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# Conformational Properties of Nine Purified Cystathionine β-Synthase Mutants

Aleš Hnízda,[†] Tomas Majtan,[‡,§] Lu Liu,[∥] Angel L. Pey,[⊥] John F. Carpenter,[∥] Milan Kodíček,[@] Viktor Kožich,[†] and Jan P. Kraus*[,‡]

[†]Institute of Inherited Metabolic Disorders, First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague, Ke Karlovu 2, 128 08 Prague 2, Czech Republic

[‡]Department of Pediatrics and Colorado Intellectual and Developmental Disabilities Research Center (IDDRC), University of Colorado at Denver, 12800 East 19th Avenue, Aurora, Colorado 80045, United States

[§]Department of Genomics and Biotechnology, Institute of Molecular Biology, Slovak Academy of Sciences, Bratislava 84551, Slovakia

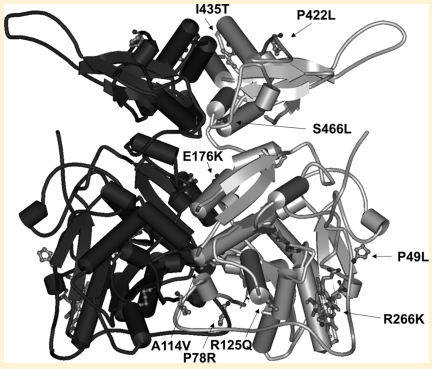
[∥]Department of Pharmaceutical Sciences, School of Pharmacy, University of Colorado at Denver, Aurora, Colorado 80045, United States

[⊥]Department of Physical Chemistry, Faculty of Sciences, University of Granada, Granada 18071, Spain

[@]Department of Biochemistry and Microbiology, Institute of Chemical Technology in Prague, Technická 5, 166 28, Prague 6, Czech Republic

Supporting Information

**ABSTRACT:** Protein misfolding due to missense mutations is a common pathogenic mechanism in cystathionine β-synthase (CBS) deficiency. In our previous studies, we successfully expressed, purified, and characterized nine CBS mutant enzymes containing the following patient mutations: P49L, P78R, A114V, R125Q, E176K, R266K, P422L, I435T, and S466L. These purified mutants exhibited full heme saturation, normal tetrameric assembly, and high catalytic activity. In this work, we used several spectroscopic and proteolytic techniques to provide a more thorough insight into the conformation of these mutant enzymes. Far-UV circular dichroism, fluorescence, and second-derivative UV spectroscopy revealed that the spatial arrangement of these CBS mutants is similar to that of the wild type, although the microenvironment of the chromophores may be slightly altered. Using proteolysis with thermolysin under native conditions, we found that the majority of the studied mutants is more susceptible to cleavage, suggesting their increased local flexibility or propensity for local unfolding. Interestingly, the presence of the CBS allosteric activator, *S*-adenosylmethionine (AdoMet), increased the rate of cleavage of the wild type and the AdoMet-responsive mutants, while the proteolytic rate of the AdoMet-unresponsive mutants was not significantly changed. Pulse proteolysis analysis suggested that the protein structure of the R125Q and E176K mutants is significantly less stable than that of the wild type and the other mutants. Taken together, the proteolytic data shows that the conformation of the pathogenic mutants is altered despite retained catalytic activity and normal tetrameric assembly. This study demonstrates that the proteolytic techniques are useful tools for the assessment of the biochemical penalty of missense mutations in CBS.




Cystathionine β-synthase (CBS) deficient homocystinuria (CBSDH; OMIM 236200) is the most common inherited defect in sulfur amino acid metabolism characterized by severely elevated levels of plasma homocysteine, methionine, and *S*-adenosyl-L-homocysteine.[1] The worldwide frequency is estimated to be around 1:330000,[1] thus classifying CBSDH as a rare disease. Interestingly, this number may be underestimated as several molecular epidemiological studies revealed the prevalence of homozygosity or compound heterozygosity for pathogenic mutations to be around 1:10000.[2−5] If untreated, CBSDH manifests clinically with connective tissue symptoms such as dislocated lenses and skeletal abnormalities, mental retardation, and vascular complications, particularly thromboembolic episodes.[1] Approximately half of CBS deficient patients respond to a treatment with pharmacological doses of pyridoxine (vitamin B6), with a significant lowering of plasma homocysteine levels and an alleviation of the clinical phenotype. The treatment of pyridoxine-nonresponsive patients involves a low-methionine diet and supplementation with betaine, which lowers the level of homocysteine by promoting its remethylation to methionine via betaine:homocysteine *S*-methyltransferase.

Cystathionine β-synthase (EC 4.2.1.22) is a pyridoxal 5′-phosphate (PLP)-dependent hemeprotein that catalyzes

