Assignment of Enzymatic Functions to Specific Regions of the PLP-Dependent Heme Protein Cystathionine β-Synthase[†]

Shinichi Taoka, Lusiana Widjaja, and Ruma Banerjee*

Department of Biochemistry, University of Nebraska, Lincoln, Nebraska 68588-0664

Received April 14, 1999; Revised Manuscript Received July 9, 1999

ABSTRACT: Cystathionine β-synthase is a unique heme protein that catalyzes a pyridoxal phosphate (or PLP)-dependent β-replacement reaction. The reaction involves the condensation of serine and homocysteine and constitutes one of the two major avenues for detoxification of homocysteine in mammals. The enzyme is allosterically regulated by *S*-adenosylmethionine (AdoMet). In this study, we have characterized the kinetic, spectroscopic, and ligand binding properties of a truncated catalytic core of cystathionine β-synthase extending from residues 1 through 408 in which the C-terminal 143 residues have been deleted. This is similar to a natural variant of the protein that has been described in a homocystinuric patient in which the predicted peptide is 419 amino acids in length. Truncation leads to the formation of a dimeric enzyme in contrast to the tetrameric organization of the native enzyme. Some of the kinetic properties of the truncated enzyme are different from the full-length form, most notably, significantly higher K_ms for the two substrates, and loss of activation by AdoMet. This is paralleled by the absence of AdoMet binding to the truncated form, whereas four AdoMet molecules bind cooperatively to the full-length tetrameric enzyme with a K_d of 7.4 μM. Steady-state kinetic analysis indicates that the order of substrate addition is important. Thus, preincubation of the enzyme with homocysteine leads to a 2-fold increase in V_{max} relative to preincubation of the enzyme with serine. Since the intracellular concentration of serine is significantly greater than that of homocysteine, the physiological significance of this phenomenon needs to be considered. Based on ligand binding studies and homology searches with protein sequences in the database, we assign residues 68−209 as being important for PLP binding, residues 241−341 for heme binding, and residues 421−469 for AdoMet binding.

Cystathionine β-synthase is an unusual heme protein that catalyzes a PLP[1]-dependent condensation of homocysteine and serine to give cystathionine. While the role of PLP is immediately suggested by the β-replacement reaction that is catalyzed, the role of the heme is not obvious. The importance of cystathionine β-synthase to human physiology is that it represents one of two major avenues for detoxification of homocysteine, an amino acid whose elevated levels are correlated with cardiovascular diseases (reviewed in ref *1*) and neural tube defects. It is estimated that up to 40% of patients with coronary or cerebrovascular atherosclerosis have hyperhomocysteinemia. The single most common cause of homocystinuria, in which homocysteine levels are catastrophically high, is mutations in cystathionine β-synthase. Despite the clinical significance of this enzyme, large gaps remain in our understanding of its organization, reaction mechanism, and the roles of the various cofactors.

Human cystathionine β-synthase is a homotetrameric enzyme with a subunit molecular mass of 63 kDa. In addition to heme and PLP, the enzyme also binds AdoMet, a V-type allosteric effector that increases V_{max} 2−3-fold but has no effect on the K_m for either substrate (*2*). Based on the homology between cystathionine β-synthase and a number of PLP binding proteins, viz., *O*-acetylserine sulfhydrylase, threonine deaminase, and tryptophan synthase, the location of the PLP binding site is predicted to be in the N-terminal third of the protein (Scheme 1). This was recently confirmed by identification of Lys119 as the residue that forms a Schiff base with PLP (*3*). The binding sites for AdoMet, heme, and the substrates have not been identified, although serine is expected to bind in the PLP pocket. Kinetic (*4*) and spectroscopic (*3*) data indicate that homocysteine binds in the proximity of heme.

Purification of the wild-type enzyme from mammalian liver has resulted in the isolation of mixtures of full-length tetrameric enzyme as well as a truncated but active dimeric form (*5*−*7*). This resulted presumably from the proteolytic susceptibility of the native enzyme. Pulse−chase experiments in human fibroblast did not provide evidence for posttranslational processing of the native cystathionine β-synthase to the truncated form (*8*). However, since skin fibroblast cells in their natural environment apparently do not express cystathionine β-synthase (*9*, *10*), in contrast to tissue culture conditions, it limits the inferences that can be drawn about the absence of the truncated form under these conditions and the extension to other tissue types. Thus, the physiological

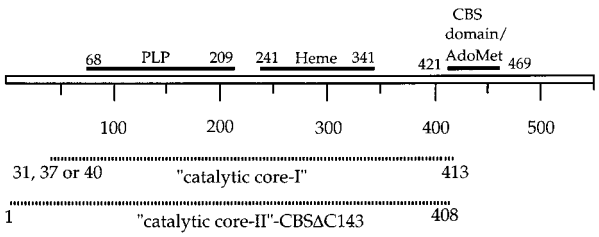
[†]Supported by grants from the National Institutes of Health (HL58984) and the American Heart Association (96-1516).

* Corresponding author. Telephone: (402)-472-2941. FAX: (402)-472-7842. Email: rbanerjee1@unl.edu.

[1] Abbreviations: PLP, pyridoxal phosphate; CBSΔC143, truncated form of cystathionine β-synthase lacking the C-terminal 143 residues; AdoMet, *S*-adenosylmethionine; AdoHCy, *S*-adenosylhomocysteine; GST, glutathione *S*-transferase; HCy, homocysteine; BSA, bovine serum albumin.

