Regulation of Human Cystathionine β-Synthase by *S*-Adenosyl-L-methionine: Evidence for Two Catalytically Active Conformations Involving an Autoinhibitory Domain in the C-Terminal Region†

Miroslav Janošík, Vladimír Kery, Mette Gaustadnes, Kenneth N. Maclean, and Jan P. Kraus*

Department of Pediatrics, University of Colorado School of Medicine, Denver, Colorado 80262

Received April 6, 2001; Revised Manuscript Received June 18, 2001

ABSTRACT: Cystathionine β-synthase (CBS), condensing homocysteine and serine, represents a key regulatory point in the biosynthesis of cysteine via the transsulfuration pathway. Inherited deficiency of CBS causes homocystinuria. CBS is activated by *S*-adenosyl-L-methionine (AdoMet) by inducing a conformational change involving a noncatalytic C-terminal region spanning residues 414−551. We report the purification of two patient-derived C-terminal mutant forms of CBS, S466L and I435T, that provide new insight into the mechanism of CBS regulation and indicate a regulatory function for the "CBS domain". Both of these point mutations confer catalytically active proteins. The I435T protein is AdoMet inducible but is 10-fold less responsive than wild-type (WT) CBS to physiologically relevant concentrations of this compound. The S466L form does not respond to AdoMet but is constitutively activated to a level intermediate between those of WT CBS in the presence and absence of AdoMet. Both mutant proteins are able to bind AdoMet, indicating that their impairment is related to their ability to assume the fully activated conformation that AdoMet induces in WT CBS. We found that I435T and WT CBS can be activated by partial thermal denaturation but that the AdoMet-stimulated WT, S466L, and a truncated form of CBS lacking the C-terminal region cannot be further activated by this treatment. Tryptophan and PLP fluorescence data for these different forms of CBS indicate that activation by AdoMet, limited proteolysis, and thermal denaturation share a common mechanism involving the displacement of an autoinhibitory domain located in the C-terminal region of the protein.

Cystathionine β-synthase (EC 4.2.1.22, CBS)[1] catalyzes a pyridoxal 5′-phosphate (PLP)-dependent β-replacement reaction condensing homocysteine (Hcy) and serine (Ser) to form cystathionine. This reaction represents the first committed step in the biosynthesis of cysteine from methionine by transsulfuration (*1*). CBS forms homotetramers of 63-kDa subunits (*2*−*5*) that may associate in solution into large multimers of undefined size (*6*). In addition to binding PLP, mammalian CBS also binds heme (*7*). Although the function of this heme group is currently unknown, recent work has indicated that it is not directly involved in the catalytic mechanism (*4*, *5*, *8*). Mammalian CBS is activated 2.5−5-fold by *S*-adenosyl-L-methionine (AdoMet) (*9*, *10*) with an apparent dissociation constant of 15 μM (*11*, *12*). Recent work in our laboratory has demonstrated that binding of AdoMet to human CBS induces a change in the CBS fluorescence spectra. This finding indicates that AdoMet binding to CBS is accompanied by a conformational change (*13*). An active core of the human CBS enzyme, derived by limited trypsinolysis and consisting of residues 37−413, forms well-defined dimers in solution. This active core is about twice as active as the native full-length enzyme but is not further activated by AdoMet. Detailed characterization of the trypsin-cleaved forms of CBS suggested that the region responsible for CBS activation and for the formation of tetramers in solution is located somewhere in the last 137 C-terminal residues (*13*, *14*). Recently, we have generated a human CBS deletion mutant that lacks these residues and, as such, constitutes a model of the protease-activated form of CBS. This truncated CBS protein, designated Δ414−551, has been expressed in *Escherichia coli*, purified to homogeneity, and crystallized (*15*, *16*).

Homocystinuria due to CBS deficiency is accompanied by a variety of clinical and pathological abnormalities (*1*). Four organ systems show major involvement: the ocular, skeletal, vascular, and central nervous systems. Close to 400 homocystinuric alleles have been studied worldwide, and over 100 pathogenic mutations in the CBS gene have been described (*17*). (For a continuously updated list, see the CBS website at www.uchsc.edu/sm/cbs.) The vast majority of human CBS mutations are missense mutations that significantly diminish CBS activity. Interestingly, a number of patient-derived mutations in the CBS catalytic region can be functionally suppressed by either deletion of the C-terminal regulatory region or specific point mutations within

† This work was supported by NIH Grants PO1HD0805 and HL65217 to J.P.K.

* To whom correspondence should be addressed: Department of Pediatrics, Box C-233, University of Colorado Health Sciences Center, 4200 E. 9th Ave., Denver, CO 80262. Tel: 303-315-7858; fax: 303-315-8080; e-mail: Jan.Kraus@uchsc.edu.

[1] Abbreviations: AdoMet, *S*-adenosyl-L-methionine; CBS, cystathionine β-synthase; DTT, dithiothreitol; PBS, phosphate-buffered saline; PLP, pyridoxal 5′-phosphate; TBS, Tris-buffered saline; Hcy, homocysteine; PAGE, polyacrylamide gel electrophoresis; WT, wild type; Ser, serine.

10.1021/bi010711p CCC: $20.00 © 2001 American Chemical Society
Published on Web 08/08/2001

this region (*18*, *19*). The mechanism by which deletion of the C-terminal region or mutation therein restores CBS activity to catalytic domain CBS mutants is currently unknown. The C-terminal regulatory region spans approximately 140 amino acid residues and contains a 53 amino acid motif known as the CBS domain (spanning residues 415−468), which is found in a wide variety of functionally unrelated proteins (*20*). The majority of CBS mutations reported to date have been found in the catalytic region of the protein. Classically, these CBS mutations have been divided into two groups based on patient response to treatment with pyridoxine (*21*, *22*). Recent work in our laboratory has identified a novel class of patient-derived CBS mutations in the CBS domain located in the C-terminal portion of the protein. Heterologous expression of the I435T, P422L, and S466L CBS mutants in both *E. coli* and transfected hamster fibroblasts indicated that these mutants are highly active but could not be induced to higher levels of activity by AdoMet.[2] Previously, the only mutation in this region of CBS that has been examined and characterized in detail by expression analysis is D444N, which was identified in a partially pyridoxine-responsive homocystinuric patient (*23*). The inability of D444N to respond to AdoMet indicates that it also belongs in this class of mutations. The behavior of all of these proteins in cell extracts indicated that this class of mutations could be further subdivided on the basis of activity. Mutations I435T and D444N appeared to have activity roughly equivalent to that of the wild-type (WT) protein assayed in the absence of AdoMet induction. The P422L and S466L forms appeared to have similar levels of activity to the AdoMet-induced WT CBS.[2] These findings lead to the hypothesis that there are two active conformations of CBS with correspondingly different levels of catalytic activity. In this work, we describe the subcloning, expression, and purification of the I435T and S466L mutant forms of human CBS. Comparison of the physical properties of these C-terminal mutant proteins with those of the full-length and truncated WT CBS sheds new light on the mechanism of CBS activation by proteolytic cleavage and AdoMet binding. Taken together, our data indicate that a portion of the C-terminal region of human CBS, located at least in part around the "CBS domain", has an autoinhibitory function. Limited proteolytic digestion, AdoMet binding, or partial thermal denaturation serve to upregulate CBS by displacing this domain from its site of inhibition.

