Kinetic Properties of Polymorphic Variants and Pathogenic Mutants in Human Cystathionine γ-Lyase[†]

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ABSTRACT: Human cystathionine-γ-lyase (CGL) is a pyridoxal-5′-phosphate (PLP)-dependent enzyme, which functions in the transsulfuration pathway that converts homocysteine to cysteine. In addition, CGL is one of two major enzymes that can catalyze the formation of hydrogen sulfide, an important gaseous signaling molecule. Recently, several mutations in CGL have been described in patients with cystathioninuria, a rare but poorly understood genetic disease. Moreover, a common single nucleotide polymorphism in CGL, c.1364G>T that converts serine at position 403 to isoleucine, has been linked to elevated plasma homocysteine levels. In this study, we have characterized the pathogenic T67I and Q240E missense mutations and the polymorphic variants at amino acid residues 403 using kinetic and spectrophotometric methods. We report that the polymorphism does not influence the cofactor content of the enzyme or its steady-state kinetic properties. In contrast, the T67I mutant exhibits a 3.5-fold decrease in V_{max} compared to that of wild-type CGL, while the Q240E mutant exhibits a 70-fold decrease in V_{max}. The K_Ms for cystathionine for both pathogenic mutants are comparable to that of wild type CGL. The PLP content of the T67I and Q240E mutants were about 4-fold and 80-fold lower than that of wild-type enzyme, respectively. Preincubation of the T67I mutant with PLP restored activity to wild-type levels while the same treatment resulted in only partial restoration of activity of the Q240E mutant. These results reveal that both mutations weaken the affinity for PLP and suggest that cystathionuric patients with these mutations should be responsive to pyridoxine therapy.

The mammalian transsulfuration pathway represents one of two major metabolic routes for the removal of homocysteine, which at elevated levels, is an independent risk factor for cardiovascular diseases and other complex disorders (*1, 2*). Cystathionine γ-lyase (CGL[1]), the second enzyme in the transsulfuration pathway, catalyzes the conversion of cystathionine to cysteine, α-ketobutyrate and ammonia in a PLP-dependent α,γ-elimination reaction (eq 1).

$$\text{Cystathionine} \rightarrow \alpha\text{-Ketobutyrate} + \text{Cysteine} + NH_3 \quad (1)$$

The first step in the pathway is catalyzed by cystathionine β-synthase and involves the condensation of homocysteine and serine to cystathionine (*3*). Human CGL has a subunit molecular mass of 44.5 kDa, purifies as a homotetramer (*4*), and belongs to the γ-family of PLP enzymes (*5*). One PLP cofactor is bound per subunit and is present in a Schiff base linkage to the active site lysine, K212.

In addition to providing an avenue for clearance of homocysteine, the transsulfuration pathway contributes in a quantitatively significant manner to the total intracellular cysteine pool. Thus, it is estimated that in liver, ~50% of the cysteine that is utilized for biosynthesis of the ubiquitous antioxidant, glutathione, is derived from the transsulfuration pathway with the remainder presumably being obtained via transport (*6*). In addition, the transsulfuration pathway provides cysteine for catabolism under conditions of methionine excess and is upregulated as a component of an allosteric switching mechanism. The product of the transsulfuration pathway, cysteine, also serves as the primary substrate for H_2S biogenesis by both cystathionine β-synthase and by CGL (*7, 8*). Traditionally regarded as a toxic gas, H_2S is the newest member of the family of biological gasotransmitters and appears to function both as a neuromodulator in the central nervous system and as a vasodilator of smooth muscle cells in the vascular system (*9, 10*). In addition, H_2S also plays an important role in inflammation, although the mechanism by which this occurs, is controversial (*11–14*).

Mutations in CGL lead to cystathioninuria, an autosomal recessive disorder that has an estimated prevalence of about 1:14,000 live births (*15*). Cystathioninuria can also be secondarily associated with a wide range of diseases including diabetes insipidus, Down's syndrome, neuoroblastoma, hepatoblastoma, and celiac disease (*15, 16*). Sequence analysis of the CGL gene in cystathionuric patients has so far identified four rare nonsynonymous CGL mutations (*15*). Of these, two are nonsense mutations leading to premature termination, and two are

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[1] Abbreviations: CGL, cystathionine γ-lyase; PLP, pyridoxal-5′-phosphate; SNP, single nucleotide polymorphism; DTNB, dithiobisnitrobenzene.

