fulfills a regulatory role and/or sensing function upon binding adenosine analogs [38-40]. Indeed, in CBS the C-terminal domain represents a regulatory domain, which inhibits catalytic activity of the enzyme. The CBS allosteric activator S-adenosyl-L-methionine (AdoMet) binds to the regulatory domain and thus releases an intrasteric autoinhibitory block and activates the enzyme [41, 42]. 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 AdoMet [16, 42].

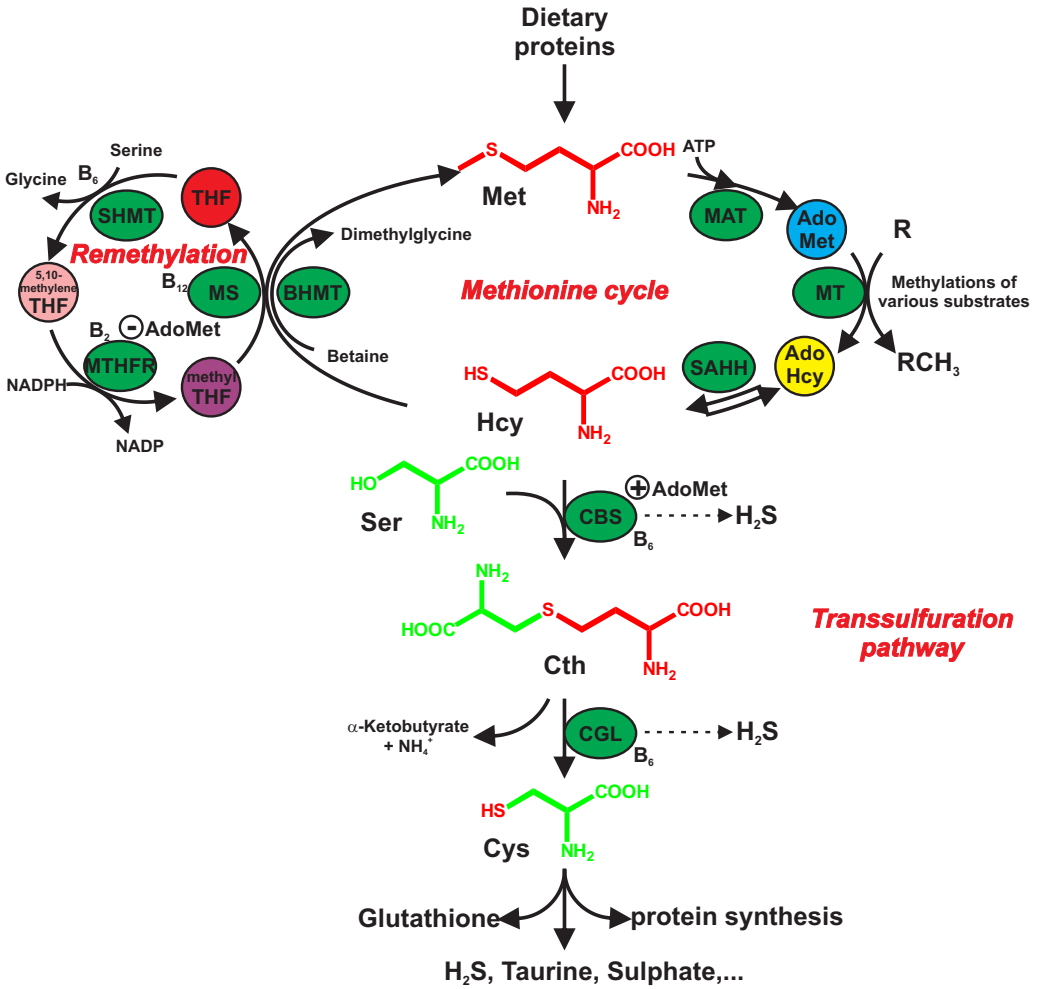

Fig. 1. Sulfur amino acid metabolism pathways. Methionine (Met), an essential amino acid taken from dietary proteins, is condensed with ATP by methionine adenosyltransferase (MAT) to form S-adenosylmethionine (AdoMet). AdoMet serves as a methyl donor for multiple methylation reactions catalyzed by various methyltransferases (MT) yielding a methylated product and S-adenosylhomocysteine (AdoHcy). AdoHcy is subsequently hydrolyzed by AdoHcy hydrolase (SAHH) into adenosine and homocysteine (Hcy). Hcy is then distributed between two competing pathways. In order to conserve Met, Hcy is remethylated back to Met by the action of either betaine homocysteine methyltransferase (BHMT) or methionine synthase (MS) using betaine and methyl tetrahydrofolate (methyl-THF), respectively, as the methyl donor. In order to generate Cys, Hcy is irreversible diverted from the methionine cycle to the transsulfuration pathway by cystathionine beta-synthase (CBS)-catalyzed condensation with serine (Ser) forming cystathionine (Cth), which is subsequently hydrolyzed by cystathionine gamma-lyase (CGL) into cysteine (Cys). Importantly, AdoMet regulates the flux of Hcy through the competing pathways by serving as an allosteric activator of CBS and exerting an opposite effect on MTHFR. Interestingly, all transsulfuration and remethylation enzymes require assistance of a member of vitamin B family: B_2 (riboflavin) in MTHFR, B_6 (pyridoxine) in SHMT, CBS and CGL or B_{12} (cobalamin) in MS.

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 [43]. In addition to its regulatory function, the C-terminal domain is responsible for CBS tetramerization and its removal leads to the formation of a highly active truncated dimers having similar specific activity as the AdoMet-stimulated full-length enzyme [44, 45].

