Deletion Mutagenesis of Human Cystathionine β-Synthase

IMPACT ON ACTIVITY, OLIGOMERIC STATUS, AND S-ADENOSYLMETHIONINE REGULATION*

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Cystathionine β-synthase is a tetrameric hemeprotein that catalyzes the pyridoxal 5'-phosphate-dependent condensation of serine and homocysteine to cystathionine. We have used deletion mutagenesis of both the N and C termini to investigate the functional organization of the catalytic and regulatory regions of this enzyme. Western blot analysis of these mutants expressed in Escherichia coli indicated that residues 497-543 are involved in tetramer formation. Deletion of the 70 N-terminal residues resulted in a heme-free protein retaining 20% of wild type activity. Additional deletion of 151 C-terminal residues from this mutant resulted in an inactive enzyme. Expression of this double-deletion mutant as a glutathione S-transferase fusion protein generated catalytically active protein (15% of wild type activity) that was unaffected by subsequent removal of the fusion partner. The function of the N-terminal region appears to be primarily steric in nature and involved in the correct folding of the enzyme. The C-terminal region of human cystathionine β-synthase contains two hydrophobic motifs designated “CBS domains.” Partial deletion of the most C-terminal of these domains decreased activity and caused enzyme aggregation and instability. Removal of both of these domains resulted in stable constitutively activated enzyme. Deletion of as few as 8 C-terminal residues increased enzyme activity and abolished any further activation by S-adenosylmethionine indicating that the autoinhibitory role of the C-terminal region is not exclusively a function of the CBS domains.

Cystathionine β-synthase (CBS, 1-1-serine hydrolyase (add homocysteine), EC 4.2.1.22) plays an essential role in homocysteine (Hcy) metabolism in eukaryotes (1). The enzyme catalyzes the pyridoxal 5'-phosphate (PLP)-dependent condensation of serine and Hcy to form cystathionine, which is then converted to cysteine by another PLP-dependent enzyme, cystathionine γ-lyase. CBS can also form cysteine directly from serum and hydrogen sulfide via the serine sulfhydrase reaction (2). In mammalian cells that possess the transsulfuration pathway, CBS occupies a key regulatory position between the remethylation of Hcy to methionine or its alternative use in the biosynthesis of cysteine. The relative flux between these two competing pathways is roughly equal and is controlled by intracellular S-adenosylmethionine (AdoMet) concentrations (3). AdoMet activates the mammalian CBS enzyme by as much as 5-fold with an apparent dissociation constant of 15 μM (4, 5). Conversely, the same compound acts as an allosteric inhibitor of Hcy remethylation by inhibiting 5,10-methylenetetrahydrofolate reductase (6, 7) and betaine-homocysteine methyltransferase (8). Deficiency of CBS is the most common cause of inherited homocystinuria, a serious life-threatening disease that results in severely elevated Hcy levels in plasma, tissues, and urine. Symptoms include dislocated optic lenses, skeletal disorders, mental retardation, and premature arteriosclerosis and thrombosis (1).

Human CBS is a member of a large family of PLP-dependent enzymes that operate almost exclusively in the metabolism of amino acids. Members of this family are of multiple evolutionary origins (9) but can be classified into four distinct families depending on their folds: the large α family, the β family, the d-alanine aminotransferase family, and the alanine racemase family (10). CBS belongs to the β family of PLP-dependent enzymes, members of which catalyze replacement and elimination reactions at Cβ. Human CBS displays significant levels of sequence and structural similarity with several other members of this family such as cysteine synthases, serine/threonine deaminases, and the β subunit of tryptophan synthase (11). The sequence and structural conservation between these enzymes is primarily confined to a region encompassed by residues 84-382 in human CBS (available at www.uchsc.edu/sm/cbsdata/cbsprotein.htm). This region includes the PLP binding domain and is similar to the previously described, catalytically activated, protease-resistant core of the enzyme spanning residues 40-413 (12).

Although the catalytic cores of cysteine synthases (CS) and CBS enzymes exhibit high levels of homology, the N- and the C-terminal non-catalytic regions of these proteins show virtually no similarity. Human CBS contains an N-terminal region of ~70 amino acids (Fig. 1), which accommodates the heme prosthetic group (11). The function of this ligand is unknown, but a number of studies indicate it may play either a regulatory or structural role (13-16). The observation that both yeast (17-19) and Trypanosoma cruzi (20) CBS lack heme indicates that it is not directly involved in catalysis (18, 19).