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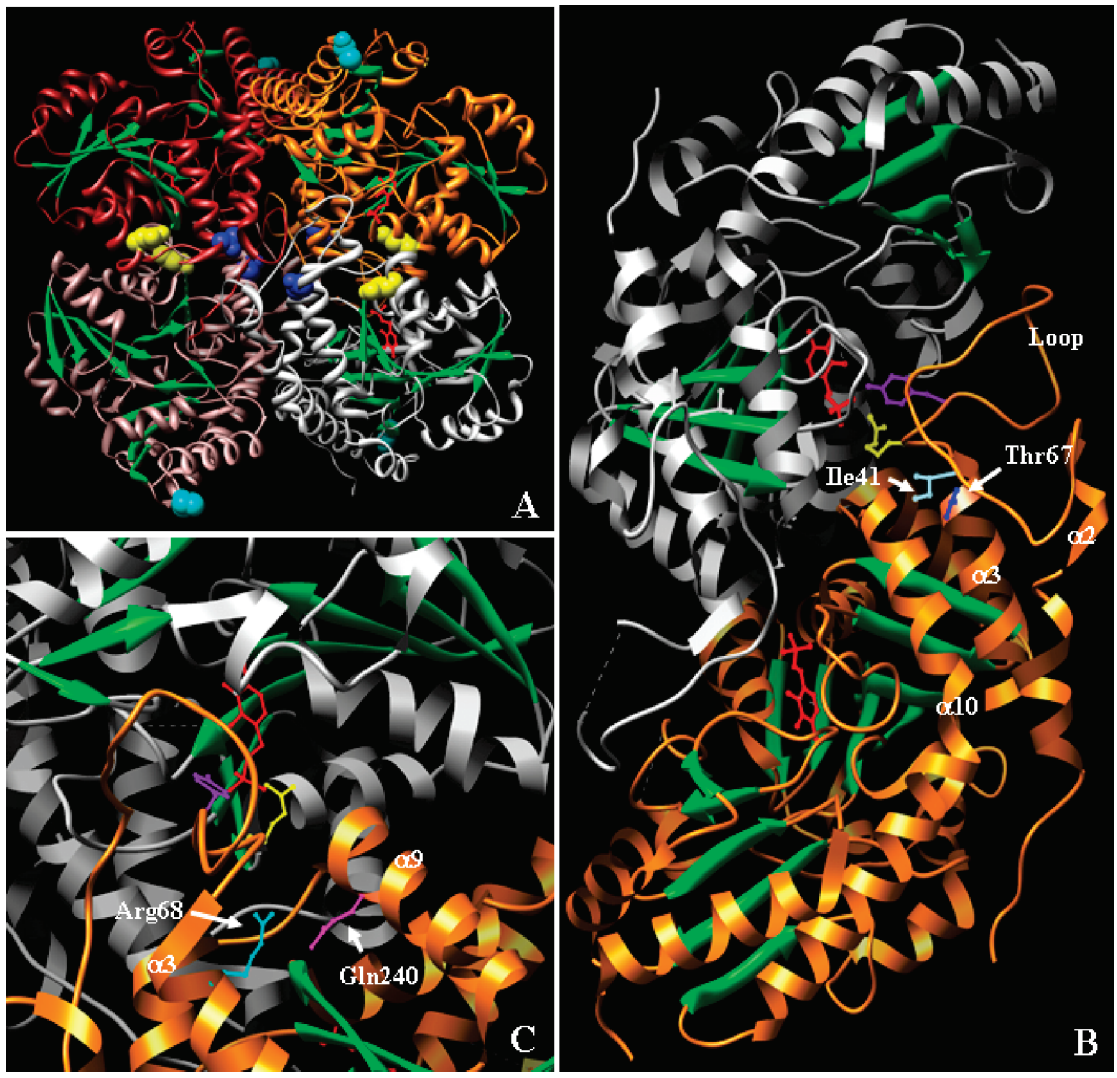