Taking into an account the crucial role of CBS in metabolism of sulfur amino acids, it is surprising that 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 [16]. More importantly, the AdoMet-mediated regulatory mechanism is not universal for CBS enzymes. For example, CBS enzymes from parasitic protozoans entirely lack both the N-terminal heme-binding domain as well as the C-terminal regulatory AdoMet-binding module suggesting that the central catalytic module represent a self-sustainable and fully catalytically competent unit [46, 47]. The lack of regulatory domain is understandably accompanied by insensitivity of the CBS enzyme to AdoMet-mediated activation. However, despite possessing the C-terminal CBS domains, CBS enzymes from insect or yeast are not regulated by AdoMet [35, 48]. Interestingly, while both enzymes are highly active and do not respond to AdoMet activation, yeast CBS, but not the one from fruit fly, binds AdoMet [16].

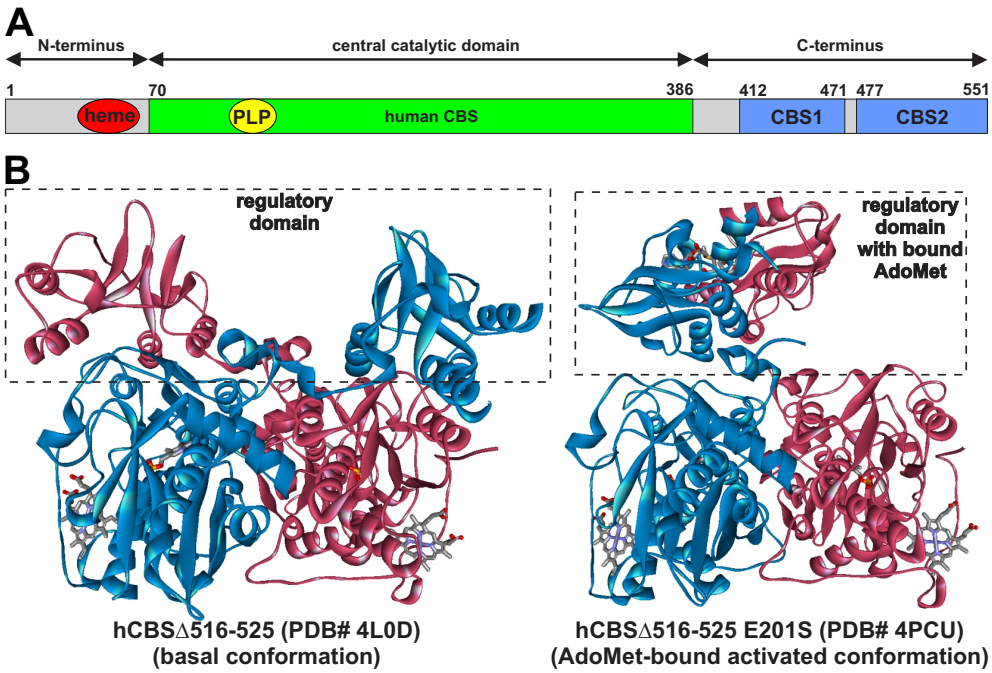

Fig. 2. Domain organization and structure of human CBS. (**A**) The CBS polypeptide consists of three functional domains. The N-terminal domain binds the heme cofactor *via* C52 and H65 as axial ligands. The central catalytic domain binds the PLP cofactor *via* Shiff bond with the ε-amino group of K119. The C-terminal regulatory domain includes a tandem of CBS domains (CBS1 and CBS2), where the CBS allosteric activator AdoMet binds. (**B**) Crystal structures of a dimeric full-length CBS lacking the flexible loop 516-525 from CBS2 domain in basal and activated conformations. In the basal conformation, regulatory domain from one subunit (turquoise) interacts with the catalytic core of the other subunit (pink) and thus exerts its auto-inhibitory effect on the enzyme's catalytic activity. Binding of AdoMet into the S2 site leads to formation of a compact, disk-shaped CBS module accompanied by kinetic stabilization of the regulatory domain and activation of the catalytic core thus yielding the AdoMet-bound activated conformation. The cofactors (heme, PLP, AdoMet) are shown as sticks. *(For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)*

1.2. Biochemical and Structural Aspects of CBS Allosteric Regulation

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 extensively 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 unclear function, the CXXC oxidoreductase motif [21, 22]. Tumor necrosis factor TNFα, which enhances ROS levels, induces a 50-60% increase in CBS activity by yielding a truncated form of the enzyme [49]. 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 [50]. While CBS has been long thought to be located solely in the cytoplasm, SUMOylated CBS has been found in the nucleus [51]. 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 H_2S production [52, 53]; 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 2-fold by S-glutathionylation at the residue C346, which needs first to be oxidized in order to be modified by GSH efficiently [54].

Despite the fact that many aspects of CBS physiological regulation remain unclear, recent advances in CBS crystallography and the utilization of calorimetric techniques have allowed for understanding the kinetic stabilization and activation of CBS by AdoMet and to unravel the molecular mechanism of AdoMet-mediated CBS allosteric regulation [41-43]. Isothermal titration calorimetric analyses are consistent with the presence of two sets of AdoMet-binding sites in the C-terminal regulatory domain with different functional features and total stoichiometry of six sites per CBS tetramer. A high affinity set of two sites (K_d ~ 10 nM) is most likely involved in the kinetic stabilization of the regulatory domain, while a lower affinity set of four sites (K_d ~ 400 nM) is responsible for the enzyme activation. The number of available AdoMet binding sites and oligomeric status of CBS result in a complex situation in which the kinetic stability of the regulatory domain and activity of the enzyme depends on AdoMet concentration and the degree of binding (Fig. **3**). Increasing the amount of bound AdoMet ligands first yields species with a highly kinetically stabilized regulatory domains, *i.e.* the denaturation rates of such species *in vitro* is significantly decreased by AdoMet. With higher concentrations of AdoMet, these species are further stabilized, but AdoMet also increases the catalytic turnover. Interestingly, such substantial changes in stability and activity are not accompanied by changes in the secondary structural elements [41, 55] as previously suggested [39, 56]. Clear differences in thermodynamic properties and functionality of the two types of AdoMet binding sites hint at the possibility of iden-