MATERIALS AND METHODS

Chemicals. Unless otherwise stated, all chemicals were obtained from Sigma. L- [U-^{14}C]Ser was obtained from NEN Life Science Products.

Construct Preparation. Previously, we had generated I435T and S466L expression constructs by replacing restriction fragment length portions of a WT CBS cDNA expression construct (*11*) with the equivalent restriction fragment isolated from the relevant mutant cDNA.[2] The I435T and S466L mutant coding sequences were subcloned from these *E. coli* expression constructs into a previously described pGEX5X-1-hCBS expression vector (*15*) as *Sph*I and *Kpn*I restriction fragments. The vector-insert junctions and the entire coding sequence were verified 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 the I435T and S466L Mutant Proteins. These forms of CBS were all expressed as fusion proteins with glutathione *S*-transferase (GST) at their NH_2 termini. An endoproteinase Xa-cleavable spacer separated the fusion partners. To enhance binding of the recombinant fusion protein to a glutathione-Sepharose column, an additional 23 amino acids were engineered between the GST tag and the CBS coding sequence. Typically, an overnight culture of *E. coli* (XL1-Blue MR, Stratagene) was resuspended at a 1:200 v/v ratio into 6 L (×1) of NZCYM broth (Life Technologies) in Fernbach flasks containing 100 μg/mL ampicillin and 0.3 mM δ-aminolevulinic acid. The expression of the CBS−GST fusion protein was induced at an OD_{600} of approximately 0.6 by the addition of IPTG to a final concentration of 0.1 mM. After induction, the cells were grown for a further 16 h at 37 °C. Cell extracts were obtained by a combination of lysozyme digestion (2 mg/g wet weight of cells) for 1 h at 4 °C and subsequent sonication for 10 min on ice in Tris-HCl buffer, pH 8.0, containing 1% Triton X-100 and 2 mM DTT (3:1 v/w of cells). Cell extracts were then cleared by centrifugation for 20 min at 18000*g* followed by centrifugation at 40000*g* for 1 h. The heme absorbance at 428 nm was used to estimate the amount of recombinant CBS protein in the cell extract (*13*). Glutathione-Sepharose (Amersham Pharmacia Biotech) was then added to the cell extract at a ratio of 1 mL of Sepharose to 5 mg of CBS fusion protein. The suspension was then rocked for 30 min at room temperature. The beads were washed in a column (1.5 × 10 cm) with 5× the column volume of 20 mM potassium phosphate buffer, pH 7.0, containing 0.5 M NaCl and 5 mM DTT. Finally, the column was eluted with 30 mL of freshly prepared 20 mM reduced glutathione in 50 mM Tris-HCl buffer at pH 8.0. The fusion protein was then cleaved with endoprotease Xa (New England Biolabs) at a working concentration of 7.5−15 μg/mg of the fusion protein overnight at 4 °C. The digest was dialyzed against PBS containing 2 mM DTT for 2 h (×2) and then loaded onto a secondary glutathione affinity column to remove the cleaved GST tag and the uncleaved fusion protein. The proteins were further purified on a Sephacryl S200 HR size-exclusion column equilibrated with Tris-buffered saline (TBS), pH 8.6.

Enzyme Characterization. Saturation of the enzyme preparations with PLP was determined by the fluorimetric method of Adams (*24*). The heme content was determined using the previously described pyridine-hemochromogen method (*25*). UV−vis spectra were measured on a Hewlett-Packard diode array model 8453 UV−vis spectrophotometer. Measurements of tryptophan fluorescence were performed at the excitation wavelength of 295 nm on a Shimadzu RF-5301 PC spectrofluorimeter using excitation and emission slits of 5 nm with samples in TBS, pH 8.6, at room temperature. All CBS concentrations for tryptophan fluorescence measurements were adjusted to $A_{428} = 0.05$, corresponding to a protein concentration of 0.03 mg/mL. For PLP fluorescence measurements, the A_{428} was adjusted to 0.5, corresponding to a protein concentration of 0.3 mg/mL. The excitation wave-

[2] Unpublished results.

lengths used were 295 and 420 nm for tryptophan and PLP fluorescence, respectively. Specific activities of the various forms of CBS were measured in the presence of 10 mM concentrations of both Hcy and Ser as previously described (*26*). Steady-state kinetic parameters of the WT and mutant enzymes were measured as described previously (*13*), using a range of concentrations between 0.05 and 0.1 mg/mL of each enzyme in the reaction mix for 4, 8, and 16 min at 37 °C. The concentration of Ser for Hcy kinetics was kept at a constant value of 10 mM, while the concentration of Hcy for Ser kinetics was 5 mM.

Native Molecular Size Determinations. Native molecular sizes of the purified proteins were determined by high-pressure size-exclusion chromatography using a Phenomenex SEC S3000 column (Phenomenex, Torrance, CA) equilibrated with PBS, pH 7.4, at a flow rate of 1 mL/min. Alternatively, we employed low-pressure size-exclusion chromatography using Sephacryl S300 HR equilibrated with TBS, pH 8.6. Protein standards such as thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), chicken ovalbumin (44 kDa), equine myoglobin, (17 kDa), and vitamin B-12 (1.35 kDa) were obtained from Bio-Rad and were used as native protein standards to calibrate the size-exclusion columns.

AdoMet Activation of CBS. The activation of CBS by AdoMet was measured by assaying for CBS activity at a number of AdoMet concentrations between 0 and 1200 μM. The concentration of AdoMet in the stock solution was determined at 260 nm using the extinction coefficient of 15.4 mM^{-1} cm^{-1}.

AdoMet Binding. AdoMet binding to CBS was measured by a novel filter-based assay. Briefly, CBS enzyme (7−15 μM) in TBS, pH 8.6, was preincubated with various concentrations of unlabeled AdoMet mixed with 1 μL of [^{14}C]AdoMet (0.025 μCi) (Amersham Pharmacia Biotech) per tube at room temperature for 10 min in a total volume of 12 μL. Subsequently, 10 μL of the assay mix was loaded onto a Millipore HAWP 02500 filter membrane and washed with 1 mL of cold TBS, pH 8.6, under gentle vacuum. Bound AdoMet was counted in a scintillation counter, and the amount of free/bound AdoMet was calculated.