FIGURE 1: Crystal structure of human CGL showing locations of the polymorphic variation and the pathogenic mutations. (A) Tetrameric wild-type CGL. Key residues are highlighted with the PLP cofactor shown in stick representation in red, T67 shown in blue spheres, and Q240 in yellow spheres. The C-terminal residues are highlighted in cyan spheres and are immediately upstream of the polymorphic site, 403, which is not resolved in the structure. The CGL tetramer is built up by two active dimers (stacked vertically). (B) Each active site is formed by two adjacent subunits. T67 (blue, located on helix 3) makes close interactions with I41 (light blue, located on the extended loop between helix 2 and helix 3). C. The active site formed by two subunits shows the important interaction between Q240 (pink, located on helix 9) and R68 (cyan, located on helix 3). The figure was produced using UCSF Chimera (*24*) and the PDB file 2NMP.

missense mutations: T67I and Q240E. The biochemical properties of the pathogenic missense mutations have not been characterized. In addition, a common single nucleotide polymorphism (SNP), c.1364G>T, resulting in the predicted replacement of serine at position 403 with isoleucine has been reported, and its prevalence in a Canadian population shows ethnic variation. Thus, the frequency for the isoleucine-encoding allele was found to range from 10% (in Africans) to 20.7% (in Chinese) to 31.5% (in East Indians) to 33% (in Europeans). The T403 allele is correlated with mild hyperhomocysteinemia under homozygous conditions (*17*).

The structure of human CGL has recently become available in the protein database from the Structural Genomics Consortium (PDB ID: 2NMP). The 2.6 Å resolution structure of human CGL reveals that it is a tetramer (Figure 1). The polymorphic site at position 403 is located two residues away from the C-terminus of the polypeptide chain, which ends at residue 405. Q240 is located at the monomer−monomer interface, whereas T67 is at the dimer−dimer interface. Thus, on the basis of the structure, both mutations could be expected to destablize the oligomeric architecture of CGL, which in turn may represent the underlying cause of their pathogenecity.

In this study, we have characterized the polymorphic variants and the two pathogenic missense mutations described in human CGL. While the steady-kinetic parameters of the polymorphic variants I403 and S403 are indistinguishable, the two patient mutants exhibit significantly lower activity and PLP content than wild-type CGL and show different degrees of PLP-responsiveness, which could be clinically relevant. The enzyme activity of the T67I mutant can be fully rescued by exogenous PLP, while that of the Q240E mutant can only be partially recovered, indicating that this mutation impairs CGL more severely.

MATERIALS AND METHODS

Chemicals and Reagents. All chemicals were purchased from Sigma or Fisher, and the oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA).

Generation of Human CGL S403, T67I, and Q240E Variants. The expression plasmid for the recombinant human I403 CGL variant was generously provided by Dr. Marcus Wahl (Max-Planck-Institute for Biochemistry (Martinsried, Germany). The S403 variant was generated by site-directed mutagenesis using the Quickchange Kit from Stratagene and the I403-encoding plasmid DNA as the template, and site-directed mutagenesis was performed using the following forward primer: 5′-CACCCTCCAAGTGGAAGTCA-CAGCTAGTAACTCGAG-3′. The mutated codon is underlined. The reverse primer had the complementary sequence. The codon change in the PCR product was confirmed by DNA sequencing.

The T67I and Q240E mutations were generated using the S403-encoding plasmid DNA as a template. The forward primers for the generation of T67I and Q240E were T67I: 5′-GCCGTTCTGGAAATCCCATTAGGAATTG-CCT-TGAAAAAGC-3′ and Q240E: 5′-CATAATAGACTTCGTT-TCTTGGAAAACTCTCTTGGAGCAGTTCC-3′. The mutated codons are underlined. The reverse primers had the complementary sequences. After the sequences were confirmed, the plasmids were transformed into the *Escherichia coli* expression strain BL21 DE3.