The C-terminal regulatory domain of human CBS consists of ~140 amino acid residues (12). This region is required for tetramerization of the human enzyme and AdoMet activation (12). The C-terminal regulatory region also encompasses the previously defined “CBS domain” (21). This hydrophobic sequence (CBS1), spanning amino acid residues 415-468, is conserved in a wide range of otherwise unrelated proteins. Its
function remains unknown, although the sharp transition of thermally induced CBS activation and the observation that mutations in this domain can constitutively activate the enzyme indicate that it plays a role in the autoinhibitory function of the C-terminal region (22, 23). Based on sequence similarity with another CBS domain-containing protein, isoinosine 5′-monophosphate dehydrogenase (IMPDH) from Streptomyces pyogenes (23), a second, less conserved CBS domain (CBS2) has recently been identified between amino acid residues 486 and 543 in the C-terminal regulatory region of human CBS (see Fig. 1). Two well conserved CBS domains are also present in the C-terminal region of the yeast CBS, which is of approximately the same length as the human enzyme (see Fig. 1). The yeast enzyme functions as a tetramer but is not activated by AdoMet (17). CBS from T. cruzi, which is also unresponsive to AdoMet, lacks the typical CBS C-terminal region and exists predominantly as a tetramer. This observation has lead to speculation that CBS tetramerization is not exclusively a function of the C-terminal region (20).

All of the CS enzymes lack both the N-terminal heme binding domain and the C-terminal regulatory region (Fig. 1). These enzymes function as dimers, do not bind heme, and are not activated by AdoMet (24, 25).

Structure/function analyses of products derived from limited trypsinolysis of human CBS provided some initial insight into the domain architecture of this protein (12). It was determined that the N-terminal 39-amino acid region does not play a significant role in the native structure of full-length CBS, because removal of this region by partial trypsin cleavage does not affect binding of AdoMet, PLP or heme, or tetramer formation (12). Further proteolysis leads to the removal of the entire C-terminal regulatory region, yielding a proteolytically resistant core, consisting of amino acid residues 40–413. The removal of the C-terminal domain causes the enzyme to dissociate from tetramers to dimers. This change in oligomeric status of the enzyme is accompanied by an increase in tryptophan fluorescence, possibly caused by exposing a tryptophan cluster at positions 408–410. The truncated protein showed no change in both its UV and visible absorption spectra indicating that it maintains the structural features of full-length CBS and is unaffected in its ability to bind both PLP and heme (12). The active core is about 2- to 3-fold more active than the full-length tetramer but cannot be further activated by AdoMet (12).

Apart from AdoMet, several other modes of CBS activation have been reported. These include partial thermal denaturation (22), limited proteolysis (12), and the presence of certain C-terminal mutations (22, 23). A possible common CBS activation mechanism has been proposed whereby the C-terminal region of CBS acts as an autoinhibitory domain and that certain mutations, binding of AdoMet, limited trypsinolysis, or partial thermal denaturation all serve to displace this domain from its zone of inhibition (22, 23).

A recombinant human CBS enzyme similar to the above described “proteolytically resistant core” has recently been expressed in Escherichia coli and purified to homogeneity (26). This truncated enzyme, comprising amino acid residues 1–413, has been crystallized, and its x-ray structure has been determined (11). The crystals contained three dimers per asymmetric unit, and each dimer contained one heme and one PLP per subunit. It was observed that the heme binding region of the enzyme is almost completely disordered; the only exception is a short β1 helix formed by amino acid residues 60–62. Two N-terminal residues, Cys-52 and His-65 were identified as thiolate and histidine ligands to the heme. The heme resides in a small hydrophobic pocket at the outer end of each dimer, distant from the PLP cofactor, which is deeply buried in the active site and accessible only via a narrow channel (11). The finding that the heme is relatively distant from the PLP and the fact that the heme iron is ligated from both sides by the amino acid residues provide further evidence against its direct catalytic involvement (11).