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condensation of serine and homocysteine to form cystathionine as the first committed step in transsulfuration and the subsequent biosynthesis of cysteine, glutathione, and taurine. The human enzyme has a modular structure and a complex regulatory behavior (reviewed in refs 6 and 7). More than 160 mutant alleles have been described in CBSDH patients (http://cbs.lf1.cuni.cz/cbsdata/cbsmain.htm), with missense mutations being the most common variants in the CBS gene accounting for 87% of analyzed patient alleles. Our previous study proposed that misfolding of CBS mutants may be responsible for pathogenicity in CBS deficiency as a majority of tested CBS mutants formed large inactive aggregates devoid of heme.[8] This proposed mechanism was later supported by several studies exploring the beneficial effect of chemical chaperones on the recovery, activity, and assembly of various CBS mutant proteins.[9−11] The presence of chemical chaperones such as ethanol or dimethyl sulfoxide during expression in bacteria permitted the purification of several CBS mutants that exhibit normal heme saturation, native tetrameric assembly, and the same or higher specific activities compared to those of wild-type CBS.[12] Although the recovery of fully active CBS mutants suggests an improved folding, the final conformation of the purified mutants most likely differs from that of the wild-type protein as the purified fully active CBS mutants varied in their response to exogenous addition of the PLP cofactor as well as in their response to *S*-adenosyl-L-methionine (AdoMet) stimulation.[12] In addition to studying the spatial arrangement of proteins by spectroscopic monitoring of their conformation, we found proteolytic techniques such as proteolysis under native conditions and pulse proteolysis proved to be useful and effective for examining some aspects of protein structure;[13,14] this approach uses thermolysin, an endoprotease that preferentially cleaves peptide bonds in regions containing hydrophobic amino acids. The rate of proteolysis with thermolysin under native conditions reveals the extent of unfolding of the studied proteins because this endoprotease can cleave only flexible regions and partially or globally unfolded structures.[15] Further, pulse proteolysis is a technique that uses thermolysin to monitor urea-induced unfolding. After a short proteolytic pulse, the fraction of folded proteins remains intact whereas the locally and/or globally unfolded species are digested. Using this premise, the protein is analyzed at varying concentrations of urea and the $c_m$ value, a measure of protein thermodynamic stability in the urea gradient, may be determined.[14] The recent study of several CBS mutants in crude extracts showed that the extent of protein unfolding inversely correlated with the catalytic activity of the mutant enzymes.[16]

In this study, we applied several spectroscopic and proteolytic techniques to gain insight into the changes in CBS protein conformation induced by disease-causing missense mutations. We studied nine purified mutants: P49L, P78R, A114V, R125Q, E176K, R266K, P422L, I435T, and S466L. The purification and biochemical characterization of these mutants has been reported previously;[12,17] the determination of their conformational properties adds additional new information, thus permitting an understanding of the pathogenicity of missense mutations in CBS.

## EXPERIMENTAL PROCEDURES

**Purification of CBS Proteins.** The wild-type and mutant CBS enzymes except for R266K CBS were expressed as fusion proteins with the N-terminal glutathione *S*-transferase (GST) in *Escherichia coli* Rosetta2(DE3) cells and purified essentially as described previously.[12] The R266K mutant enzyme was expressed in *E. coli* Rosetta2(DE3) cells with a C-terminal six-His tag and isolated following the previously reported procedure.[17]

**Circular Dichroism (CD) and Fluorescence Spectroscopy.** The CD spectra of 0.5 mg/mL CBS proteins [in 50 mM phosphate buffer (pH 7.5)] were recorded with a Jasco J-810 chiroptic spectrometer. The intrinsic fluorescence of CBS proteins was measured in the same buffer using a Perkin-Elmer LS55 fluorescence spectrometer. The excitation wavelength was 295 nm (slit width of 5 nm) for tryptophans and 420 nm (slit width of 10 nm) for internal aldimines with an emission signal scanned from 300 to 450 nm (slit width of 5 nm) and from 430 to 700 nm (slit width of 10 nm), respectively.

**Second-Derivative UV Spectroscopy.** The UV spectra were recorded between 10 and 90 °C in 2.5 °C increments and a 3 min equilibration time at each temperature. Measurements were taken on an Agilent diode array model 8453 UV−visible spectrophotometer equipped with a Peltier temperature controller. Two milliliters of the protein sample (0.2 mg/mL) were prepared by diluting the stock protein with the appropriate amount of buffer [20 mM HEPES (pH 7.4), 1 mM tris(2-carboxyethyl)phosphine, and 0.01% Tween 20] and then placed in a quartz cuvette with a 1 cm path length. A micro stir bar (100 rpm) was put into the cuvette to improve the heat exchange in the sample. The second-derivative UV spectra at each temperature were used to compare the changes in the tertiary structure of proteins. The peak positions for aromatic amino acids tryptophan, tyrosine, and phenylalanine were plotted as a function of temperature.

**Proteolysis with Thermolysin under Native Conditions and Pulse Proteolysis.** Proteolytic techniques were performed and evaluated according to the previously published procedures.[18] To conduct the proteolytic experiments in the presence of AdoMet, we preincubated the CBS proteins (0.5 mg/mL) with 300 $\mu$M AdoMet at room temperature for 10 min. Each experiment was repeated at least twice.

The rate of proteolysis under native conditions was expressed as $k_p$, the constant in a single-exponential equation.

Pulse proteolysis revealed the $c_m$ value, i.e., concentration of urea at which the fraction of folded proteins comprises 50% of the entire protein population. To assess the accuracy of the determined $c_m$ values, we observed proteolytic cleavage of the CBS proteins in a 2 M urea solution. The kinetic constant for proteolysis of all proteins with a determined $c_m$ was lower than 0.2 $min^{-1}$, indicating negligible cleavage of the protein during the proteolytic pulse at the beginning of the transition zone and thus the absence of a systematic error.[19]

**Protein Structure Modeling.** Model of the full-length human CBS was built by homology modeling package Modeler 9v4 (Discovery Studio version 2.5, Accelrys) using the structure of *Drosophila melanogaster* CBS [Protein Data Bank (PDB) entry 3PC2] as a template. The input sequence alignment was constructed with Align123 (Discovery Studio version 2.5, Accelrys). Among the five resulting models, the model with the lowest probability density function (PDF) total energy was selected. Because *D. melanogaster* CBS is highly active and does not respond to AdoMet stimulation,[31] the presented model most likely corresponds to an activated conformation of human CBS.