Protein Purification. Recombinant human CGL was expressed in the *E. coli* strain BL21(DE3). Following inoculation, cells were grown to OD_{600} of 0.8 at 37 °C. CGL was induced at 25 °C by adding 1 mM isopropyl-β-D-thiogalacto-pyranoside, and growth was continued at 25 °C for 18 h. Cells were harvested by centrifugation at 11,000*g* for 15 min. To purify CGL, the *E. coli* cell pellet from a 6 L culture was resuspended in 100 mL of 1× binding buffer (20 mM Tris, pH 7.9, containing 0.5 M NaCl) supplemented with protease inhibitors and lysozyme. The resuspended cells were stirred at room temperature for 30 min and then sonicated with an Ultrasonic Processor (Misonix) at 4 °C using a macrotip with a pulse sequence of 15 s on, 45 s off (8 min total pulse time). The broken cells were centrifuged at 27,000*g* for 40 min. The resulting supernatant was subsequently added to a 3 × 8 cm Ni-NTA fast flow (Qiagen) column equilibrated with 1× binding buffer. CGL was eluted with a gradient from 0−0.2 M imidazole in binding buffer. Fractions exhibiting enzyme activity in the dithiobisnitrobenzene (DTNB) assay (see below) were pooled and dialyzed overnight against 70 mM Tris buffer at pH 8.1 containing 2 mM EDTA (Buffer A). Further purification was performed on a 3 × 12 cm Q-Sepharose fast-flow column (Pharmacia Biotech) equilibrated with buffer A. A gradient of 0−0.3 M KCl in buffer A was employed to elute CGL. Fractions exhibiting enzyme activity were pooled and concentrated down to ∼10 mg/mL using an Amicon ultrafiltration cell. The concentrated pool was then applied to a 500-ml Superdex S-200 (Sigma) size exclusion column and chromatographed in 70 mM Tris at pH 8.1 containing 150 mM NaCl at a flow rate of 0.5 mL/min. The active fractions were pooled and concentrated as above, and then stored at −80 °C. The concentration of CGL was determined using the Bradford reagent (Bio-Rad) with bovine serum albumin as a standard.

Enzyme Activity. The activity of CGL was measured by monitoring the generation of cysteine from cystathionine or the generation of H_2S from homocysteine or cysteine. The dithiobisnitrobenzene (DTNB) assay was used to measure cysteine produced from cystathionine as described previously (*4*). Production of H_2S from different substrates was measured in a spectrophotometric assay in which the reaction of H_2S with lead acetate to form lead sulfide was monitored continuously by the increase in absorption at 390 nm. After the reaction mixture (980 μL) containing 40 mM borate (pH 7.0), 50 μM PLP, 0.4 mM lead acetate, and varying concentrations of substrate (homocysteine or cysteine) was preincubated at 37 °C for 4 min, 20 μL of 1 mg/mL purified CGL was added to the assay mixture to initiate the reaction, and the reaction was monitored at 37 °C for 3 min. Lead acetate (0.4 mM) did not inhibit CGL as determined in the DTNB assay described above. The molar extinction coefficient for lead sulfide under these conditions was determined to be 6294 M^{-1} cm^{-1}using Na_2S as standard.

PLP Dependence of CGL Activity. To measure the PLP responsiveness of the CGL variants, 100 μL of 1 mg/mL CGL in 70 mM Tris buffer (pH 8.1, containing 150 mM NaCl) was preincubated with 20 μL of 10 mM PLP in the same buffer (or just buffer alone in the control sample) at room temperature. Aliquots (10 μL) of preincubated CGL were removed at different time points to perform activity assays. The activity was measured in the DTNB assay with 1 mM cystathionine, and additional PLP was not added.

PLP Analysis. The PLP content of the CGL variants was measured fluorimetrically using a modification of a published procedure (*18*). PLP was released from the enzyme by treating 0.5 mg/mL of CGL in 1.5 mL of 0.1 M potassium phosphate buffer at pH 7.2 with 0.5 M hydroxylamine at room temperature for 3−5 h followed by centrifugation in a Centricon concentrator (P30, Amicon). Fluorescence emission of the PLP oxime was detected at 446 nm following excitation at 353 nm. A standard curve was generated using PLP samples of known concentrations, which had been determined spectrophotometrically using $\epsilon_{295} = 5.1$ mM^{-1} cm^{-1} in 0.1 N HCl.

Determination of the Oligomeric State. To determine the oligomeric state of wild-type CGL and the variants, ∼0.1 mg of the protein was loaded on a 2 × 70 cm Sephacryl 200 column in 70 mM Tris buffer, (150 mM NaCl at pH 8.1) and eluted at a flow rate of 2 mL min^{-1}. The column was calibrated using gel filtration standards from Bio-Rad.