In this work, we have studied the role of both the N- and C-terminal regions of human CBS by generating 11 sequential truncation mutants spanning residues 1–70 and 401–551 (Fig. 2). We expressed all of these truncation mutants in E. coli and determined the effect of each deletion on activity, response to
CBS deletion mutants were constructed using PCR amplification of the relevant portions of human CBS cDNA followed by restriction digestion and subsequent sub-cloning into either pHCS3, pKK 388.1, or pGEX-6P-1 vectors as required. The primer sequences employed in the PCR amplifications are shown at the bottom.

| Construct | Sense primer | Restriction site | Antisense primer | Restriction site | Amplified CBS cDNA region |
|-----------|--------------|-----------------|-----------------|-----------------|--------------------------|
| pKK CBS Δ551<sup>a</sup> | 126 SphI | 285 KpnI | 1010–1650 |
| pKK CBS Δ544–551<sup>a</sup> | 126 SphI | 355 KpnI | 1010–1029 |
| pKK CBS Δ534–551<sup>b</sup> | 126 SphI | 284 KpnI | 1010–1599 |
| pKK CBS Δ497–551<sup>e</sup> | 126 SphI | 257 KpnI | 1010–1569 |
| pKK CBS Δ488–551<sup>e</sup> | 126 SphI | 256 KpnI | 1010–1488 |
| pKK CBS Δ442–551<sup>e</sup> | 126 SphI | 210 KpnI | 1010–1464 |
| pKK CBS Δ414–551<sup>e</sup> | 126 SphI | 216 KpnI | 1010–1233 |
| pKK CBS Δ1–39<sup>f</sup> | 389 NcoI | 127 None | 210–1010 |
| pKK CBS Δ1–70<sup>f</sup> | 384 NcoI | 127 None | 210–1010 |
| pKK CBS Δ1–70 Δ401–551<sup>b</sup> | 384 NcoI | 378 XhoI | 213–1200 |
| pGEX-6P-1 CBS Δ1–70 Δ401–551<sup>e</sup> | 377 ApaI | 378 XhoI | 213–1200 |

<sup>a</sup> pHCS3.
<sup>b</sup> pKK 388.1.
<sup>c</sup> pGEX-6P-1.
<sup>d</sup> Primers are written from 5’ to 3’. Underlined bases indicate where the restriction site was introduced.

| Primer | Primer sequence<sup>d</sup> |
|--------|-----------------------------|
| 126    | CGTGAATTCATCCCTTGCGGCTGACAA |
| 127    | TACGATCGATGGCCCTCCTGCAGCTC |
| 210    | TACGGGTACCTCACAGCGTGTCCGT |
| 283    | TACGGGTACCTCACAGCGTGTCCGT |
| 256    | TACGGGTACCTCACAGCGTGTCCGT |
| 257    | TACGGGTACCTCACAGCGTGTCCGT |
| 258    | TACGGGTACCTCACAGCGTGTCCGT |
| 259    | TACGGGTACCTCACAGCGTGTCCGT |
| 284    | TACGGGTACCTCACAGCGTGTCCGT |
| 285    | TACGGGTACCTCACAGCGTGTCCGT |
| 286    | TACGGGTACCTCACAGCGTGTCCGT |
| 287    | TACGGGTACCTCACAGCGTGTCCGT |
| 377    | TACGGATCCATCCCTTGCGGCTGACAA |
| 378    | TACGGATCCATCCCTTGCGGCTGACAA |
| 384    | TACGGATCCATCCCTTGCGGCTGACAA |
| 389    | TACGGATCCATCCCTTGCGGCTGACAA |

CBS Activity Assay—CBS activity was determined by a previously described radiolotope assay using [14C]serine as the labeled substrate (29). Protein concentrations were determined by the Lowry procedure (30) using bovine serum albumin as a standard. One unit of activity is defined as the amount of CBS that catalyzes the formation of 1 μmol of cystathionine in 1 h at 37 °C.

Western Blotting—Western blot analysis of crude cell lysates under...
mutant subunits were detected in the soluble fraction. More extensive deletions from the C terminus (mutants Δ524–551, Δ497–551, Δ489–551, Δ442–551, and Δ414–551) lead to a pronounced decrease in the amount of detectable CBS mutant subunits compared with the wild type control. Although diminished relative to the wild type control, it can be seen that the Δ414–551 construct, which lacks the entire C-terminal regulatory region, is present in a significantly higher concentration than the preceding deletion clones that lack between 18 and 109 C-terminal residues. Taken together, these findings indicate that partial deletions of the CBS C-terminal regulatory region that extend into and past the CBS2 domain are considerably more deleterious than removal of this entire region. The relative amount of the double-deletion mutant (Δ1–70 Δ401–551) was also severely diminished, indicating that removal of these relatively large areas of the CBS protein compromises the ability of the protein to assemble correctly.

