Alleviation of Intrasteric Inhibition by the Pathogenic Activation Domain Mutation, D444N, in Human Cystathionine β-Synthase†

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ABSTRACT: Human cystathionine β-synthase is a heme protein that catalyzes the condensation of serine and homocysteine to form cystathionine in a pyridoxal phosphate-dependent reaction. Mutations in this enzyme are the leading cause of hereditary hyperhomocysteinemia with attendant cardiovascular and other complications. The enzyme is activated ∼2-fold by the allosteric regulator *S*-adenosylmethionine (AdoMet), which is presumed to bind to the C-terminal regulatory domain. The regulatory domain exerts an inhibitory effect on the enzyme, and its deletion is correlated with a 2-fold increase in catalytic activity and loss of responsiveness to AdoMet. A mutation in the C-terminal regulatory domain, D444N, displays high levels of enzyme activity, yet is pathogenic. In this study, we have characterized the biochemical penalties associated with this mutation and demonstrate that it is associated with a 4-fold lower steady-state level of cystathionine β-synthase in a fibroblast cell line that is homozygous for the D444N mutation. The activity of the recombinant D444N enzyme mimics the activity of the wild-type enzyme seen in the presence of AdoMet and can be further activated ∼2-fold in the presence of supraphysiolgical concentrations of the allosteric regulator. The mutation increases the K_{act} for AdoMet from 7.4 ± 0.2 to 460 ± 130 μM, thus rendering the enzyme functionally unresponsive to AdoMet under physiological concentrations. These results indicate that the D444N mutation partially abrogates the intrasteric inhibition imposed by the C-terminal domain. We propose a model that takes into account the three kinetically distinguishable states that are observed with human cystathionine β-synthase: "basal" (i.e., wild-type enzyme as isolated), "activated" (wild-type enzyme + AdoMet or the D444N mutant as isolated), and superactivated (D444N mutant + AdoMet or wild-type enzyme lacking the C-terminal regulatory domain).

Mutations in human cystathionine β-synthase are the single most common cause of hereditary hyperhomocysteinemia in which circulating levels of homocysteine are severely elevated (*1*). Cystathionine β-synthase deficiency presents pleiotropic clinical symptoms, and four major organ systems are affected. The clinical manifestations include ectopia lentis, skeletal deformities, mental retardation, and vascular disease with life-threatening thromboembolisms (*2*). Over 100 mutations, mostly private, have been described in cystathionine β-synthase deficient patients and are dispersed over the entire length of the coding sequence (*3*).

Cystathionine β-synthase catalyzes the condensation of homocysteine and serine to give cystathionine and represents one of two major metabolic routes for the removal of homocysteine. Mildly elevated homocysteine is a risk factor for cardiovascular diseases and is also correlated with neural tube defects and Alzheimer's disease (*4*−*6*). The enzyme is a unique heme protein that requires the cofactor pyridoxal phosphate (PLP)[1] and is activated by the allosteric effector AdoMet. The human enzyme is a homotetramer composed of subunits with a molecular mass of 63 kDa. It appears to have a modular organization in which N-terminal, middle, and C-terminal regions of the protein represent the binding sites for heme, PLP, and AdoMet, respectively (Figure 1) (*7*). Limited proteolysis results in the separation of the N-terminal "catalytic core" from the C-terminal AdoMet regulatory domain (*8*). The catalytic core (referred to as the ΔC143 form of the enzyme) retains high levels of activity but loses sensitivity to AdoMet (*7*). The crystal structure of the catalytic core has been reported recently (*9*, *10*).

The structure of the full-length enzyme or of the C-terminal regulatory domain is not available. However, residues 415−468 within this domain are predicted to adopt a β−α−β−β−α fold-containing "CBS" domain, which is found in a diverse set of proteins including inosine monophosphate dehydrogenase, 5′-AMP activated protein kinase, chloride channels, and ABC transporters (*11*). The role of the CBS domain in these proteins is not known, and it is postulated to provide binding sites for effector molecules and/or to play a targeting role for compartmental localization.

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[1] Abbreviations: PLP, pyridoxal phosphate; AdoMet, *S*-adenosylhomocysteine; PBS, phosphate-buffered saline; TLCK, tosyllysine chloromethyl ketone.