10.1021/bi990865t CCC: $18.00
Published on Web 09/17/1999

Scheme 1: Postulated Locations of Cofactor and AdoMet Binding Sites in Cystathionine β-Synthase[a]



[a] The boundaries of the two truncated forms, catalytic cores I and II (or CBSΔC143), are indicated.

relevance of the dimeric form observed in the liver is still open to question.

Recently two groups have reported the isolation of a truncated core of the protein that displays a high level of catalytic activity using recombinant cystathionine β-synthase (*11*, *12*). In the first study, limited proteolysis resulted in the generation of a 45 kDa subunit and an accompanying change in the oligomerization status from α_4 to α_2 (*11*). The resulting catalytic core (Scheme 1) extends from amino acids 31, 37, or 40 through 413, and is 2-fold more active than the native enzyme. The N-terminus of this polypeptide is heterogeneous due to multiple susceptible proteolysis sites at lysines 30, 36, and 39. The second catalytic core (*12*) was generated by the introduction of an opal mutation at position 409 in the open reading frame, and the resulting protein extends from residues 1 through 408 (designated hereafter as CBSΔC143). The activity of this enzyme, whose oligomeric structure was not determined, was also observed to increase 2-fold relative to the native form. Both forms lost sensitivity to allosteric activation by AdoMet. Interestingly, a natural variant similar to CBSΔC143 has recently been described in a homocystinuric patient where a nonsense codon is predicted to lead to premature termination at residue 420 (*13*).

These observations raise a number of interesting questions about how the interactions between the N- and C-terminal domains in cystathionine β-synthase modulate the activity of the enzyme. In this study, we have characterized catalytic core II missing the C-terminal 143 residues. The influence of the C-terminal domain on the activity of the full-length protein has been evaluated by comparison of the kinetic properties of the two enzyme forms. In addition, based on homology searches and ligand binding analysis, the regions of the protein involved in binding heme and AdoMet have been identified. These studies provide interesting insights into the organization and steady-state kinetic mechanism of cystathionine β-synthase.

METHODS

Materials. The following materials were obtained from Sigma: BSA, 5,5′-dithiobisnitrobenzene, L-homocysteine thiolactone, PLP, dithionite, and thrombin. [^{14}C]Serine (158 mCi/mmol) and [C^{3}H$_3$]AdoMet (80 Ci/mmol) were purchased from Amersham Life Science Co. Glutathione Sepharose 4B and Q-Sepharose (fast flow) were purchased from Pharmacia. Homocysteine was prepared from homocysteine thiolactone as described previously (*14*). AdoMet 1,4-butanedisulfonate was a generous gift from Knoll Farmaceutici Spa (Milano, Italy).

Enzyme Purification and Assays. Full-length human CBS was purified from a recombinant expression system (pGEX4T1/hCBS) described previously (*12*) that produces a fusion protein with glutathione *S*-transferase (GST) at the N-terminus. The truncated human CBS lacking 143 amino acids at the C-terminus (CBSΔC143) was also purified as a fusion protein containing GST using the expression vector pGEXCBSN (*12*) that was generously provided by Warren Kruger (Fox Chase Cancer Center, Philadelphia). The cells were cultured, and the protein was purified as described previously (*2*). In the last step of purification, the GST domain is cleaved by limited proteolysis using thrombin (*2*).

The enzymes were assayed under aerobic conditions using radiolabeled serine as described before (*2*). One unit of CBS catalyzes the formation of 1 μmol of cystathionine per hour at 37 °C. Protein concentrations were determined by the Bradford method, using reagents from Bio-Rad and bovine serum albumin as standard. For steady-state kinetic analyses, the concentration of the stock homocysteine solution was determined immediately before the experiments by titration with the thiol reagent dithiobisnitrobenzene and calculated using an ϵ_{412} value of 13 600 M^{-1} cm^{-1}.

Kinetic Studies. Steady-state kinetic analyses were performed as follows: the reaction mixture (0.2 mL) containing 0.34 M Tris, pH 8.6, 0.25 mM PLP, 0.25 mg/mL BSA, 7.4 μg of CBSΔC143, and varying concentrations of the first substrate (either [^{14}C]serine or homocysteine) was preincubated for 5 min at 37 °C. A fixed concentration of the second substrate (either homocysteine or [^{14}C]serine) was added to initiate the reaction. The mixture was incubated for 30 min at 37 °C, and quenched by the addition of 0.2 mL of 10% trifluoroacetic acid. The radiolabeled product, [^{14}C]cystathionine, was separated from [^{14}C]serine as described previously (*2*). The specific substrate concentrations that were employed are indicated in the figure legends.

AdoMet Binding. Equilibrium dialysis was employed to measure the dissociation constant of AdoMet using commercially available [^{3}H]AdoMet. One chamber of the dialysis unit contained 20−30 μM cystathionine β-synthase in 0.2 M potassium phosphate buffer, pH 7.2. The other compartment contained 4−400 μM [^{3}H]AdoMet in 0.2 M potassium phosphate buffer, pH 7.2. The solutions were allowed to equilibrate for 2−3 days at 4 °C. Control samples lacking enzyme were run in parallel to confirm that [^{3}H]AdoMet had achieved equilibrium during that time. Aliquots were removed from the two compartments, and the radioactivity was determined by scintillation counting.

L-Serine Binding. Two alternative methods were employed to measure the dissociation constant for serine: equilibrium dialysis using [^{14}C]-L-serine and fluorescence spectroscopy as described in (*2*).