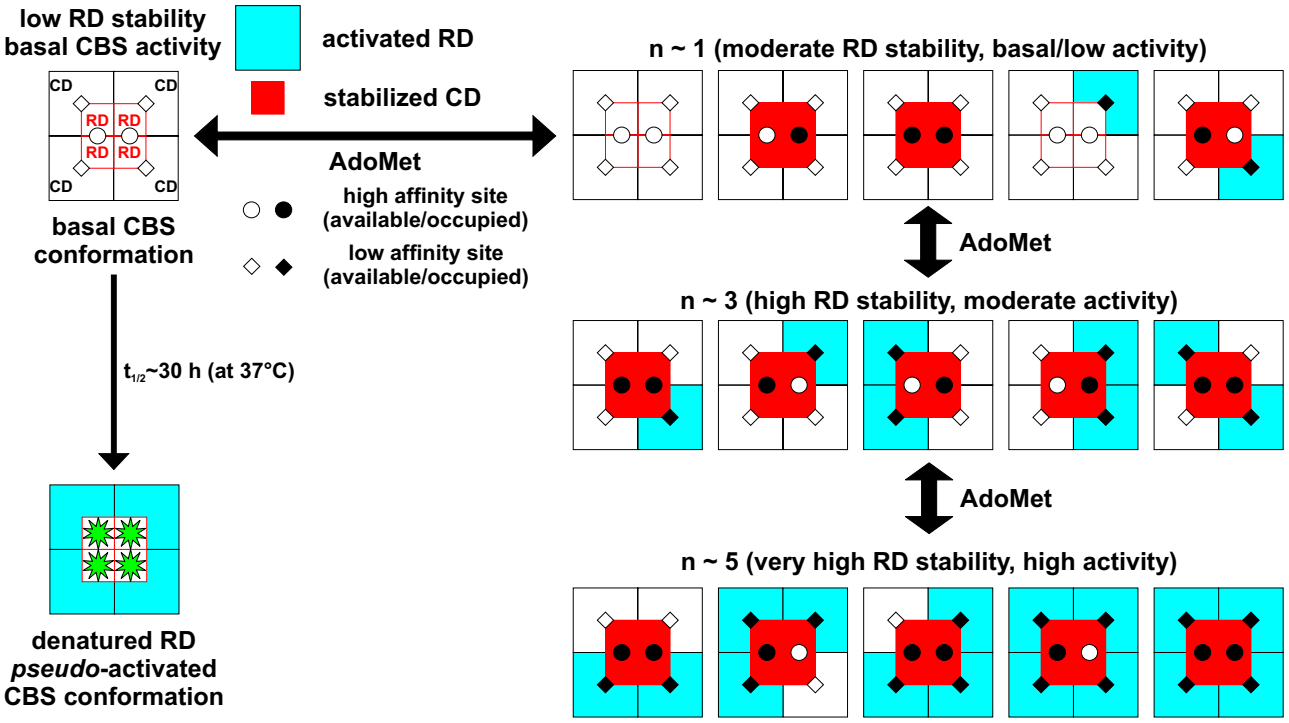

Fig. 3. Model of AdoMet-mediated kinetic stabilization and activation of human CBS. Isothermal titration calorimetric measurements identified two types of binding sites for AdoMet in the human CBS regulatory domain (RD): two high affinity (K_d ~ 10 nM, circle) and four low affinity sites (K_d ~ 400 nM; diamond). Differential scanning calorimetry experiment supported the notion that the half-life of RD denaturation at the physiological temperature is ~30 hours yielding a *pseudo*-activated conformation with denatured RD. Depending on the degree of AdoMet binding to RD (number of AdoMet ligands per tetramer being ~ 1, ~ 3 or ~ 5), average properties of the ensemble evolves from a moderately stabilized RD and basal activity of the catalytic domain (CD) with n ~ 1, through a highly stabilized RD and partially activated CD with n ~ 3 to a highly stabilized RD and highly activated CD with n ~ 5. Open and closed circle or diamond represents vacant and occupied high or low affinity AdoMet binding site, respectively. Open and filled red squares denote kinetically unstable and stabilized RD, respectively, while the open or filled blue squares designate CD having low or high catalytic activity [41]. *(For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)*

tification of pharmacological ligands that would specifically and independently bind and modulate the properties of one type, but not the other type of AdoMet binding site [57].

Structural insight into AdoMet-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 AdoMet, except for its dimeric oligomeric status [43]. The construct was successfully crystalized and yielded the crystal structure of human CBS in the basal, AdoMet-free conformation (Fig. **2**) [43]. The structure revealed a unique and quite unexpected arrangement of regulatory CBS domains as well as suggested the molecular mechanism of the intrasteric inhibition exerted by the regulatory domain. The long and flexible connecting linker allows the CBS domains from one subunit to interact with the catalytic site of the complementary subunit. In addition, the structure allowed for proposing a model of a tetrameric enzyme, where two dimers assembly together like two complementary pieces of a puzzle *via* the main cavity between their Bateman modules. The model also explains the crucial role of the deleted loop in maintaining the tetrameric status by functioning as a hook locking the two dimers together [43].

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 AdoMet and yielded the activated conformation of CBS with bound AdoMet (Fig. **2**) [42]. The structure of CBS in its basal conformation suggested the presence of two plausible AdoMet 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 AdoMet. Indeed, the structure of the activated state revealed one AdoMet ligand per monomer nested only within S2 site, despite the S1 site being solvent-accessible [42]. The availability of structural information on CBS resulted in the proposal of a molecular mechanism of CBS allosteric regulation (Fig. **4**). Binding of AdoMet to solvent-exposed S2 site in the basal conformation triggers the rotation of CBS1 and CBS2 domains thus disrupting the interaction between the Bateman module and the catalytic core of the complementary subunit. Thanks to the flexible connecting linker, the Bateman module moves away thus allowing for a free movement of the loops delineating the entrance to the catalytic center. Comparable conformations can be achieved by binding of AdoMet, by partial thermal denaturation of the enzyme or by the presence of an activat-

