Solvent-Accessible Cysteines in Human Cystathionine β-Synthase: Crucial Role of Cysteine 431 in *S*-Adenosyl-L-methionine Binding†

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ABSTRACT: Cystathionine β-synthase (CBS) is a tetrameric heme protein that catalyzes the PLP-dependent condensation of serine and homocysteine to cystathionine. CBS occupies a crucial regulatory position between the methionine cycle and transsulfuration. Human CBS contains 11 cysteine residues that are highly conserved in mammals but completely absent in the yeast enzyme, which catalyzes an identical reaction, suggesting a possible regulatory role for some of these residues. In this report, we demonstrate that in both the presence and absence of the CBS allosteric regulator *S*-adenosyl-L-methionine (AdoMet), only C15 and C431 of human CBS are solvent accessible. Mutagenesis of C15 to serine did not affect catalysis or AdoMet activation but significantly reduced aggregation of the purified enzyme *in vitro*. Mutagenesis of C431 resulted in a constitutively activated form of CBS that could not be further activated by either AdoMet or thermal activation. We and others have previously reported a number of C-terminal CBS point mutations that result in a decreased or abolished response to AdoMet. In contrast to all of these previously investigated CBS mutants, the C431 mutant form of CBS was unable to bind AdoMet, indicating that either this residue is directly involved in AdoMet binding or its absence induces a conformational change that destroys the integrity of the binding site for this regulatory ligand.

Cystathionine β-synthase [L-serine hydrolyase [adding homocysteine (Hcy)], EC 4.2.1.22] (CBS)[1] is a pyridoxal 5′-phosphate (PLP) dependent heme protein which catalyzes the condensation of serine and homocysteine to form cystathionine (*1*, *2*). Cystathionine is then used as a substrate for cystathionine γ-lyase with the formation of cysteine. CBS is a key regulatory enzyme at the homocysteine (Hcy) branch point between the methionine cycle and cysteine biosynthesis by the transsulfuration pathway. The relative flux between these two competing pathways is regulated by the intracellular concentration of AdoMet. AdoMet is an allosteric activator of CBS activity and acts to repress the alternative remethylation pathway by acting as an allosteric inhibitor of betaine−homocysteine methyltransferase (*3*) and 5,10-methylenetetrahydrofolate reductase (*4*, *5*). CBS and its regulatory interaction with AdoMet are, thus, key regulators of plasma total Hcy (tHcy) levels (*6*, *7*).

CBS is a homotetramer composed of identical subunits of 551 amino acids each and an apparent molecular mass of 63 kDa. In addition to the cofactor PLP, each CBS subunit binds an equimolar amount of heme whose function is unknown. The enzyme has a modular organization consisting of an N-terminal heme binding domain, a highly conserved catalytic core (amino acid residues 70−413), and a regulatory C-terminal domain (Figure 1). The C-terminal regulatory region contains two hydrophobic domains named CBS1 and CBS2 (*8*), recently found to form allosteric binding sites for AdoMet (*9*). A portion of the C-terminal domain has an autoinhibitory function, and binding of AdoMet, proteolytic cleavage, deletion of the C-terminal residues, thermal activation, or specific mutations act to displace the autoinhibitory domain from the catalytic site causing an elevation in CBS catalytic activity (*10*). Deletion of the C-terminal regulatory domain consisting of ∼140 amino acids, originally defined by limited proteolysis, converts the native tetrameric enzyme to a 45 kDa dimer which is constitutively activated and unresponsive to AdoMet (*11*) (Figure 1).

Complete deficiency of CBS in humans results in homocystinuria (MIM number 236200), a disease involving the ocular, skeletal, vascular, and central nervous systems (*12*, *13*). Since partial CBS deficiency may lead to mild hyperhomocysteinemia, which is a known risk factor for premature peripheral and cerebral occlusive arterial disease (*14*, *15*), neural tube defects (*16*), and Alzheimer's and Parkinson's disease (*17*, *18*), interest has increased in the mechanisms by which CBS is regulated. A number of lines of evidence have indicated that CBS expression is regulated coordinately with changes in intracellular redox potential (*19*−*21*). Many redox-regulated proteins vary in conformation and/or function because of changes in oxidant-sensitive cysteine thiols. Common mechanisms underlie the sensitivity of cysteines to redox, such as solvent accessibility and proximity to polar and charged groups, and signal transduction is exerted via conformational changes that are induced by the formation

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[1] Abbreviations: CBS, cystathionine β-synthase; PLP, pyridoxal 5′-phosphate; AdoMet, *S*-adenosyl-L-methionine; GST, glutathione *S*-transferase; wt, wild type; TBS, Tris-HCl-buffered saline; NEM, *N*-ethylmaleimide; IAA, iodoacetic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoate).

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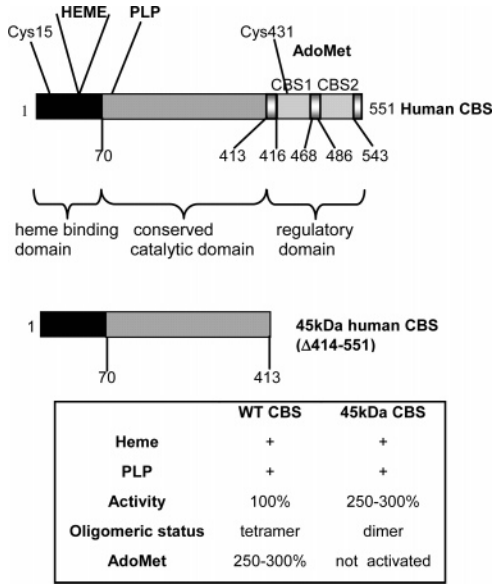

	WT CBS	45kDa CBS
Heme	+	+
PLP	+	+
Activity	100%	250-300%
Oligomeric status	tetramer	dimer
AdoMet	250-300%	not activated

FIGURE 1: Domain organization of human wild-type CBS and 45 kDa CBS.

of disulfide and cyclic sulfenamide covalent bonds and sulfenic and sulfonic acids (*22*).