The reduced levels of some of the deletion mutant forms of CBS in the E. coli-soluble fractions poses a question regarding the possible influence of these truncations upon the solubility of the resultant mutant forms of CBS. In the past, we have repeatedly observed that, during the expression of wild type CBS in E. coli, a significant portion of the protein is produced as insoluble aggregates.2 Full-length wild type CBS also exhibits a strong tendency toward aggregation during the course of purification as well as in the purified state, although this tendency is somewhat ameliorated by removal of the C-terminal regulatory region (12, 26). To examine the possible effects of the various deletions upon CBS solubility, aliquots of the insoluble cell fractions obtained from the E. coli expression analysis described above were examined by Western blotting (Fig. 3, bottom). With two exceptions, the insoluble fractions were found to contain roughly the same amounts of the various CBS mutant proteins as the soluble fractions. Notably, both of the deletion mutants that lack the heme co-factor due to the absence of the Cys-52 and His-65 residues (Δ1–70 and Δ1–70 Δ401–551) and whose amounts were severely diminished in the soluble fraction were ~5-fold more abundant in the insoluble fractions. It appears that the removal of the N-terminal region acts to decrease the solubility of these mutant forms of CBS and that this is responsible for the diminished amounts of these proteins in the soluble fractions. The Δ551–551-truncated protein was absent in both soluble and insoluble fractions, indicating that this deletion exerts a completely destructive effect upon CBS.

**Effects of Truncations on CBS Activity**—Relative enzyme activities were determined for all of the various deletion mutants by assaying crude cell lysate-soluble fractions for CBS activity in the presence and absence of 1 mM AdoMet. The activities were normalized to the amounts of expressed CBS protein as determined by the densitometric scanning analysis of a Western blot of the cell lysate-soluble fractions. Specific activities of all deletion mutants were expressed as a percentage of the wild type activity determined in the absence of AdoMet, which was considered to be 100% (Table II). The calculated specific activity for the wild type enzyme derived from this analysis was 213 ± 19 units/mg, which is strikingly consistent with the value of 220 units/mg that we determined previously for the purified recombinant wild type enzyme (12).

While having no effect on the enzyme response to AdoMet, deletion of the non-conserved 39-amino acid N-terminal domain causes a 2-fold decrease of specific activity (Table II). Even though this mutant form of CBS was still induced nearly

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2 J. Oliveriusová, V. Kery, K. N. Maclean, and J. P. Kraus, unpublished results.
4-fold by AdoMet, the scale of the impairment of CBS activity was conserved, indicating that the absence of this sequence confers an intrinsic catalytic deficiency. Previous work has shown that this deficiency is not present when this domain is removed after the protein has been assembled (12) indicating that this region plays an important role in ensuring the correct folding of the enzyme.

The effect of deleting the first 70 residues from the CBS N-terminal region was particularly interesting, because this region includes the heme-binding residues. Despite the fact that the resulting truncation mutant lacked the heme cofactor, it retained 20% of wild type activity. Interestingly, although still catalytically active, this mutant is AdoMet non-responsive indicating that this region may play a role in the conformational response of the autoinhibitory C-terminal domain to AdoMet.

The removal of the C-terminal lysine (mutant Δ551) had no effect on either specific activity or the scale of AdoMet activation observed. However, the relatively subtle deletion of the last 8 C-terminal amino acids (mutant Δ544–551) caused a small constitutive activation of enzyme activity (36% higher than uninduced wild type) while rendering the enzyme immune to further activation by AdoMet. In terms of the location of the CBS domains, the deletion of these 8 C-terminal residues removed the non-conserved region downstream of the CBS2 domain (Fig. 1). Larger C-terminal deletions that involved the removal of significant portions of the CBS2 domain (mutants Δ534–551 and Δ524–551) resulted in a dramatic decrease in specific activity, which could not be further elevated by the addition of AdoMet. By extending the deletions up to amino acid 442, the truncations gradually removed up to half of the CBS1 motif. All of these mutants were constitutively activated by ~3-fold (Table II). The mutant Δ414–551, lacking both of the proposed CBS1 and CBS2 domains, was ~5-fold more active than the wild type enzyme control in the absence of AdoMet. None of these constitutively activated deletion mutants could be further activated by AdoMet. These findings are consistent with the proposed autoinhibitory function of the C-terminal domain and our data described above, regarding the effect of partial deletion of the C-terminal region upon CBS protein stability. The double-deletion mutant, missing both 70 N-terminal amino acids and the entire C-terminal regulatory region, was essentially inactive with activity values as low as 2% of that of the wild type control. This heme-free mutant CBS enzyme was also found to be AdoMet non-responsive.