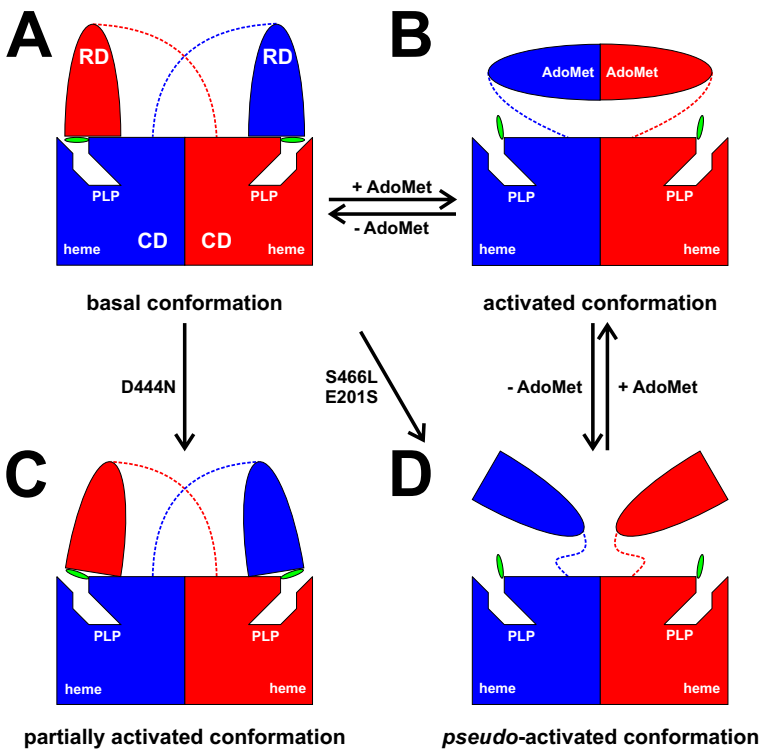

Fig. 4. Model of AdoMet-mediated CBS activation. The optimized full-length CBS lacking the flexible loop of residues 516-525 from the CBS2 domain is biochemically indistinguishable from the native WT except that it assembles into dimers (blue and red subunits). (**A**) In the absence of AdoMet, CBS is in the basal conformation with low specific activity (~ 200 U/mg of protein), where the regulatory domain (RD) of one the subunit interacts with the loops delineating entrance to the catalytic cavity of the other subunit (green oval) and thus inhibits the activity of the complementary catalytic domain (CD). (**B**) Binding of AdoMet leads to a displacement of the regulatory domain away from the catalytic cavity and formation of a disk-shaped CBS module. Thus auto-inhibition is released and the enzyme is activated up to 5-fold. Activated conformation is stabilized by the presence of AdoMet in the S2 site. (**C**) Pathogenic mutations, such as D444N, may disrupt interaction between the regulatory and the catalytic domain as well as impair AdoMet binding thus yielding partially activated conformation. Such structural perturbation enables increased flexibility of the loops near the catalytic cavity and leads to an increased catalytic activity of the mutant enzyme. (**D**) Other mutations, such as the pathogenic S466L or artificial E201S, completely abolish interaction between the Bateman module of the regulatory domain and the catalytic core yielding a *pseudo*-activated conformation. If AdoMet binding is not impaired by the mutation, the presence of AdoMet may result in the formation of CBS module and thus "true" activated conformation (**B**) [42].

ing missense mutation, such as the artificial E201S or the pathogenic S466L [42, 58]. However, such activated conformations are relatively unstable in the absence of AdoMet. The presence of AdoMet in the S2 site of each subunit triggers formation of a head-to-tail antiparallel CBS module, a disk-shaped structural motif, which represents the most typical association manner of Bateman modules among various CBS domain proteins [43]. Such conformation of activated CBS with bound AdoMet is stable and remarkably resembles the crystal structure of the AdoMet-insensitive insect CBS [35, 42]. Identification of the molecular mechanism of CBS activation by AdoMet allows to propose mechanisms of how the pathogenic missense mutations impair the regulation and activation of CBS.

1.3. CBS Deficiency As a Conformational Disorder

HCU is primarily caused by the presence of a point mutation within the *cbs* gene sequence [1]. So far, 164 mutations in the *cbs* gene have been reported with the overwhelming majority (85%) being missense mutations (http:// medschool.ucdenver.edu/krauslab).

Even though the mutations are detected in all functional domains of the CBS enzyme, the most abundant ones, such as I278T, T191M G307S or R336C, affect the catalytic domain. While the most disease-causing mutations do not target critical residues involved in catalysis, missense mutation can interfere with proper folding of the enzyme into its native form, its stability, conformational flexibility or cause other structural perturbations all leading to a misfolded protein [41, 59]. Protein misfolding results in either gain-of-function phenotype due to toxic aggregation of a misfolded protein (*e.g.* Alzheimer's, Huntington's or Parkinson's diseases) or loss-of-function phenotype due to protein destabilization or folding defect causing non-toxic protein aggregation or accelerated degradation. Accelerated degradation is characteristic for many inherited metabolic disorders, such as phenylketonuria, Gaucher diseases as well as HCU. Thus conformational or protein misfolding disease can be defined as any condition, which is caused by misfolding of the individual proteins complemented by proteostasis including altered aggregation, degradation and trafficking. Indications that CBS deficiency is a conformational disorder were present almost from the moment of

