Small aminothiol compounds improve the function of Arg to Cys variant proteins: effect on the human cystathionine β-synthase p.R336C

Marisa I.S. Mendes¹,³, Desirée E.C. Smith³, João B. Vicente¹,², Isabel Tavares De Almeida¹,², Tawfeg Ben-Omran⁴, Gajja S. Salomons³, Isabel A. Rivera¹,², Paula Leandro¹,²,† and Henk J. Blom³,†,‡,*

¹Metabolism and Genetics Group, Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, ²Department of Biochemistry and Human Biology, Faculty of Pharmacy, University of Lisbon, Lisbon 1649-003, Portugal, ³Metabolic Unit, Department of Clinical Chemistry, VU University Medical Centre, Amsterdam 1081 HV, The Netherlands and ⁴Section of Clinical and Metabolic Genetics, Department of Pediatric, Hamad Medical Corporation, Doha, Qatar

*To whom correspondence should be addressed at: Laboratory of Clinical Biochemistry and Metabolism, Department of General Pediatrics, Adolescent Medicine and Neonatology, University Medical Centre Freiburg, Freiburg, Germany. Tel: +49 761 270 43710; Fax: +49 761 270 45270; Email: henk.blom@uniklinik-freiburg.de

Abstract

The key regulatory point of L-methionine (Met) and L-homocysteine (Hcy) degradation is catalyzed by cystathionine beta-synthase (CBS). CBS deficiency is caused by mutations in CBS gene, often resulting in protein misfolding. The prevalence of CBS deficiency in Qatar is 1/1800, ~200-fold higher than the worldwide prevalence of 1/344 000. Almost all patients bear the CBS p.R336C variant. More than 20 years ago, it was shown in vitro that two unrelated protein variants with a substitution of an arginine (Arg) residue by cysteine (Cys) could be rescued by cysteamine (mercaptoethylamine), likely via formation of a disulfide between Cys and cysteamine, functionally mimicking the wild-type (WT) Arg side-chain. Based on these findings, we aimed to study whether cysteamine was able to improve the function of p.R336C CBS variant. Additionally, we tested the effect of mercaptoethylguanidine (MEG), a compound with a guanidino and a thiol function that may resemble Arg structure better than cysteamine. Three purified recombinant CBS proteins (p.R336C, p.R336H and WT) were pre-incubated with cysteamine and MEG or Cys (as negative control), and CBS activity and stability were measured. Pre-incubation with cysteamine and MEG increased the enzymatic activity of the p.R336C protein, which was absent upon pre-incubation with Cys. The WT and the p.R336H variant enzyme activity presented no increase with any of the tested compounds. Our results show that cysteamine and MEG are able to specifically improve the function of the CBS p.R336C variant, suggesting that any Arg-to-Cys substitution accessible to these small molecules may be converted back to a moiety resembling Arg.

†These authors should be regarded as joint last Authors.
‡This author is currently working at the Laboratory of Clinical Biochemistry and Metabolism, Department of General Pediatrics, Adolescent Medicine and Neonatology, University Medical Centre Freiburg, Freiburg, Germany.

Received: August 14, 2015. Revised and Accepted: October 7, 2015

© The Author 2015. Published by Oxford University Press. All rights reserved. For Permissions, please email: journals.permissions@oup.com
Introduction

Classical Homocystinuria (OMIM#236200), the most frequent inborn error of the sulfur amino acid metabolism (1), is caused by autosomal recessive mutations in the cystathionine beta-synthase (CBS)-encoding gene. CBS deficient patients present with intellectual disability, connective tissue problems and vascular symptoms (2). About half of the patients respond to high dose of B6 (the precursor of the pyridoxal 5′-phosphate cofactor, PLP), and B6-responsiveness is usually associated with milder symptoms (2,3). The average incidence of CBS deficiency is calculated to be ∼1/344,000 worldwide, but it varies dramatically from 1/1,000,000 in Japan (4) to 1/1800 in Qatar (5) and even to 1/240 in an isolated Austronesian population of the Orchid Island (south of Taiwan) (6).

The CBS protein (EC 4.2.1.22) is a cytoplasmatic homotetrameric enzyme with 63 kDa monomers (551 amino acid residues) (7). Each monomer is organized in three domains: an N-terminal homodimeric enzyme with 63 kDa monomers (551 amino acid residues) (6).