Effect of Truncations on the Oligomeric Status of CBS—Previous work from our laboratory and others has indicated that the region responsible for the assembly of CBS subunits into tetramers is located in the C-terminal region of CBS (12, 32). Recently, work from another group has suggested that the formation of CBS tetramers may involve residues outside of the C-terminal domain (20). The series of deletion mutants generated in this present study provides us with a useful tool to further delineate the one or more regions of CBS associated with the formation of tetramers. To determine the oligomeric status of all of our deletion mutants, soluble fractions of cell extracts were analyzed by native PAGE followed by Western blotting (results not shown). A cell lysate derived from E. coli containing no expression plasmid was used as a negative control. Our results indicated that deletion of the N-terminal 70 amino acids did not affect the oligomeric status of the enzyme. Similarly, the removal of either one or eight amino acids from the C terminus was accompanied by the normal formation of tetrameric CBS. Investigation of the oligomeric status of the two subsequent deletion mutants (Δ534–551 and Δ524–551) was complicated by the degradation of the former and aggregation of the latter. However, the properties of the enzyme improved as larger regions of the C-terminal regulatory region were removed. All of the mutants missing between 55 and 138 amino acids from the C terminus assemble into dimers, whose stability seems to increase with the extent of the deletion. The deletion mutant lacking the 70 N-terminal residues and the 151-amino acid C-terminal regulatory region also forms dimers. Our finding that removal of 70 N-terminal residues did not convert the enzyme from a tetramer to a dimer indicates that the residues associated with tetramer formation reside exclusively in the C-terminal region and are located between residues 497 and 543.

Effect of Truncations on the Heme Content—To determine whether any of the deletions impaired heme binding, soluble fractions of cell extracts were run on a native polyacrylamide gel and analyzed by Western blotting. The blot was stained for the presence of heme (31) using a method relying on heme peroxidase activity (results not shown). As a negative control, lysate from E. coli containing no expression plasmid was used. The experiment demonstrated that all of the C-terminal deletion mutants, with the exception of those that were undetectable on the denaturing Western blot, were capable of binding...
the heme cofactor. The level of heme staining correlated well with the amount of CBS protein detected in the soluble fraction indicating that there has been no obvious change in the ability of the stable deletion mutants to bind and retain heme. The N-terminal deletion mutant Δ1–39 also contained heme. As expected, both mutants that had the heme binding residues deleted (Δ1–70 and Δ1–70 Δ401–551) completely lacked this cofactor.

Expression of the Δ1–70 Δ401–551 CBS Deletion Mutant as a GST Fusion Protein—Analysis of the Δ1–39 and Δ1–70 N-terminal mutants indicated that these mutant forms of CBS were decreased in terms of their relative stability and activity. Because removal of a further 150 C-terminal residues is likely to augment this instability, the observed inactivity of the Δ1–70 Δ401–551 mutant is not surprising. However, as previous work (12) has shown that the removal of the 39 N-terminal and 138 C-terminal residues from full-length CBS does not diminish the resultant protein's activity, it appears that these domains are only required during the folding and assembly of the protein. Consequently, it is conceivable that stabilization of residues 71–400 during the folding and assembly process could lead to catalytically active CBS protein. In an effort to further delineate the catalytic region of CBS, we expressed the Δ1–70 Δ401–551 CBS deletion mutant as a fusion protein with the GST affinity tag at the N terminus. The activity of this mutant was measured in the soluble fraction of a crude cell lysate, both in the presence and absence of 1 mM AdoMet. Surprisingly, and in direct contrast to our previous finding described above, this deletion mutant was found to be catalytically active when expressed as a GST fusion protein. When measured in the crude cell lysate, the activity of this deletion mutant was equivalent to 25% of that of the wild type enzyme. Although catalytically active, this mutant remained AdoMet non-responsive, which is consistent with the previous observation that the presence of the C-terminal regulatory region is required for AdoMet activation of the enzyme.