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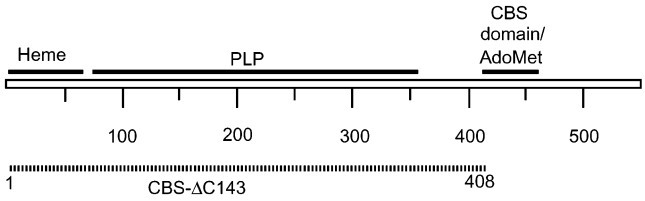


FIGURE 1: Domain organization of human cystathionine β-synthase.

Intrasteric regulation by a domain in the protein that can reversibly modulate the activity of the catalytic domain has been well studied in protein kinase C (*12*). In cystathionine β-synthase, the C-terminal regulatory domain plays an important, but as yet poorly understood, role in modulating activity. In addition to harboring the presumed binding site for AdoMet, it also appears to inhibit enzyme activity. The catalytic core of the protein (ΔC143) lacking the C-terminal domain displays a 2-fold and ~4-fold higher k_{cat} than the full-length enzyme in the presence and absence of AdoMet, respectively. Curiously, deletion of the C-terminal domain alleviates the catalytic penalties associated with several missense mutations in the catalytic core of the protein and points to its importance in activities other than AdoMet binding (*13*).

Despite the relatively large number of missense mutations that have been described in homocystinuric patients with impaired cystathionine β-synthase activity, the biochemical penalties associated with very few of these have been characterized (*14*−*16*). The point mutation D444N was the first regulatory domain mutation to be characterized (*17*). In *Escherichia coli* cell extracts the activity associated with the recombinant D444N protein was found to be comparable to that of the wild-type enzyme. However, the enzyme was unresponsive to AdoMet at the concentrations that were tested. In this study, we have examined the biochemical characteristics associated with this mutation with purified recombinant protein and in a patient fibroblast cell line that is homozygous for the D444N expressing allele. We report that the mutation reduces the steady-state levels of the protein 4-fold and drastically increases the K_{act} for AdoMet (by ~100-fold) so that it can no longer be activated at physiological concentrations of this effector. The combination of these effects explains the diminished cystathionine β-synthase activity observed in fibroblast extracts and is likely to account for the homocystinuric phenotype associated with this genotype. These studies provide interesting insights into the role of the C-terminal domain in intrasteric regulation of the N-terminal catalytic core.

MATERIALS AND METHODS

Materials. Redivue [^{32}P]dCTP (250 μCi) and [^{14}C]serine (158 mCi/mmol) were purchased from Amersham Pharmacia. Serine, ampicillin, TLCK, leupeptin, pepstatin, aprotinin, benzamidine, and D,L-homocysteine were purchased from Sigma. AdoMet 1,4-butanedisulfonate was a generous gift from Knoll Farmaceutici Spa (Milano, Italy).

Generation of the D444N Mutant and Protein Purification. The D444N mutant was generated by site-directed mutagenesis using the Quickchange Kit from Stratagene. The template for mutagenesis was pGEX4T1/hCBS carrying the wild-type human cystathionine β-synthase gene fused in-frame with glutathionine *S*-transferase (*13*). The forward and reverse primers that were employed are as follows: forward, 5′-CCGGGAGAAGGGCTTTAACCAGGCGCCCGTGG; reverse, 3′-CCACGGGCGCCTGGTTAAAGCCCTTCTCCCGG.

The mutation was confirmed by DNA sequencing. The mutant enzyme was purified, and the GST tag was removed by partial proteolysis by thrombin using the procedure described for the wild-type enzyme (*18*).

Enzyme Assays and Kinetic Studies. Specific activity was measured using the fixed-time radiolabeled assay described previously (*18*) except that 30 mM D,L-homocysteine was employed instead of 15 mM L-homocysteine. The use of the D,L-mixture versus the L-isomer generated by hydrolysis of L-homocysteine thiolactone does not appear to affect enzyme activity. Reactions were initiated by addition of homocysteine. Protein concentrations were determined by the Bradford method (Bio-Rad) with bovine serum albumin as a standard. Heme concentration was determined by the pyridine hemochrome assay as described previously (*19*). PLP was measured fluorometrically using a modification of a published procedure (*20*) as described previously (*7*). CO binding studies were performed as described before (*21*).