According to the CBS database as of September 2015 (http://cbs.lf1.cuni.cz/mutations.php), the CBS mutation c.1006C>T (p. R336C), in exon 9, is one of the most common mutations (fourth most common; 83 of 924 analyzed alleles). This mutation was first identified in a patient of English descent (10), and as then it was observed in patients from Australia (11), Iberian Peninsula (12), Korea (13), and Qatar. Indeed, CBS deficiency shows a very high incidence (1/1800) in Qatar (5), mainly due to the c.1006C>T CBS mutation, probably due to a founder effect in combination with a high incidence of consanguineous marriage in this country (14). This mutation occurs at a CpG dinucleotide known to be hypermutable and it is likely that this mutation has reoccurred independently in different populations (14). This mutation causes a severe B6 non-responsive phenotype and, if untreated, homozgyous patients develop severe mental retardation as the most prominent feature, in addition to other complications. Current treatment options include the use of betaine and a life-long methionine-restricted diet. However, compliance of the diet is rather difficult, in particular during puberty and adulthood.

In 1985, it was hypothesized that variant proteins with the replacement of an arginine (Arg) by a cysteine (Cys) may be amenable to treatment with cysteamine (mercaptoethyliamine) (15). This hypothesis was based on the observations that either in vitro in plasma of a type III hyperlipoproteinemic patient carrying an Arg-to-Cys substitution in apolipoprotein E (ApoE) or in vitro in two cystinotic patients treated with cysteamine, this thiol caused a charge shift in the ApoE isoelectric focusing pattern. Substitution of the wild-type (WT) Arg1689 for Cys in Factor VIII light chain (FVIII-EH) prevents thrombin cleavage at that site, thereby blocking FVIII-EH cofactor function (16,17). The incubation of the variant FVIII-EH with cysteamine resulted in an ∼14-fold increase in FVIII-EH activity, which was enough to restore light chain cleavage by thrombin (18). These studies triggered our interest to explore whether the CBS p.R336C variant could be repaired by cysteamine. As the mixed disulfide formed between the variant Cys residue and cysteamine resembles the Lys side-chain more than that of Arg, we searched for alternative aminothiol compounds that would result in a complex resembling more closely the Arg guanidine moiety. We selected the commercially available mercaptoethylguanidine (MEG), which contains a guanidine group in addition to the thiol function (Fig. 1). This hypothesis was based on the observations that either a charge shift in the ApoE isoelectric focusing pattern.

In this paper, we produced in a prokaryotic expression system the p.R336C variant and, as controls, the WT CBS and the disease-
causing p.R336H variant. To study whether cysteamine or MEG could represent an alternative treatment strategy for patients with CBS deficiency caused by p.R336C, we investigated the effects of cysteamine and MEG on the activity and stability of purified CBS proteins.

Results

Characterization of the CBS p.R336C variant

To characterize the expression levels of the CBS proteins and the relative aggregation propensity, the soluble and insoluble fraction of the bacterial lysates were subjected to western blot analysis. The WT protein was present predominantly in the soluble fraction (Fig. 2A and B). The total expression level of the p.R336C protein was ~60% of the WT, equally divided between the soluble and insoluble fractions.

After purification, aliquots of the WT and p.R336C proteins were subjected to electrophoresis in order to determine the protein purity (Fig. 2C, Supplementary Material, Fig. S1 for complete gel picture). For both proteins, the main band corresponded to the CBS monomer (63 kDa).

To study the oligomeric profile, both purified proteins were loaded into a native gel (Fig. 2D). They both presented predominantly the band corresponding to the CBS tetramer (~245 kDa).