PLP Analysis. The PLP content of the enzyme was determined using a fluorimetric method in which the enzyme containing the internal aldimine is treated with hydroxylamine to generate an oxime that is subsequently separated from the protein by filtration (*2*, *15*). The fluorescence emission of the oxime at 446 nm was measured following excitation at 353 nm. A calibration curve was established using varying concentrations of PLP and 5 mM hydroxylamine in 0.25 M potassium phosphate buffer, pH 7.2. The concentration of PLP used to generate the standard curve

was determined spectroscopically using $\epsilon_{295} = 5.1$ mM^{-1} cm^{-1} in 0.1 N HCl.

Metal Analysis. Enzyme preparations were analyzed for their total metal content by plasma emission spectroscopy (*16*) at the Chemical Analysis Laboratory, University of Georgia, Athens. This method detects 20 metal ions including the common transition metals. Samples were sent for analysis along with the appropriate buffer blanks.

Sequence Analysis. Homology of the cystathionine β-synthase to other proteins in the Swiss protein database was searched using the position specific iterated (PSI) blast program (*17*) available at the National Center for Biotechnology Information website. Overlapping fragments (150 amino acids in length) of cystathionine β-synthase were used as query sequences for the searches.

RESULTS

Purification of CBSΔC143. The truncated form of CBS was purified using the procedure developed for the full-length enzyme (*2*). CBSΔC143 eluted from the Q-Sepharose column at 0.1−0.2 M NaCl with 50 mM Tris buffer, pH 8, and from the DEAE-Sepharose column at 0.2 M potassium phosphate buffer, pH 7.2. From a 6 L culture, 70 mg of cystathionine β-synthase of ~95% purity was obtained in high yield. CBSΔC143 eluted as a single peak from a calibrated size exclusion column (Superose 12, Pharmacia) with a retention time consistent with it being a dimer (data not shown).

Heme and PLP Content. The iron stoichiometry of the dimeric enzyme was determined in two independent samples by plasma emission spectroscopy and was found to be 1.7 ± 0.3 irons per dimer. This is surprising since the iron stoichiometry of the tetrameric enzyme has been measured to be 2 per tetramer (*2*). Therefore, metal analysis was repeated on the tetrameric enzyme. Based on three independent enzyme preparations, the iron stoichiometry for full-length cystathionine β-synthase was confirmed to be 2.1 ± 0.1 irons per tetramer.

The PLP content of the enzyme was determined by a fluorimetric method described before (*4*). A stoichiometry of 1.8 ± 0.1 PLPs per dimer was obtained following treatment with 5 mM hydroxylamine for 14 h at 4 °C. This is consistent with the presence of four PLPs in the tetramer (*4*).

UV−Visible Spectrum of CBSΔC143. The heme spectrum of CBSΔC143 is very similar to that of the full-length protein (Figure 1) with an $A_{428\ nm}/A_{280\ nm}$ ratio of 1.35. The presence of the Soret maximum at 428 nm together with broad α and β bands indicates the presence of low-spin six-coordinate heme in ferric CBS. The extinction coefficient at 428 nm is 185 ± 15 mM^{-1} cm^{-1} at pH 8.6 and is similar to that for the native enzyme (*2*).

Steady-State Kinetic Studies on CBSΔC143. The steady-state kinetic parameters for CBSΔC143 were determined in two separate sets of experiments where the enzyme was premixed with one of the substrates and the concentration of the second substrate was varied. When the enzyme is preincubated with homocysteine, saturation kinetics are observed at low concentrations of serine (Figure 2). However, at high concentrations of serine (≥15 mM), saturation conditions are more difficult to achieve. Lineweaver−Burk plots of these data (not shown) reveal substrate inhibition by homocysteine when the latter is present at high concentrations, and the serine concentration is low (<15 mM). This inhibition is alleviated at higher concentrations of serine. Slope and intercept replots of data from the Lineweaver−Burk analysis yield the following values for the kinetic parameters: $V_{max} = 1800 \pm 1200$ μmol h^{-1} mg^{-1}, $K_{m_{Ser}} = 14 \pm 3$ mM and $K_{m_{Hcy}} = 67 \pm 48$ mM, $K_{i_{HCy}} = 4.3 \pm 0.9$ mM (Table 1). The K_m for homocysteine under these conditions is too high to be determined accurately and leads to a correspondingly large uncertainty in the value of V_{max}.

When the enzyme is preincubated with serine, a different set of kinetic parameters is obtained. In the concentration range employed for homocysteine (7−40 mM), hyperbolic kinetics are observed (Figure 3). Secondary plots yield the following estimates for the kinetic parameters: $V_{max} = 750 \pm 330$ μmol h^{-1} mg^{-1}, $K_{m_{Hcy}} = 9.7 \pm 4.3$ mM and $K_{m_{Ser}} = 18 \pm 8$ mM, and $K_{i_{Ser}} = 2.9 \pm 1.1$ mM (Table 1). Again, the relatively high K_m for the substrates leads to uncertainty in the estimated value as well as in the extrapolated value for V_{max}. The combined kinetic data (Table 1) indicate that the order of substrate addition has a marked influence on the V_{max} of the reaction, with serine pretreatment resulting in a 2-fold lower rate.