Thermal Denaturation Assays. The purified recombinant enzymes were diluted to a final concentration of 5 mg/mL with Tris buffer, pH 8.6. The enzyme solution was warmed directly in a 1 mL quartz cuvette in a water-jacketed holder connected to a thermostated water bath. The temperature was raised from 30 to 65 °C in 5 °C increments, and the sample was maintained at the desired temperature for 5 min to achieve thermal equilibration. UV−visible absorption spectra were recorded between 345 and 445 nm, and the values at the Soret aborption peak at 429 nm were plotted against the respective temperatures.

Cell Culture. The human fibroblast cell line homozygous for the D444N expressing allele of cystathionine β-synthase was from a Dutch patient as described (*17*). The *cbl*G fibroblast cell line WG1892, purchased from the Repository for Mutant Human Cells, Montreal Children's Hospital, was used as a control. Cells were grown in 100 × 15 mm tissue culture dishes in MEM supplemented with 10% antimycotic, 10% v/v fetal bovine serum, and 2.2 g/L sodium bicarbonate until they reached 60−80% confluency. The culture medium was changed every 4 days.

Western Blot Analysis. After the medium was removed by aspiration, the monolayer of cells was rinsed with 2 × 3 mL of ice-cold PBS. Lysis buffer (containing 0.1 M potassium phosphate buffer, pH 7.4, 25 μg/mL TLCK, 0.1% Triton X-100, 50 μg/mL phenylmethanesulfonyl fluoride, 25 μg/mL leupeptin, 10 μg/mL pepstatin A, and 27.5 μg/mL aprotinin) was added to cover the cells which were incubated on ice for 30 min. Cell membranes were separated by centrifugation at 14000*g* for 10 min at 4 °C, and the supernatant was used for Western analysis.

Cell lysates containing 15 μg of protein were electrophoresed on a 7.5% polyacrylamide gel under denaturing conditions and transferred to an Immuno-Blot poly(vinylidene difluoride) membrane. Cystathionine β-synthase was detected using a primary antibody to cystathionine β-synthase at a 1:500 dilution and a secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG) diluted 5000-fold. Chemiluminescent detection was performed using the Immun-Star substrate (Bio-Rad). To confirm equal loading,

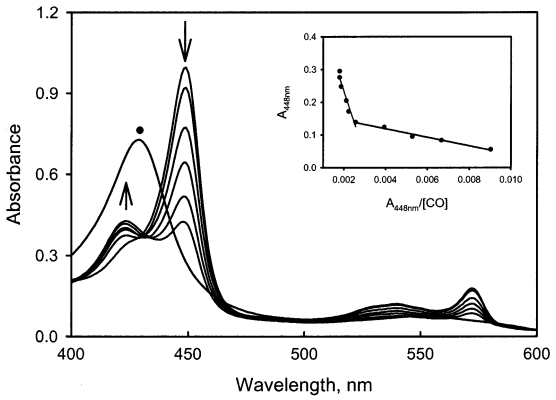


FIGURE 2: Binding of CO to the ferrous form of the D444N mutant. An anaerobic solution of enzyme (2.5 μM) in 25 mM Tris, pH 8.6, was reduced with 2 mM dithionite. Aliquots of CO-saturated water were added to the enzyme solution, and the spectrum was recorded after 2 min of equilibration. The lines from top to bottom at 448 nm represent CO concentrations of 1, 12, 50, 91, 130, and 150 μM. The spectrum marked with a dot represents the ferric form of the enzyme as isolated. Inset: Eadie−Hofstee plot showing biphasic binding isotherms with K_d's corresponding to 13 ± 1 μM and 194 ± 25 μM, respectively.

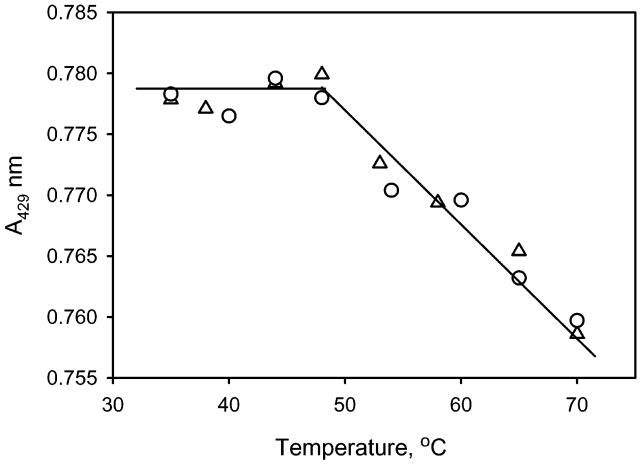


FIGURE 3: Thermal denaturation of wild type (○) and D444N mutant (△) forms of cystathionine β-synthase. Absorbance changes at 429 nm were plotted versus increasing temperature.

the membranes were reprobed with antibodies to methylmalonyl-CoA mutase. Western blots were performed in triplicate.