Effect of cysteamine and MEG on the enzymatic properties of the WT and variant p.R336C and p.R336H CBS

The enzymatic activities of the WT and variant p.R336C and p.R336H proteins were determined after different incubation periods (1, 4 and 8 h) at 37°C with different concentrations of cysteamine and MEG. Control assays, performed in the absence of compounds, showed that at time zero the p.R336C variant presented a specific activity of 0.4% of WT. As expected, incubation of the WT enzyme at 37°C for 8 h did not lead to enzyme inactivation (19), whereas both p.R336C and p.R336H variants exhibited a strong decrease of enzyme activity. In the presence of 250 or 1000 µmol/l cysteamine, a stabilization of the specific activity of p.R336C was observed (Fig. 3A). Regarding MEG, incubation with 25 µmol/l sufficed to stabilize the specific activity of p.R336C, whereas incubations with 250 and 1000 µmol/l led to an increase in the specific activity (Fig. 4A). In the presence of Cys, the aminothiol compound used as negative control, there was no effect on the enzymatic activity of the p.R336C variant protein (Fig. 3A). For the p.R336H variant, addition of cysteamine and MEG had no protective effect regarding the specific activity (Figs 3C and 4C), because a faster enzyme inactivation was observed in all conditions. The enzyme activity of the WT CBS did not show significant changes in the presence of the tested aminothiols (Figs 3B and 4B), suggesting that these compounds had negligible effects on the WT enzyme activity.

The effect of the two aminothiol compounds was directly proportional to the duration of incubation, with longer incubations resulting in a larger stabilization in residual activity, compared with incubation without added compound. Regarding the kinetic studies, an increase in the catalytic efficiency for both substrates was observed for the variant p.R336C after incubation with MEG, mainly due to the higher V_max obtained. In the presence of MEG, the p.R336C also showed an increased affinity for Ser. Concerning the WT protein, the presence of MEG did not affect the kinetic parameters for either substrate (Table 1).

Figure 2. Characterization of expression levels, purity grade and oligomeric assembly of recombinant WT and p.R336C variant CBS proteins. (A) Western blot analysis of soluble and insoluble fractions of the E. coli lysate. After centrifugation, soluble and insoluble fractions were obtained and 60 µg of total protein was loaded. (B) Percentage of CBS proteins present in the soluble and insoluble fractions of E. coli lysates as monitored by western blot analysis. (C) SDS-PAGE analysis of the purified CBS proteins on a 12% stain-free gel (Bio-Rad Laboratories); the gel was visualized directly after 5 min activation with UV light; the 63 kDa full-length CBS is indicated by an arrow. Supplementary Material, Figure S1 depicts complete picture of the gel. (D) Oligomeric profile of the purified proteins; 7 µg of protein was loaded in a native 3-8% gel; after electrophoresis the gel was stained with Coomassie brilliant blue. M: NativeMark Unstained Protein Standard (Life Technologies).
Owing to the fact that Cys431 is a relevant residue for the activation of CBS by SAM, we checked if pre-incubation with MEG affected SAM activation. Using the assay conditions described previously (20), no changes in the activation profile were found (results not shown).

Thermal inactivation profile

The p.R336C and the WT proteins were incubated with 1000 µmol/l cysteamine or 1000 µmol/l MEG for 4 h at different temperatures (ranging from 4 to 55°C) to study the effect of these compounds on thermal stability. The activity measured after 4 h incubation at 4°C was considered 100%. As shown in Figure 5, both proteins were inactive after prolonged incubation at 55°C. For the WT CBS, the activity curves obtained in the presence of cysteamine or MEG overlapped with the curve obtained in the absence of these compounds, suggesting that neither thiol compound affected thermal stability of the WT (Fig. 5). Conversely, in the presence of cysteamine, the p.R336C variant showed a clear increase in thermal stability. This suggests that, in the presence of cysteamine, the variant protein was more resistant to increases in temperature, especially at the higher temperatures tested (37 and 42°C). Using MEG, we observed an increase in the relative activity at temperatures 22°C (1.6-fold) and 37°C (1.8-fold).

Effect of cysteamine and MEG on the WT and p.R336C proteolysis profiles

The WT and p.R336C variant CBS proteins were incubated for 1 h at 25°C in the presence and absence of cysteamine and MEG, after which trypsin was added. Aliquots were collected at different time points and analyzed by SDS-PAGE. The profiles in the absence of the thiol compounds showed a faster proteolysis of the p.R336C variant ($k_p = 0.36 \pm 0.07 \text{ min}^{-1}$) with respect to the WT CBS ($0.16 \pm 0.02 \text{ min}^{-1}$), which might indicate a change in the native folding and/or conformational flexibility of the enzyme caused by the amino acid change (Table 2). A change in the $k_p$ of the p.R336C to values similar to the WT was obtained only in the presence of MEG ($k_p = 0.17 \pm 0.04 \text{ min}^{-1}$), neatly fitting with the increase in the residual activity observed (Supplementary Material, Fig. S2 depicts two representative gels).