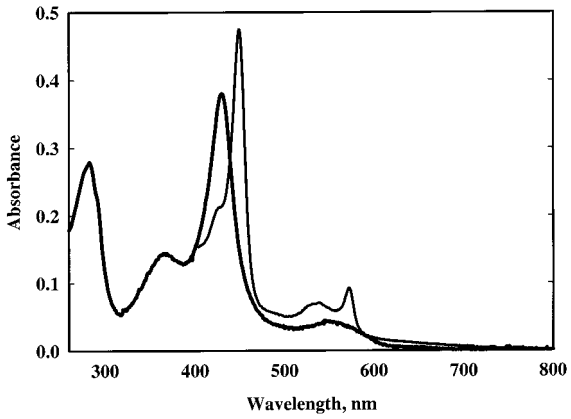


FIGURE 1: UV−visible spectrum of CBSΔC143 in the ferric (thick line) and ferrous (thin line) states. The reduced form was generated by addition of 1 mM titanium citrate to an anaerobic solution of enzyme (0.17 μg of protein) in 0.25 M Tris buffer, pH 8.6.

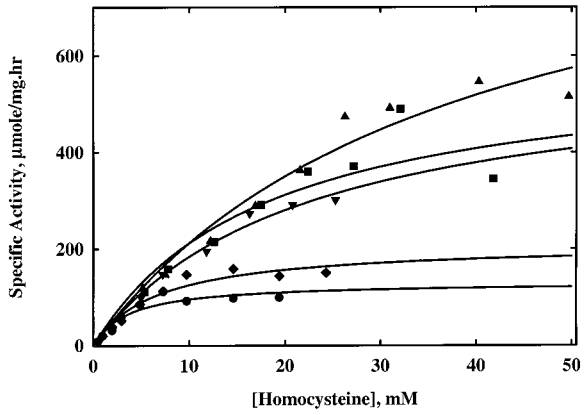


FIGURE 2: Steady-state initial velocity kinetics showing dependence of the reaction velocity on homocysteine concentrations at different fixed concentrations of serine. The symbols represent experimental data points whereas the lines were obtained from Michaelis−Menten fits to the data at each fixed concentration of serine. The concentrations of serine beginning from the lowermost line are 2.5, 3.75, 7.5, 11.3, and 15 mM.

Table 1: Summary of Kinetic Parameters of CBSΔC143 and Full-Length Enzyme

order of substrate addition	CBSΔC143 Hcy pretreatment	CBSΔC143 Ser pretreatment	full-length CBS Hcy pretreatment	full-length CBS Ser pretreatment[a]
V_{max} (μmol h^{-1} mg^{-1})	1800 ± 1300	750 ± 330	564 ± 315	295 ± 23
k_{cat} (s^{-1})	45 ± 33	19 ± 8	39 ± 22	21 ± 1.6
$K_{m_{Ser}}$ (mM)	14 ± 3	18 ± 8	5.5 ± 3.9	2 ± 0.3
$K_{m_{HCy}}$ (mM)	67 ± 48	9.7 ± 4.3	15 ± 8.4	5 ± 0.9
$k_{cat}/K_{m_{Ser}}$ (M^{-1} s^{-1})	3.2 × 10^3	1.1 × 10^3	7 × 10^3	10.5 × 10^3
$k_{cat}/K_{m_{HCy}}$ (M^{-1} s^{-1})	0.7 × 10^3	2 × 10^3	2.6 × 10^3	4.2 × 10^3
$K_{i_{Ser}}$ (mM)	−	2.9 ± 1.0	−	−
$K_{i_{HCy}}$ (mM)	4.3 ± 0.9	−	2.7 ± 1.2	−

[a] These values were taken from (*2*).

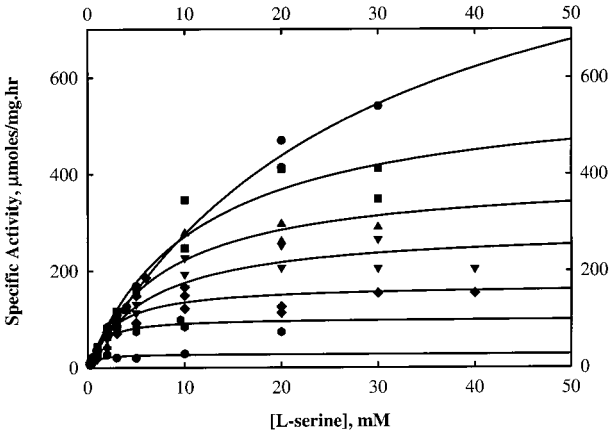


FIGURE 3: Steady-state initial velocity kinetics showing dependence of the reaction velocity on serine concentrations at different fixed concentrations of homocysteine. The symbols represent the experimental data points, and the lines represent fits to the data using the Michaelis−Menten equation at each fixed concentration of homocysteine. The concentrations of homocysteine beginning from the lowermost line are 2.3, 3.62, 7.24, 14.8, 23, 29.8, and 40 mM.

Steady-State Kinetics of Full-Length Cystathionine β-Synthase. The importance of the order of substrate binding on the reaction velocity of the truncated enzyme led us to examine its significance in the native enzyme. In the standard assay, the reaction is initiated by addition of homocysteine (*5*). Thus, the specific activities that have been reported previously have been measured under conditions of serine preincubation (*2*, *5*). The activity of the full-length enzyme, like that of the truncated form, is sensitive to the order of substrate addition. The V_{max} for the reaction increases from 295 ± 23 μmol h^{-1} mg^{-1} when the enzyme is premixed with serine (*2*) to 564 ± 315 μmol h^{-1} mg^{-1} when the enzyme is premixed with homocysteine (Table 1).