Human CBS has 11 cysteine residues that are highly conserved in mammals. The possibility that some of these residues play a noncatalytic regulatory role in higher organisms is suggested by the fact that the amino acid sequence of the mechanistically identical yeast CBS is conserved with 38% identity and 72% similarity to the human CBS protein (*23*) and contains only one cysteine residue in a position that is not conserved with any of the mammalian forms of the enzyme.

In this paper, we investigate the possible function of these conserved cysteine residues in human CBS. Specifically, we show that full-length human CBS contains two solvent-accessible cysteine residues, which we identified as C15 and C431, located in the noncatalytic N- and C-terminal regulatory domains, respectively. Mutagenesis of C15 caused no apparent effect upon catalysis or AdoMet activation but did appear to prevent the formation of high molecular weight aggregates of purified CBS. Mutagenesis of C431 resulted in a constitutively activated form of CBS that could not be further activated by either AdoMet or thermal activation and was unable to bind AdoMet.

EXPERIMENTAL PROCEDURES

Materials. L-[U-^{14}C]Serine and *N*-[1-^{14}C]ethylmaleimide were obtained from Perkin-Elmer Life Sciences (Boston, MA). [1-^{14}C]Iodoacetic acid was purchased from ICN Biomedicals, Inc. (Irvine, CA), and *S*-adenosyl-L-[methyl-^{14}C]methionine was purchased from Amersham Biosciences (Piscataway, NJ). Unless otherwise stated, all other chemicals were purchased from Sigma (St. Louis, MO).

CBS Expression Construct Preparation. The C431S CBS mutant expression construct was generated with a wild-type CBS template in the pGEX6P-1 vector using the overlapping PCR method described by Higuchi et al. (*24*). The primers used can be found in Table S1 (Supporting Information). The PCR product (spanning nucleotides 1012−1674 of the CBS coding sequence) containing the C431S mutation was digested with *Sph*I and *Kpn*I and cloned into the pGEX6P-1-hCBS expression vector (Amersham Biosciences, Piscataway, NJ) digested with *Sph*I and *Kpn*I. The C15S mutant construct was generated using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions and oligonucleotides 397 and 267 (Table S1). The vector-insert junctions and the entire CBS coding sequence were verified for each mutant by DNA sequencing using the Thermo Sequenase Cy5.5 sequencing kit (Amersham Pharmacia Biotech) and the Visible Genetics Long Read Tower System-V 3.1 DNA sequencer according to the manufacturer's instructions.

Purification of Recombinant Human wt CBS and Mutant Protein. The purification of wt CBS, C15S, S466L, 45 kDa, and C431S was performed as described previously (*10*). The purification procedure for the C431S and S466L was modified slightly. After the GST-CBS fusion proteins were cleaved with PreScission protease (Amersham Pharmacia Biotech), the GST tag was subsequently removed by size-exclusion chromatography as described previously (*25*).

CBS Assay. The CBS activity was determined by a previously described radioisotope assay using [^{14}C]-L-serine as the labeled substrate (*26*). One unit of enzyme activity is defined as the amount of CBS that catalyzes the formation of 1 μmol of cystathionine in 1 h at 37 °C. AdoMet activation was found in preliminary experiments to be independent of pH in the range 6.5−9.0 and independent of ionic strength in the range 10−150 mM NaCl.

AdoMet Binding. To measure AdoMet binding to CBS, two independent methods were used; the first was fluorescence and the second was a radioactive filter binding assay.

Fluorescence Measurements. Measurements of the internal protonated aldimine of PLP in CBS were done by fluorescence performed at an excitation wavelength of 420 nm on a Shimadzu RF-5301 PC spectrofluorometer using excitation and emission slits of 5 nm with samples in a 1× solution of Tris-HCl-buffered saline, pH 8.6 (1 × TBS; a 1× solution consisted of 10 mM Tris-HCl, pH 8.6, and 150 mM NaCl) at 25 °C. For CBS measurements, the protein absorbance was adjusted to $A_{428} = 0.5$, using a Hewlett-Packard diode array model 8453 UV−visible spectrophotometer. The A_{428} value of 0.5 gives a protein concentration of 0.3 mg/mL. AdoMet was added in small increments to achieve gradually increasing concentrations. Preliminary experiments were done with each CBS variant to determine a valid range of AdoMet concentrations to work with for K_d determination. The initial AdoMet concentrations tested ranged from 0 to 1000 μM. To determine the K_d for each variant, we used a range of concentrations which went 15 points above and below the estimated K_d ranging approximately 10−20-fold in concentration. Specifically, for the K_d determination of wt CBS, 0−170 μM AdoMet was used, for S466L, 0−460 μM, for C431S, 0−1000 μM, for C15S, 0−130 μM, and for 45 kDa, 0−1000 μM AdoMet.

AdoMet Filter Binding Assay. AdoMet binding to CBS was measured by a previously described filter binding assay (*10*). The range of AdoMet concentrations used in this analysis was determined by a similar procedure as above, but the highest concentration was limited by the specific activity of the radioactively labeled AdoMet. AdoMet (0−500 μM) was used initially, then wt CBS, 0−100 μM, 45 kDa, 0−500 μM, and C431S, 0−500 μM.