**In-Gel Digestion and Mass Spectrometric Analysis.** To identify fragments observed during native proteolysis, we

excised the bands of interest and performed in-gel digestion according to a previously described procedure.[20] Mass spectra were recorded using MALDI-TOF MS (Autoflex II, Bruker Daltonics) and processed as described previously.[18]

## RESULTS

**Mutants Analyzed in This Study.** All studied mutants were purified to homogeneity. They retained catalytic activity, and their saturation with heme and tetrameric assembly were similar to those of wild-type CBS as previously reported.[12,17] All of the studied CBS mutants, except R266K and I435T, were expressed in the presence of a particular chemical chaperone included in the growth medium as described previously.[12] Parallel expression of the selected mutants (R125Q, E176K, P422L, and S466L) in the absence or presence of a chemical chaperone in the medium and their subsequent purification did not show significant differences between "chaperoned" CBS mutants and their "nonchaperoned" counterparts in terms of catalytic activity, AdoMet response, oligomeric status, or heme saturation (data not shown). The only detectable difference was in the increased yield and recovery of the purified enzymes (data not shown). The studied mutations are located (a) in the proximity of the heme-binding pocket (P49L, R125Q, and R266K), (b) at the dimer interface (P78R, A114V, and E176K), and (c) in the regulatory domain (P422L, I435T, and S466L) (Figure 1).

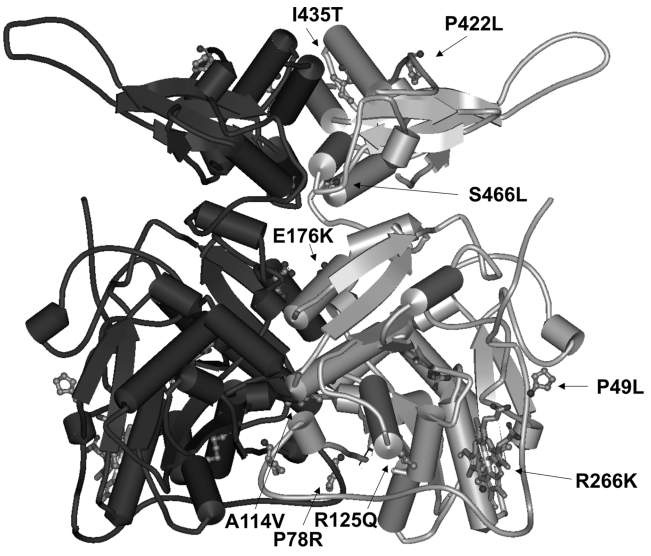


**Figure 1.** Model of hCBS. Model of the full-length human CBS based on the crystal structure of dimeric *D. melanogaster* CBS (PDB entry 3PC2). The CBS cofactors (heme and PLP) are displayed as sticks. Arrows point to the mutated residues (displayed as scaled balls and sticks) in one of the subunits of WT CBS. The analyzed pathogenic mutations are located in the proximity of the heme-binding pocket (P49L, R125Q, and R266K), at the dimer interface (P78R, A114V, and E176K), and in the regulatory AdoMet-binding domain (P422L, I435T, and S466L).

**Far-UV CD Spectroscopy.** To assess the impact of mutations on the formation of the secondary structure of the protein, we analyzed CBS mutants by far UV-CD spectroscopy. Far-UV CD spectra (Figure S1 of the Supporting Information) showed maxima at ~208 and ~222 nm indicating a high helical content. No noticeable differences in the content of secondary structure were observed among the studied CBS mutants compared to the wild-type enzyme with a single exception of the E176K mutant. This mutant exhibited a differently shaped CD spectrum, indicating a partial decrease in the proportion of helical structures together with formation of a different type of secondary structure; nevertheless, these changes are subtle, and the overall helical content is retained. Far-UV CD spectra recorded in the presence of AdoMet did not show any change in the overall secondary structure compared to that revealed by the CD spectra in the absence of AdoMet (data not shown).

These observations are consistent with the structural topology of the studied mutations. Only the R125Q and E176K mutations could affect hydrogen bonding with polar residues that form the adjacent helices; the available structural data show that the R125 residue forms a hydrogen bond with E234 and E176 interacts with the hydroxyl moiety of T383.[21,22] Our data demonstrate that the E176K mutation, causing the significant changes in charge and sterical disproportions, leads to a partial destabilization of the helical structures in the CBS enzyme, and in contrast, the R125Q mutation does not affect substantially the helical content of the CBS protein. Alternatively, the presence of either pathogenic mutation might lead to a local rearrangement or fluctuations resulting in the loss of stability as detected in our previous work.[12] Taken together, the CD results suggest that the majority of CBS mutants possessed unaltered secondary structure compared to that of the wild-type enzyme, and the presence of AdoMet does not alter the secondary structure of the CBS enzymes.

**Fluorescence Spectroscopy.** Using fluorescence spectroscopy, we studied the conformation of the CBS mutants by analyzing the microenvironment of the tryptophans. Tryptophan fluorescence spectra for all CBS enzymes exhibited broad maxima resulting from the fluorescence of eight tryptophan residues in each CBS subunit (Figure 2). These spectra did not show any major differences in the position of emission maxima, indicating an unaltered polarity in the tryptophan environment. However, significant changes were observed for the relative emission intensities, namely, increased intensity for P49L, R125Q, and S466L and, on the other hand, decreased intensity for R266K in comparison to that of the wild type. The altered emission intensity may be caused by a different microenvironment of tryptophans. The altered relative orientation of one or more tryptophans toward possible quenching groups, such as polar amino acid residues, and/or the mutual position of tryptophan and tyrosine residues, affecting the transfer of resonance energy from hydroxyphenyl to indolyl groups, could have caused the observed intensity changes.[23]

Fluorescence spectroscopy was further used for analyses of PLP that is covalently bound in the active site of CBS in the form of aldimine. These measurements revealed that the majority of the mutants exhibited decreased emission intensity (Figure 2). These changes may be caused by an increased extent of quenching by surrounding amino acid residues or by the heme moiety.[24] Alternatively, the lower fluorescence intensity may be attributed to a decreased level of saturation of CBS proteins with PLP that was previously observed for the R125Q and E176K and partially also for the C-terminal mutants P422L, I435T, and S466L.[12] In summary, both fluorescence-based measurements showed that the majority of the studied mutants are similar to wild-type CBS with minor differences in the microenvironment of the fluorophores.

