Effects of Heme Ligand Mutations Including a Pathogenic Variant, H65R, on the Properties of Human Cystathionine β-Synthase†

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ABSTRACT: Human cystathionine β-synthase is a hemeprotein that catalyzes a pyridoxal phosphate (PLP)-dependent condensation of serine and homocysteine into cystathionine. Biophysical characterization of this enzyme has led to the assignment of the heme ligands as histidine and cysteinate, respectively, which has recently been confirmed by crystal structure determination of the catalytic core of the protein. Using site-directed mutagenesis, we confirm that C52 and H65 represent the thiolate and histidine ligands to the heme. Conversion of C52 to alanine or serine results in spectral properties of the resulting hemeprotein that are consistent with the loss of a thiolate ligand. Thus, the Soret peak blue-shifts from 428 to 415 and 417 nm in the ferric forms of the C52S and C52A mutants, respectively, and from 450 to 423 nm in the ferrous states of both mutants. Addition of CO to the dithionite-reduced ferrous C52 mutants results in spectra with Soret peaks at 420 nm. EPR spectroscopy of the ferric C52 variants reveals the predominance of a high-spin species. The H65R mutant, a variant described in a homocystinuric patient, has Soret peaks at 424, 421, and 420 nm in the ferric, ferrous, and ferrous CO states, respectively. EPR spectroscopy reveals predominance of the low-spin species. Both C52A and C52S mutations lead to protein with substoichiometric heme (19% with respect to wild type); however, the PLP content is comparable to that of wild-type enzyme. The heme and PLP contents of the H65R mutant are 40% and 75% that of wild-type enzyme. These results indicate that heme saturation does not dictate PLP saturation in these mutant enzymes. Both H65 and C52 variants display low catalytic activity, revealing that changes in the heme binding domain modulate activity, consistent with a regulatory role for this cofactor.

Cystathionine β-synthase is a key enzyme involved in homocysteine metabolism in mammals and catalyzes the condensation of serine and homocysteine to form cystathionine. Elevated levels of homocysteine are correlated with an increased risk for cardiovascular diseases (*1*), neural tube defects (*2*, *3*), and Alzheimer's disease (*4*). The enzymes from both yeast and mammalian sources have been characterized, and are dependent on PLP[1] for activity, which is expected based on the β-replacement chemistry that is catalyzed. The mammalian enzymes contain an additional cofactor, iron protoporphyrin IX (*5*), whose role in the reaction is unclear.

Human cystathionine β-synthase belongs to the relatively small family of hemeproteins that includes CooA (*6*), cytochrome P450 (*7*, *8*), chloroperoxidase (*9*, *10*), and NO synthase (*11*−*13*), characterized by the presence of an axial cysteinate ligand. A combination of biophysical approaches including EPR spectroscopy (*14*, *15*), resonance Raman spectroscopy (*16*), and extended X-ray absorption fine structure spectroscopy (*14*) has led to the assignment of the axial ligands in mammalian cystathionine β-synthase as histidine and cysteine, respectively. This assignment has been confirmed in the recently reported crystal structure of the catalytic core of human cystathionine β-synthase, which revealed that C52 and H65 serve as the axial ligands (*17*). The heme is six-coordinate in both the ferric and ferrous states, and the endogenous ligands are difficult to replace by exogenous ligands that are commonly used to interrogate both oxidation states of heme. Thus far, only the strongest ligands, viz., CO (*18*), NO (*19*), cyanide (*18*), and isothiocyanates (*20*), have been reported to affect the ferrous heme spectra.

The role of the heme in cystathionine β-synthase is an open question. Studies from our laboratory that have characterized the effect of changes in the heme ligand and oxidation states on enzyme activity point to a regulatory role for this cofactor. NMR spectroscopy, in which the environment of the phosphorus nucleus of PLP in the active site of the protein was probed as a function of the heme oxidation state, revealed that the two cofactors are distant from each other since paramagnetic effects on the relaxation of phosphorus were not observed (*21*). However, chemical shift changes of the phosphorus nucleus accompanied a change in the oxidation state from the paramagnetic ferric form to the diamagnetic ferrous form, indicating transmission of information between the two cofactor binding sites. These observations lend support to the hypothesis that the heme in mammalian cystathionine β-synthases may play a regulatory

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[1] Abbreviations: PLP, pyridoxal phosphate; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline; DTT, dithiothreitol.

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role by modulating the catalytic function of the enzyme.

To understand the role of the heme, it is important to characterize its binding site, to establish its relation to the N-terminal catalytic domain and the C-terminal regulatory domain where the allosteric effector, *S*-adenosylmethionine, is presumed to bind. In this study, we have employed site-directed mutagenesis to create variants of cystathionine β-synthase in which the axial ligands have been altered. One, H65R, mimics a variant described in a homocystinuric patient[2] Both the cysteine and histidine mutations have significantly lower heme but not PLP content as compared to the wild-type enzyme and display low activity. These results are consistent with a regulatory role for the heme and demonstrate that changes in the heme binding domain modulate catalysis at the active site which is distant.