CBS Activation by Thermal Activation. CBS activity was measured in response to thermal activation as described previously (*10*). Briefly, the wt CBS, C431S, and C15S forms were all diluted to a final concentration of 0.1 mg/mL in 1 × TBS, pH 8.6. Samples were analyzed in a Hewlett-Packard diode array model 8453 UV−visible spectrophotometer with a connected Peltier temperature controller. The enzyme was loaded in a 50 μL quartz cuvette (Helma) and heated in 0.5 °C increments with 1 min hold intervals from 25 to 60 °C. Aliquots (10 μL) of enzyme were taken at different temperatures and chilled on ice, and the CBS activity was subsequently determined at 37 °C. The unfolding of the enzyme has been shown to be irreversible for up to 6 h on ice.

Size-Exclusion Chromatography. The oligomeric status of mutant derivatives of CBS was investigated using a Beckman HPLC Gold system (Beckman Instruments, Inc., Fullerton, CA) with a SEC-250 size-exclusion column (Bio-Rad, Hercules, CA). The column was equilibrated in TBS at a flow rate of 1 mL/min. Protein standards thyroglobulin (670 kDa), immunoglobulin G (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and cyanocobalamin (1.35 kDa) were used for column calibration.

Determination of Accessible Protein Sulfhydryls. All cysteine determinations were performed using a freshly reduced enzyme because nonreduced enzyme has a tendency to aggregate. CBS was reduced by preincubation with 10 mM dithiothreitol (DTT) for 20 min at 37 °C. DTT was then removed using a 1 mL spin column filled with Bio-Rad P-10 in 20 mM Tris-HCl, pH 8.0. The number of reactive sulfhydryl groups was immediately determined spectrophotometrically either with 100 μM DTNB at 412 nm using the extinction coefficient of 5-thio-2-nitrobenzoate of 14150 M^{-1} cm^{-1}, by carboxymethylation with [^{14}C]OOH-iodoacetic acid, or by modification with *N*-[1-^{14}C]ethylmaleimide followed by separation of the free label from the protein using Bio-Rad P-10 in a 1 mL spin column (*27*). Protein concentrations were determined spectrophotometrically using the combined absorbance of the heme and PLP ligands in CBS with ϵ_{428} = 107.1 mM^{-1} cm^{-1}. Reactions using each of the three modifying agents were initially run for up to 2.5 h, and in each case an end point was reached at 30 min.

Isolation, Purification, and NH_2-Terminal Sequencing of Peptides Containing Accessible Cysteines. Native CBS, 1 mg (15.8 nmol), was reduced in 10 mM Tris-HCl buffer, pH 8.6, with 10 mM DTT for 30 min at 37 °C. The DTT was removed using a 1 mL spin column, and the enzyme was carboxymethylated with 3 mM [^{14}C]iodoacetic acid in 0.1 M Tris-HCl buffer, pH 8.6, for either 4 or 30 min at 37 °C. The carboxymethylated enzyme was subjected to a preparative 9% SDS−PAGE. The enzyme band, visualized by autoradiography, was cut out of the gel and electroeluted in an Elutrap chamber (Schleicher & Schuel, Inc.) at 10 W for 1 h into Tris−glycine electrode buffer, pH 8.3. The ^{14}C-carboxymethylated protein solution was then dialyzed against 0.1 M ammonium bicarbonate, pH 7.8, and vacuum-dried. The purified carboxymethylated CBS protein was subsequently dissolved in 50 μL of 8 M urea in 0.1 M ammonium carbonate buffer and reduced with 40 mM DTT for 15 min at 50 °C, and remaining sulfhydryl residues were carboxymethylated with 100 mM cold iodoacetic acid. The sample was diluted to 2 M urea and digested with 1:25 (w/w) endoproteinase Glu C for 6 h at 37 °C. Finally, the Glu C digest was loaded on a C18 reverse-phase column 218TP54 (Vydac, Hesperia, CA) equilibrated in 0.1% trifluoroacetic acid (TFA). The peptides were eluted with a gradient of 0−80% acetonitrile in 0.1% TFA. Fractions containing radioactivity were pooled, vacuum-dried, dissolved in 20 mM triethylamine hydrochloride buffer, pH 7.0, and rechromatographed on a C8 column 228TP104 (Vydac, Hesperia, CA) in a gradient of 0−80% acetonitrile in 20 mM triethylamine hydrochloride buffer, pH 7.0. The separated peptides were then subjected to NH_2-terminal amino acid sequencing by Edman degradation (*28*), which revealed the identity of the two separated peptides by the presence of the ^{14}C label.

Protein Gel Electrophoresis and Activity Staining. Denatured proteins were separated by SDS−PAGE (*29*) using a 9% separating gel with a 4% stacking gel. Blue native polyacrylamide gels (BN-PAGE) were prepared as described previously (*30*, *31*) using a 1 mm thick 8.6 × 6.8 cm, 5−13% gradient gel for separation. Electrophoresis was carried out at 4 °C for 6−7 h. A high molecular weight calibration kit for electrophoresis (Amersham Biosciences) was used as the protein standards for BN-PAGE. For visualization, the gels were stained with Simple Blue (Invitrogen, Carlsbad, CA). CBS activity staining of the BN-PAGE was performed as described previously (*32*).