**Second-Derivative UV Spectroscopy.** To determine possible changes in the microenvironment of chromophores,


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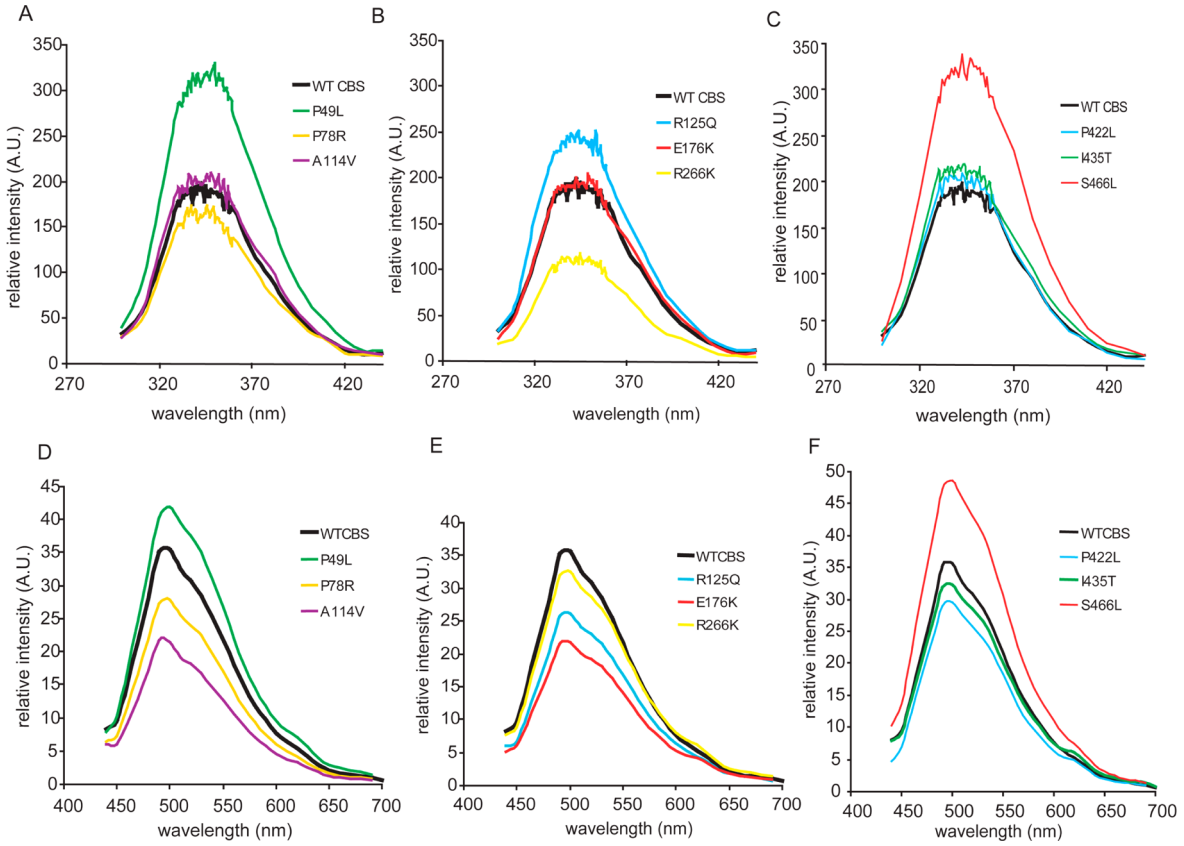


**Figure 2.** Fluorescence spectroscopy of CBS proteins. (A–C) Emission spectra of tryptophan residues (excitation at 295 nm). (D–F) Emission spectra of internal aldimines (excitation at 420 nm).

we also performed a second-derivative UV spectroscopy that may reveal information about the microenvironments of tryptophan, tyrosine, and phenylalanine residues.[27] Positions of the maxima were unaltered for the majority of the studied mutants (see P49L as an example for the unaltered mutant CBS spectrum in Figure 3). Decreases in wavelength maxima for tryptophan and tyrosine were recorded for A114V, R125Q, and E176K (Figure 3; spectral traces are shown in Figure S2 of the Supporting Information), suggesting possible differences in the microenvironment of these chromophores. It should be noted that these changes were subtle, typically around 0.1 nm. Nevertheless, the altered positions of maxima were consistently observed for all temperatures below the melting point, i.e., from 10 to 55 °C (Figure 3). These data further suggest that some of the studied mutants may differ from the wild type in the local spatial arrangement of the CBS protein, such as the A114V, R125Q, and E176K, and/or in the mutual position of the catalytic core and the C-terminal AdoMet-binding regulatory domain, such as the P422L and S466L.

**Proteolysis with Thermolysin under Native Conditions.** To determine the structural flexibility and tendency toward unfolding of the CBS mutants, we assessed their proteolytic susceptibility to thermolysin under native conditions.

Three mutants, namely, P49L, P78R, and R266K, were resistant to proteolysis to an extent similar to or even greater than that of the wild type. The six remaining CBS mutants exhibited greater susceptibility to proteolysis, and extremely rapid cleavage was observed for R125Q and E176K (Table 1, Figure 4, and Figure S3 of the Supporting Information). In the case of mutant I435T, we were not able to determine proteolytic kinetics because this mutant was almost instantly, within several seconds, cleaved to a fragment having a molecular mass of ≈40 kDa (Figure 4B). This proteolytic fragment corresponds to the catalytic core of CBS as was determined by in-gel digestion followed by MALDI-TOF mass spectrometry (Table S1 and Figure S4 of the Supporting Information). These data show that the I435T mutant is cleaved predominantly in the connecting loop between the catalytic core and the C-terminal regulatory domain. Thus, one of the requirements for this technique, i.e. cleavage without formation of major fragments, has not been met.