Thermal Denaturation. CBS was diluted to a final concentration of 0.1 mg/mL in TBS, pH 8.6. The enzyme solutions were heated directly in a 50-μL quartz cuvette (Helma), using a Peltier temperature controller connected to a Hewlett-Packard diode array model 8453 UV−vis spectrophotometer. The temperature was raised from 35 to 70 °C in 0.5 °C increments at 1-min hold intervals. Enzyme samples (10 μL) were taken at different temperatures and chilled on ice, and the CBS activity was subsequently determined.

RESULTS

Purification and Characterization of WT and Mutant CBS. Purification of the full-length WT, I435T, and S466L mutant CBS proteins was performed using a slightly modified version of our previously published procedure (*15*). For all three of these forms of CBS, the level of recombinant protein in *E. coli* was approximately 5% of total protein (15 mg/L of cell culture). Although we ultimately obtained the proteins at greater than 95% homogeneity as judged by SDS−PAGE (result not shown), we observed relatively poor yields

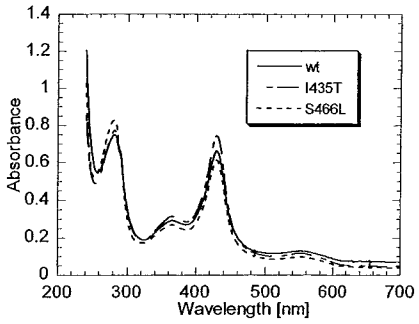

FIGURE 1: UV−vis spectra of WT and the I435T and S466L mutant forms of human CBS measured in TBS, pH 8.6, at 25 °C. Concentrations of all proteins were adjusted to 0.4 mg/mL.

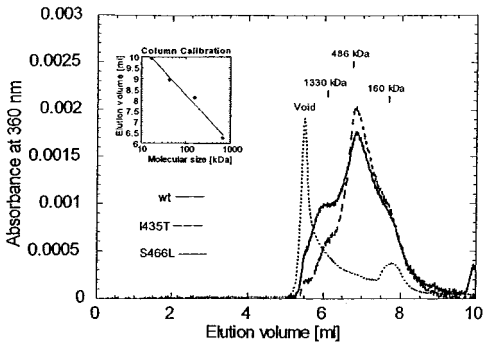

FIGURE 2: Size-exclusion HPLC chromatography of WT and the I435T and S466L mutant forms of human CBS. The purified proteins were run on a SEC 3000 column equilibrated in TBS, pH 8.6, at 25 °C at a flow rate of 1 mL/min. The amount of protein loaded was 10 μg. Inset shows the calibration of the column using defined protein standards.

(<15%). The low yields observed were primarily due to a strong tendency of all three of the full-length forms of CBS toward aggregation and surface adsorption. We determined that the WT and mutant enzymes were about 70−80% saturated with the PLP and heme cofactors as described previously (*7*). These findings were subsequently confirmed by analysis of the UV−vis spectra of the proteins, where similar ratios of absorbance at 428 to 280 nm were observed (Figure 1). The failure of either the I435T or the S466L mutations to induce any shift in the visible region absorption spectra, as compared to WT CBS, indicates that neither mutation induces any changes in the electrostatic environment of the heme or PLP group.

Native Size. The native sizes of the purified enzymes were determined by high-pressure size-exclusion chromatography (HPLC). Interpretation of these data was complicated by the tendency of both the WT and the mutant forms of CBS to aggregate into a number of different complexes (Figure 2). WT CBS is distributed between large assemblies of a 1330-kDa multimer and a 486-kDa octamer with a shoulder at 160 kDa. The I435T mutant protein has a similar native size distribution to the WT protein, while the S466L mutant protein elutes from the size-exclusion column as a sharp peak in the void volume. This latter finding indicates that the S466L protein is not a dimer as might have been predicted, given that it has catalytic and regulatory properties that are similar to the dimeric form of CBS generated by limited protease digestion (*13*).

Enzyme Kinetics. To further characterize the purified proteins, we measured steady-state kinetics for WT CBS and

Table 1: Characterization of WT and Mutant CBS Activities

		Hcy				Ser			
Enzyme	AdoMet	K_m (mM)	V_{max} (U/mg)	k_{cat} (s^{-1})	k_{cat}/K_m ($\times 1000$)	K_m (mM)	V_{max} (U/mg)	k_{cat} (s^{-1})	k_{cat}/K_m ($\times 1000$)
WT	−	0.17	354	6.2	36.8	4.9	310	5.4	1.1
	+	0.08	1946	34	425	3.6	1121	19.7	5.5
I435T	−	0.1	339	5.9	59	4.2	339	5.9	1.4
	+	0.2	1838	32.1	150	4.5	905	15.8	3.5
S466L[a]	−	0.05	883	15.5	310	7.2	835	14.6	2

[a] The S466L mutant showed absolutely no response to the addition of 1 mM AdoMet. Thus, the kinetic data for this mutant only include values obtained in the absence of AdoMet.

three different C-terminal mutant proteins in the presence and absence of 1 mM AdoMet. Previous work has indicated that this level of AdoMet is effectively a saturating concentration (*11*, *12*). While WT CBS and the I435T mutant can be activated up to 5-fold in the presence of AdoMet, the S466L mutant exhibits no AdoMet activation. Table 1 summarizes the steady-state kinetic data calculated using Lineweaver−Burk plots. We did not observe any significant effect upon the K_m values determined for either Ser or Hcy due to either the I435T or the S466L mutations. However, the k_{cat} values for both substrates, representing the turnover number per catalytic site, indicated significant differences between these enzymes. The I435T k_{cat} value determined for both Ser and Hcy in the absence of AdoMet is almost identical to the value determined for WT CBS. More significantly, the Hcy k_{cat} values for these two enzymes are significantly increased by the presence of saturating concentrations of AdoMet. It is interesting to note that there is a significant difference between the k_{cat} values determined for Hcy and Ser for both WT and I435T in the presence of AdoMet. It has been noted previously that Hcy at concentrations greater than 2 mM can inhibit CBS activity, and such an effect would provide some explanation for the observed differences in k_{cat} values. However, why such an inhibitory effect should only be visible in the presence of AdoMet is unknown and is currently the subject of further investigation in our laboratory. Consideration of the observed k_{cat}/K_m ratios, which represent an index of the catalytic efficiency of the enzymes, reveals a subtle impairment in the response of the I435T form to AdoMet as compared to the WT in terms of catalytic efficiency. The addition of AdoMet induces an approximate 2−3-fold increase in the k_{cat}/K_m value determined for the I435T form as compared to an 11-fold increase for WT CBS. The S466L form has a k_{cat} value of approximately 15 that is intermediate between the values for the WT and I435T CBS measured in the absence ($k_{cat} \sim 6$) and the presence of AdoMet ($k_{cat} > 30$ for Hcy). On this basis, the mutant S466L appears to be already activated in the absence of AdoMet, and the addition of AdoMet is unable to induce any further activation. However, it should be noted that the S466L form is not as active as the AdoMet-induced WT.