RESULTS

Purification and Kinetic Properties of the Polymorphic Variant S403. The recombinant S403 variant was purified using a combination of Ni-NTA chromatography, anion exchange chromatography, and size-exclusion chromatography steps, as used previously for the I403 polymorphic variant. The resulting S403 protein has a purity of >95% based on separation on a denaturing polyacrylamide gel (not shown). The spectrum of S403 is identical to that of I403 CGL with a ratio of A_{280}/A_{428} of 7 (Figure 2). In addition, a minor peak at ∼500 nm, which is also seen in the I403 protein preparations (*4*), was observed and could be removed by reconstituting CGL with PLP following the generation of apoprotein (data not shown). The 500 nm absorption could be due to a quinonoid species formed by a tightly bound amino acid that copurifies with CGL. The identity of the amino acid is presently not known. The specific activities for cystathionine cleavage and for H_2S generation from homocysteine or cysteine were compared for the polymorphic

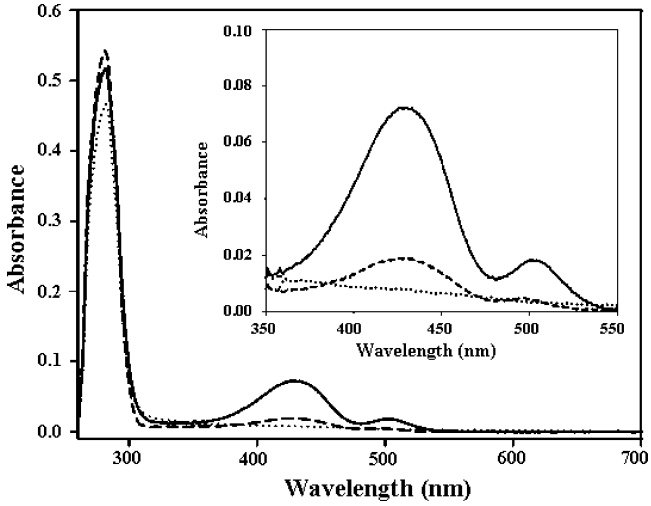

FIGURE 2: UV−visible absorption spectra of purified wild-type (—), mutant CGL (T67I (---), and Q240E (•••). Protein concentrations used were wild-type, 0.82 mg/mL; T67I, 0.95 mg/mL; and Q240E, 0.80 mg/mL in Tris-HCl buffer at pH 8.1 containing 150 mM NaCl. The inset zooms in on the PLP absorbance region.

variants using at least three independent preparations of each variant. The specific activities of the S403 and I403 CGL were comparable in the standard assay using cystathionine as substrate (Table 1). In fact, no differences in the kinetics parameters were seen between the polymorphic variants regardless of whether cystathionine, homocysteine, or cysteine was used as substrate.

Purification and Kinetic Properties of the Patient Mutations T67I and Q240E. Since the S403-encoding allele is the more prevalent of the two polymorphic variants, we chose the S403 variant as the background for expressing the two pathogenic mutants, T67I and Q240E. The mutant proteins were purified through three chromatographic steps like wild-type CGL. However, the expression level of both the T67I and the Q240E mutant proteins were lower, and they were purified in lower yields (~12 mg of T67I and 5 mg of Q240E per liter of culture compared to ~ 25 mg of wild type (S403) CGL per liter of culture). Both the T67I and Q240E mutants were obtained in >90% purity based on Coomassie blue staining of denaturing polyacrylamide gels (not shown).

The specific activity of the T67I CGL for α,γ-cleavage of cystathionine was 0.6 μmol min^{-1} mg^{-1} protein, i.e., 3.5-fold lower than that of wild-type enzyme. The specific activities for H_2S generation from homocysteine and cysteine showed similar decreases of 4- and 2-fold, respectively, while the K_Ms for all three substrates were comparable (Table 1). In contrast, the activity of the Q240E mutant was 70-fold lower with cystathionine as substrate (0.03 μmol min^{-1} mg^{-1}) and H_2S generation could not be detected using either homocysteine or cysteine as substrate.

The UV−visible absorption spectrum of the two mutants (Figure 2) revealed that the T67I CGL has an A_{280}:A_{427} ratio of 29 compared to 7 for wild-type CGL indicating substoichiometric saturation with PLP. In contrast, the absorbance of the Q240E mutant in the 427 nm region was barely detectable. These data indicate that the lower activity associated with both mutants is associated with their lower PLP content with the Q240E mutant being more severely affected than the T67I one.