Northern Blot Analysis. Total RNA from fibroblast cells was isolated with the use of RNAqueous Kit (Ambion, Inc) following the vendor's protocol. RNA (13 μg) was electrophoresed on a 0.7% agarose gel containing 2.2 M formaldehyde. The gel was transferred in 20× SSC (where 1× SSC contains 0.15 M NaCl with 0.015 M sodium citrate, pH 7) to a Hybond-XL nylon membrane (Amersham Pharmacia) and then UV cross-linked. Prehybridization was carried out in 0.25 M sodium phosphate, pH 7.4, 20% SDS, 0.1 mM EDTA, and 10% bovine serum albumin. Further hybridization was carried out for ~24 h at 65 °C with cystathionine β-synthase cDNA labeled with [α-^{32}P]dCTP by random priming. The membrane was washed four times for 30 min each in 2× SSC and 0.1% SDS at 65 °C and was exposed overnight to a Biomax MS film (Kodak) at −80 °C. Radioactivity associated with the cystathionine β-synthase mRNA was normalized to the intensity of the 28S and 18S rRNA in each sample.

RESULTS

Purification and Kinetic Properties of the D444N Mutant. The recombinant D444N mutant was purified using the procedure developed for the wild-type enzyme (*18*). The specific activity of the mutant in the absence of AdoMet was 237 μmol h^{-1} (mg of protein)$^{-1}$ at 37 °C and is 1.5-fold higher than that of wild-type enzyme under the same conditions [159 μmol h^{-1} (mg of protein)$^{-1}$]. The K_m's for the substrates, serine and homocysteine, were unaffected, and the heme and PLP contents of the mutant and wild-type enzymes were identical (data not shown). The spectrum of the enzyme as isolated was of ferric heme with a Soret absorption maximum at 428 nm which shifted to 448 nm on reduction with dithionite (Figure 2), as reported previously for the wild-type enzyme (*18*). Denaturation of the enzyme can be monitored by changes in the heme absorption spectrum, which diminishes in intensity across the entire spectral range (not shown) and is inconsistent with a change in the axial ligation state as suggested previously (*15*). The denaturation profiles for the wild-type enzyme and the D444N mutant were identical, as shown in Figure 3, and did not exhibit the multiphasic behavior (initial decrease followed by an increase which is likely to have been contributed by turbidity) reported previously (*15*). The Soret absorption remained fairly constant up to 48 °C and diminished linearly in intensity above that temperature.

The native molecular masses of the D444N and wild-type enzymes were found to be identical. Each showed a broad elution profile when separated by HPLC on a gel filtration column. This has been seen previously for the wild-type enzyme separated by FPLC on a Superose 12 column, in which the peak of the broad elution band was consistent with the presence of a tetramer (*18*).

AdoMet Responsiveness of D444N versus Wild-Type Cystathionine β-Synthase. Under the standard assay conditions, 380 μM AdoMet is added to the reaction mixture and elicits an ~2-fold increase in activity with the wild-type enzyme and resulted in a slightly more modest 1.5-fold increase in the D444N mutant. Since the activity of this mutant was reported to be unresponsive to AdoMet in cell extracts up to a concentration of 200 μM AdoMet, we decided to further investigate the altered response of the mutant. The concentration dependence for AdoMet activation for wild-type and D444N enzymes is compared in Figure 4. In contrast to the wild-type enzyme, which is reported to have a K_{act} for AdoMet of 7.4 μM (*7*), the D444N mutant showed a modest response up to a concentration of ~200 μM AdoMet. Beyond this, the activity increased linearly and doubled at 1 mM AdoMet. The K_{act} for AdoMet for the D444N mutant was estimated to be 460 ± 130 μM.