Serine Binding to CBSΔC143. PLP in CBSΔC143 is weakly fluorescent as in the full-length enzyme (*4*). The excitation spectrum shows a peak at 400 nm when the emission wavelength is set at 500 nm (Figure 4). This peak is assigned as the protonated internal aldimine. In contrast to the full-length form, the neutral enolimine tautomer of the aldimine (emission maximum 450 nm, excitation maximum 330 nm) is not detected in CBSΔC143. Addition of serine to the truncated enzyme results in enhancement of fluorescence emission in the 420−450 nm region and a corresponding quenching in the 500 nm peak. The 500 and 450 nm fluorescence emission peaks have been assigned previously as corresponding to the protonated aldimine and the neutral enolimine tautomer, respectively (*4*). In native enzyme, a new emission peak is observed at 400 nm upon addition of serine and is tentatively assigned as the aminoacrylate based on its excitation maximum (*4*). With CBSΔC143, the new fluorescence emission peak is red-shifted to 418 nm and has an excitation maximum at 328 nm (Figure 4A). Serine binding was also determined by equilibrium dialysis using [^{14}C]serine (Figure 5). Analysis of these data in an Eadie−Hofstee plot yielded $K_{d_{Ser}}$ = 22.9 ± 1.9 μM and n = 0.9 ± 0.05. Thus, one serine binds per dimer, and is consistent with two serines binding per tetramer (*4*). These data suggest that both the tetrameric and dimeric forms of the enzyme exhibit half-of-sites activity.

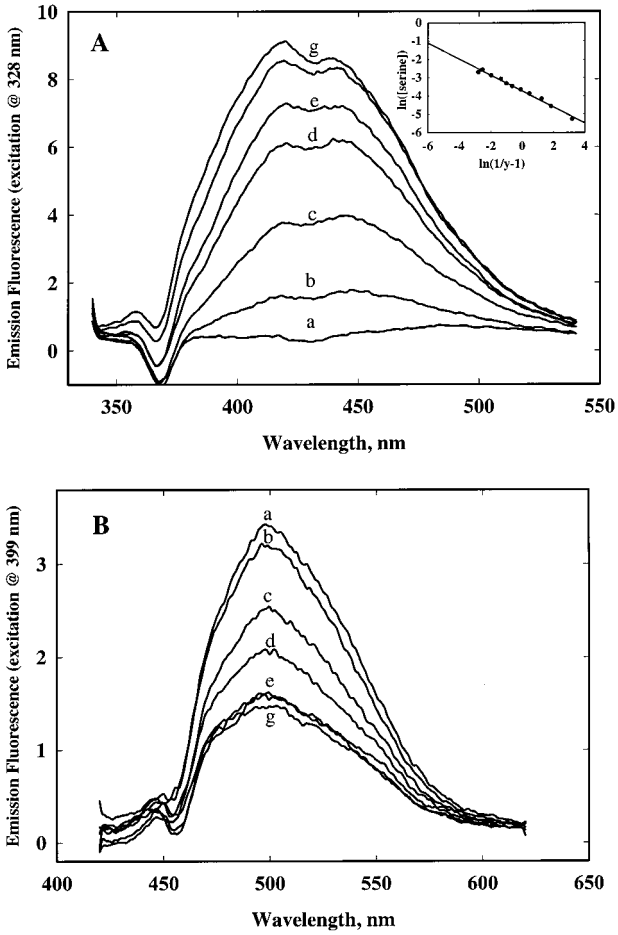


FIGURE 4: Fluorescence changes in PLP bound to CBSΔC143 induced by serine. The excitation wavelengths are indicated on the *y*-axes. (A) Enhancement of fluorescence emission by serine at 418 and 450 nm. The concentrations of serine in successive spectra are as follows: (a) 0 μM, (b) 10.4 μM, (c) 20.7 μM, (d) 31.1 μM, (e) 46.4 μM, (f) 66.8 μM, and (g) 87 μM. (B) Quenching of fluorescence emission by serine at 500 nm. The concentrations of serine in successive spectra are the same as in (A).

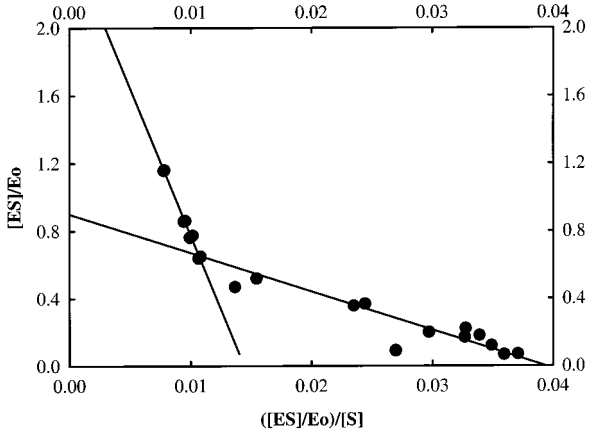


FIGURE 5: Eadie−Hofstee plot of equilibrium dialysis data for [^{14}C]-serine binding to CBSΔC143. The values obtained from this analysis are $K_{d_{Ser}} = 22.9 \pm 1.9$ μM and $n = 0.9 \pm 0.05$, where n is the stoichiometry of serine bound per dimer.