Differential scanning fluorimetry

The conformational stability of the recombinant WT and p.R336C proteins was evaluated by differential scanning fluorimetry (DSF). The extrapolated melting temperatures for the WT and p.R336C protein are within the range of 42–45°C for the regulatory domain ($T_{m1}$) and 57°C for the catalytic domain ($T_{m2}$) (Table 2). The p.R336C variant exhibited very similar $T_m$ values to the WT protein, which appeared unaffected by either tested aminothiol.

Discussion

In this paper, we explored the potential repairing effect of two aminothiols, cysteamine and MEG, on the function of a clinically relevant CBS variant carrying an arginine to cysteine substitution at position 336 of the catalytic domain (p.R336C). Although similar studies were carried out for cysteamine in the 1980’s and
1990’s using unrelated proteins, with successful results, to our knowledge, this is the first study of MEG with such purpose. Our data show that both aminothiol compounds were able to partially improve the function of the variant CBS p.R336C. Although the functional improvement remains below the WT activity levels, an increase of residual enzyme activity to a few percent of the normal activity may already be sufficient in the treatment of inborn errors of metabolism.

The CBS p.R336C variant was previously studied in in vitro prokaryotic expression systems, where it showed a residual enzymatic activity of ∼6% of the WT (13) or a complete absence of activity (21), whereas the expression level was only slightly decreased when compared with the WT protein (10). In our model, the p.R336C protein presented an expression level of ∼60% of the WT, although half was recovered in the insoluble fraction. This variant protein showed a dramatically decreased specific enzymatic activity of 0.4% of the WT after 1 h incubation at 37°C, consistent with the severe phenotype of the respective homozygous patients. The data collected from the proteolysis assay suggested that the variant protein presents a slightly altered conformation and/or flexibility, as it showed to be more prone to cleavage by trypsin. Interestingly, when analyzed by DSF, the variant protein showed no changes in the Tm for the catalytic and the regulatory domains, suggesting an overall decreased conformation at these temperatures.

### Table 1. Steady state kinetic parameters of CBS WT and p.R336C variant for Ser and Hcy in the absence (−) and presence (+) of 250 µmol/l MEG

<table>
<thead>
<tr>
<th></th>
<th>p.R336C (−MEG)</th>
<th>p.R336C (+MEG)</th>
<th>WT (−MEG)</th>
<th>WT (+MEG)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ser Km (mM)</td>
<td>0.89 ± 0.3</td>
<td>0.62 ± 0.2</td>
<td>2.4 ± 0.1</td>
<td>2.7 ± 0.1</td>
</tr>
<tr>
<td>Vmax (µmol Cth h⁻¹ mg⁻¹)</td>
<td>0.23 ± 0.01</td>
<td>0.33 ± 0.01</td>
<td>90.6 ± 1.7</td>
<td>90.0 ± 1.4</td>
</tr>
<tr>
<td>Kcat/Km (mm⁻¹ min⁻¹)</td>
<td>0.27</td>
<td>0.56</td>
<td>39.6</td>
<td>35.0</td>
</tr>
<tr>
<td>Hcy Km (mM)</td>
<td>0.057 ± 0.02</td>
<td>0.072 ± 0.02</td>
<td>0.22 ± 0.04</td>
<td>0.22 ± 0.04</td>
</tr>
<tr>
<td>Vmax (µmol Cth h⁻¹ mg⁻¹)</td>
<td>0.10 ± 0.007</td>
<td>0.17 ± 0.01</td>
<td>54.5 ± 2.2</td>
<td>52.8 ± 2.4</td>
</tr>
<tr>
<td>Kcat/Km (mm⁻¹ min⁻¹)</td>
<td>1.84</td>
<td>2.48</td>
<td>260</td>
<td>252</td>
</tr>
</tbody>
</table>

The catalytic activity was measured at 37°C in the presence of 2.7 mmol/l Hcy and variable concentrations of Ser (0.32–15 mmol/l) or 5 mmol/l Ser and variable concentrations of Hcy (0.005–9 mmol/l). Kcat/Km represents the catalytic efficiency, which was calculated on the basis of the CBS monomer molecular mass (63 kDa).