The GST tag enabled us to rapidly purify large amounts of the Δ1–70 Δ401–551 CBS deletion mutant in a two-step procedure. The GST Δ1–70 Δ401–551 CBS fusion protein was purified from bacterial lysate by affinity chromatography using glutathione-Sepharose 4B. The GST tag was then released from the fusion protein by cleavage with PreScission protease. Due to an uncharacterized interaction between the cleaved Δ1–70 Δ401–551 CBS and the GST tag, we were not able to remove the affinity tag by applying the digest onto a secondary glutathione-Sepharose 4B column. Instead, we separated the cleaved GST tag on a Sephadex G-100 size exclusion column. The protein composition and the degree of purity after each step of the purification procedure were analyzed by SDS-PAGE (Fig. 4C, inset). From a total of 6 liters of culture, we were able to obtain 31 mg of the Δ1–70 Δ401–551 CBS protein at ~95% purity with an overall yield of 28%. After removal of the GST tag, the purified Δ1–70 Δ401–551 CBS mutant exhibits 15% of the activity typically observed for the wild type enzyme. This result indicates that, although CBS cannot tolerate the combined absence of the N-terminal 70 residues and the C-terminal domain during the folding and/or assembly process, these regions are not essential for catalysis once the protein has folded and assembled.

To determine the degree of PLP saturation, we measured the Δ1–70 Δ401–551 CBS activity in the presence and absence of this cofactor. The activity of the wild type enzyme, fully saturated with this cofactor, does not increase in the presence of additional PLP. In contrast, the activity of the truncation mutant increased from 7.3 to 32.1 units/mg of protein upon the addition of PLP to the assay mixture at a final concentration of 0.5 mM. Based on this result, the degree of enzyme saturation with PLP appears to be only 23%. Because the enzyme is kept in 50 μM PLP throughout the purification procedure, it is likely that the loss of PLP occurs when the enzyme is diluted upon addition to the activity assay mixture. Consequently, it appears that, in common with yeast CBS (33), the Δ1–70 Δ401–551 deletion mutant has decreased affinity for PLP relative to the wild type form of CBS.

Fig. 4 shows the absorption spectrum of the truncated enzyme compared with that of the wild type human and yeast enzymes. All three enzymes were presaturated with 50 μM PLP, and the unbound PLP was removed on a Bio-Spin 6 Column (Bio-Rad) equilibrated with 0.1 mM sodium acetate buffer, pH 7.4. The absorption spectrum of the deletion mutant (Fig. 4C) exhibited major peaks at 280 and 412 nm, closely resembling the absorbance profile of yeast CBS (17, 18). The ratio of the A280 nm/A412 nm was determined as 1:0.18, which is somewhat greater than the one reported for yeast CBS (17, 34). The spectra of both enzymes lack the Soret peak at 428 nm, reflecting the absence of the heme cofactor (Fig. 4, B and C). Similar absorption spectra were observed for crystals of the CBS active core from which the heme cofactor had been removed by carbon monoxide (35).

DISCUSSION

Previous information regarding the functional domain organization of CBS has been derived from studies involving limited trypsinolysis (12) and the effects of various regulatory mutations (23, 26, 28). The determination of the crystal structure of full-length CBS is problematic and complicated by the tendency of the protein to aggregate. In the absence of this information, we have used deletion studies to extend our knowledge of the functional domain organization of this protein.

Analysis of the GST Δ1–70 Δ401–551 CBS double-deletion mutant has allowed us to further delineate the region of human CBS that is essential for catalysis. The fact that replacement of the N-terminal 70 residues with the unrelated GST fusion partner is sufficient and necessary for the formation of a catalytically active form of this mutant indicates that the function of the N-terminal domain is primarily steric in nature. Similarly, the observation that this mutant protein remains stable and active after the subsequent removal of the GST fusion partner indicates that this region probably plays an essential role during the CBS folding pathway and/or assembly. The observed role of the GST fusion partner in stabilizing the double-deletion mutant has important implications for studies designed to characterize the behavior of CBS mutants by heterologous expression in *E. coli*. A number of recent studies have investigated various CBS mutants solely as GST fusion proteins (36, 37). The results presented in this report indicate that this approach may generate artifactual results, because the GST fusion partner is clearly capable of masking defects in CBS by assisting with folding and/or assembly and that its stabilizing effects can persist even after its removal from the assembled protein. Consequently, the behavior of the purified mutant protein may differ significantly from that observed in vivo.