CO Binding to Ferrous Heme in D444N versus Wild-Type Cystathionine β-Synthase. Wild-type cystathionine β-synthase binds CO in its ferrous state and displays a biphasic binding isotherm with K_d's of 1.5 ± 0.1 and 68 ± 14 μM, respectively (*21*). Binding of CO to the ferrous form of the D444N mutant was characterized as shown in Figure 2. As with the wild-type enzyme, binding of CO resulted in a blue shift in the Soret peak from 448 to 422 nm. Two distinct binding isotherms were also observed with the D444N

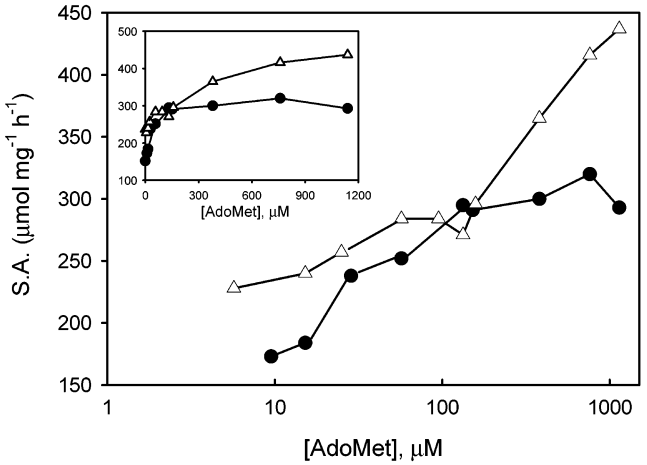


FIGURE 4: Dependence of cystathionine β-synthase activity on the concentration of AdoMet in the wild-type (●) and mutant (△) enzymes. The K_{act} for wild-type cystathionine β-synthase is reported to be 7.4 ± 0.2 μM. It is estimated to be 460 ± 130 μM for the D444N mutant. SA denotes specific activity.

mutant with K_d's corresponding to 13 ± 1 and 194 ± 25 μM, respectively (Figure 2, inset).

Characterization of D444N Cystathionine β-Synthase in a Patient Fibroblast Cell Line. The high level of enzyme activity associated with the D444N mutant even in the absence of AdoMet begs the question as to why the mutation is correlated with hyperhomocysteinemia in the homozygous state. To address this question, the steady-state mRNA and enzyme levels in a fibroblast cell line homozygous for the D444N-encoding allele were compared to that of a control cell line. As shown in Figure 5A, Northern blot analysis revealed comparable levels of cystathionine β-synthase-specific mRNA in both patient and control cell lines. In contrast, Western blot analysis revealed a 4-fold lower level of cystathionine β-synthase in the patient cell line (Figure 5B).

DISCUSSION

The D444N mutation was the first C-terminal regulatory mutant to be described in a homocystinuric patient (*17*). Cystathionine β-synthase activity in cultured fibroblasts from a patient who is homozygous for this mutation has been reported to lie in the range that is usually seen in heterozygotes in contrast to the severely elevated homocysteine levels that were observed in this patient. Furthermore, although the 1330A → G transition in cystathionine β-synthase encoding the D444N missense mutation was associated with severe hyperhomocysteinemia, the activity associated with the recombinant enzyme in *E. coli* cell extracts was comparable to that of the wild-type enzyme (*17*). However, the recombinant mutant showed only a modest increase in activity in the presence of up to 200 μM AdoMet, in contrast to the wild-type enzyme that showed a 2-fold increase (*17*). These observations are enigmatic and raise the following question. Why is loss of allosteric regulation by AdoMet in the face of higher basal activity in the D444N mutant versus wild-type enzyme pathogenic? To address this question, we have examined the biochemical properties associated with this mutation in a fibroblast cell line and with the purified recombinant protein.