Mutant I435T Is Activated by AdoMet with Decreased Cooperativity as Compared to the WT CBS. To further investigate the AdoMet activation of WT CBS and the I435T mutant, we measured AdoMet activation curves in the presence of 10 mM Ser and Hcy. Figure 3 shows that the response of I435T to AdoMet is significantly impaired. Both proteins exhibit cooperativity in AdoMet activation represented by a nonresponsive phase at the lower concentrations

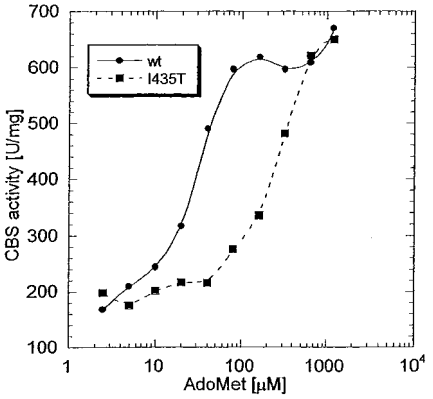

FIGURE 3: AdoMet activation of WT and the I435T mutant form of human CBS. The enzymes (0.1 mg/mL) were assayed in a reaction mix consisting of 10 mM Ser and 10 mM Hcy (in TBS, pH 8.6) containing different concentrations of AdoMet. AdoMet concentration was determined at 260 nm using the extinction coefficient 15.4 mM^{-1} cm^{-1}.

of AdoMet. This nonresponsive phase extends to 100 μM for I435T as compared to about 10 μM for WT CBS. Similarly, maximal activation for the WT was achieved at about 100 μM AdoMet, while the mutant required the presence of 1 mM AdoMet to reach the same activity. Consequently, at lower concentrations of AdoMet, the I435T mutant appears to be 10-fold less sensitive to the activating effects of this compound. This reduced response for AdoMet is reflected in the Michaelis−Menten constants, K_{act} for AdoMet activation, calculated by nonlinear regression as being 17.7 ± 3.6 and 163 ± 30 μM for the WT and the I435T mutant, respectively.

AdoMet Binding to CBS Is Not Cooperative. We measured direct binding of AdoMet to the purified enzymes using a novel filter-based assay, described in the Materials and Methods. These data were then analyzed using Scatchard plots to determine that the WT, I435T, and S466L forms of CBS have similar dissociation constants for AdoMet binding of 13.5, 22.7, and 8.9 μM, respectively (data not shown). A linear plot of bound AdoMet per mole of CBS subunit against the concentration of free AdoMet (Figure 4 inset) indicated that the WT, I435T, and S466L forms of CBS all bind one molecule of AdoMet per subunit. This finding indicates that the lack of AdoMet regulation observed for the S466L mutant is not due to this mutation, inducing structural changes that prevent AdoMet binding. There is no sigmoid pattern apparent on the binding curves, indicating a lack of cooperativity in terms of AdoMet binding to all three forms of CBS. This finding was consistent with the Hill plots generated from the same data (Figure 4). Here, the data exhibit greater than 99% linearity with slopes close to one.

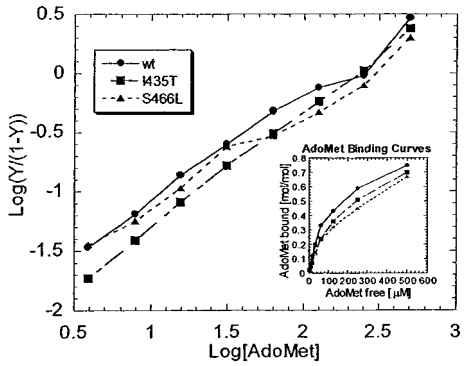

FIGURE 4: Hill plots of AdoMet binding to WT and the I435T and S466L mutant forms of CBS. The enzymes (7 μM) were mixed with AdoMet in 12 μL of TBS, pH 8.6, at 25 °C containing [^{14}C]AdoMet (0.025 μCi). After 10 min, 10 μL of the reaction mixture was loaded onto a membrane filter and washed with 1 mL of ice cold TBS under gentle vacuum. The amount of bound ligand was calculated from radioactivity retained on the filter using liquid scintillation. The inset shows the linear dependence between AdoMet concentration and AdoMet bound to CBS expressed in moles of AdoMet per mole of CBS subunit.

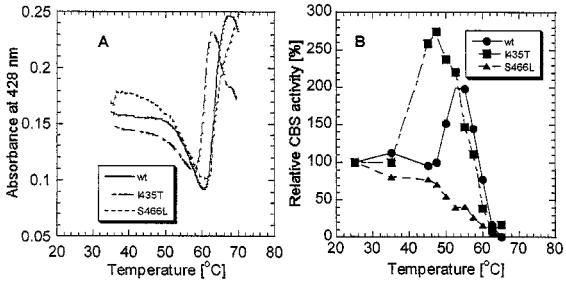

FIGURE 5: Thermal denaturation of normal and mutant CBS. (A) Spectral changes were recorded at 428 nm. (B) Profiles of relative enzyme activities. For the activity measurements, 10-μL aliquots were taken out of the cuvette during the scanning of the thermal denaturation curve. The aliquots were chilled on ice, and the CBS activity was measured as described in the Materials and Methods section.

The deletion mutant Δ414−551 does not bind AdoMet at any of the AdoMet concentrations tested (results not shown).

Activation of CBS by Partial Thermal Denaturation. To characterize the mutant forms of CBS further, we used thermal denaturation followed by UV−vis spectrophotometry to compare their relative thermal stability. During the course of thermally induced denaturation, all three forms of CBS undergo dramatic changes in their visible spectra due to changes in heme and PLP environment. We observed a noticeable decrease in the absorbance peak at 428 nm that was further used to measure thermal denaturation curves (data not shown). This decrease is most likely attributed to uncoupling of one of the two amino acid residues bound to the axial heme positions in the course of thermal denaturation.