HCU discovery; however, this aspect of CBS deficiency has attracted scientists' curiosity only relatively recently. Without any knowledge about CBS structure and/or function, Drs. Barber and Spaeth in 1967 reported that three homocystinuric patients normalized their biochemical parameters after being given very high daily doses of pyridoxine (250-500 mg) for a period of 2-4 weeks of continuous therapy [60]. A while later, it was found that not all patients responded even up to 1000 mg of vitamin B_6 daily (higher doses are neurotoxic) and thus patients were divided into pyridoxine responders or non-responders [61]. It was clear from the beginning that pyridoxine responsiveness was not due to correction of vitamin B_6 deficiency and later studies suggested a correlation between measurable residual CBS activity (typically 1-9%) and clinical response to pyridoxine [62]. Approximately half of the patients can benefit from vitamin B_6 treatment and there is a strong link between responsiveness to pyridoxine and pathogenic mutation(s) carried by a patient [1]. Mutant CBS enzymes, such as the most frequent one I278T, A114V, R266K or R336H, appear to confer vitamin B_6 clinical responsiveness, while other missense mutants, such the second and third most frequent ones T191M and G307S, R125Q, E176K or T262M, seem to be insensitive to such treatment. 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 [63]. Western blot analysis of patient fibroblast extracts showed normally assembled tetrameric CBS only in WT control fibroblasts and was not detectable for any of the mutants. In patient samples, the CBS antigen, if any, was only detected as a high molecular weight aggregate and correlated with little or no CBS activity. Heterologous expression of selected CBS mutants in *E. coli* followed by Western blot detection and heme determination confirmed their aggregation tendencies, the lack of CBS activity and the absence of heme. 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 [64].

2. MOLECULAR AND CHEMICAL CHAPERONES IN HCU

CBS deficiency due to the presence of a missense mutation can be considered a conformational disorder and as such could benefit from a type of treatment devised for other misfolding diseases. In general, there are three different small molecule treatment strategies currently available to rescue misfolded mutant proteins and to restore their homeostasis [59]. These small molecule therapeutics are often called chaperones as they help the mutated protein to adopt native active conformation and thus share this function with cellular molecular chaperones, the proteins of cellular quality control and repair machinery responsible for proper folding and assembly of expressed proteins [65]. Chemical chaperones representing the first group of therapeutics belong to a diverse group of small, 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 [66, 67]. However, as it will be discussed below in more detail, chemical chaperones sometimes work indirectly *via* induction of expression of molecular chaperones or otherwise enhancing their activity [58, 68]. A second group includes the so-called pharmacological chaperones, which often resemble natural ligands or cofactors of the target protein and thus bind specifically to mutant native states, stabilize their conformation and prevent their early degradation or aggregation [69-71]. Such effects lead to an increase of steady state levels of the mutant active protein. The last third group of small molecule chaperones consists of proteostasis modulators, which regulate the pathways leading to improved folding of mutant proteins, enhanced degradation of misfolded nonfunctional species or amended trafficking [72]. Essentially, proteostasis regulators specifically impact the function and steady-state levels of molecular chaperones and other components of cellular protein quality control apparatus.

2.1. CBS Cofactors and Their Precursors As Pharmacological Chaperones

2.1.1. PLP and its Precursor Pyridoxine

As detailed above, CBS fulfills its role in sulfur amino acid metabolism with the assistance of three cofactors: heme, PLP and AdoMet. Even though only PLP is catalytically active, heme has been shown to be important for proper folding and optimal activity, while AdoMet activates and kinetically stabilizes the enzyme. The idea of supplementation of pyridoxine hydrochloride as a precursor of PLP in inborn errors affecting PLP-dependent enzymes can be traced back to 1963 [73]. However, pyridoxine responsiveness varies greatly among inherited metabolic diseases involving PLP-dependent enzymes [74].

The biochemical and molecular basis for pyridoxine responsiveness in HCU remains unknown, particularly due to the weak correlation among data obtained from *in vitro*, bacterial and eukaryotic systems, animal models of HCU and homocystinuric patients. The most obvious hypothesis to explain pyridoxine responsiveness in HCU is an effect of mutations on PLP binding. 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 [75]. This study concluded that the B_6 non-responsiveness 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. Even though there is a quite consistent correlation between pyridoxine response *in vivo* and at least some measurable residual CBS enzyme activity, some of the largest improvement of CBS activity by supplementation of PLP *in vitro* have been detected with mutants from clinically nonresponsive patients [1]. This discrepancy originates from supra-physiological concentrations of pyridoxine used in *in vitro* studies, which can never be reached *in vivo* [75]. In addition, contrasting behavior of two CBS mutants associated with the pyridoxine response in patients suggests inter-individual differences in the response to vitamin B_6 supplementation [76].

2.1.2. Heme

Human CBS binds, in addition to PLP and AdoMet, the heme cofactor [77]. Although the heme in CBS is not directly involved in catalysis [34], its importance for the proper function and folding of human CBS has been very well documented. The role of heme in CBS folding came from expression studies in heme biosynthesis-deficient strains of *E. coli* and *S. cerevisiae* that demonstrated substantial CBS misfolding and aggregation in the absence of heme or protoporphyrin supplementation [19, 78]. In combination with other studies showing a correlation between the heme content and aggregation propensity of CBS mutants [63], these data suggest that heme incorporation is crucial for proper CBS folding, and support the use of heme, heme precursors or heme analogs to treat HCU.

One interesting possibility is that the pathogenic mutations in CBS may affect heme binding, thus leading to defects in PLP binding. Supplementation with δ-aminolevulinate (500 μM) affects the folding, assembly and activity of CBS mutants heterologously expressed in *E. coli* [79]. About half of the mutants showed improvement in formation of native tetramers, and often promoted the rescue of CBS activity. Mutations responsive to treatment with the heme precursor were located across the whole CBS polypeptide. Interestingly, δ-aminolevulinate had substantially different effect on two mutations in the heme-binding pocket. Whereas tetramerization and activity of the R266K CBS mutant was significantly improved, the H65R mutation failed to show any changes in response to the treatment. To test whether these results may be reproduced in a mammalian proteostasis environment, the catalytic and conformational properties of the same set mutants has been recently revisited after expression in a mammalian cell system [80]. Five pathogenic CBS mutants, which in the bacterial expression system showed residual CBS specific activity of at least 10% of the WT and were responsive to treatment with δ-aminolevulinate [79] together with the I278T and H65R mutations were expressed in CHO-K1 cells in the presence of 77 μM heme arginate in order to directly supply the CBS cofactor [80]. The results essentially verified the findings from the *E. coli* expression system, suggesting that there might be a limited set of CBS mutants, which could benefit from δ-aminolevulinate or heme arginate supplementation.