The behavior of the Δ1–39 mutant is also consistent with a role for the N-terminal domain in facilitating the correct folding of human CBS. This tetrameric mutant enzyme is induced ~4-fold by AdoMet but is intrinsically impaired in catalysis with only half the activity of wild type CBS. This impairment is unlikely to be due to a regulatory function, because the scale of impairment is perfectly conserved in the AdoMet-induced form of the protein. The deleterious effect of this deletion must be exerted during the folding and/or assembly of the protein. Pre-
Previously, the function of the evolutionarily non-conserved 70 N-terminal residues of mammalian CBS was unknown. The behavior of the Δ1–70 N-terminal deletion mutant described in this report offers some insight into the function of this region and a possible explanation for its absence in CBS found in lower eukaryotes. We have shown that, when the Δ1–70 Δ401–551 deletion mutant is expressed as a GST fusion protein and the GST fusion partner is removed, the resultant protein has decreased affinity for PLP relative to that of the wild type. Previously, we have expressed CBS as a GST fusion protein lacking only the C-terminal domain, and no such deficiency in PLP binding was observed (26), indicating that it is the lack of the 70 N-terminal residues that is responsible for the observed decrease in PLP binding. Yeast CBS lacks the N-terminal domain and has a marked decrease in affinity for PLP relative to the human enzyme (18). This decreased affinity for PLP may reflect the fact that yeast are capable of endogenous de novo synthesis of pyridoxine (the metabolic precursor of PLP), whereas humans are dependent on dietary sources. It is conceivable that the N-terminal domain represents an evolutionary adaptation in mammals designed to augment retention of PLP by CBS. Further work on the purified N-terminal deletion mutants is currently in progress in our laboratory and will provide a definitive answer to this hypothesis.

The observation that the heme-free Δ1–70 mutant is significantly impaired in catalysis (~20% of wild type) is consistent with a recent report on the activities of mutant forms of CBS that were severely depleted of heme by mutagenesis of either (but not both) of the heme binding residues Cys-52 or His-65 (36). The function of the heme group is currently unknown. An early hypothesis indicated that the heme group played a role in substrate activation by direct coordination of Hcy (13). However, discovery that the yeast enzyme does not require heme for catalysis (18, 19) and the subsequent observation that crystallized CBS is capable of catalysis after the displacement of the heme group by carbon monoxide (35) both strongly indicate that the heme ligand is not directly involved in the condensation reaction catalyzed by CBS. The catalytic activity of the heme-free Δ1–70 and Δ1–70 Δ401–551 deletion mutants also serve to disprove any direct catalytic role for heme indicating that the function of this ligand is likely to be regulatory and/or structural. Recent work from our laboratory has indicated that deleterious oligomerization of mutant CBS molecules is associated with a concomitant loss of heme (38). Data presented in this report indicate that the heme-free deletion forms of CBS are also considerably less soluble than the wild type form and that the heme group has a significant influence upon the correct assembly of CBS.

The structural instability demonstrated by some of the partial C-terminal deletion mutants is consistent with the presence of a second CBS domain in the C-terminal regulatory region. The presence of this CBS2 domain was originally proposed on the basis of sequence similarity with the CBS domains present in the otherwise unrelated IMPDH enzyme (23). The recent determination of the structure of the S. pyogenes IM-

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**Fig. 4.** Comparison of spectroscopic properties of (A) purified human wild type CBS, (B) yeast CBS, and (C) Δ1–70 Δ401–551 human CBS deletion mutant. UV-visible absorption spectra of all three enzymes (>95% purity in all cases) were recorded on a Hewlett-Packard diode array spectrophotometer (model 8453 UV-vis) in 0.1 M sodium acetate buffer, pH 7.4, at 25 °C. All enzymes were presaturated with 50 μM PLP. The unbound PLP was then removed on a Bio-Spin 6 column (Bio-Rad). The concentration of all proteins examined was adjusted to 0.7 mg/ml. The inset (C) illustrates the purification of the Δ1–70 Δ401–551 CBS deletion mutant. The expression and purification of the Δ1–70 Δ401–551 CBS deletion mutant was performed as described previously (38) with some modifications. To improve the folding and solubility of the mutant protein, the expression was performed at 30 °C. After cell lysis, the soluble fraction was incubated for 10 min at room temperature in the presence of 2 mM ATP and 10 mM MgSO4 to prevent any nonspecific interaction between the E. coli 70-kDa DNA K protein and the affinity resin. Isolated GST Δ1–70 Δ401–551 fusion protein was cleaved with PreScission protease (Amersham Biosciences) in 1× cleavage buffer (50 mM Tris-HCl, pH 7.0, 150 mM NaCl, 1 mM EDTA, 1 mM DTT) at 5 °C for 12 h at a final concentration of 0.5 unit/mg of protein. The GST tag was subsequently removed by size exclusion chromatography on a Sephadex G-100 column equilibrated with 25 mM MOPS, pH 7.5, 1 mM DTT, and 50 μM PLP. Ten μg of protein was loaded per lane, run on a 9% SDS-polyacrylamide gel, and stained with Coomassie Brilliant Blue. Aliquots from each step were assayed for activity. **Lanes:** 1, molecular weight marker (Premixed Protein Molecular Weight Marker, low range, Roche Molecular Biochemicals); 2, cell lysate soluble fraction; 3, eluate from the glutathione-Sepharose 4B affinity column; 4, PreScission protease digestion products; 5, eluate from gel filtration chromatography. Specific activity (footnote a) and percent yield (footnote b) for each step are shown below the gel. **Footnote a** and percent yield (footnote b) for each step are shown below the gel.
Functional Domains of Human CBS