Thermal denaturation curves recorded at 428 nm are relatively complex (Figure 5A). A sharp decrease in absorbance around the melting points of the proteins is followed by a rapid increase of absorbance at 428 nm. This increase is due to a spontaneous precipitation of CBS around its melting point. The absorbance at 428 nm drops again after the precipitated protein starts to aggregate and falls to the bottom of the cuvette. The melting points of both the WT and the S466L mutant are around 55 °C. The denaturation

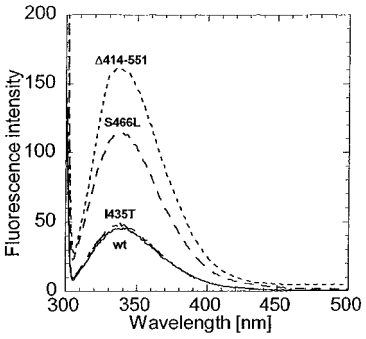

FIGURE 6: Tryptophan fluorescence spectra of WT CBS and of the I435T, S466L, and Δ414−551 mutants. The spectra were measured in TBS, pH 8.6, at 25 °C, and the enzyme concentrations were adjusted to a constant absorbance at 428 nm of 0.05. Samples were excited at 295 nm using 5-nm emission slits.

curve of I435T is essentially identical to that of the WT and S466L forms of CBS except that the melting point and the various changes in the curve consistently occur at roughly 5 °C lower temperatures. The presence of AdoMet does not have any significant effect on the thermal denaturation curves (results not shown).

During the course of our investigation into the effect of incremental elevations in temperature on CBS activity, we noticed a previously unreported effect that gives further insight into the nature of CBS activation by both AdoMet and partial proteolysis. We monitored changes in the level of catalytic activity during the course of thermal denaturation and found that, at temperatures just below the previously determined melting points, WT and I435T mutant CBS activities are increased approximately 2- and 3-fold, respectively (Figure 5B). HPLC analysis indicated that thermal activation was not accompanied by any change in aggregation status (data not shown). The addition of AdoMet to thermally activated WT CBS did not induce any further activation of activity (data not shown). The constitutively activated C-terminal S466L mutant does not show any increase of enzyme activity during the course of thermal denaturation. This finding indicates that in the WT and I435T forms of CBS thermal activation is inducing a functionally equivalent conformational change to that induced by AdoMet binding. When this experiment was repeated using the CBS deletion mutant Δ414−551, which lacks the C-terminal regulatory region and is also constitutively activated, we observed no further increase in activity (result not shown). These results are the first report that CBS can be activated by partial thermal denaturation and indicate that activation of CBS due to AdoMet binding, the CBS domain mutation S466L, heating, and limited proteolysis proceed through a common mechanism involving the C-terminal region of the protein.

CBS Fluorescence Data Indicate a Common Mechanism of CBS Activation. Tryptophan fluorescence is a sensitive tool to determine changes in protein conformation (*27*). We measured tryptophan fluorescence induced at the excitation wavelength of 295 nm to characterize the putative conformational changes associated with the I435T, the S466L, and the Δ414−551 forms of CBS as compared to the WT. Figure 6 shows the tryptophan fluorescence of all of these forms of CBS. The I435T mutant exhibits a tryptophan fluorescence spectrum almost identical to that of the WT CBS with a maximum at 338 nm. Although the S466L mutant exhibits a fluorescence maximum at the same wavelength, the signal

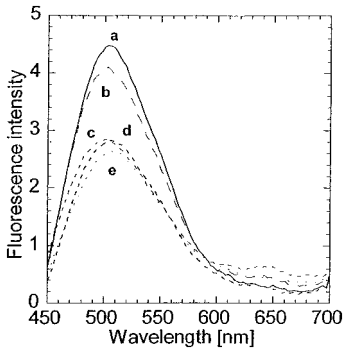

FIGURE 7: Fluorescence spectra of the internal protonated aldimine of PLP in normal and mutant forms of CBS. The spectra were measured in TBS, pH 8.6, at 25 °C, and the enzyme concentrations were adjusted to a constant absorbance at 428 nm of 0.5. Samples were excited at 420 nm using 5-nm emission slits. Fluorescence spectra are shown for the WT CBS (a); I435T (b); S466L (c); WT CBS + 100 μM AdoMet (d); and the deletion mutant Δ414−551 (e).

intensity as compared to the WT and the I435T mutant increases more than 2-fold. The fluorescence intensity of the S466L mutant is close to that of the deletion mutant in which the entire regulatory domain has been deleted. These results indicate that, while the mutant I435T remains in a conformation close to that of the WT, the mutant protein S466L folds into a conformation close to that of the permanently activated Δ414−551 deletion mutant.

To investigate this hypothesis further, we investigated the effect of these conformational changes upon the CBS PLP environment. Because of the role that PLP plays in substrate aldimine formation, it is a suitable marker to monitor changes in the environment around the CBS catalytic site. We measured PLP-derived fluorescence for the WT and activated forms of CBS to see if there were any changes in the architecture of the CBS catalytic site consistent with a common mechanism of CBS activation (Figure 7). As heme quenches most of the PLP-related fluorescence in CBS (*28*), we could only measure a weak fluorescence signal due to the protonated internal PLP aldimine with a maximum at 508 nm. We observed that the I435T protein has an almost identical PLP fluorescence spectra to that of the WT protein. This finding further indicates that in terms of conformation, this protein is essentially identical to nonactivated WT CBS. The S466L mutation induces an approximate 40% drop in the intensity of PLP-derived fluorescence and an 8-nm hypsochromic shift of the maximum toward the shorter wavelengths as compared to the WT. The binding of AdoMet to WT CBS or the removal of the C-terminal regulatory domain in the Δ414−551 form of CBS causes an identical drop in the fluorescence intensity without a shift. These findings indicate that these different activation processes have had a functionally equivalent effect upon the CBS active site. Taken together, the tryptophan and PLP fluorescence data strongly support the hypothesis that there are two distinct active CBS conformations with differing catalytic activities and of a common mechanism for AdoMet, thermal and proteolytic activation of CBS involving the C-terminal portion of the protein.

DISCUSSION

I435T and S466L Mutants Are the First CBS Regulatory Mutants To Be Purified and Characterized. Our previous work on the S466L and I435T mutants in crude cell extracts indicated that these proteins were as catalytically active and as stable as the WT protein. The only difference that could be detected was that at least one mutant (S466L) appeared to be constitutively activated, and both forms were impaired in their regulation by AdoMet. Consequently, it became clear that purification of these mutant forms of CBS could provide us with unique investigative tools with which to study the mechanism of CBS activation by AdoMet.