2.1.3. S-adenosylmethionine

As Figure **1** illustrates, the methyl group of Met becomes activated by ATP with the addition of adenosine to the sulfur of methionine thus forming AdoMet. This stereospecific reaction is catalyzed by methionine adenosyltransferase and generates only the S-diastereoisomer [81]. AdoMet is an important biological sulfonium compound and the second most often used substrate in enzymatic reactions after ATP [82]. A majority of methylation reactions occurring in the cell are catalyzed by methyltransferases, which utilize AdoMet as the methyl donor forming methylated product and S-adenosylhomocysteine (AdoHcy). AdoHcy is subsequently hydrolyzed by AdoHcy hydrolase to generate Hcy. AdoHcy is a potent inhibitor of methylation reactions catalyzed by methyltransferases [83]. In addition, AdoHcy hydrolase catalyze the formation of AdoHcy in the excess of Hcy. Therefore, AdoMet activation of CBS represents a critical mechanism for maintaining the balanced methylation as well as redox potential. This fine balance is disturbed by the lack of CBS activity and leads to an increase formation of Hcy, AdoHcy, inhibition of methylation reactions and normal or increased AdoMet plasma concentrations in HCU patients [84].

Kozich *et al.* have explored the possibility of AdoMet or AdoHcy stimulating the residual CBS activity in a large set of missense mutants using *E. coli* expression system [85]. 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 AdoHcy was observed [85]. The study suggested that AdoHcy hydrolase inhibitors could decrease AdoHcy 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 AdoMet to stimulate residual activity of several mutants. However, reactive properties, intrinsic instability and charged character of AdoMet makes such an approach likely unsuccessful.

2.2. 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 [86, 87]. 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 [66, 67]. 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 [87]. Some of the most widely used chemical chaperones are for example glycerol, dimethylsulfoxide (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 group of Dr. Warren Kruger [88]. Earlier he developed a yeast complementation assay, where yeast lacking endogenous CBS gene (*cys*4) 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 [89, 90]. 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 [88]. 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 [68]. 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 towards treatment of HCU.

A remarkably different effect of chemical chaperones and co-solvents was described by Majtan *et al.* [58]. 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 AdoMet 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 was 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 CBS mutants expressed in *E. coli* representing about 70% of known CBS alleles [79]. 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 [80]. PBA is an FDA-approved drug for treatment of urea cycle disorders; however, many studies reported its positive effect on other diseases as well [91]. 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 endoplasmatic reticulum stress as well as acts as a chemical chaperone [91]. However, PBA only mildly increased the specific activity in a small set of CBS mutants [80]. This result suggests that a generic effect of PBA does not have any noticeable impact on CBS mutants unlike on other misfolded proteins.

2.2. Molecular Chaperones & Proteasome Inhibitors

As reviewed above, 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 [92]. 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 [68]. 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 to 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. In agreement with this notion, 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 [93]. 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 [94]. 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 [95], 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 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 responders *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.

3. RATIONAL APPROACH IN A SEARCH FOR PHARMACOLOGICAL CHAPERONES FOR HCU

Except for the use of high doses of vitamin B_6 in pyridoxine-responsive homocystinurics, 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 H_2S biosynthesis. While the physiological relevance of CBS alternative reactivity leading to an *in vivo* generation of H_2S remains to be answered, a multitude of pathological and physiological effects of H_2S has attracted a lot of attention in recent years and lead to a development of many H_2S probes and CBS activity assays employing alternative substrates suitable for high-throughput screening and a search for CBS-specific activity modulators [96-98]. Second, high-resolution crystal structures of human full-length CBS has recently been solved in both the basal and activated AdoMet-bound conformations [42, 43, 99]. Structural information about the PLP-containing catalytic center as well as the AdoMet-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 towards rational design of small molecules targeting CBS folding, stability or activity.

3.1. CBS Inhibitors and High-Throughput Screening

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-deoxynojirimycin; NB-DNJ) is a substrate reduction therapy for Gaucher disease type 1 patients, who showed anaphylactic reactions to the available enzyme replacement therapies [100]. In addition, miglustat acts as an active site inhibitor and chaperone-like compound preventing misfolding and rapid degradation [101]. 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 [102]. 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 (i) 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, (ii) high cellular permeability and subcellular distribution, which is particularly relevant for post-translationally modified enzymes, such as those responsible for lysosomal storage disorders, and (iii) smooth dissociation of the inhibitor from the enzyme's catalytic center, so it can be replaced by a natural substrate [102]. Screening for identification of such 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 IC_{50} values for best hits. Second, cell-based chaperone enhancement assay is used for evaluation of hits (typically with IC_{50} 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) [103]. 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 [80]. 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 [97]. 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 [96]. 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 a close analog of AdoMet, S-adenosylethionine, on Hcy levels and H_2S production in mice [104], thus one could assume that the structures of the identified CBS activators were closely related to AdoMet.

The growing interest in H_2S 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_2S detection [98]. An HTS-capable assay using 7-azido-4-methylcoumarine as a novel H_2S probe producing robust fluorescent signal has been recently developed [105] 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 [106]. 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.

3.2. Towards Structure-Guided 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 the 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.