We also studied the effect of AdoMet on the proteolytic kinetics (Table 1). We have demonstrated previously that allosteric activation of CBS is associated with opening of the protein conformation and that wild-type CBS is cleaved in the presence of AdoMet more rapidly with a doubling of the kinetic constant.[18] A similar increase in the rate of cleavage was also observed in this study for all AdoMet-responsive CBS mutants, namely, P49L, P78R, A114V, and R266K. In the case of the A114V mutant, the presence of AdoMet in the proteolytic assay promoted an extensive opening of conformation accompanied by a dramatic 9-fold increase in the rate of cleavage compared to the rate in the absence of the ligand. In contrast, virtually no change in the rate of cleavage in the presence of AdoMet was observed for the mutants with the highest cleavage rates (R125Q and E176K) and for the C-terminal mutants (P422L and S466L). Interestingly, the same mutants did not show any response to AdoMet allosteric stimulation of catalytic activity in our previous study.[12]

Data from proteolysis with thermolysin under native conditions suggest that the majority of the studied CBS


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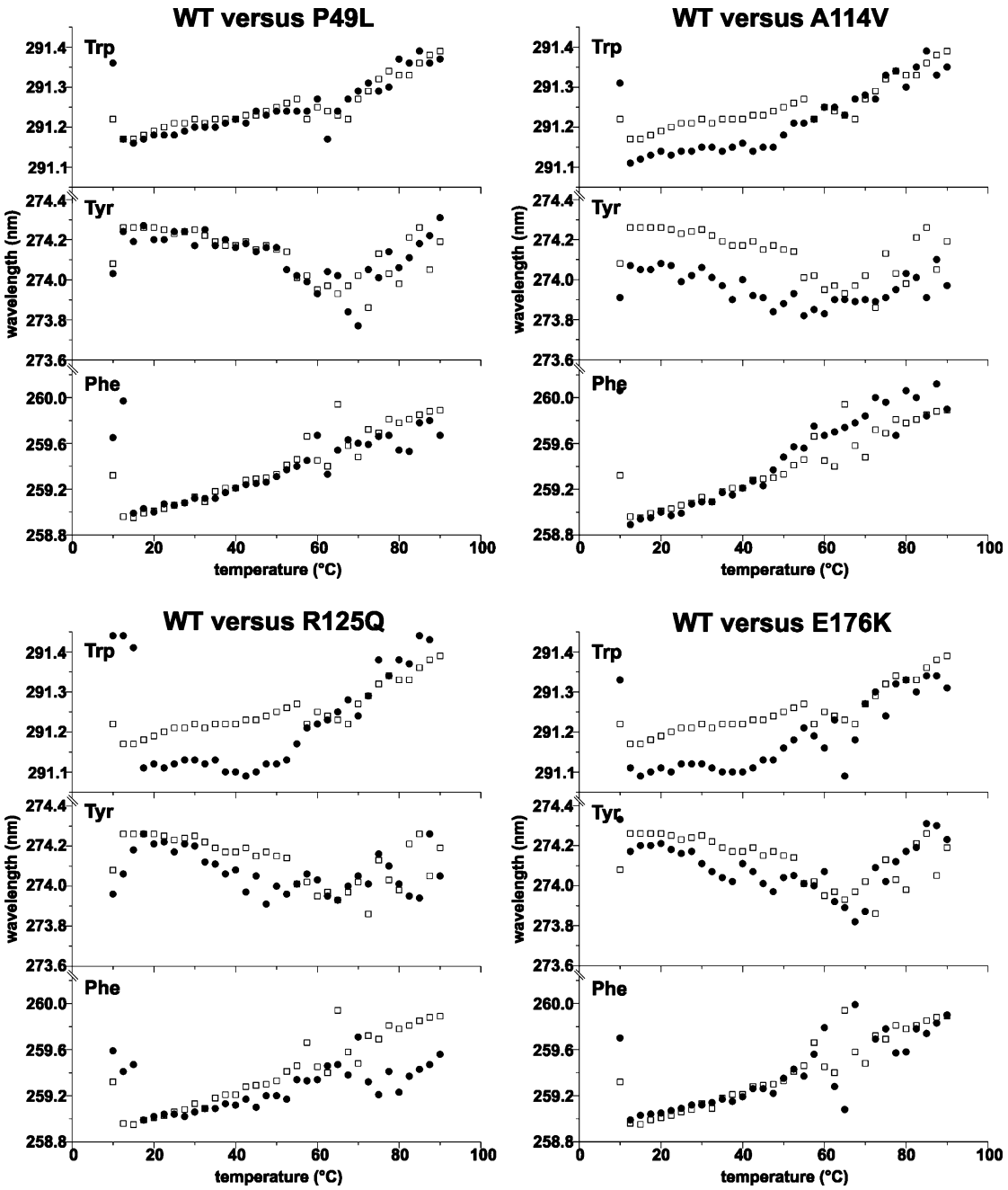


**Figure 3.** Second-derivative UV spectroscopy reveals the peak position for aromatic amino acids (Phe, Tyr, and Trp) as a function of temperature. The exact peaks for a particular aromatic amino acid were determined by using second-derivative UV spectra recorded every 2.5 °C for temperatures from 10 to 90 °C (see the Supporting Information for raw spectra) and subsequently plotted as a function of temperature. The wild-type enzyme [WT (□)] is separately compared with P49L, A114V, R125Q, and E176K CBS mutants (●). P49L represents the CBS mutants that were similar to the wild-type enzyme. On the other hand, A114V, R125Q, and E176K exhibited blue shifts for tyrosines and tryptophans.

mutants have higher structural flexibility and are more susceptible to proteolytic cleavage than the wild-type enzyme despite only subtle changes in protein conformation observed by spectroscopic techniques. Additionally, increased proteolytic rates of the wild-type and AdoMet-responsive mutant enzymes in the presence of AdoMet are indicative of conformational rearrangement upon AdoMet binding. On the other hand, the rates of cleavage of AdoMet-unresponsive mutants were not altered, suggesting their inability to undergo a change in conformation upon AdoMet binding and thus explaining their inability to be allosterically stimulated by AdoMet.