We have previously tried to express several mutant CBS proteins in *E. coli* and purify them to homogeneity. However, many of the proteins carrying point mutations in the CBS active core region, spanning amino acid residues 37−413 (*13*), do not fold properly when expressed in *E. coli* and were found to be insoluble and located predominantly in inclusion bodies. Our recent report has indicated that mutations in the CBS catalytic region yield proteins that are unstable in human skin fibroblasts. Further investigation showed that these CBS mutant forms are extremely prone to aggregation and heme loss in both fibroblasts and *E. coli* (*29*). The only mutant form of CBS that has been purified previously is the V168M form (*30*). To our knowledge, the work presented in this paper constitutes the first time that C-terminal mutant forms of CBS that are deficient in their ability to be regulated by AdoMet have been successfully purified to near homogeneity and characterized.

Functional Changes Induced by I435T and S466L Are Not Due to Changes in Aggregation Status. The HPLC analysis shown in Figure 2 indicates that the different forms of CBS investigated in this paper differ in terms of their aggregation status with the S466L form, showing significantly altered mobility as compared to the WT and I435T forms. The inherent variability of purified full-length CBS in terms of aggregation behavior is a persistent complication of its analysis, and it is impossible to completely eliminate a possible influence of this factor in structural studies. However, it is very unlikely that the observed differences in CBS activity in the S466L form are due to a difference in aggregation status as compared to that of the WT and I435T forms of CBS. All batches of purified full-length CBS suffer from some minor degree of aggregation over time without any significant accompanying changes in activity or fluorescence properties. More profound increases in aggregation typically lead to a reduction in CBS activity rather than the activation exhibited by the S466L form. Additionally, in terms of activity and response to AdoMet, *E. coli* cell extracts expressing these mutants show very similar behavior to their purified counterparts. When these cell extracts were analyzed by native PAGE, the I435T and WT forms clearly ran as tetramers with no evidence of aggregation.[2] This observation indicates that the constitutive activation and failure to respond to AdoMet that we observe for purified S466L is not related to the apparent change in aggregation status seen in the HPLC analysis. One possibility is that at least part of the apparent aggregation observed in the HPLC analysis may be due to the altered conformation of the S466L form of CBS affecting its mobility in the column.

S466L and I435T Shed New Light on the Conformational Changes Associated with AdoMet Activation of CBS. Investigation of the biochemical properties of these proteins essentially agreed with our previous findings in the crude cell extracts with two noticeable exceptions. Although we

found that the I435T form was altered in terms of AdoMet regulation, our analysis of the purified I435T protein indicated that this impairment is subtler than we had previously supposed. In contrast to our results in crude extracts where I435T was not responsive to AdoMet, purified I435T is activated in the presence of saturating concentrations of AdoMet. However, the I435T form of CBS is clearly impaired in its response to AdoMet as can be seen from the enzyme kinetic data in Table 1. In this analysis, it is evident that as compared to WT CBS, the I435T form of the enzyme has a 3-fold reduced increase in catalytic efficiency due to the presence of AdoMet. Similarly, at lower concentrations of AdoMet, the I435T mutant is approximately 10-fold less sensitive to the activating effects of this compound (Figure 3). Previous investigations of the level of hepatic AdoMet have estimated a concentration of between 35 and 70 μmol/kg (*31*). At these concentrations of AdoMet, it seems highly likely that the I435T form would be unable to convert to the activated conformation, and AdoMet regulation would be dramatically impaired, if not abolished completely.

As the observed AdoMet binding is essentially unaffected, it is clear that the impaired response of I435T to AdoMet cannot be explained on the basis of AdoMet binding. Instead, it appears that the I435T mutation impedes the conversion of this form of CBS to the more activated conformation. Similarly, our work with the purified S466L form of CBS confirmed that this enzyme is locked in a constitutively activated conformation and is not further activated by AdoMet. In contrast to our cell-extract analysis, comparison of the activities of purified WT and S466L indicated that the mutant is less active than the AdoMet-activated WT. This mutant was originally observed in a patient with homocystinuric levels of Hcy, suggesting impaired CBS activity. However, heterologous expression of the S466L cDNA in both prokaryotic and eukaryotic expression systems showed very high levels of CBS activity, posing the question as to why this patient suffered from such high levels of Hcy. The finding that purified S466L is not capable of attaining the maximal activity of WT CBS fully induced with AdoMet provides some explanation for the impaired Hcy metabolism that was observed in the patient[2] and illustrates the functional importance of AdoMet regulation of CBS in vivo. The importance of the AdoMet response for normal cellular CBS function is also made more apparent by consideration of the effect this inducing compound has on the catalytic efficiency of the WT enzyme. Table 1 contains the first report of the effect of AdoMet upon the k_{cat}/K_m values of WT CBS, and it can be seen that this compound increases the catalytic efficiency of this enzyme by approximately 11-fold. This finding is consistent with the observation that impairment of the CBS AdoMet response has a profound effect upon the metabolism of Hcy in vivo.

It could be argued that the failure of AdoMet to further upregulate S466L is because the nature of the mutation effectively destroys the AdoMet binding site. However, our data indicate that S466L is not affected in its ability to bind AdoMet (Figure 4). This finding makes the point that it is not the binding of AdoMet per se that activates CBS but the conformational change that the binding induces. Consequently, it seems likely that the failure of AdoMet to increase S466L catalytic activity is due to the fact that S466L is already in an activated conformation that, if not identical, is certainly similar to that induced by AdoMet binding. Support for the involvement of a conformational change in the activation of CBS can also be drawn from our observation that the effect can be mimicked by partial thermal denaturation (Figure 5B). Additional supporting evidence is obtained from both the tryptophan and the PLP fluorescence data where S466L is significantly shifted relative to the uninduced WT and I435T forms of CBS. Taken together, these results strongly indicate that there are two catalytically active conformations of CBS and that AdoMet activates CBS by converting the protein into the more active conformation.

Common Mechanism for CBS Activation. The observation that specific C-terminal mutations, partial thermal denaturation (this work), limited proteolysis, and AdoMet (*9*−*11*, *13*) all induce a very similar level of activation of WT CBS suggests that these different forms of activation are acting through a common mechanism. None of these three methods of CBS activation can further activate CBS after it has previously been activated by one of the other methods. The observation that partial thermal denaturation, limited proteolysis, and AdoMet are mutually exclusive in their ability to upregulate CBS strongly supports the hypothesis that they are acting through a functionally analogous mechanism. However, comparison of the kinetic parameters for WT activated by either limited proteolysis or AdoMet indicates some differences in their respective mechanisms of activation. The AdoMet activation is mainly reflected in an increase of the V_{max} values for both substrates (this work and ref *13*). Activation due to limited proteolytic cleavage is predominantly attributable to a decrease of K_m for Hcy (*13*). This difference does not exclude a common mechanism for the two types of activation but may instead represent subtle differences in the course of these respective activation processes that are reflected in their respective K_m values.