To evaluate the effect of MEG in the catalytic efficiency, the proteins were pre-incubated prior to determination of enzymatic activity in the absence (−) and presence (+) of 250 µmol/l MEG. Values represent the mean ± SD of three independent experiments. For more details, see ‘Materials and Methods’ section.

Figure 4. Effect of MEG on the specific activity of purified proteins p.R336C (A), WT (B) and p.R336H (C) after incubation with no compound (open squares), 25 µmol/l (open circles), 250 µmol/l (open triangles) or 1000 µmol/l MEG (filled circles) for 1, 4 or 8 h, at 37°C.
equivalent resistance to thermal denaturation at the tertiary structure level.

Human CBS has 11 Cys residues that are highly conserved in mammals. To study whether the observed effects of incubation with cysteamine and MEG were specific to p.R336C, and also if incubation with these compounds would not result in unwanted interactions with other Cys residues in CBS, we included in our studies the WT and the p.R336H variant. The tested compounds only slightly reduced CBS WT activity, indicating that the aminothiol compounds appear to be harmless for the normal function of CBS in the studied concentration range. Also, the activity of the CBS p.R336H variant remained unaffected by incubation with the aminothiols. Cysteamine and MEG also do not seem to affect the thermal stability or conformation/flexibility of the WT, because no differences were observed in the thermal inactivation or proteolysis assays.

To explore the mechanism by which cysteamine and MEG were able to selectively influence the activity of the p.R336C variant, we employed DSF to test whether the increase in activity was due to a stabilization of the variant protein. Surprisingly, both aminothiol compounds did not alter the p.R336C protein conformational stability.

As MEG increased the activity of the CBS p.R336C variant the most, we decided to study in more detail its effect on catalytic efficiency. The results revealed that MEG was indeed able to influence the catalytic efficiency of the p.R336C protein. For Ser, the catalytic efficiency increased by $\sim 2$-fold. Regarding Hcy, a more modest increase in the catalytic efficiency was observed.

Taken together, our data suggest that the two aminothiol compounds are able to specifically and partially repair the activity of the CBS p.R336C variant, whereas modestly affecting the conformational stability without altering its thermal stability. The lack of effect observed for the WT suggests that both compounds have no deleterious effects on the activity of the protein. Interestingly enough, when the accessible surface area (ASA) is

Figure 5. Thermal inactivation profile of WT (A) and p.R336C variant (B) CBS proteins in the absence and in the presence of aminothiol compounds. Data represent the mean of three independent experiments. The proteins (0.165 µg for the WT or 2.5 µg for the variant) were incubated with 1000 µmol/l cysteamine (circles) or 1000 µmol/l MEG (triangles) at different temperatures for 4 h, cooled on ice for 5 min, and the enzymatic activity was measured as described in the text. The activity measured after 4 h incubation at 4°C was considered 100%. Data obtained in the absence of compound are represented for the WT and the p.R336C (diamonds).

Table 2. Effect of the aminothiol compounds cysteamine (1 mmol/l) and MEG (1 mmol/l) on the proteolysis rate ($k_p$) and thermal stability ($T_m$) of the CBS WT and p.R336C variant