**Pulse Proteolysis.** Using pulse proteolysis, we have complemented the proteolytic data under native conditions by determining the resistance of wild-type and mutant CBS proteins against urea-induced denaturation. Interestingly, the majority of the mutants were found to be more resistant to urea-induced denaturation than the wild type (Table 2; a representative gel with the corresponding curve of the P49L mutant is shown in Figure S3 of the Supporting Information). On the other hand, mutants R125Q and E176K, sensitive to rapid proteolytic cleavage, exhibited abnormal behavior in pulse proteolysis that resulted in the nonsigmoidal curves of urea-induced unfolding (Figure S4 of the Supporting Information);


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**Table 1. CBS Specific Activities in the Absence and Presence of AdoMet and Rate Constants ($k_p$) of Proteolytic Kinetics under Native Conditions[a]**

| protein | CBS specific activity (units/mg of protein)[b] without AdoMet | CBS specific activity (units/mg of protein)[b] with AdoMet | $k_p$ ($min^{-1}$) | $k_p$ in the presence of AdoMet ($min^{-1}$) | with Adomet/without AdoMet ratio of $k_p$ |
|---|---|---|---|---|---|
| CBS | 148 ± 21 | 530 ± 45 | 0.026 ± 0.005 | 0.056 ± 0.005 | 2.15 |
| P49L | 122 ± 12 | 388 ± 39 | 0.025 ± 0.001 | 0.057 ± 0.005 | 2.28 |
| P78R | 110 ± 15 | 501 ± 27 | 0.0097 ± 0.001 | 0.031 ± 0.002 | 3.20 |
| A114V | 100 ± 11 | 401 ± 31 | 0.054 ± 0.011 | 0.5 ± 0.08 | 9.26 |
| R125Q | 139 ± 18 | 106 ± 9 | 0.87 ± 0.18 | 1.105 ± 0.008 | 1.27 |
| E176K | 138 ± 14 | 111 ± 8 | 0.97 ± 0.18 | 1.19 ± 0.18 | 1.22 |
| R266K | 191 ± 17 | 386 ± 26 | 0.014 ± 0.001 | 0.041 ± 0.02 | 2.93 |
| P422L | 226 ± 28 | 238 ± 41 | 0.036 ± 0.003 | 0.041 ± 0.001 | 1.14 |
| I435T | 564 ± 44 | 582 ± 60 | ND[c] | ND[c] | ND[c] |
| S466L | 626 ± 48 | 639 ± 43 | 0.109 ± 0.008 | 0.063 ± 0.007 | 0.58 |

[a]The values were determined using nonlinear fitting of data to a single-exponential equation. [b]For comparative purposes, the specific activities were taken from refs 12 and 17. [c]Not determined.

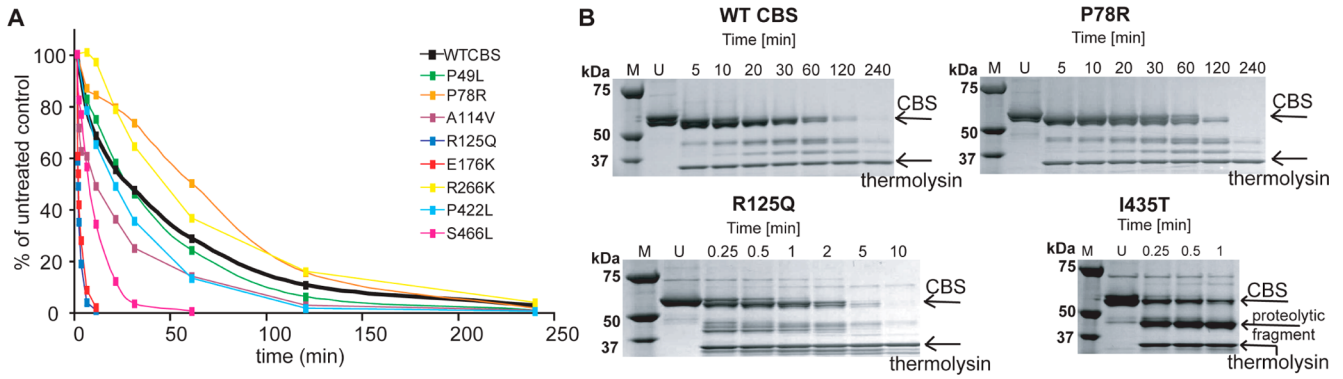


**Figure 4.** Proteolysis of CBS mutants with thermolysin under native conditions. (A) Comparison of wild-type CBS with mutant proteins. Each point represents a mean from at least two independent experiments. (B) Representative gels depicting proteolytic cleavage of selected mutants. P78R represents the proteolytically resistant mutants, while R125Q belongs to the group of more rapidly cleaved proteins. The mutant I435T is rapidly cleaved with the formation of the major fragment with a molecular mass of ≈40 kDa. M refers to molecular mass markers, and U refers to the untreated control without added thermolysin.

this finding indicates very low cooperativity of the tertiary structure of the proteins. Furthermore, parallel pulse proteolysis of R125Q and E176K mutant proteins expressed in the absence or presence of a chemical chaperone showed essentially the same profiles. This result further supports our previous notion that a chemical chaperone present during mutant CBS expression does not alleviate the impact of the pathogenic mutation on the CBS protein but rather results in the enrichment of the mutant protein in the crude extract, thus increasing the total yield and total recovery during the purification.