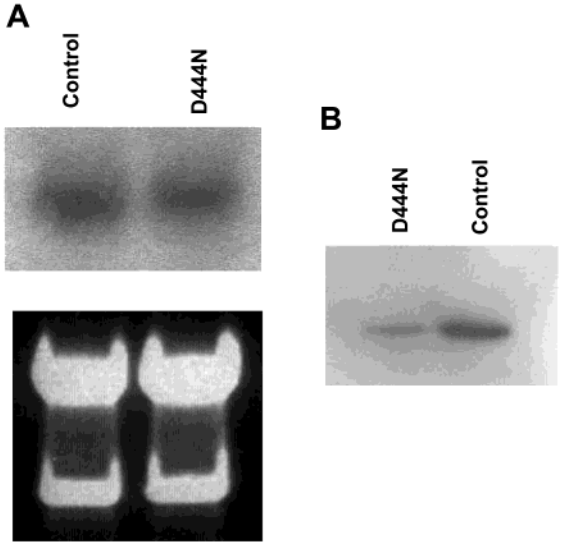


FIGURE 5: Comparison of steady-state cystathionine β-synthase mRNA and protein levels in control versus patient fibroblast cell lines. (A) Northern blot analysis of cystathionine β-synthase transcripts in control (WG1892) and D444N fibroblast cell lines. Upper: Total cellular RNA (13 μg) from each cell line was separated electrophoretically, blotted, and hybridized to a ^{32}P-labeled cystathionine β-synthase probe as described under Materials and Methods. Lower: Ethidium bromide staining was used to visualize the 28S and 18S rRNAs in each sample to ensure equal loading. (B) Western blot analysis of cystathionine β-synthase in cell extracts of control (WG1892) and D444N fibroblast cell lines. Cell extracts (15 μg of protein) from WG1892 and D444N fibroblast cells were loaded in each lane, and cystathionine β-synthase was detected with polyclonal antibodies as described under Materials and Methods. To ensure equal loading, the membrane was reprobed with methylmalonyl-CoA mutase antibody (not shown).

In a fibroblast cell line homozygous for the 1330A → G cystathionine β-synthase genotype we find that the mutation is associated with a 4-fold decrease in the steady-state levels of cystathionine β-synthase although the mRNA levels are unaffected (Figure 5). This corresponds very well with the ~4-fold lower cystathionine β-synthase activity in fibroblast extracts homozygous for the D444N mutation compared to the wild-type enzyme in the absence of AdoMet (see Figure 2 in ref *17*). Furthermore, although the D444N mutant is capable of being activated by AdoMet, the response is elicited at unphysiologically high concentrations of the effector (Figure 4). Thus, in an intracellular milieu where the AdoMet concentration is estimated to be ~50−80 μM (*22*), the D444N would be functionally unresponsive to AdoMet.

The properties of the recombinant enzyme expressed in *E. coli* were also characterized in this study. The V_{max} for the D444N mutant in the absence of AdoMet is high [237 μmol h^{-1} (mg of protein)$^{-1}$ at 37 °C] and more similar to that of the wild-type enzyme in the presence [295 μmol h^{-1} (mg of protein)$^{-1}$] rather than absence [159 μmol h^{-1} (mg of protein)$^{-1}$] of AdoMet (Table 1). This is in contrast to an earlier report in which the activities of the recombinant D444N mutant and wild-type enzymes were reported to be comparable in the absence of AdoMet (*17*). However, these measurements were made with *E. coli* cell extracts, and possible differences in the expression levels of the wild-type versus mutant proteins were not taken into account.

The observation that a mutation in the C-terminus affects the affinity of ferrous heme for CO in the N-terminus is

Table 1

parameter	wild type	D444N	ΔC143[c]
specific activity[a] (−AdoMet)	159	237	750
specific activity (+AdoMet)	295	365	750
V_{max} (+AdoMet)	295	549	750
k_{cat}[b] (−AdoMet) (s^{-1})	2.8	4.2	10
k_{cat} (+AdoMet) (s^{-1})	5.2	9.6	10
$K_{act(AdoMet)}$ (μM)	7.4	460	

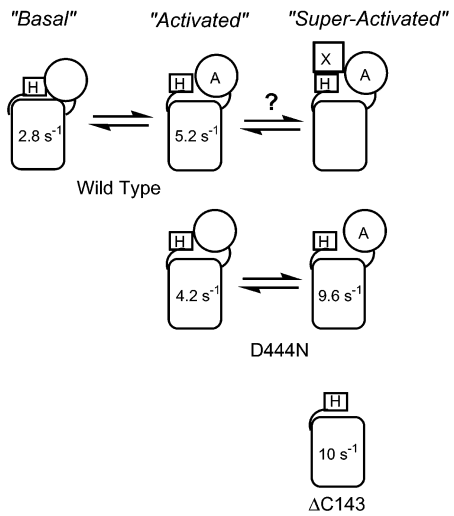
[a] Specific activity is represented in units of μmol h^{-1} (mg of protein)$^{-1}$ at 37 °C. The concentration of AdoMet in these assays was 380 μM. [b] k_{cat} is calculated per mole of active site (63 and 48 kDa molecular masses for the full-length and ΔC413 enzymes, respectively) to avoid assumptions about the oligomerization state, particularly for the full-length enzyme that exists as higher order aggregates of tetramers. [c] These values are taken from ref *7*.