RESULTS

Determination of the Number of Solvent-Accessible Cysteines in Human CBS. Cysteine residues in proteins can be the site of irreversible modification by reactive oxygen and/or nitrogen species causing a permanent loss of function. Alternatively, reversible modifications of cysteine residues may protect those residues from irreversible oxidation or lead to modulation of protein function serving as a form of redox regulation. Not all cysteine residues are equivalent, and any residue that is sensitive to modification and/or plays a role in signal transduction is likely to be solvent accessible. In order to determine which, if any, of the 11 cysteine residues in CBS are solvent accessible, we used Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoate) (DTNB), for the initial estimation of solvent-accessible sulfhydryl groups. This preliminary analysis of CBS reduced with DTT and then treated with DTNB resulted in the detection of two accessible sulfhydryl groups (Table 1). In order to verify this finding, the experiment was repeated using ^{14}C-labeled iodoacetic acid ([^{14}C]IAA) and ^{14}C-labeled *N*-ethylmaleimide ([^{14}C]NEM). Both IAA and NEM showed an average of two solvent-accessible cysteine residues (Table 1). Interestingly, the 45 kDa CBS enzyme (residues 1−413) shows only one accessible cysteine, indicating that one of the solvent-accessible cysteine residues is located in the C-terminal regulatory region of CBS. Previous work in our laboratory has shown that AdoMet binding to CBS induces a conformational change (*10*). In order to investigate if this conformational change acts to expose additional cysteine residues to solvent, we repeated the above experiments with CBS in the presence of AdoMet. No additional cysteine residues were modified in the presence of this compound.

We have investigated the extent of cysteine modification with iodoacetic acid in the presence and absence of DTNB. In the absence of DTNB, two cysteines were modified within

Table 1: Number of Solvent-Exposed Cysteines in the Presence of Three Different Cysteine-Modifying Agents

Cys-modifying agent	CBS enzyme	no. of solvent-accessible Cys modified	relative activity (%) of wt CBS[a]
DTNB	wt	2.12 ± 0.09	N/A[b]
IAA	wt	1.67 ± 0.34[c]	115
	45 kDa	1.10 ± 0.03	*d*
NEM	wt	2.05 ± 0.29	135
	C15S	1.11 ± 0.25	163

[a] 100 wt % CBS activity of unmodified full-length 63 kDa CBS is 135 units/mg of protein ± 16 SEM (standard error of the mean). [b] N/A, not applicable since DTNB modification is reversed during activity assay. [c] Modification of the first cysteine residue is very fast; modification of the second cysteine is slower and not always 100%. [d] Since the 45 kDa enzyme is constitutively activated, we did not compare it to wt activity in this table; however, with modification of one cysteine, there was no change in activity. All values are the average of four to six independent experiments ± SEM.

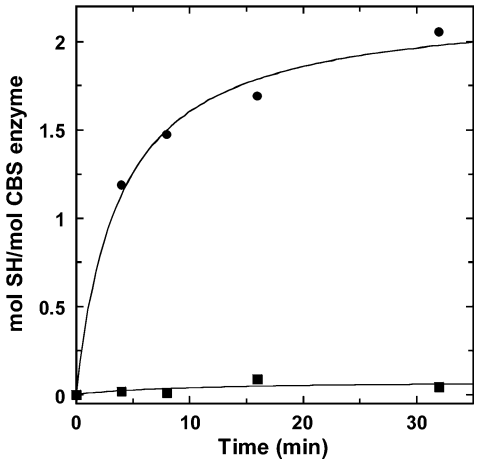

FIGURE 2: Modification of accessible cysteine residues in CBS by [^{14}C]iodoacetic acid. Freshly reduced CBS was carboxymethylated in the presence (■) or in the absence (●) of 10 mM DTNB. CBS reduced with 10 mM DTT in 1 × TBS, pH 8.6, for 10 min at 37 °C was filtered through a 1 mL spin column. A freshly prepared solution of IAA was subsequently added to a final concentration of 3 mM. Aliquots containing 10 μg of protein were precipitated with 10% cold TCA, and the amount of [^{14}C]IAA bound to the protein was determined by scintillation counting. Upon modification with IAA essentially no free SH can be observed with DTNB.

30 min (Figure 2). When reduced CBS was pretreated with DTNB, no further modification by [^{14}C]IAA was observed. This result indicates that modification was occurring specifically on cysteine residues and that both reagents are reacting with the same two residues. Taken together, these three independent cysteine modification methods all come to the same conclusion that there are two solvent-accessible cysteine residues in full-length wild-type CBS.

Identification of Modified Solvent-Accessible Cysteines by N-Terminal Peptide Sequencing. Having determined that there are two solvent-accessible cysteines in CBS, a number of experiments were performed in order to identify their location in the CBS protein sequence as described in Experimental Procedures. Our strategy to achieve this aim involved the labeling of the solvent-accessible cysteine residues followed by proteolysis and subsequent purification of peptides containing the labeled residues which we identified by protein sequencing. Full-length CBS was labeled by carboxymethylation with [^{14}C]IAA as described above, and endopeptidase Glu C was then used in an ammonium carbonate buffer to cleave the reduced and 8 M urea-denatured enzyme into a mixture of peptides. Final separation of the two ^{14}C-carboxymethylated peptides after the second reverse-phase column is shown in Figure 3.

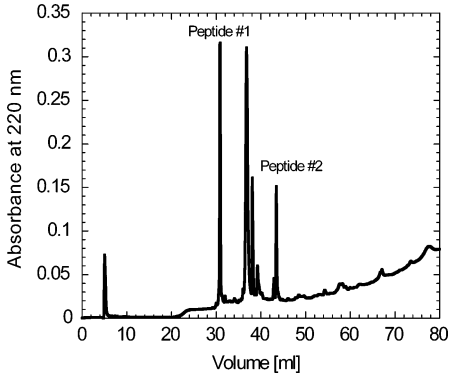

FIGURE 3: HPLC C_{18} chromatography of ^{14}C-carboxymethylated peptides of a trypsin digest of human CBS. Separation of the peptides on the second reverse-phase column C_8 in a gradient of 0−80% acetonitrile in 20 mM triethylammonium hydrochloride buffer, pH 7.0, at a flow rate of 0.5 mL/min (see Experimental Procedures for details).