AdoMet Binding to Full-Length and Truncated Cystathionine β-Synthase. AdoMet has been reported to cause fluorescence quenching of full-length cystathionine β-synthase (*11*). To determine the K_d for AdoMet, we monitored quenching of fluorescence emission at 346 nm (excitation wavelength = 282 nm) using full-length and truncated enzymes (data not shown). Eadie−Hofstee analysis of these data provided excellent linear fits, yielding a K_d for AdoMet of 0.27 ± 0.02 mM for the full-length enzyme. However, AdoHCy also causes fluorescence quenching of the native enzyme, yielding an identical value for K_d. This is inconsistent with our observation that AdoHCy has no effect on cystathionine β-synthase activity under standard assay conditions, and argues against it binding to the enzyme as tightly as AdoMet. In addition, AdoMet caused fluorescence quenching when added to the dimeric enzyme, and analysis of these data yielded a very similar value for K_d (data not shown). This is surprising since AdoMet has no effect on the activity of CBSΔC143 (*12*). These discrepancies led us to suspect that the observed changes in the fluorescence spectra elicited by AdoMet and AdoHCy result from nonspecific interactions of the nucleotides with tryptophan residues in the protein.

The binding of AdoMet to cystathionine β-synthase was therefore studied by equilibrium dialysis using [^{3}H]AdoMet. Scatchard analysis of the data yielded nonlinear plots indicating cooperativity and provided a stoichiometry of 4 AdoMet molecules per tetramer (Figure 6). From a Hill plot (Figure 6, inset), the K_d for AdoMet was estimated to be 7.4 ± 0.2 μM with a Hill coefficient of 1.6 ± 0.1. In two independent determinations, no binding of radiolabeled AdoMet was observed to the dimeric enzyme. These results illustrate the importance of independent confirmation of protein fluorescence titration data before assignment of the changes to specific ligand−protein interactions is made.

Homology of CBS to Other Heme Proteins. PSI blast search using a query sequence extending from amino acids 200 through 350 revealed weak homologies with three heme proteins (Scheme 2). These included chick cytochrome P450 (sp P20678), sulfide dehydrogenase or flavocytochrome *c* (sp Q06530), and cytochrome *c*-550 precursor (sp P51199). All three sequences aligned to a region of the cystathionine β-synthase sequence ranging from residues 241 through 341.

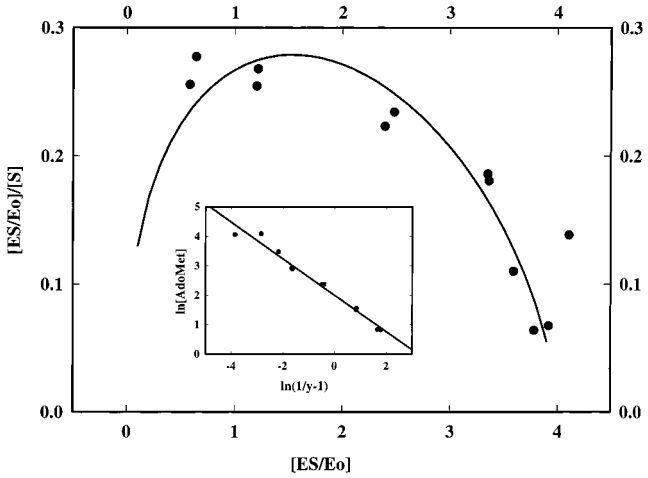


FIGURE 6: Binding of [^{3}H]AdoMet to CBSΔC143. A Scatchard plot of the data indicated curvature consistent with cooperative binding. The line represents a fit to the experimental data (circles) obtained using the following equation: $K^{\alpha H} = [S]^{\alpha H}[(n/\nu) - 1]$ where [S] is the concentration of free AdoMet, α_H is the Hill coefficient, $\nu = [ES]/[E_o]$, where [ES] and $[E_o]$ are the concentrations of enzyme-bound AdoMet and total enzyme, respectively, and n is the stoichiometry of bound AdoMet per tetramer, which is 4. Inset: Hill plot of the same data set. Values of 7.4 ± 0.2 μM for K_d and 1.6 ± 0.1 for α_H were obtained from the fits.

Within this limited region, the identities were 19% for flavocytochrome *c*, 25% for cytochrome P450, and 26% for cytochrome *c*-550 precursor, and the homologies varied from 40% to 42%. No other region in the cystathionine β-synthase sequence was found to be homologous to any heme proteins. Based on this sequence alignment, the region extending from residues 241 to 341 in cystathionine β-synthase is predicted to be important for heme binding.

DISCUSSION

The monomeric subunit size of cystathionine β-synthase is a mere 63 kDa within which the determinants for binding of the cofactors, PLP and heme, the substrates, homocysteine and serine, and the allosteric effector, AdoMet, are apparently encoded. Despite the central importance of this enzyme to homocysteine metabolism, little is known about its organization or its reaction mechanism. In this study, we have characterized the kinetic properties of an active core of cystathionine β-synthase lacking the C-terminal 143 amino acid residues. This has allowed us to assign functions to individual regions of the protein.

The C-terminal domain of cystathionine β-synthase has a profound influence on the organization and activity of the enzyme as judged by the loss of functions correlated with the loss of this domain. Thus, elimination of the C-terminal domain results in the oligomerization state changing from tetrameric to dimeric. Hence, this region of the protein appears to be involved in the tetramerization of the native enzyme. Also lost is the responsiveness of the enzyme to AdoMet (*11, 12*), which coincides with the lack of binding of this effector to CBSΔC143. This C-terminal region encompasses the so-called CBS domain which is predicted to adopt a $\beta-\alpha-\beta-\beta-\alpha$ fold and is conserved in proteins from organisms ranging from eubacteria to archae and humans (*18, 19*). It has been identified in a diverse group of proteins including inosine monophosphate dehydrogenase,