<table>
<thead>
<tr>
<th></th>
<th>Limited proteolysis</th>
<th>DSF</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_p$ (min$^{-1}$)</td>
<td>$T_m1$ (°C)</td>
<td>$T_m2$ (°C)</td>
<td>$k_p$ (min$^{-1}$)</td>
<td>$T_m1$ (°C)</td>
<td>$T_m2$ (°C)</td>
<td>$k_p$ (min$^{-1}$)</td>
</tr>
<tr>
<td>No compound</td>
<td>0.16 ± 0.02</td>
<td>42.16 ± 0.39</td>
<td>43.97 ± 0.22</td>
<td>0.32 ± 0.09</td>
<td>43.97 ± 0.22</td>
<td>40.63 ± 0.15</td>
<td>0.17 ± 0.04</td>
</tr>
<tr>
<td>Cysteamine</td>
<td>0.32 ± 0.09</td>
<td>44.43 ± 0.21</td>
<td>43.97 ± 0.11</td>
<td>0.11 ± 0.002</td>
<td>43.97 ± 0.11</td>
<td>43.56 ± 0.18</td>
<td>0.34 ± 0.02</td>
</tr>
<tr>
<td>MEG</td>
<td>0.17 ± 0.04</td>
<td>44.43 ± 0.21</td>
<td>43.97 ± 0.11</td>
<td>0.11 ± 0.002</td>
<td>43.97 ± 0.11</td>
<td>43.56 ± 0.18</td>
<td>0.34 ± 0.02</td>
</tr>
<tr>
<td>No compound</td>
<td>0.16 ± 0.02</td>
<td>42.16 ± 0.39</td>
<td>43.97 ± 0.22</td>
<td>0.32 ± 0.09</td>
<td>43.97 ± 0.22</td>
<td>40.63 ± 0.15</td>
<td>0.17 ± 0.04</td>
</tr>
<tr>
<td>Cysteamine</td>
<td>0.32 ± 0.09</td>
<td>44.43 ± 0.21</td>
<td>43.97 ± 0.11</td>
<td>0.11 ± 0.002</td>
<td>43.97 ± 0.11</td>
<td>43.56 ± 0.18</td>
<td>0.34 ± 0.02</td>
</tr>
<tr>
<td>MEG</td>
<td>0.17 ± 0.04</td>
<td>44.43 ± 0.21</td>
<td>43.97 ± 0.11</td>
<td>0.11 ± 0.002</td>
<td>43.97 ± 0.11</td>
<td>43.56 ± 0.18</td>
<td>0.34 ± 0.02</td>
</tr>
</tbody>
</table>

*a$k_p$ was determined using non-linear fitting of data to a single-exponential equation.

*b$T_m1$ and $T_m2$ refer to the melting temperature of the regulatory and catalytic domain, respectively and were determined by DSF. See text for details. Proteins were pre-incubated with the tested compounds for 1 h at 25°C.
determined for the 11 Cys residues found in the WT, only Cys52 presents 32% of solvent exposure (Cupsat; http://cupsat.tu-bs.de/). The remaining Cys residues present mean ASA values of buried residues (∼3%; 0–10% range), suggesting that low accessibility to the aminothiol compounds is probably governing the absence of a drastic negative impact over the WT CBS catalytic activity.

The Arg336 residue is located in helix α12 of the catalytic domain and is mildly exposed to solvents (ASA value of 29%). In the 3D structure of the CBS dimer (PBD ID 4L0D), Arg336 lies at the periphery of the interface between the two monomers and its guanidinium group takes part of a complex network of interactions with residues located in the connecting linker, namely a salt-bridge with Asp388, a H-bond with Phe385 and van der Waals contacts with Met391 (22). These interactions will probably be disrupted when Arg is replaced by Cys (Fig. 6). Taken together, the data support the postulate that these aminothiol compounds are able to form disulfides with the variant Cys residue. MEG may show a better effect when compared with cysteamine, because a postulated Cys-MEG residue mimics better the structure of the WT Arg. The resulting disulfide may enable the formation of the lost hydrogen bonds, thus resulting in a better function of CBS.

Cysteamine bitartrate (Cystagon) is currently approved for the treatment of cystinosis and significantly improves patients’ quality of life (23,24). Cystinosis is characterized by accumulation of cystine in lysosomes due to mutations in the CTNS gene encoding cystinosin, a lysosomal membrane protein transporting cystine (25). The mechanism of action of cysteamine involves a thiol-disulfide exchange reaction with cystine, producing a cysteamine–cysteine mixed disulfide. This disulfide leaves the lysosome via the lysine transport system (26), resulting in depletion of lysosomal cystine (27). Although cysteamine presents a wide variety of toxic effects when given at very high doses (28), the administration of 60 mg/kg/day to patients with cystinosis appears to be well tolerated over long periods of time (15). This dose results in plasma concentrations of cysteamine between 50 and 100 µmol/l (18).

MEG is a potent scavenger of peroxynitrite and inhibits multiple peroxynitrite-induced oxidative processes (29,30). This compound showed a dramatic protective effect in many experimental models of inflammation, including ischemia/reperfusion injury (29), periodontitis (31), hemorrhagic, endotoxic and septic shock (32), and inflammatory bowel disease (33), although there is an absence of reports on its administration to humans. Intraperitoneal administration to rats was tested for different purposes, including the treatment of caustical esophageal injury (34) and reduction of doxorubicin and trastuzumab cardiotoxicity (35). In both cases, 10 mg/kg were administered in one or two daily doses. Both papers report no negative effects directly related with the administration of MEG.