To investigate this further, we have used the S466L form of CBS as a model of CBS activated with AdoMet and the Δ414−551 form as a model of CBS after limited proteolysis. Strikingly, both of these forms of CBS show similar increases in their tryptophan fluorescence spectra relative to uninduced WT or the I435T form of CBS, indicating that a similar conformational change has occurred in these proteins. Previously, we have shown similar increases in tryptophan fluorescence concomitant with AdoMet binding to WT CBS (*13*). Additionally, the PLP fluorescence data show that the C-terminal S466L mutant, the Δ414−551, and the WT CBS stimulated with AdoMet all experience an identical change in signal magnitude as compared to uninduced WT. All of these results are consistent with a common activation mechanism involving the C-terminal portion of CBS.

CBS Appears To Contain an Autoinhibitory Domain in the C-Terminal Region. The Δ414−551 mutant has had the C-terminal regulatory region removed and may thus be offering a clue to the nature of the conformational change associated with CBS activation. However, it could be argued that the constitutively activated Δ414−551 mutant has also lost regions associated with the tetrameric organization of CBS and that the observed activation is as a consequence of the shift from the tetrameric to the dimeric form. Our HPLC data (Figure 2) indicate that the S466L form of CBS, which is also constitutively activated and shows similar changes in fluorescence spectra, is not a dimer. This finding, together with the observation that neither AdoMet or heat activation

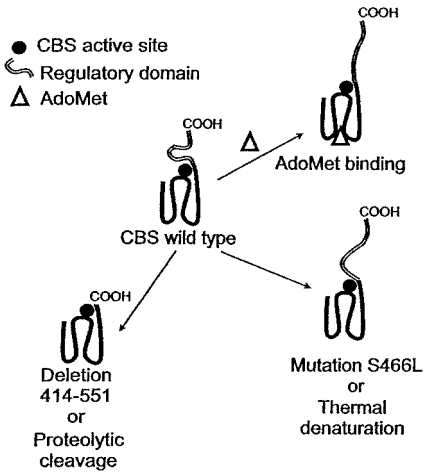

FIGURE 8: Proposed mechanisms of CBS activation. In nonactivated CBS, the catalytic site is partially occluded by the C-terminal autoinhibitory domain. Binding of AdoMet, proteolytic cleavage, thermal denaturation, or the S466L mutation act to displace the autoinhibitory domain from the catalytic site, causing an elevation in CBS catalytic activity. In this figure, the positioning of the AdoMet ligand is purely arbitrary as the exact AdoMet binding site is currently unknown.

converts CBS to a dimeric form, strongly indicates that it is the removal of the C-terminal region and not the conversion of CBS to a dimer that is responsible for the observed activation.

Our data indicate that the C terminus of CBS acts as an autoinhibitory regulatory domain and that the conformational change induced by AdoMet binding, specific point mutations, or heating displaces this domain from its site of inhibition. This hypothesis is illustrated in Figure 8. Regulatory interactions between the catalytic and the C-terminal regions of CBS have been observed before where removal of the C-terminal portion of CBS serves to suppress the effect of mutations in the catalytic domain (*14*, *30*). Very recent work has indicated that point mutations in the CBS domain and elsewhere in the C-terminal region of CBS can also serve to suppress certain kinds of mutations in the catalytic domain (*19*). One possibility is that the CBS C-terminal region acts to autoinhibit by partial occlusion of the catalytic site and that AdoMet binding induces the displacement of the autoinhibitory domain leading to higher levels of CBS activity. The implication of this theory is that some catalytic region mutations such as I278T cause unwelcome interaction with the C-terminal domain. This interaction may serve to completely occlude the CBS catalytic site, thus either rendering the protein inactive or so misfolded that it is subsequently degraded. This possibility would then account for why removal of the C-terminal portion of the protein restores activity.

Autoinhibition Allows for Rapid Regulation of CBS Activity. The organization of CBS into a distinct catalytic region that is modulated by an inhibitory regulatory domain is not unique. There are a growing number of reports of proteins that share this functional organization including the endothelial and neuronal isoforms of nitric oxide synthase (*32*, *33*), calcineurin A (*34*), and protein kinase C (*35*). Although proteins with autoregulatory functions may differ in terms of their domain organization and the nature of their regulatory reactants/ligands, they often have a common need for rapid moment-by-moment regulation in response to changes in cellular conditions. As transcriptional and translational mechanisms are too slow for this kind of response, cells appear to have evolved mechanisms by which the activities of already existing pools of certain enzymes can be rapidly modulated. CBS is coordinately regulated with proliferation, and many of the conditions that regulate CBS through this mechanism also have an effect upon the intracellular concentrations of AdoMet (refs *4* and *36* and our unpublished results).[2] Consequently, the modulation of CBS activity by AdoMet may have evolved to enable rapid regulation of this enzyme in response to changes in cellular proliferation status.

CBS Domain Has a Key Role in the Autoinhibition of CBS. The occurrence of the AdoMet regulatory mutations S466L, I435T, D444N (*23*), and P422L[2] in the CBS domain indicate that this structure plays a key role in the autoinhibitory behavior of the C-terminal portion of CBS. Further evidence to support this conclusion can be drawn from the hydrophobic nature of the CBS domain and consideration of the manner in which CBS is activated during thermal denaturation. The sharp transition in activation at temperatures above 54 °C is characteristic of a hydrophobic interaction, where temperatures below this level typically reinforce hydrophobic interactions and further increases in temperature subsequently break these bonds (*37*). Although the CBS domain has been found in a large number of different proteins with diverse functions, no definitive role for this domain has ever been described. Amino acid sequence comparisons of CBS domains in isofunctional proteins across species boundaries can show relatively little conservation but appear to be structurally conserved and retain a hydrophobic character, indicating that this characteristic is intrinsic to the function of the CBS domain (*38*).

The only previous work that suggested a role for the CBS domain was concerned with the yeast chloride channel protein gef1p, which contains two CBS domains in the C-terminal portion of the protein. Alanine scanning mutagenesis of either of these domains leads to loss of activity and failure of this protein to localize in the golgi (*39*). Although disruption of the CBS domain in the yeast chloride channel protein seems to affect the intracellular localization of this protein, it is unlikely to be the primary role of this domain as most of the proteins that contain CBS domains are not localized to the golgi. Instead, it is more likely that this finding is a secondary effect due to a loss of the correct protein conformation, which then prevents correct transport of the protein into the desired organelle. The data presented in this paper indicate that the natural function of CBS domains is involved in regulatory hydrophobic interactions.