interesting and points to interactions between the two ends of the protein in the three-dimensional structure. This is also consistent with the observation that the redox sensitivity of the protein, which is correlated with heme oxidation state change, is lost upon truncation of the C-terminus (*23*). The physiological significance of the high affinity of ferrous cystathionine β-synthase for CO and the consequent inhibition of catalytic activity is presently not known (*21*, *23*). The D444N mutation alters the affinity of the enzyme for CO, and this may contribute to the phenotype associated with this mutation.

The role of the C-terminal domain in regulating the activity of the catalytic core is poorly understood. Its deletion enhances the k_{cat} of the truncated enzyme 2-fold relative to the AdoMet-activated state of the full-length enzyme (Table 1), indicating that this domain functions as an intrasteric repressor (*7*). Furthermore, truncation results in a change in the oligomerization state from tetrameric to dimeric, revealing a role for the C-terminal domain in organizing the quaternary structure of the protein. Curiously, deletion of this domain alleviates the catalytic penalties associated with a number of catalytic core mutations that are pathogenic and points to the importance of interactions between the two domains in modulating enzyme activity (*13*). In the case of the V168M variant, which is an example of this class of pathogenic mutants (*13*), deletion of the C-terminal regulatory domain increased the PLP saturation from 15% to 100% (*14*). This results in the truncated enzyme carrying the V168M mutation becoming catalytically indistinguishable from the truncated enzyme with the wild-type sequence (*13*, *14*).

In yeast cystathionine β-synthase, truncation of the C-terminal domain similarly elicits a 2-fold increase in the V_{max} of the enzyme and a change in the oligomerization state from octameric to dimeric (*24*). However, the C-terminal domains of the yeast and human enzymes, unlike the catalytic cores, are not highly conserved at a primary sequence level, and the yeast enzyme is not responsive to AdoMet (*24*, *25*). Our observation that the D444N mutant behaves like the AdoMet-activated state of the wild-type enzyme (and does not change its oligomeric state) suggests that the mutation shifts the conformational equilibrium of the enzyme from a "basal" to an "activated" state (Scheme 1). Addition of high concentrations of AdoMet elicits a superactivated state that is similar to that of the truncated enzyme. In the model presented in Scheme 1, we present the possibility that an endogenous small molecule effector and/or one or more proteins, "X", could shift the conformational equilibrium of the wild-type enzyme to the superactivated state which is mimicked by the D444N mutant at very high AdoMet concentrations. This model is consistent with the observation of three kinetically distinguishable states of cystathionine β-synthase (Table 1), wild-type enzyme in the absence of AdoMet (basal state), wild-type enzyme in the presence of AdoMet (activated state), and wild-type enzyme missing the C-terminus (superactivated state). Our observation that the D444N mutation at supraphysiological concentrations of AdoMet mimics the superactivated state raises the possibility that the full-length enzyme is also capable of accessing this conformation.

Scheme 1: Model Showing Three Kinetically Distinguishable States of Human Cystathionine β-Synthase[a]



[a] H, A, and X denote heme, AdoMet, and an unknown effector, respectively. The number associated with each enzyme form represents its k_{cat} value calculated per mole of active site.

In summary, characterization of the biochemical penalties associated with the D444N missense mutation, first described in a Dutch homocystinuric patient, provides interesting insights into the properties of the native enzyme. The mutation mimics an activated conformation that is elicited in the wild-type enzyme by the V-type allosteric effector, AdoMet. Although the enzyme can be further activated in vitro at very high concentrations of AdoMet, the D444N mutant would be functionally unresponsive in an intracellular milieu. The high activity associated with the D444N mutant is countered by a diminished steady-state level of the mutant protein and accounts for the 4-fold lower cystathionine β-synthase activity reported in cultured fibroblast cell extracts. The cystathionine β-synthase activity measured in the patient nevertheless falls within the range measured in heterozygotes rather than in homozygotes, which is in contrast to the high homocysteine levels measured in this patient (*17*). These findings point to the important role of the C-terminal domain, either independently or via interactions with one or more proteins, in regulating the intracellular activity of cystathionine β-synthase.

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