Once isolated, we proceeded to identify the labeled peptides using NH_2-terminal amino acid sequencing by Edman degradation. This approach unambiguously revealed the identity of the two separated peptides. Peptide 1 was found to consist of CBS residues 10−21, containing the carboxymethylated C15. Peptide 2 was found to consist of residues 413−436 and contains the modified C431 (Table 2). Repeating this procedure for the enzyme carboxymethylated with [^{14}C]IAA for only 4 min yielded peptide 1 and thus clearly identified C15 as the more reactive residue of the two cysteine residues.

Cysteine Mutagenesis. In order to further investigate the role of cysteine residues in CBS, we used site-directed mutagenesis as described in Experimental Procedures to generate bacterial expression constructs designed to express CBS with either C15 or C431 substituted by a serine residue. After expression of these CBS mutant derivatives in *Escherichia coli* (*E. coli*), we performed an initial characterization of mutant enzyme activities in bacterial crude cell extracts using the wild-type CBS expression construct as a positive control. Western blotting analysis of the crude extracts showed that all of the mutant forms of CBS were stably expressed at roughly equivalent levels in *E. coli* as compared to wild type (results not shown). In terms of enzyme activity, the C15S mutant was essentially identical to that of the wild type in both the presence and absence of AdoMet. Interestingly, the C431S mutant was more active than wild-type CBS but did not show any further increase in activity in the presence of AdoMet.

Purification and Characterization of C15S and C431S. The C15S and C431S mutants, which displayed activity at wild-type levels or better, were purified to near homogeneity and further characterized. The C15S mutant protein and the wild-type protein were purified as described previously (*10*). Because the C431S mutant was found to suffer from an increased tendency toward aggregation compared to wild-type CBS, particularly during concentration of the enzyme, this mutant protein was purified using a slightly modified

Table 2: NH_2-Terminal Sequencing of Peptides 1 and 2

peptide 1[a]																				
position in CBS	10	11	12	13	14	15	16	17	18	19	20	21								
amino acid	Val	Gly	Pro	Thr	Gly	CMCys	Pro	His	Arg	Ser	Gly	Pro								
yield (pmol)	1471	1380	966	590	817	1041	452	121	109	159	187	166								
radioactivity (cpm)	11	13	15	17	20	2397	1001	262	96	61	38	33								
peptide 2																				
position in CBS	417	418	419	420	421	422	423	424	425	426	427	428	429	430	431	432	433	434	435	436
amino acid	Leu	Gly	Leu	Ser	Ala	Pro	Leu	Thr	Val	Leu	Pro	Thr	Ile	Thr	CMCys	Gly	His	Thr	Ile	Glu
yield (pmol)	594	468	490	140	446	289	272	183	241	224	181	594	468	490	140	446	289	272	183	241
radioactivity (cpm)	10	10	12	30	37	32	36	61	35	31	34	41	34	28	247	169	84	85	53	37

[a] An identical sequence of peptide 1 was obtained after only 4 min of carboxymethylation

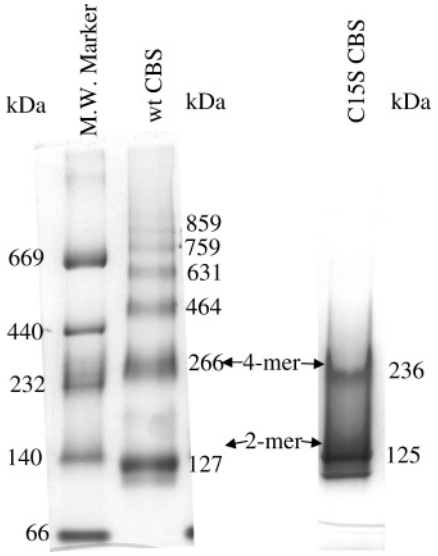

FIGURE 4: Native blue gel comparison of wild-type (wt) CBS with C15S. Native blue gels were run as described in Experimental Procedures. The C15S shows complexes no larger than a tetramer, while the wild-type enzyme shows complexes larger than a tetramer. Both the wild type and C15S mutant also show a dimer on this gel. The molecular weight marker consisted of thyroglobulin from porcine thyroid (669 kDa), ferritin from equine spleen (440 kDa), catalase from bovine liver (232 kDa), lactate dehydrogenase from bovine heart (140 kDa), and albumin from bovine serum (66 kDa).

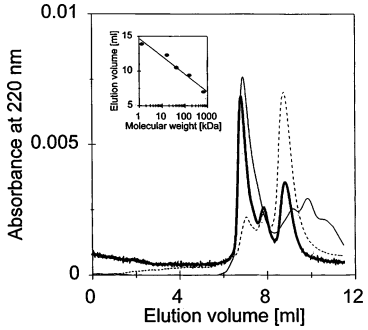

FIGURE 5: Wild-type CBS carboxymethylation of C15 prevents multimerization of the enzyme driven by oxidation. The freshly reduced enzyme (bold line) was oxidized with 1 mM diamide for 20 min (thin line). Reduced wt CBS was first carboxymethylated for 4 min under the standard conditions (see Experimental Procedures) and then oxidized with 1 mM diamide for 20 min (dashed line). All incubations were made in TBS at 37 °C and an enzyme concentration of 0.1 mg/mL. Inset: A calibration curve was generated using thyroglobulin (670 kDa), immunoglobulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and cyanocobalamin (1.35 kDa) as protein standards.

version of this procedure (see Experimental Procedures). SDS−PAGE analysis of the purified proteins indicated that they were all present at approximately 90% purity (Figure S1, Supporting Information).

Upon purification of the C15S enzyme, its oligomeric status was investigated by native blue gel electrophoresis (Figure 4) and HPLC size-exclusion chromatography (results not shown). On the native blue gel, the wild-type enzyme showed several different complexes larger than a tetramer as we have observed previously. Interestingly, the C15S mutant did not show any complexes larger than a tetramer (Figure 4), confirming our notion that C15 is involved in the formation of higher molecular weight oligomers and aggregated forms of wild-type CBS.