The present study demonstrates that Arg to Cys substitutions can be partially repaired by cysteamine or MEG, likely via formation of mixed disulfides with the variant Cys residue. Our results are in line with reports of more than 20 years ago, describing the repair by cysteamine of Arg to Cys substitutions in other proteins. The next challenge will be to explore in cultured cells and animal models if these compounds will be able to form the same intracellular mixed disulfides and restore enzyme activity to levels allowing reversion of disease phenotype.

Material and Methods

Production of WT and mutant CBS expression constructs

To produce the recombinant WT and variant CBS proteins, the pET28b expression vector (Clontech, Mountain View, CA, USA) was used as described previously (36).

The c.1007G>A (p.R336H) mutation was introduced by site-directed mutagenesis using the XL Quick Change Kit (Stratagene,
were analyzed by SDS–PAGE and protein concentrations were determined using the BCA method (37). Protein concentration was evaluated by densitometric scanning, followed by analysis using the Image Lab software version 4.1 (Bio-Rad Laboratories). Protein purity was assessed after 5 min activation with UV light. Protein purity was assessed by densitometric scanning, followed by analysis using the Image Lab software version 4.1 (Bio-Rad Laboratories). Protein concentrations were determined using the BCA method (37).

**Expression and purification of recombinant human WT and variant CBS proteins**

Unless otherwise stated, all experiments were performed in triplicate. The WT and variant forms of CBS were expressed as fusion proteins in Escherichia coli (BL21 DE3) using the hexa-histidyl (6xHis) peptide as the purification tag, as described earlier (36). Protein purification was performed by immobilized metal affinity chromatography according to Mendes et al. (20). Purified proteins were analyzed by SDS–PAGE 12% criterion TGX stain-free gel (Bio-Rad Laboratories, Hercules, CA, USA). Proteins were visualized after 5 min activation with UV light. Protein purity was assessed by densitometric scanning, followed by analysis using the Image Lab software version 4.1 (Bio-Rad Laboratories). Protein concentrations were determined using the BCA method (37).

**Western blotting of the soluble and insoluble fractions of the E. coli lysates**

To monitor the expression level of recombinant CBS proteins, after cell lysis and centrifugation at 10 000 g for 30 min, an aliquot of the supernatant (soluble fraction) and pellet (insoluble fraction) was subjected to SDS–PAGE in 12% criterion TGX stain-free gel (Bio-Rad laboratories) as described earlier (36). Stain-free gels allow the quantification of the total protein transferred to the membrane without the use of any antibodies, and this was used as a loading control. After electrophoresis, the proteins were transferred to a polyvinylidene fluoride membrane using the iBlot Gel Transfer Device (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions, and visualized by immunodetection using mouse polyclonal anti-CBS (Abnova, Taipei City, Taiwan) as the primary antibody and the anti-mouse IgG–HRP (Promega, Madison, WI, USA) as the secondary antibody. Immunoblots were developed with enhanced chemiluminescent agents (Lumi-Light Plus Western Blotting Substrate, Roche Applied Science, Indianapolis, IN, USA) and the images were acquired in a charge-coupled device imager ChemiDoc XRS (Bio-Rad Laboratories) using the Image Lab software version 4.1.

**Analysis of oligomerization**

To evaluate the oligomeric profile of the recombinant proteins, 7 µg of the purified proteins were analyzed by native gel electrophoresis using 3–8% gradient native polyacrylamide gels (NuPAGE Novex 3–8% Tris-Acetate Gel, Invitrogen). Gels were stained with Coomassie brilliant blue.