PDH protein indicates that these two intrinsically hydrophobic domains are separate from the catalytic domain of the protein and are juxtaposed to minimize their hydrophobic interaction with the polar solvent (39). The relative instability of the partial C-terminal deletion mutants, compared with that of the larger deletion lacking both of the CBS domains, is consistent with a similar arrangement in CBS. In this scenario, removal of one of the CBS domains would induce destabilizing structural deformation of the protein as the remaining CBS domain seeks to minimize its interaction with the aqueous solvent, whereas removal of both CBS domains would result in a stable active protein. The presence of dual CBS domains is evolutionarily conserved in the yeast CBS C-terminal region, which also appears to serve an autoinhibitory function albeit independent of AdoMet regulation (19, 40).

It has previously been reported that AdoMet is likely to bind CBS in a region localized between residues 421 and 469 in the previously defined CBS1 domain (33). The rationale behind this assertion was that the D444N mutation contained within this region interfered with the CBS AdoMet response (41). Subsequent work from our laboratory has shown that mutations in this region can impair the CBS response to AdoMet without affecting the ability of the protein to bind AdoMet. Instead it was seen that CBS mutants containing these mutations, e.g. S466L, have already undergone the conformational change that is typically induced by AdoMet binding (22). Additionally, point mutations in the second CBS2 domain located between residues 415 and 468 have been shown to impair AdoMet regulation, although the effect of these mutations upon AdoMet binding was not investigated (23). The fact that deletion of C-terminal residues can abolish the CBS response to AdoMet and constitutively activate the protein in a manner analogous to the previously described S466L mutation (22) indicates that the autoinhibitory function of the C-terminal region is not exclusively a function of the CBS domains. Further work is required to investigate if the deletion of these terminal residues acts to interfere with the ability of CBS to bind AdoMet and whether or not mutation of any or all of these residues is capable of either disrupting the CBS AdoMet response or inducing constitutive activation.

The fold of the 1–413 truncated CBS enzyme belongs to the β-family of PLP enzymes (10). Another PLP-containing enzyme, threonine deaminase (TD), shares the same fold of the catalytic domain, and its domain organization seems to be analogous to CBS (42). TD is also a homotetramer, with each subunit organized into an N-terminal catalytic domain (residues 1–320) and a C-terminal regulatory region (residues 321–514). The catalytic and regulatory domains are connected by a short “neck” that functions as a hinge, enabling the regulatory domain to rotate. It is possible that this arrangement allows the regulatory region to partially occlude the active site and thus modulate the catalytic efficiency of TD (42). Superposition of both TD and CBS enzyme structures suggests that the regulatory domains of both TD and CBS are located at similar positions. ClustalW alignment (available at www.ebi.ac.uk/clustalw/) of the TD and CBS amino acid sequences reveals that the region in human CBS corresponding to the TD neck is represented by amino acids 383–394. These residues form an α-helix (helix 11) in CBS (11) that may also serve a hinge function during the AdoMet-induced removal of the C-terminal domain from its zone of inhibition leading to greater active site accessibility.

Consideration of the oligomeric status of the various deletion mutants adds further evidence to the notion that the C-terminal domain is responsible for the assembly of CBS dimers into higher molecular weight oligomers. A recent report regarding CBS from T. cruzi found that this protein, although clearly related to mammalian CBS, lacks the typical CBS C-terminal region and exists predominantly as a tetramer. As a result of these findings, these authors suggested that the C-terminal region of CBS is not exclusively responsible for the formation of trimers (20).

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