The finding that C15 may play a role in CBS aggregation is consistent with a number of independent observations made in our laboratory regarding purified wild-type CBS. When this enzyme is separated on an HPLC size-exclusion column and equilibrated in a nonreducing buffer, we observe a number of different native size molecular forms identified from the calibration curve as an undefined multimer (elution volume 6.8 mL), an octamer (elution volume 7.8 mL), and a tetramer (elution volume 8.9 mL). Typically, if the purified protein is kept in the absence of a thiol-reducing reagent, the majority of the enzyme is in the higher molecular weight undefined multimeric form (Figure 5).

In order to investigate the possible contribution of C15 to this observed phenomenon, freshly reduced wild-type CBS was carboxymethylated for 4 min as described in Experimental Procedures, allowing specific modification of the C15 residue. Subsequent size- exclusion chromatography analysis of this modified enzyme was performed before and after reaction with diamide (Figure 5). While the noncarboxymethylated CBS showed the typical shift from a tetramer to a multimer, the enzyme carboxymethylated at C15 was prevented from aggregating. Taken together, these results strongly implicate cysteine thiols and specifically C15 in the in vitro aggregation into high molecular weight multimers observed for purified CBS in vitro.

The Impact of C15 and C431 upon Activity, AdoMet Activation, and AdoMet Binding. After purification of the C15S and C431S mutant enzymes, we investigated their CBS activity levels and compared these values to that of purified wild-type CBS enzyme (Figure 6). The C15S mutant had activities essentially identical to that of purified wild-type CBS in both the presence and absence of AdoMet. The purified C431S mutant form of CBS exhibited more than 2-fold higher catalytic activity than that of the wild-type control protein but could not be further activated in the presence of up to 10 mM AdoMet. The level of CBS activity observed for the C431S form of CBS is comparable to that seen for the wild-type enzyme activated either by AdoMet

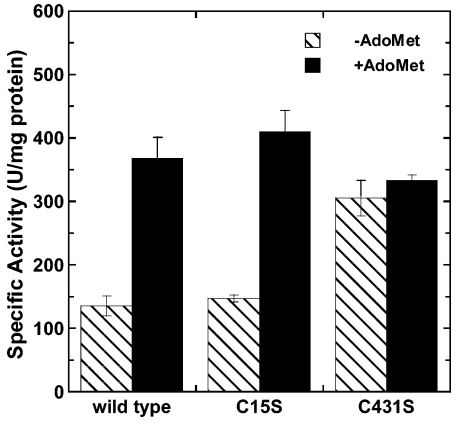

FIGURE 6: CBS activity of purified wt CBS, C15S, and C431S enzymes in the presence and absence of 1 mM AdoMet.

Table 3: Binding of AdoMet to wt and Mutant CBS Determined by Fluorescence and Filter Binding Assay

	K_d (μM)	
enzyme	fluorescence	filter binding
wt CBS	11.4 ± 0.6	11.2 ± 3.1
C15S	16.3 ± 1.8	ND[a]
C431S	not det[b]	not det
45 kDa	not det	not det
S466L	2.9 ± 0.6	ND

[a] ND, not determined. The S466L and C15S were not further analyzed by AdoMet filter binding assay because the fluorescence assay suggested no significant decrease in AdoMet affinity. [b] Not det, not detected. The fluorescence signal or the radioactive counts were insufficient to evaluate a binding constant.

or by partial protease digestion (*11*) and that previously observed in the constitutively activated S466L mutant form of CBS (*10*). These results strongly indicate that mutagenesis of C431 results in an altered activated conformation acting to displace the C-terminal autoinhibitory domain of CBS from the active site. To further investigate this possibility, we studied the AdoMet binding properties of the C431S mutant. Because previous work in our laboratory has shown that AdoMet binding to wild-type CBS results in a change in the PLP-derived fluorescence intensity (*10*), we investigated possible changes in the environment around the CBS catalytic site in C431S CBS using fluorescence spectra of the internal protonated aldimine of PLP in the presence and absence of AdoMet (*10*). By looking at fluorescence changes in this region, we are able to observe the effects of AdoMet on the CBS active site. The PLP fluorescence spectrum of the C431S mutant was compared to those of wild-type CBS and C15S CBS, both of which are activated by AdoMet. In addition, the C431S PLP fluorescence spectrum was compared to that of the CBS mutant S466L that binds AdoMet but is not further activated by this compound and the 45 kDa C-terminal deletion mutant that lacks the C-terminal autoinhibitory domain and is thus unable to bind AdoMet. The wild-type and C15S forms of CBS both show a change in fluorescence intensity upon addition of AdoMet, allowing for the calculation of K_d values (Table 3). The K_d value for AdoMet binding to the wild-type enzyme was 11.4 ± 0.6 μM, and Figure 7 shows the PLP fluorescence spectra of wild-type CBS with increasing concentrations of AdoMet along with an inset showing the K_d determination. The wild-type CBS K_d value is in good agreement with the previously

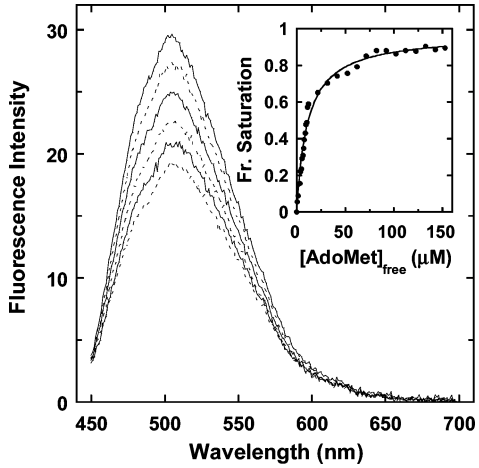

FIGURE 7: Fluorescence spectrum and K_d graph for wild-type CBS alone and for wild-type CBS with increasing concentrations of AdoMet. The highest curve represents wild-type CBS, and each of the curves below represent wt CBS + 5, 10, 15, 45, and 145 μM AdoMet, respectively. The inset is a graph showing the binding constant determination for wt CBS.