**Table 2. Midpoint Values of Urea-Induced Unfolding ($c_m$) for CBS Mutants in the Absence and Presence of AdoMet[a]**

| protein | $c_m$ in the absence of AdoMet (M) | $c_m$ in the presence of AdoMet (M) |
|---|---|---|
| CBS | 2.7 ± 0.08 | 3.26 ± 0.08 |
| P49L | 3.31 ± 0.13 | 3.40 ± 0.15 |
| P78R | 3.39 ± 0.06 | 3.42 ± 0.05 |
| A114V | 2.94 ± 0.20 | 3.46 ± 0.13 |
| R125Q | ND[b] | ND[b] |
| E176K | ND[b] | ND[b] |
| R266K | 3.08 ± 0.10 | 2.62 ± 0.11 |
| P422L | 3.85 ± 0.12 | 4.00 ± 0.09 |
| I435T | NA[c] | NA[c] |
| S466L | 3.39 ± 0.18 | 3.18 ± 0.12 |

[a]The values were determined using pulse proteolysis followed by nonlinear fitting of data to a sigmoidal equation. [b]Not determined because of the nonsigmoidal behavior of the mutant proteins (see Figure S3 of the Supplementary Information). [c]Not applicable. Pulse proteolysis could not have been used because of a rapid cleavage in the absence of urea.

The presence of AdoMet increased the stability of wild-type CBS as reported previously,[18,26] whereas the $c_m$ values for the majority of mutants were not changed (Table 2). Taken together, the stability of the CBS mutants in the absence of AdoMet did not change after addition of AdoMet and was comparable to the stability of wild-type CBS incubated with AdoMet. Different behavior was observed only for the R266K mutant that, unlike the remaining wild-type and mutant CBS enzymes, exhibited a significantly lowered protein stability upon AdoMet binding with a concomitantly decreased $c_m$ value.

## DISCUSSION

The cause of the pathogenicity of the eight disease-causing CBS mutant enzymes that have normal catalytic activity was not apparent from our initial study.[12] Purification and initial characterization of the CBS mutants expressed in the presence of various chemical chaperones showed that the inclusion of a


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chemical chaperone induced a specific increase in the level of the DnaJ molecular chaperone.[12] The likely involvement of the DnaJ/DnaK/GrpE machinery resulted in an enhanced folding efficiency of the mutants, thus shifting the equilibrium between the native active and misfolded inactive forms in favor of the folded fraction. However, a parallel expression of the selected mutants in the absence and presence of a chemical chaperone resulted in lower yields and lower total recoveries of CBS from nonchaperoned samples compared to chaperoned ones, supporting the notion that the presence of a chemical chaperone during expression of the mutant CBS mainly affects the equilibrium between folded and misfolded forms (data not shown). Additionally, our previous results suggest that the properly folded active conformations of these CBS mutants were enriched in the crude extracts, when expressed in the presence of a chaperone.[12] Furthermore, our analyses of several CBS mutants in bacterial lysates (P49L, P78R, A114V, and R125Q) revealed that their expression in the presence of various chemical chaperones did not significantly alter their proteolytic susceptibility (data not shown).

In this study, we compared the conformational properties of the nine purified mutant CBS enzymes with those of the wild-type enzyme using far-UV CD, fluorescence, and second-derivative UV spectroscopy. In the next step, we determined their structural flexibility by proteolysis with thermolysin under native conditions as well as their sensitivity to urea-induced denaturation by pulse proteolysis. Only subtle conformational differences between the wild type and the mutants studied were detected by the spectroscopic methods used. These findings are consistent with our previous work showing that the studied mutants did not exhibit dramatic abnormalities in the specific activities, heme saturation, or native tetramer formation.[12]

The major differences in the properties of CBS mutants were observed when the mutants were tested for their proteolytic susceptibility under native conditions. These data suggest that the mutant proteins adopted a conformation, which differs from that of wild-type CBS, that is more flexible and has more exposed hydrophobic residues for the thermolysin to attack. Moreover, the less compact structure of mutants A114V, R125Q, and E176K can also be assumed from their second-derivative UV spectra, which revealed subtle decreases in the wavelength maxima assigned to tryptophan and tyrosine residues; a similar blue shift was observed in the unfolding of several model proteins.[27]

Interestingly, the increased structural mobility of the mutants is accompanied by impaired protein stability in urea only for the extensively flexible CBS mutants, R125Q and E176K, while the other CBS mutants exhibit unaffected global protein stability as demonstrated by pulse proteolysis. The same or even higher resistance against urea-induced unfolding compared to that of the wild type is also consistent with the thermostability of these mutants previously determined by absorption spectrophotometry.[12] Analogous increased proteolytic susceptibility associated with subtle conformational changes and with unaltered thermodynamic stability was reported for the yeast phosphoglycerate kinase compared to that of its ortholog from *E. coli*.[15] It was proposed that the discrepancies in the phosphoglycerate kinase orthologs were caused by a divergent interdomain cooperativity and consequently different mechanism of unfolding in these modular proteins. It is tempting to speculate that the increased structural mobility of a majority of the CBS mutants is not caused by their low thermodynamic stability but more likely by the lower kinetic barrier of protein unfolding; the altered interdomain communication, despite only minor conformational changes in each particular domain, may be responsible for the increased rates of unfolding of these mutants.