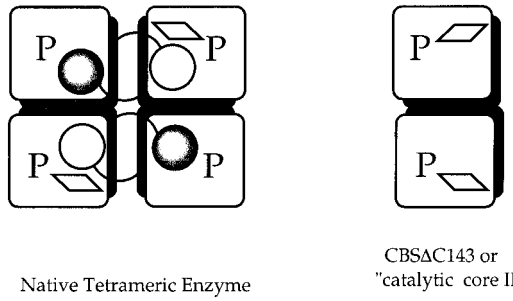
Scheme 2: Sequence Homology between Human Cystathionine β-Synthase and Three Heme Proteins[a]

```
HCBS:          LDMLVASVGTGGTITGIARKLKEKCPGC---------RIIGVDPEGSILAEPEELNQTEQTTYEVEGIGYD
CytC550:                                                VGLDPESLGLATPQRDNIEALVDYMKDPTSYD
CytP450:       LDLFLAGTGTTSTTLRYGLLILLKHPEIEEKMHKEIDRVVGRD-RSPCMADRSQLPYTDAVIHEIQRF-ID
SD:              VVVVGGGTGGAT--AAKYIKLADPSIEVTLIEP
                 LD•-VA--GTGG-----A--•K-K-P----------R••G-DPE---LA--P--LN-T----YE-•---YD


HCBS:          -------FIPTVLDRTVVDKWFKSNDEEAFTFARMLIAQ
CytC550:     GAESIAELHPSIKSAEIFPKMRNLTDEDLFTIAGHILLQ
CytP450:             FLPVNLPRAVI
                     F•P--L-R-V--K-----DE•-FT-A--••-Q
```

[a] SD is sulfide dehydrogenase. The residues that are identical between cystathionine β-synthase and one of the three heme protein sequences are indicated in the bottom line; the conservative changes are indicated by dots.

Scheme 3: Postulated Organization of the Native Tetrameric and Truncated Dimeric Forms of Cystathionine β-Synthase[a]



[a] Although alternative organizations of the dimeric units in the tetramer are possible as are different relative orientations of the N- and C-terminal domains, only one is presented here for clarity.

voltage-gated chloride channels, 5′-AMP-activated protein kinase, and ABC transporters. While its role in these proteins is not known, it has been postulated to provide binding sites for effector molecules and/or to play a targeting role during compartmental localization. The CBS domain in human cystathionine β-synthase extends between residues 421 and 469. Based on the loss of AdoMet binding to CBSΔC143, we predict that this region is involved in AdoMet binding in the native enzyme (Scheme 1). This is consistent with the loss of AdoMet regulation by a point mutation in this region, D444N, described in a homocystinuric patient (*20*). Four AdoMet molecules bind in a cooperative fashion to the full-length tetrameric enzyme.

In the absence of the C-terminal domain, the heme to PLP stoichiometry is 1:1. This is in contrast to a 1:2 stoichiometry in the native enzyme. This puzzling observation is reconciled in a tentative model proposed in Scheme 3, in which tetramerization is predicted to result in the loss of one of the two hemes from each dimer, and leads to the half-of-sites activity reported for the full-length enzyme (*4*). While the orientations of and interactions between the N- and C-terminal domains in the dimer and tetramer interfaces are unknown, it is interesting to note the potential similarity between the organization of cystathionine β-synthase and a related PLP-dependent enzyme, threonine deaminase. The crystal structure of the latter enzyme reveals a modular organization of the N- and C-terminal domains that house the catalytic and regulatory functions, respectively, and are separated by a thin neck formed by an α-helix (*21*).

The steady-state kinetic properties of CBSΔC143 are distinctly different from those of the native enzyme. The K_m's for both substrates are high relative to those for the native enzyme (Table 1), making saturation conditions difficult to achieve. This accounts for the high standard deviations in the estimated values for V_{max} and K_m for the respective substrates. This is in contrast to the kinetics of the catalytic core I in which the K_m's for the two substrates are reported to be identical to that of the native enzyme, although no primary kinetic data were shown (*11*). If confirmed, the differences in the kinetic properties between the full-length protein, catalytic core I, and CBSΔC143 would be narrowed to interactions between the 29−38 N-terminal residues and the C-terminus. In catalytic core I, heterogeneity at the N-terminus results from multiple cleavage sites at lysines 30, 36, and 39. An extreme example of the kinetic properties of an enzyme being modulated by its N-terminus is glycine *N*-methyltransferase, in which loss of the N-terminal acetyl group results in the kinetic behavior of the enzyme changing from hyperbolic to sigmoidal (*22*).

Despite the high K_m's for the substrates which are in a nonphysiological concentration range, significant enzyme activity is observed under standard assay conditions due to the higher V_{max} for the truncated enzyme (Table 1). In fact, the $k_{cat}/K_{m_{Ser}}$ and $k_{cat}/K_{m_{HCy}}$ for CBSΔC143 are only 2−10-fold lower than for the native enzyme.

Both the full-length and truncated enzymes are sensitive to the order of substrate addition. Under standard assay conditions, the reaction is initiated by addition of homocysteine (*2, 5*). The V_{max} of the reaction is 2-fold lower under these conditions versus when homocysteine is added first. Serine binds to both native and CBSΔC143 forms of cystathionine β-synthase with a low K_d [7.5 μM for full-length (*4*) and 23 μM for the truncated form], leading to a binary complex whose formation can be followed fluorometrically and by equilibrium dialysis. It is interesting to note that the K_d for serine in the binary complex is significantly lower than the K_i (2.9 ± 1 mM) obtained under turnover conditions (Table 1). Kinetic evidence, i.e., lower V_{max} when enzyme is preincubated with serine, suggests the enzyme may display hysteretic behavior.