PLP Responsiveness of Patient Mutations. We determined the PLP content of each variant by releasing PLP from holoenzyme with hydroxylamine and measuring the fluorescence of the resulting PLP-oximine. Both wild-type polymorphic variants I403 and S403, exhibited PLP content of ~80% based on four PLPs bound per tetramer, while the PLP content of the T67I mutant was 19%. The PLP content of the Q240E mutant was strikingly lower and corresponded to ~0.3%−1% saturation, consistent with the UV−visible absorption spectrum indicating that the enzyme is predominantly present as apoenzyme. Since the two mutants exhibit significantly lower PLP content than wild-type CGL, their responsiveness to exogenous PLP was assessed. As shown in Figure 3, when preincubated with PLP at room temperature, the activity of T67I CGL increased, approaching wild-type levels within 8 h (specific activity of 0.97 ± 0.04 μmol/min/mg protein). While the Q240E mutant also exhibited PLP responsiveness, its activity after 8 h of preincubation with the cofactor was still ~8−10 fold lower (specific activity is 0.15 ± 0.03 μmol/min/mg protein) than that of wild-type CGL. Hence, PLP preincubation cannot fully rescue the activity of the Q240E mutant.

Oligomeric States of CGL Variants. Wild-type human CGL purifies as a homotetramer with a predicted molecular mass of 178 kDa. In the three-dimensional structure of human CGL, two monomers contribute residues to each of the two active sites in the dimer, and two dimers come together to form a tetramer (Figure 1A). Since the locations of the missense mutations at the monomer−monomer interface (T67I) and at the dimer−dimer interface (Q240E) suggested a priori that they might disrupt structural integrity, their oligomeric states were analyzed by size-exclusion chromatography (Figure 4). The estimated molecular masses of both the T67I CGL (156 kDa) and the Q240E mutant (170 kDa) were comparable to that of wild-type CGL (167 kDa), indicating that both mutants exist as tetrameric proteins.

DISCUSSION

The transsulfuration pathway enzymes cystathionine β-synthase and CGL comprise an avenue for the synthesis of cysteine and disposal of excess methionine, and play a role in H_2S biogenesis and in homocysteine homeostasis in mammals. In contrast to cystathionine β-synthase, which has been studied quite extensively over many years (for reviews see refs *19 and 20*), CGL has been the subject of sparse attention, and significant gaps exist in our understanding of the reaction mechanism and regulation of this enzyme. The recombinant human enzyme has been expressed in *E. coli*, and the basic kinetic parameters for the reaction with cystathionine have been reported (*4*). More recently, our laboratory has used the recombinant human enzyme to evaluate the relative importance of cysteine versus homocysteine as substrates for CGL-catalyzed H_2S biogenesis (Zhu et al., unpublished results).

In this study, the biochemical properties of a common polymorphism, S403I, have been examined. The SNP in exon 12, c.1364G>T, results in the replacement of serine at residue 403 (S403) with isoleucine (I403) (*15*). The I403 variant has an allele frequency varying from 0.1 to 0.33 in different ethnic groups. Moreover, homozygosity of this less frequent allelic variant was found to be associated with

Table 1: Comparison of the kinetic parameters for the polymorphic variants and pathogenic mutants of CGL

substrate	parameter	CGL variant or mutant			
		S403	I403	T67I	Q240E
Cst[a]	V_{max}[b]	2.3 ± 0.2	2.6 ± 0.1	0.6 ± 0.1	0.03 ± 0.005
	K_M, mM	0.4 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.72 ± 0.16
	k_{cat}, s^{-1}	1.7 ± 0.2	1.9 ± 0.08	0.45 ± 0.07	0.02 ± 0.004
	k_{cat}/K_M[c]	4.2	3.2	0.86	0.028
Hcy	V_{max}	4.7 ± 0.3	5.0 ± 0.2	1.1 ± 0.2	N.D.[d]
	K_M, mM	5.4 ± 1.0	7.0 ± 1.3	8.0 ± 2.1	N.D.
	k_{cat}, s^{-1}	3.5 ± 0.2	3.7 ± 0.2	0.82 ± 0.16	N.D.
	k_{cat}/K_M	0.65	0.53	0.10	N.D.
Cys	V_{max}	0.9 ± 0.1	0.9 ± 0.1	0.4 ± 0.03	N.D.
	K_M, mM	3.5 ± 1.2	3.4 ± 1.4	4.1 ± 1.7	N.D.
	k_{cat}, s^{-1}	0.67 ± 0.07	0.67 ± 0.07	0.30 ± 0.02	N.D.
	k_{cat}/K_M	0.19	0.20	0.07	N.D.