The findings of increased proteolytic susceptibility of CBS mutants toward thermolysin are consistent with our previous study conducted directly in bacterial lysates for a different set of CBS mutants.[16] In the previous study,[16] it was proposed that the higher proteolytic susceptibility of the misfolded CBS mutants in vitro may mirror their accelerated turnover in vivo, indicating possibly an important role of proteolysis in the pathophysiology of CBS deficiency.[10,28] However, the higher proteolytic susceptibility of all studied mutants in the previous study was associated with their increased sensitivity to urea-induced denaturation. This discrepancy may be due to a different panel of studied CBS mutations and a different degree of purity of CBS proteins in each study. In the study presented here, we analyzed catalytically active CBS mutants that were successfully purified to homogeneity after expression in *E. coli*, whereas the previous work[16] was conducted mainly with mutants exhibiting decreased levels of catalytic activity that were not amenable to purification because of excessive aggregation. It indicates that decreased global protein stability may be observed only for the severely affected CBS mutants but not for the mutants exhibiting subtle conformational changes. This notion is also supported by the study presented here showing that only the R125Q and E176K CBS mutants exhibited impaired protein stability in a urea gradient. This study indicates that even subtle changes in protein conformation of the catalytically active CBS mutants with normal structural stability in vitro may lead to more rapid degradation of these variants in vivo.

Because the altered response to AdoMet was proposed as one of the possible pathogenic mechanisms in CBS deficiency, particularly for the C-terminal missense CBS mutations, we compared the kinetics of proteolytic cleavage of wild-type and mutant CBS enzymes in the presence of this allosteric activator. The most rapidly cleaved CBS mutants, namely R125Q and E176K, exhibited unaltered $k_p$ values in the presence of AdoMet compared to that obtained in the absence of AdoMet. This indicates that these mutants cannot bind AdoMet and/or are locked in a specific conformation that prevents allosteric change upon AdoMet binding. This suggestion is also supported by the previously reported lack of stimulation of the catalytic activity by AdoMet and by heating of mutants.[12] More surprisingly, the extremely increased proteolytic susceptibility of the A114V mutant in the presence of AdoMet suggests that its allosteric activation is likely associated with an extensive opening of the folded structure exposing naturally buried hydrophobic residues on the protein surface. Interestingly, the effect of AdoMet on native proteolysis of the R266K mutant was similar to that observed for wild-type CBS, but different behavior of this mutant was observed using pulse proteolysis. The presence of AdoMet led to a lower $c_m$, indicating that this CBS ligand decreases the thermodynamic stability of the R266K mutant. This result correlates well with the previously observed decreased thermal stability and AdoMet activation of this mutant compared to those of wild-type CBS.[17] The impaired response to AdoMet activation was also observed for the C-terminal mutants. The rate of proteolytic cleavage of the P422L mutant was not significantly increased, and moreover, the S466L mutant was cleaved even less rapidly in the presence of AdoMet. These findings further support the previous notion

that the C-terminal mutants are locked in a specific conformation, which results in permanently activated mutant CBS enzymes lacking the proper response to AdoMet stimulation.[29,30] Interestingly, our results indicate that these mutant proteins may be more flexible in the absence of AdoMet than the wild type. Even though the S466L mutant does not respond to AdoMet, it is still capable of binding it as reported previously.[29] As CBS domains in the C-terminal region very likely fold in a manner that is independent of the catalytic core,[31] these mutants may still bind AdoMet but are apparently unable to rearrange their conformation. It is tempting to speculate that this locked conformation is in vivo recognized as a misfolded structure by the cellular control machinery and consequently targeted for degradation.[32]

It should be noted that mutants P49L and P78R did not exhibit structural abnormalities by the approaches used in this study, which correlates well with their biochemical properties being very similar to those of the wild-type enzyme.[12] P49L exhibited high catalytic activity when expressed in the pKK expression vector without any additional tags or fusion partners.[33] On the other hand, P78R possessed a decreased enzyme activity in the same study using the pKK construct, which is consistent with a study analyzing the purified mutant reported by the Banerjee group.[34] Data of P78R indicate that the pathogenicity of these mutants may be revealed by employing a different expression system or using specific conditions. Nevertheless, the P49L mutation is often associated with very mild clinical manifestions [CBS Mutation Database (http://cbs.lf1.cuni.cz/cbsdata/cbsmain.htm) and oral communication with S. Stabler at the 8th Conference on Homocysteine Metabolism, Lisbon, Protugal, 2011].

It should be noted that the application of bacterial expression systems that produce the target protein with an affinity tag may have an artificial effect on the quality of the purified proteins. For instance, the GST tag that was used for the majority of the mutants may increase protein stability during the expression and subsequent purification.[35] The dramatic effect of the type and position of the employed purification affinity tag (i.e., bulky fusion partner, such as GST, vs short flexible tag, such as a six-His tag) and its position on the proper folding was recently demonstrated in the expression studies of the R266K mutant.[17] Nevertheless, using affinity tags is necessary for the production of target proteins in sufficient yields for their conformational analysis.

In this study, we demonstrated that protein structures of the studied CBS mutants are more locally flexible than that of the wild type despite their normal catalytic activity and unaffected sensitivity toward urea-induced denaturation. In conclusion, the conformational analysis of the mutants using spectroscopic and proteolytic approaches proved to be a useful tool for the assessment of the biochemical penalty of the CBS mutations.

## ■ ASSOCIATED CONTENT

### Supporting Information

Detailed results from CD and second-derivative UV spectroscopy and pulse proteolysis. This material is available free of charge via the Internet at http://pubs.acs.org.

## ■ AUTHOR INFORMATION


### Corresponding Author

*Telephone: (303) 724-3812. Fax: (303) 724-3838. E-mail: jan.kraus@ucdenver.edu.


### Author Contributions

A.H. and T.M. contributed equally to this work.


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### Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS

AdoMet, *S*-adenosylmethionine; CBS, cystathionine $\beta$-synthase; CBSDH, cystathionine $\beta$-synthase deficient homocystinuria; CD, circular dichroism; $c_m$, midpoint of urea-induced unfolding determined by pulse proteolysis; $k_p$, rate constant of proteolysis under native conditions; PLP, pyridoxal 5′-phosphate.

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## NOTE ADDED AFTER ISSUE PUBLICATION

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