3.2.1. CBS Catalytic Center and its Inhibition

The currently available structural information on the human CBS makes it feasible for one to embark on a path towards CBS drug discovery and development *via* rational design. Although the crystal structure of the human CBS catalytic core has been solved more than a decade ago [21, 22], 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 the 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), in aminoacrylate intermediate (PDB ID 3PC3) and in complex with serine (PDB ID 3PC4) [35]. 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. While the conformational flexibility in this loop was later found relevant for accessibility of the catalytic center in human enzyme as well, other three loops, namely L171-174, L191-202 and L296-316 (Fig. **5**), have been found crucial for the formation of an entrance to the catalytic cavity in the full-length human CBS (PDB IDs 4L0D, 4L3V and 4COO for the wild-type CBS and 4L27 and 4L28 for the pathogenic D444N mutant enzyme) [43, 99]. These loops were found collapsed only in the case of the substrate present in the catalytic cavity. 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 auto-inhibitory function. Particularly the loop L191-202 was found compressed into the catalytic cavity and rigid by the presence of the regulatory domain from the complementary subunit [43]. The point mutation E201S disrupts the blocking effect of this interaction between the regulatory and the catalytic domain and activates the enzyme. The crystal structure of the activated E201S CBS mutant bound to AdoMet (PDB ID 4PCU) suggested that the activation of the enzyme is caused by a significant rearrangement of the regu-

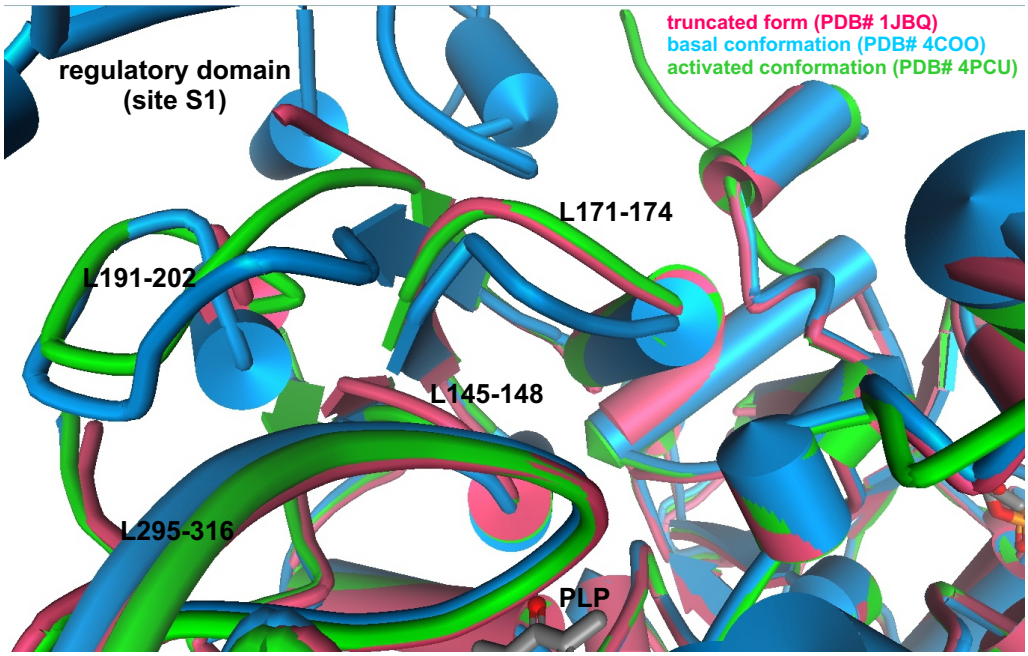

Fig. 5. Entrance to the CBS catalytic cavity. The PLP-containing catalytic center of CBS is delineated by several loops whose conformation was found affected by their interaction with the regulatory domain as well as by the absence or presence of a substrate in the catalytic cavity. Superimposition of the truncated CBS lacking the regulatory domain (pink; PDB ID 1JBQ), the basal conformation (blue; PDB ID 4COO) and the AdoMet-bound activated conformation of the full-length enzyme (green; PDB ID 4PCU) shows the variable conformation of loops forming the entrance to the catalytic center: L145-148, L171-174, L191-202 and L295-316. Particularly, the loops L171-174 and L191-202 are compressed towards the catalytic core by the presence of a regulatory domain in the basal conformation of CBS thus limiting the flow of substrate(s) and product(s) in and out. At the same time, structural elements from the catalytic domain block the S1 AdoMet-binding site in the basal conformation. However, the site S1 was found empty in the activated conformation, AdoMet was found only in the S2 site. (*For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.*)

latory domain accompanied by the release of a compression and concomitant increase in a flexibility of the loops delineating entrance to the catalytic cavity, such as L191-202, thus allowing for unrestricted flow of substrate(s) and product(s) [42]. Taken together, available structural information allowed us (i) to understand the formation and binding of CBS reaction intermediates, (ii) to identify important residues and overall structure of the catalytic cavity and (iii) to recognize the importance of conformational flexibility of the loops defining the entrance to the catalytic site.

As described above, currently there is no specific inhibitor for CBS. It is our belief that increased interest in H_2S 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. Such ligand would presumably stabilize the region enough to allow a CBS missense mutant to refold and/or to fold into a native-like conformation.

3.2.2. Role of CBS Allosteric Site in Enzyme Activation and Stabilization

In addition to the catalytic center as binding site for substrates and possible inhibitors, crystal structures of the full-length CBS enzymes unveiled the conformation of the regulatory domain and its interaction with the catalytic core. Although *Drosophila* CBS does not bind AdoMet and thus its activity is not regulated by it [16, 28], its structure revealed potential binding sites for the CBS allosteric ligand [35]. The CBS domains of the two subunits in a dimer arranged in a head-to-tail fashion form a disk-like CBS module showing up to 4 potential canonical binding sites for AdoMet. Lower sequence homology between the AdoMet-independent insect CBS and AdoMet-regulated human enzyme and the proximity of the locus corresponding to the D444N pathogenic mutation impairing AdoMet binding and partially activating human CBS to site A suggested that only the site A can bind AdoMet in human CBS [35]. Indeed, we found that site B (corresponding to site S1 in human CBS) is spatially blocked by structural elements from the catalytic core of a complementary monomer and occupied by bulky hydrophobic residues, which all preclude the binding of AdoMet to this site in a basal conformation [43]. On the other hand, site S2 (corresponding to site A in insect CBS) was found solvent exposed and suitable to accommodate AdoMet (Fig. **6**). Crystal structure of the E201S CBS mutant with bound AdoMet confirmed such prediction. Insight into AdoMet binding into S2 site was further improved by higher resolution 3D crystal structure of CBS regulatory domain with bound AdoMet (PDB ID 4UUU) [99].