Recent work in our laboratory has determined the crystal structure of the Δ414−551 form of CBS (*15*, *16*). Currently, we are working on determining the three-dimensional structure of both the S466L and the full-length WT CBS proteins. The results of this analysis should provide a final proof of the regulatory scheme proposed in this paper.

ACKNOWLEDGMENT

The authors thank Dr. Frank Frerman and Dr. Sudhindra Rao for useful discussions.

REFERENCES

1. Mudd, S. H., Levy, H. L., and Kraus, J. P. (2001) in *The Metabolic and Molecular Bases of Inherited Disease* (Scriver,

C. R., Beaudet, A. L., Sly, W. S., Valle, D., Childs, B., Kinzler, K., Vogelstein, B., Eds.) pp 2007−2056, McGraw-Hill, New York.
2. Skovby, F., Kraus, J. P., and Rosenberg, L. E. (1984) *J. Biol. Chem. 259*, 588−593.
3. Kraus, J. P., and Rosenberg, L. E. (1983) *Arch. Biochem. Biophys. 222*, 44−52.
4. Maclean, K. N., Janosik, M., Oliveriusova, J., Kery, V., and Kraus, J. P. (2000) *J. Inorg. Biochem. 81*, 161−171.
5. Jhee, K. H., McPhie, P., and Miles, E. W. (2000) *J. Biol. Chem. 275*, 11541−11544.
6. Kraus, J. P., Packman, S., Fowler, B., and Rosenberg, L. E. (1978) *J. Biol. Chem. 253*, 6523−6528.
7. Kery, V., Bukovska, G., and Kraus, J. P. (1994) *J. Biol. Chem. 269*, 25283−25288.
8. Bruno, S., Schiaretti, F., Burkhard, P., Kraus, J. P., Janosik, M., and Mozzarelli, A. (2001) *J. Biol. Chem. 276*, 16−19.
9. Finkelstein, J. D., Kyle, W. E., Martin, J. J., and Pick, A.-M. (1975) *Biochem. Biophys. Res. Commun. 66*, 81−87.
10. Koracevic, D., and Djordjevic, V. (1977) *Experientia 33*, 1010−1011.
11. Kozich, V., and Kraus, J. P. (1992) *Hum. Mutat. 1*, 113−123.
12. Roper, M. D., and Kraus, J. P. (1992) *Arch. Biochem. Biophys. 298*, 514−521.
13. Kery, V., Poneleit, L., and Kraus, J. P. (1998) *Arch. Biochem. Biophys. 355*, 222−232.
14. Shan, X., and Kruger, W. D. (1998) *Nat. Genet. 19*, 91−93.
15. Janosik, M., Meier, M., Kery, V., Oliveriusova, J., Burkhard, P., and Kraus, J. (2001) *Acta Crystallogr., Sect. D*: *Biol. Crystallogr. 57*, 289−291.
16. Meier, M., Janosik, M., Kery, V., Kraus, J. P., and Burkhard, P. *EMBO J.*, in press.
17. Kraus, J. P., Janosik, M., Kozich, V., Mandell, R., Shih, V., Sperandeo, M. P., Sebastio, G., de Franchis, R., Andria, G., Kluijtmans, L. A. J., Blom, H., Boers, G. H. J., Gordon, R. B., Kamoun, P., Tsai, M. Y., Kruger, W. D., Koch, H. G., Ohura, T., and Gaustadnes, M. (1999) *Hum. Mutat. 13*, 362−375.
18. Shan, X., and Kruger, W. D. (1997) *Am. J. Hum. Genet. 61* (Suppl.), 2420.
19. Shan, X., Dunbrack, R. L., Jr., Christopher, S. A., and Kruger, W. D. (2001) *Hum. Mol. Genet. 10*, 635−643.
20. Bateman, A. (1997) *Trends Biochem. Sci. 22*, 12−13.
21. Lipson, M. H., Kraus, J., and Rosenberg, L. E. (1980) *J. Clin. Invest. 66*, 188−193.
22. Fowler, B., Kraus, J., Packman, S., and Rosenberg, L. E. (1978) *J. Clin. Invest. 61*, 645−653.
23. Kluijtmans, L. A. J., Boers, G. H. J., Stevens, E. M. B., Renier, W. O., Kraus, J. P., Trijbels, F. J. M., van den Heuvel, L. P. W. J., and Blom, H. J. (1996) *J. Clin. Invest. 98*, 285−289.
24. Adams, E. (1979) *Methods Enzymol. 62*, 407−410.
25. Morrison, M., and Horie, S. (1965) *Anal. Biochem. 12*, 77−82.
26. Kraus, J. P. (1987) *Methods Enzymol. 143*, 388−394.
27. Eftink, M. R. (1997) *Methods Enzymol. 278*, 221−257.
28. Kery, V., Poneleit, L., Meyer, J. D., Manning, M. C., and Kraus, J. P. (1999) *Biochemistry 38*, 2716−2724.
29. Janosik, M., Oliveriusova, J., Janosikova, B., Sokolova, J., Kraus, E., Kraus, J. P., and Kozich, V. (2001) *Am. J. Hum. Genet. 68*, 1506−1513.
30. Kabil, O., and Banerjee, R. (1999) *J. Biol. Chem. 274*, 31256−31260.
31. Mudd, S. H., Ebert, M. H., and Scriver, C. R. (1980) *Metab., Clin. Exp. 29*, 707−720.
32. Nishida, C. R., and Ortiz de Montellano, P. R. (1999) *J. Biol. Chem. 274*, 14692−14698.
33. Lane, P., and Gross, S. S. (2000) *Acta Physiol. Scand. 168*, 53−63.
34. Klee, C. B., Ren, H., and Wang, X. (1998) *J. Biol. Chem. 273*, 13367−13370.
35. Gopalakrishna, R., and Jaken, S. (2000) *Free Radical Biol. Med. 28*, 1349−1361.
36. Maclean, K. N., Janosik, M., Kraus, E., and Kraus, J. P. (1999) *American Society of Microbiology*: *Yeast Genetics and Human Disease II*, Abstract 122, ASM, Vancouver.
37. Nemethy, G. (1967) *Angew. Chem., Int. Ed. Engl. 6*, 195−206.
38. Zhang, R., Evans, G., Rotella, F. J., Westbrook, E. M., Beno, D., Huberman, E., Joachimiak, A., and Collart, F. R. (1999) *Biochemistry 38*, 4691−4700.
39. Schwappach, B., Stobrawa, S., Hechenberger, M., Steinmeyer, K., and Jentsch, T. J. (1998) *J. Biol. Chem. 273*, 15110−15118.

BI010711P