Sequence comparisons of cystathionine β-synthase with proteins in the Swiss protein database reveal homology with other PLP-dependent enzymes that belong to the fold type II family (*23*). High-resolution structures for two members of this family, threonine deaminase (*21*) and tryptophan synthase (*24*), are available so far. The folding patterns of the N-terminal catalytic domains are virtually identical in both proteins. This leads to the prediction that the N-terminal

regions of cystathionine β-synthase extending from residues 68 through 209, which is homologous to the PLP binding domains of tryptophan synthase and threonine deaminase, will share a similar fold.

In contrast to the obvious sequence homologies between cystathionine β-synthase and other PLP enzymes, its relation to heme proteins is not obvious and has not been reported so far. It is therefore exciting that a PSI blast search reveals homologies with three heme proteins. Although the identity between any given pair of sequences is low, it is compelling because all three sequences are homologous to the same region of cystathionine β-synthase extending from residues 241 through 341. Based on the homologies presented in Scheme 2, we posit that this region is involved in heme binding in cystathionine β-synthase.

Conclusions. Kinetic, spectroscopic, and ligand binding studies of the catalytic core, CBSΔC143, have allowed us to attribute specific functions to different regions of the cystathionine β-synthase polypeptide (Scheme 1). In addition, our kinetic studies demonstrate the importance of the order of substrate addition to the steady-state behavior of the enzyme. Preincubation of the enzyme with serine results in the formation of a binary complex, but under these conditions the turnover rate is reduced ~2-fold for both the full-length and the truncated enzymes. Since the intracellular concentration of serine is significantly greater than that of homocysteine, the physiological significance of this phenomenon to the regulation of enzyme activity needs to be considered. The K_ms for both substrates are very high in the truncated enzyme compared to the full-length form. A mutation in cystathionine β-synthase leading to a stop codon at serine 420 has been described in a homocystinuric patient (*13*). The resulting protein has not been characterized, but its properties may be expected to be similar to CBSΔC143 described in this study.

REFERENCES

1. Refsum, H., Ueland, P. M., Nygard, O., and Vollset, S. E. (1998) *Annu. Rev. Med. 49*, 31−62.
2. Taoka, S., Ohja, S., Shan, X., Kruger, W. D., and Banerjee, R. (1998) *J. Biol. Chem. 273*, 25179−25184.
3. Kery, V., Poneleit, L., Meyer, J. D., Manning, M. C., and Kraus, J. P. (1999) *Biochemistry 38*, 2710−2724.
4. Taoka, S., West, M., and Banerjee, R. (1999) *Biochemistry 38*, 2738−2744.
5. Kraus, J., Packman, S., Fowler, B., and Rosenberg, L. E. (1978) *J. Biol. Chem. 253*, 6523−6528.
6. Kraus, J. P., and Rosenberg, L. E. (1983) *Arch. Biochem. Biophys. 222*, 44−52.
7. Skovby, F., Kraus, J. P., and Rosenberg, L. E. (1984) *J. Biol. Chem. 259*, 588−593.
8. Skovby, F., Kraus, J. P., and Rosenberg, L. E. (1984) *J. Biol. Chem. 259*, 583−587.
9. Mudd, S. H., Finkelstein, J. D., Irreverre, F., and Laster, L. (1964) *Science 143*, 1443−1445.
10. Uhlendorf, R. B., and Mudd, S. H. (1968) *Science 160*, 1007−1009.
11. Kery, V., Poneleit, L., and Kraus, J. (1998) *Arch. Biochem. Biophys. 355*, 222−232.
12. Shan, X., and Kruger, W. D. (1998) *Nat. Genet. 19*, 91−93.
13. Coude, M., Aupetit, J., Zabot, M. T., Kamoun, P., and Chadefauz-Vekemans, B. (1998) *J. Inherited Metab. Dis. 21*, 823−828.
14. Drummond, J. T., Jarrett, J., Gonzalez, J. C., Huang, S., and Matthews, R. G. (1995) *Anal. Biochem. 228*, 323−329.
15. Srivastava, S. K., and Beutler, E. (1973) *Biochim. Biophys. Acta 304*, 765−773.
16. Jones, J. B. J. (1977) *Commun. Soil. Sci. Plant Anal. 8*, 349−365.
17. Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) *Nucleic Acids Res. 25*, 3389−3402.
18. Pointing, C. P. (1997) *J. Mol. Med. 75*, 160−163.
19. Bateman, A. (1997) *Trends Biochem. Sci. 22*, 12−13.
20. Kluitjmans, L. A. J., Boers, G. H. J., Stevens, E. M. B., Renie, W. O., Kraus, J. P., Trijbels, F. J. M., Heuvel, L. P. W. J. v. d., and Blom, H. J. (1996) *J. Clin. Invest. 98*, 285−289.
21. Gallagher, D. T., Gilliland, G. L., Xiao, G., Zondlo, J., Fisher, K. E., Chinchilla, D., and Eisenstein, E. (1998) *Structure 6*, 465−475.
22. Ogawa, H., Gomi, T., Takata, Y., Date, T., and Fujioka, M. (1997) *Biochem. J. 327*, 407−412.
23. Grishin, N. V., Phillips, M. A., and Goldsmith, E. J. (1995) *Protein Sci. 4*, 1291−1304.
24. Hyde, C. C., and Miles, E. W. (1988) *J. Biol. Chem. 263*, 17857−17871.

BI990865T