**Assay of CBS activity**

The enzyme activity of purified recombinant CBS proteins was determined in a reaction volume of 110 µl, as previously described (38), with some minor modifications. Briefly, 0.165 µg of the WT or 2.5 µg of the variant CBS enzymes were incubated in a mixture containing 90 mmol/l Tris–HCl pH 8.6, 7.2 mmol/l Ser and 4.5 mmol/l EDTA. The reaction was started by the addition of 2.7 mmol/l Hcy solution, freshly prepared from Hcy thiolactone hydrochloride in the absence of DTT. After 30 min, at 37°C, the enzymatic reaction was stopped by the addition of 50 µl 6 mol/l hydrochloric acid. The total reaction volume was used to determine the amount of the produced Cth by LC-MS/MS. Specific enzyme activities were expressed as µmol Cth formed per hour per mg CBS protein, at 37°C (µmol Cth/h/mg).

**Effect of cysteamine, MEG and Cys on enzymatic activity**

In order to investigate the effect of the aminothiol compounds on enzyme activity, the purified proteins (63.5 and 793.7 nmol/l monomer for the WT and the variants, respectively) were incubated with cysteamine (250 or 1000 µmol/l), MEG (25, 250 or 1000 µmol/l) or Cys (1000 µmol/l) as a negative control. After 1, 4 and 8 h incubation at 37°C, an aliquot of the sample mixture was used to measure the enzymatic activity as described earlier. The enzyme assay of 30-min incubation at 37°C precluded having a time zero in the presence of the tested aminothiols. Therefore, the incubation points with no compound were used as zeros, using an equivalent volume of vehicle (buffer) in place of the aminothiol compounds.

**Effect of MEG on enzyme kinetics**

The effect of MEG on the kinetic properties of the WT and p.R336C proteins was evaluated. Proteins were incubated in the absence or presence of 250 µmol/l MEG for 1 h at 37°C, after which enzyme activities were determined at increasing Ser and Hcy concentrations, as previously described (20). The catalytic efficiency ($K_{cat}/K_{m}$) was determined using the CBS monomer molecular weight (63 kDa).

**Thermal inactivation profile**

Thermal inactivation profiles were performed with the purified p. R336C and WT CBS proteins in the absence or presence of 1000 µmol/l cysteamine or 1000 µmol/l MEG. Aliquots of 15 µl (containing 2.5 µg of p.R336C or 0.165 µg of WT enzyme) were pre-incubated at different temperatures (4, 22, 37, 45 and 55°C) for 4 h and chilled on ice for 5 min, after which enzymatic assays were performed as described earlier.

**Limited proteolysis by trypsin**

Limited proteolysis of WT and variant p.R336C CBS proteins was performed at 37°C in 50 mmol/l potassium phosphate buffer, 300 mmol/l KCl, pH 7.0, as previously described (20). The assay was performed in the absence or presence of 1000 µmol/l cysteamine or 1000 µmol/l MEG. Proteins were pre-incubated with cysteamine or MEG for 1 hour at 25°C. After this time-period trypsin was added to CBS at a 1:100 ratio (by mass). The final protein concentration was 135 µg/ml. At time intervals (up to 45 min), aliquots of the reaction were mixed with soybean trypsin inhibitor, at 1:1.5 trypsin to inhibitor ratio (by mass), and subjected to SDS–PAGE analysis. Gels were stained with Coomassie brilliant blue and the band corresponding to the full-length protein was quantified by densitometry.

**Differential scanning fluorimetry**

DSF was performed in a C1000 Touch thermal cycler equipped with a CFX96 optical reaction module (Bio-Rad) employing the FRET channel ($\lambda_{em} = 450–490$ nm and $\lambda_{ex} = 560–580$ nm) as previously described (20). Before analysis, protein samples were centrifuged (40 000g for 30 min, at 4°C) and diluted to 50 µg/ml in 50 mmol/l phosphate buffer, 300 mmol/l KCl, pH 7.0. Sypro
Orange (Invitrogen; 5000× stock solution) was diluted to yield a 5× working concentration. The temperature was ramped from 20 to 80°C, at 1°C/min. Assays were performed with or without pre-incubation with 1000 µmol/l of the tested aminothiols for 1 h at 25°C. The temperature scan curves were fitted to a biphasic dose-response function, and the $T_{m}$ values were obtained from the midpoint of the first and second transitions.

Supplementary Material
Supplementary Material is available at HMG online.

Conflict of interest statement. None declared.

Funding
This work was supported by Fundação para a Ciência e a Tecnologia, Portugal, (grant SFRH/BD/43934/2008 to M.I.S.M., UID/DTP/04138/2013 to iMed.UL Lisboa).

References