reported value of 13.5 μM (*10*). The C15S mutant enzyme gave a dissociation constant for AdoMet binding of 16.3 ± 1.8 μM. Although the S466L CBS mutant is not activated by AdoMet, it is clearly capable of binding this compound, exhibiting a K_d value of 2.9 ± 0.6 μM. Although this was somewhat different from our previously published K_d of 8.9 μM (*10*), both values consistently show that S466L has a higher affinity for AdoMet than wt CBS.

The 45 kDa C-terminal deletion mutant on the other hand, which shows no activation by AdoMet, displays no shift in fluorescence upon addition of AdoMet. Similarly, C431S also shows no change in relative fluorescence intensity upon addition of AdoMet, suggesting that either the fluorescence signal is insufficient to evaluate a binding constant or that the binding constant is too large. This latter possibility suggests that AdoMet may not be binding to the C431S CBS mutant. To investigate this possibility, we used a previously described AdoMet filter binding assay (*10*) to determine the level of AdoMet binding for the C431S mutant form of CBS (Table 3). As a positive control, the K_d value for AdoMet binding to the wild-type enzyme was determined in parallel and found to be 11.2 ± 3.1 μM, a value nearly identical to that determined by fluorescence measurements (11.4 ± 0.6 μM) as described above. The 45 kDa C-terminal deletion mutant was used as a negative control and did not show any detectable binding of AdoMet. In direct contrast to the constitutively activated S446L mutant form of CBS, the constitutively activated C431S CBS enzyme exhibited no detectable binding by AdoMet. Although a number of CBS mutants with aberrant AdoMet regulation and increased AdoMet binding constants have previously been described (*6*, *9*, *10*, *33*), C431 is the first reported point mutant form of CBS that is not capable of binding this ligand.

Thermal Activation Studies. We have previously reported that as a result of thermal activation, at temperatures just below the melting point (55 °C) of wild-type CBS, the catalytic activity of the enzyme increases ~2-fold. The S466L mutant does not show any increase in activity with heating, and similarly, the addition of AdoMet to the thermally activated wild-type CBS does not show any

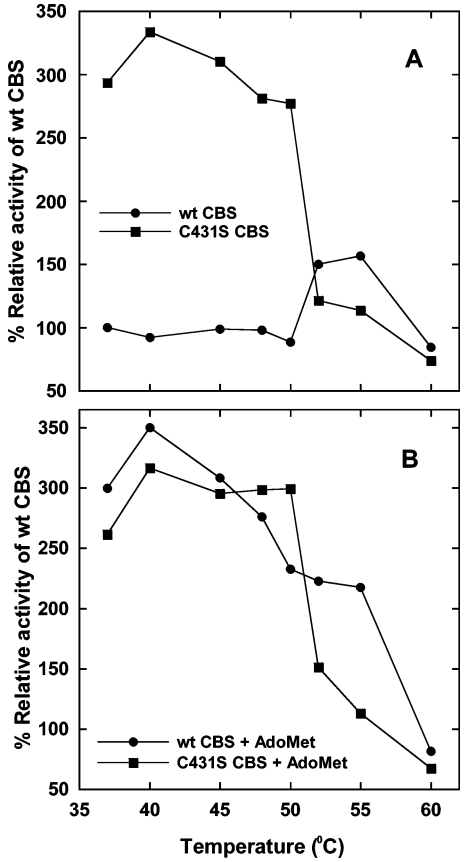

FIGURE 8: Comparative analysis of C431S and wild-type CBS thermal activation. 10 μL aliquots of enzyme were taken at different temperatures ranging from 37 to 60 °C and chilled on ice. Wild-type CBS (wt CBS) and C431S were subsequently assayed for activity in the absence of AdoMet as shown in (A) and in the presence of AdoMet as shown in (B). The activities are shown as a percentage of the wild-type enzyme as determined at 37 °C without any preincubation at higher temperatures.

increase in activity. These results were an indication that the thermal activation was inducing a functionally equivalent conformational change to that induced by AdoMet binding to the wild-type CBS enzyme (*10*). If our hypothesis regarding the mechanism by which C431S CBS is constitutively activated is correct, then one would logically predict that this mutant form of CBS would be insensitive to further activation by treatments involving the CBS autoinhibitory C-terminal domain. Having already demonstrated the inability of AdoMet to further activate the C431S mutant, we proposed to extend this investigation by looking at the ability of this protein to respond to thermal activation. In this analysis, wild-type CBS and the C15S and C431S CBS mutants were preincubated at a range of temperatures varying between 37 and 60 °C, followed by chilling on ice and subsequently assaying for activity at 37 °C.

As one might reasonably predict from the results described above, the C15S mutant behaved like wild-type CBS, showing an approximate 2-fold increase in activity just below the melting temperature (55 °C) (data not shown). Conversely, the constitutively activated C431S mutant could not be further activated by partial thermal activation (Figure 8). Interestingly, it would appear that the C431S mutation induces a significant change in the thermal stability of CBS as the transition from 50 to 52 °C is accompanied by a very significant drop from 275% to 125% of wild-type CBS activity for the C431S mutant. By way of contrast, this same temperature change is accompanied by a 50% increase in activity for wild-type CBS. Taken together, the mutually exclusive nature of thermal, AdoMet, and C431S induced activation of CBS indicates that they are acting through a functionally analogous mechanism involving the displacement of the C-terminal autoinhibitory domain from the active site.