[a] Cst, Hcy, and Cys refer to cystathionine, homocysteine, and cysteine, respectively. [b] V_{max} is expressed in units of μmol of cysteine or H_2S formed min^{-1} mg^{-1} of protein at 37 °C. [c] k_{cat}/K_M is expressed in units of mM^{-1} s^{-1}. [d] N.D. is not detected.

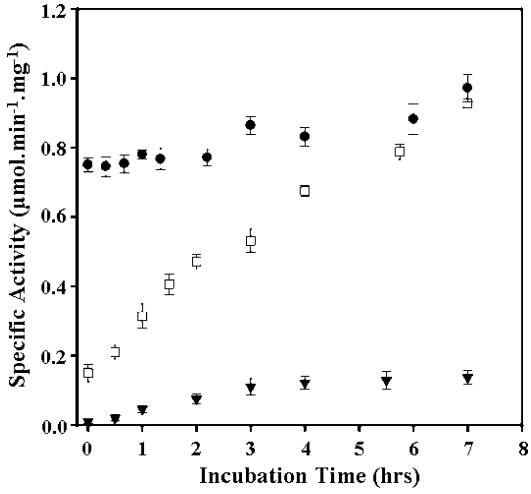

FIGURE 3: PLP dependence of enzymatic activity of CGL variants. The CGL variants (1 mg/mL) were preincubated with 1.7 mM PLP at room temperature, aliquots (5−10 μL) were removed at the indicated times, and enzyme activity was measured using the DTNB assay as described in Materials and Methods. ●, wild-type CGL (S403); □, T67I; ▼, Q240E.

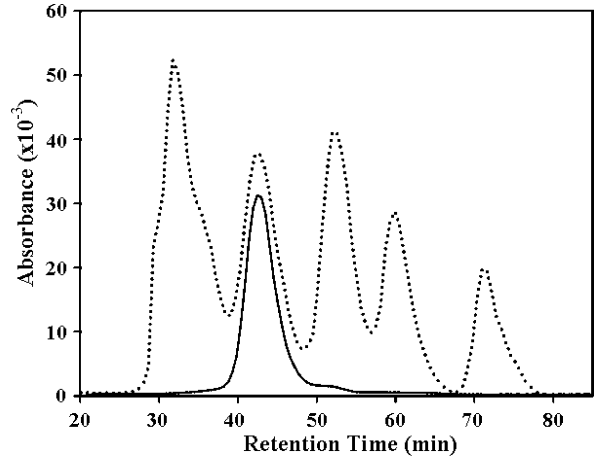

FIGURE 4: Oligomeric structure of CGL. CGL (100 μg) was separated on a Sephacryl S200 size exclusion column and eluted isocratically with 70 mM Tris at pH 8.1 containing 150 mM NaCl as described in Materials and Methods. A representative elution profile of CGL (the T67I mutant is shown by the solid line) is compared to that of protein molecular weight standards (dotted line, from Bio-Rad) separated under the same conditions. The molecular weight standards used were thyroglobulin (670 kDa), bovine gamma-globulin (158 kDa), chicken ovalbumin (44 kDa), equine myoglobin (17 kDa), and vitamin B12 (1.35 kDa). A molecular mass of 156 kDa was estimated for CGL from its retention time of 42.8 min.

elevated plasma homocysteine (*17*). This pathogenic association impelled us to evaluate the biochemical phenotypes associated with the two variants. The purified I403 and S403 do not have obvious difference in the UV−visible spectrum, and exhibit the same ratio of PLP absorbance (at 428 nm) to polypeptide absorbance (at 280 nm) of 1:7, indicating that the PLP content of these variants are the same. Steady-state kinetic analysis revealed that the polymorphic variants are indistinguishable in their kinetic parameters, both with respect to the primary CGL activity and in H_2S generation from homocysteine or cysteine (Table 1).