The potential 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 AdoMet has been known for a long time [107] and its molecular mechanism has been recently uncovered [42, 99], the role of the regulatory domain in kinetic stabilization of the enzyme has been just recently discovered [41]. Differential scanning calorimetry analyses of WT and several pathogenic CBS mutants have shown that

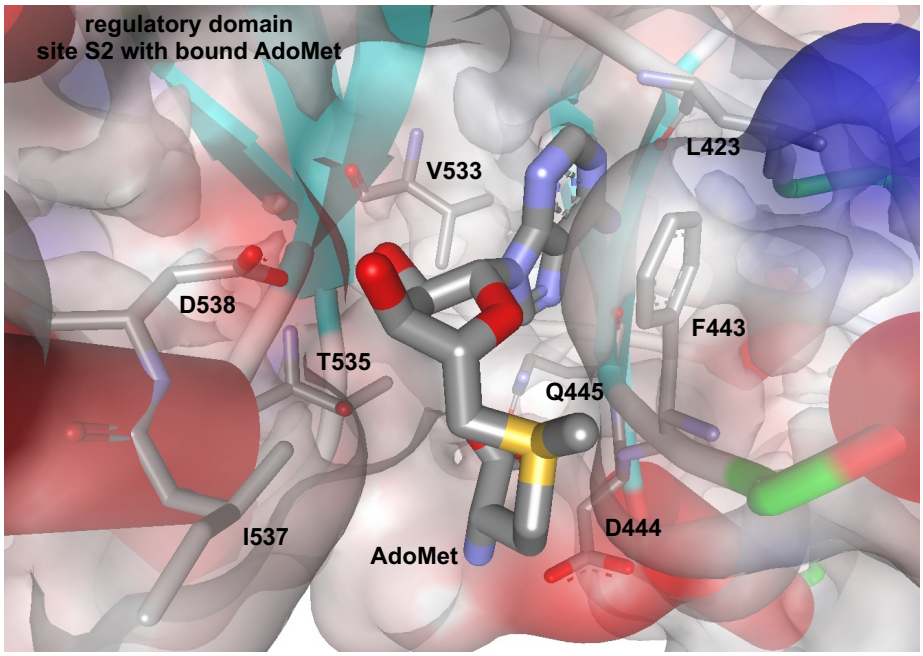

Fig. 6. Structural insight into AdoMet binding site in CBS. In the crystal structures of AdoMet-bound full-length CBS (PDB ID 4PCU) or just the regulatory domain itself (PDB ID 4UUU; shown), a ligand was found only in the proposed site S2 in the CBS regulatory domain. Here, AdoMet (thick sticks) is accommodated in a hydrophobic pocket *via* a number of key residues (thin sticks). Residues F443, L423 and V533 stabilize the adenine ring *via* stacking hydrophobic interactions deeper in the binding pocket. Residue D538 forms hydrogen bonds with the hydroxyl groups of the ribose moiety. The residue I537 stabilizes the alkyl chain of the methionine, while the residues T535, D444 and Q445 interact with carboxyl and amino group of the methionine through hydrogen bonds. In addition, negatively charged residue D444 compensates positive charge of sulfonium of AdoMet ligand.

denaturation of the regulatory and the catalytic domains are independent and kinetically controlled processes. Therefore, their stabilities must be considered from their *in vitro* denaturation half-lives 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 underline the importance of stabilization of the regulatory domain in CBS-deficient homocystinuria [41].

More importantly, current knowledge implies that specific ligands targeting CBS allosteric sites could be found or designed in order to independently modulate CBS activity and kinetic stability. Ligand-induced kinetic stabilization of the regulatory domain 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 [41]. We hypothesize that ligand-induced stabilization of its regulatory domain would remedy its abnormal susceptibility towards denaturation thus rescuing the CBS activity *in vivo*. On the other hand, ligand-induced activation similar to a natural CBS ligand AdoMet would increase the residual activity or ameliorate the impaired regulation in certain mutants. The majority of CBS pathogenic mutations do not prevent AdoMet binding but rather interfere with the molecular mechanism of the regulatory domain rearrangement and formation of the CBS module [41, 42]. As an example, binding affinity of AdoMet to D444N CBS mutant is significantly lower, thus increasing the K_{act} for AdoMet ~100 times [108] and, at the same time, partially increasing the enzyme's activity 2-fold [43]. Furthermore, the D444 residue was found to be an important residue involved in AdoMet binding and its accommodation within the allosteric binding site [42, 99]. We hypothesize that there may be identified and/or designed a ligand with higher affinity for the D444N mutant than AdoMet thus rescuing the physiological regulation and activation of the enzyme [57].

OUTLOOK

Misfolding due to the presence of missense mutations represents an increasingly better understood pathogenic mechanism in HCU. CBS mutants 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 the HCU patients, novel treatments need to be devised to address an unmet need of the remaining affected individuals. Studies using various CBS cofactors or their analogs, chemical chaperones or proteostasis regulators suggest that development of a small molecule treatment for HCU is 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 to 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 AdoMet 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.

LIST OF ABBREVIATIONS

AdoHcy	=	S-adenosylhomocysteine
AdoMet	=	S-adenosylmethionine
AOAA	=	Aminooxyacetic acid
CBS	=	Cystathionine beta-synthase
CGL	=	Cystathionine gamma-lyase
Cth	=	Cystathionine
Cys	=	Cysteine
DMSO	=	Dimethylsulfoxide
HA	=	Hydroxylamine
HCU	=	Classical homocystinuria
Hcy	=	Homocysteine
HTS	=	High-throughput screening
Met	=	Methionine
PBA	=	4-phenylbutyric acid
PLP	=	Pyridoxal-5'-phosphate
Ser	=	Serine
TMAO	=	Trimethylamine-N-oxide

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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