DISCUSSION

Our findings described here represent the first analysis of solvent-exposed cysteines in CBS, along with their functional and structural characterization. The results of our studies show that 2 out of the 11 cysteine residues in wild-type CBS are solvent exposed.

Although our results show that the C15 residue of CBS is not crucial for catalytic activity, characterization of this mutant revealed an interesting correlation between unpaired solvent-accessible cysteine residues and the tendency of purified CBS to form high molecular weight aggregates in vitro. The HPLC and native blue gel results together show that when the C15 residue is modified or mutated, the CBS enzyme is stabilized in the tetrameric form compared to the wild-type enzyme. These findings highlight the possibility that the C15 modified or mutant form of CBS may represent a more stable form of CBS for future biochemical and structural studies.

Our previously described crystal structure of a truncated form of CBS indicated that residues C272 and C275 are solvent accessible and are part of a CXXC motif followed by an α-helix, alluding to a potential oxidoreductase function where these residues would be responsible for the formation and reduction of disulfide bonds during the catalytic process (*34*). However, in our current analysis of the full-length CBS these residues were not found to be solvent accessible even when the protein was induced into the activated conformation by AdoMet binding, thus making it unlikely that C272 and C275 function as an oxidoreductase domain in full-length CBS.

Characterization of wild-type and mutant CBS responsiveness to AdoMet has led to speculations regarding the location of the AdoMet binding site. The predictions by different groups are relatively similar and focus on the tandem CBS domains located within the C-terminal portion of CBS between residues 415−469 (*8*, *9*, *35*, *36*). These "CBS domains" are a previously defined group of hydrophobic sequence motifs that are found in a wide range of functionally unrelated proteins. These domains are invariably present in tandem pairs within the same polypeptide chain and appear to interact with each other to confer regulatory functions (*8*).

A very recent report by Scott et al. (*9*) has provided evidence suggesting that twin CBS domains form a binding site for the adenosine group in a variety of allosteric regulator compounds including AdoMet. Scott et al. investigated binding of these ligands to subcloned and expressed regulatory fragments of a number of different proteins including human CBS that all contained tandem CBS domains. They used our previously described AdoMet filter binding method (*10*) to show that the isolated C-terminal portion of human CBS is capable of binding AdoMet with an apparent K_d of 34 μM. In the presence of the D444N mutation, which is

unresponsive to physiological concentrations of AdoMet (*33*, *37*), the binding affinity was significantly reduced with an apparent K_d of 500 μM. Although the work by Scott et al. was the first direct experimental proof of AdoMet binding to the CBS C-terminal region, this analysis suffers a certain degree of limitation, as this region is being expressed and folded in the absence of both the catalytic portion of the protein and the N-terminal regulatory region. In addition, Evande et al. (*33*) purified the full-length CBS containing the D444N mutation and found that comparable to wild-type CBS it was induced about 2-fold in the presence of AdoMet. In contrast to the wild-type CBS which had a K_{act} of ~7.4 μM AdoMet, the D444N mutant had a K_{act} of ~460 μM AdoMet when assayed under otherwise identical conditions (*33*). Although the D444N form of CBS is clearly altered in its response to AdoMet, it is very clearly still capable of binding and responding to this ligand.

We have previously modeled the conformational change of CBS upon the binding of AdoMet and concluded that the measured K_d describes AdoMet binding to the wt CBS followed by isomerization of the enzyme into the activated form. The thermal activation data in Figure 8 support this model. An independent biophysical method employed in a recent paper (*38*) supports this conclusion. The study used the D444N mutant to determine whether this CBS mutant was in the basal or activated conformation. Hydrogen deuterium exchange mass spectrophotometric analysis was performed on the mutant and wt CBS in the presence and absence of AdoMet. The kinetic data for the mutant in the absence of AdoMet were found to be essentially the same as the data for the wt CBS in the presence of AdoMet. This analysis indicated that the D444N mutant is also locked in an activated conformation even in the absence of AdoMet, thus imitating the wt CBS conformation in the presence of AdoMet.

Several of the C-terminal mutants are constitutively activated. Therefore, we can assume that the autoinhibitory region in all of the mutants is being displaced from the active site. However, these mutations may not cause the same type of conformational change in the AdoMet binding region. Thus, the S466L repeatedly shows tighter binding to AdoMet than the wt CBS (Table 3 and ref *10*) while the D444N shows a lower binding affinity (*9*) and the C431S has no detectable binding in the presence of up to 1 mM AdoMet. In the case of S466L we believe that the AdoMet is binding to the activated conformation without inducing a further conformational change.

Our finding that the CBS C431S mutant is completely deficient in AdoMet regulation is particularly interesting as this mutation is located within the CBS1 domain, but in contrast to all of the previously studied C-terminal AdoMet regulatory mutations (*8*, *9*, *35*, *36*), this is the only CBS mutant described to date where AdoMet binding is completely abolished. There are several possible explanations for the lack of AdoMet binding. The first is that AdoMet directly binds to this cysteine residue. Another possibility is that the C431 residue may be directly involved in interaction of the inhibitory CBS domain with the active site. The mutation at this residue therefore might have allowed uncoupling of the inhibitory domain from the active site leading to increased activity in the absence of AdoMet. Finally, C431 mutation may induce a conformational change that destroys the functional integrity of the AdoMet binding site. Clearly, the C431 mutant form of CBS merits further investigation, and these studies are currently ongoing in our laboratory.

SUPPORTING INFORMATION AVAILABLE

A figure containing an SDS gel showing purified recombinant wild-type, C15S, and C431S human CBS (Figure S1) and a table detailing the oligonucleotides used for construction of plasmids containing Cys → Ser mutations (Table S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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