Comparison of CGL sequences from different organisms reveals that the S403 residue is conserved in several mammals, worms, and yeast, but not in bacteria (data not shown). Unfortunately, the C-terminal end harboring the S403 residue is not visible in the crystal structure of either the yeast (*5*) or human CGL. However, residues that are immediately upstream (P399, P400, and S401) are resolved in the human CGL structure (Figure 1A), and the C-terminus of each subunit (highlighted in cyan) protrudes into the solvent, which raises the possibility that it may be involved in interaction with an as yet unidentified partner protein. The location of the C-terminus distal to the active site is consistent with the insensitivity of the reaction kinetics to the identity of the residue at position 403. We note, however, that although the CGL variants display virtually identical kinetic properties in vitro, differences may exist in vivo in individuals who are homozygous for the polymorphic variation. A good example of this is seen in human catechol *O*-methyltransferase, an enzyme involved in the catabolism of catechol estrogens and the catecholamine neurotransmitters (*21*). Human catechol *O*-methyltransferase has a common SNP that leads to a V108M variation, and the purified variants have similar kinetic properties. However, homozygosity for the M108 allele in comparison to the V108 allele, is associated with a 2−4-fold lower enzyme activity in erythrocytes, in liver biopsy tissues, and in postmortem dorsolateral prefrontal cortex tissues (*21*).

In contrast to the polymorphism in CGL, the pathogenic mutants exhibit substantial differences from the wild-type

enzyme. To date, only two missense mutations have been reported in CGL from cystathioninuric patients, T67I and Q240E (*15*). In this study, both mutants were expressed and purified and found to have relatively low PLP content (Figure 3) and correspondingly lower activity compared to those of the wild-type enzyme. These data indicate that both T67 and Q240 play important roles in PLP binding. Neither mutation appears to alter the K_M for cystathionine.

Patients with inborn errors of metabolism in cofactor-dependent enzymes are often responsive to treatment with the specific cofactor (*22*). For instance, a subclass, representing ~40% of patients with mutations in cystathionine β-synthase, shows responsiveness to therapy with pyridoxine, a precursor of PLP (*23*). We therefore examined the effect of preincubation of the mutant CGLs with excess PLP on enzyme activity and found that this treatment resulted in recovery of T67I CGL activity to wild-type levels (Figure 4). In contrast, the Q240E mutant could only be partially rescued by preincubation with exogenous PLP. These results suggest that cystathionuric patients with either of these mutations would be responsive, at least to some measure, to pyridoxine therapy.

The X-ray structure of human CGL reveals that the architecture of each active site incorporates structural elements from two adjoining monomers (Figure 1). As in the structure of yeast CGL (*5*), the PLP in one subunit is engaged in several hydrogen-bonding interactions with residues in the adjacent subunit (Y60* and R62*), and the phosphate moiety is a major anchor of the cofactor in the active site. The environment of T67 and Q240 may explain why their mutations to an isoleucine and glutamate respectively changes the affinity of CGL for PLP binding. As shown in Figure 1B, T67 is located at the N-terminal end of helix 3, which connects to an extended loop on which the PLP-anchoring residues, Y60 and R62, are located. Helix3 makes close contact with both the loop and the N-terminal part of the extended helix 10, which kinks at residue T261. T67 is within van der Waals distance of I41 on the extended loop. Introduction of another isoleucine at position 67 could destabilize this local conformation and alter the distance between the phosphate group and the Y60/R62 residues, thereby resulting in decreased PLP affinity. Q240 is located on helix 9 and points toward the neighboring helix3 (Figure 1C). Mutation of this residue to a glutamate could introduce an electrostatic interaction with R68 on helix3, lead to shortening of the distance between the two helices, and impact the conformation of helix3 and its adjoining loop. In this way, the positions of the PLP phosphate anchoring residues could be changed leading to a loss in the PLP binding affinity. Although both mutations impaired cofactor binding of CGL, the phenotype of the T67I is milder than that of the Q240E mutant. The interaction between I67 and I41 in the T67I mutant could be transferred to the PLP-anchoring residues through a flexible loop, while the interaction between E240 and R68 in the Q240E mutant is predicted to involve a more rigid helix. We speculate that this difference may be related to the magnitude of impairment associated with each mutation.

In summary, our study predicts that high doses of pyridoxine could be an effective therapy for cystathioninuric patients harboring the T67I mutation and be somewhat less effective in ameliorating the symptoms associated with the Q240E mutation. The relatively prevalent polymorphic variation in CGL at position 403 appears to be silent at least as evaluated by steady-state kinetic assays in vitro.

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