MATERIALS AND METHODS

Materials. Serine, TLCK, TPCK, leupeptin, aprotinin, hemin chloride, PLP, and D,L-homocysteine were purchased from Sigma. [^{14}C]-Serine (158 mCi/mmol) was purchased from Amersham. GST Sepharose was purchased from Pharmacia.

Construction of Site-Specific Mutants. Mutations were introduced using the "Quick Change" approach (Stratagene) employing the following specific mutagenic probes for construction of the C52 and H65 variants, respectively (the base changes are indicated in capital letters): C52S sense: 5′-gatgctccgagcaggAgcacctggcagctg-3′; C52A sense: 5′-gatgctccgagcaggGCcacctggcagctg-3′; H65R sense: 5′-gcctccgagtccccacGtcaccacactgccccggc-3′. Each antisense mutagenic primer had the respective complementary sequence and boundaries that were identical to those of the sense primers. The template plasmid DNA [pGEX4T1/hCBS (*22*)] was isolated from BL-21 *E. coli* cells, and *Pfu* DNA polymerase (Stratagene) was employed. Each mutation was verified by DNA sequence analysis of the entire coding sequence of cystathionine β-synthase performed at the Biotechnology Core Facility at the University of Nebraska, Lincoln.

Purification of Mutant Enzymes. Recombinant human cystathionine β-synthase has been expressed and purified previously as a GST fusion protein from which it is released by limited thrombin digestion (*23*). The C52S and C52A variants were found to be unstable relative to the wild-type enzyme, and were susceptible to heme loss and extensive proteolysis during the thrombin treatment step that is necessary to release of the N-terminal GST tag from recombinant cystathionine β-synthase. Since changes in the growth conditions, addition of hemin to the sonication buffer, choice of protease inhibitor cocktails in the buffers, and varying thrombin cleavage conditions did not alleviate this problem, it was decided to characterize the properties of the C52 and H65 mutations in the fusion protein, prior to the limited proteolysis step.

E. coli cells containing the mutant plasmids were grown and harvested as described previously (*23*), and the mutant proteins were purified as follows. *E. coli* cells (30 g) were resuspended in 300 mL of 100 mM Tris, pH 8.0, containing 1 mM PLP, 35 mg of lysozyme, 20 mg of TLCK, 20 mg of TPCK, 1 mg of leupeptin, 1 mg of pepstatin, and 1 mL of aprotinin and stirred gently at 4 °C for 2 h. The cells were disrupted by sonication as described previously (*23*), and cell wall debris was removed by centrifugation at 12000*g* for 25 min. The supernatant was loaded onto a 5 × 4 cm Glutathionine Sepharose (Pharmacia) column previously equilibrated with PBS buffer (140 mM NaCl, 2.7 mM KCl, 100 mM potassium phosphate, pH 7.4). The column was washed with 500 mL of PBS, and the bound protein was eluted with a solution containing 50 mM Tris, pH 8. 0, and 10 mM glutathonine.

Enzyme Assay and Protein Determination. Enzyme activity was measured using [^{14}C]-serine as described previously (*23*). Protein concentration was determined by the Bradford method, using reagents from Bio-Rad, and bovine serum albumin as a standard.

Determination of Heme and PLP Content. Heme concentration was determined by the pyridine hemochrome assay as described previously (*24*). PLP was measured fluorometrically using a modification of a published procedure (*25*). PLP was released from the enzyme by treating 0.5 mg of cystathionine β-synthase in 1.5 mL of 0.1 M potassium phosphate buffer, pH 7.2, with 5 mM hydroxylamine at 4 °C for ~72 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.

EPR Spectroscopy. EPR spectra (Figure 4A) were recorded on a Bruker ESP 300E spectrometer equipped with an Oxford ITC4 temperature controller, a model 5340 automatic frequency counter from Hewlett-Packard, and a gaussmeter. The EPR spectrum in Figure 4B was obtained using a Bruker Elexsys E580 spectrometer, equipped with a TE_{102} standard rectangular cavity, an Oxford ESR 900 liquid helium flow cryostat, and an ITC4 temperature controller. The specific conditions employed for spectral recording are described in the figure legends.

RESULTS AND DISCUSSION

Identification of the Cysteine Residue That Serves as a Heme Ligand. When these studies were initiated, the identities of the cysteine and histidine ligand to the heme were unknown. Homology searches between cystathionine β-synthase and all other proteins in the database had previously revealed very limited sequence similarity with three other hemeproteins (*26*). Based on this information, the region extending between amino acids 241 and 341 was predicted to be important for heme binding. Within this stretch of amino acids, there are three cysteine residues at positions 244, 272, and 275, respectively. To determine if one of these three cysteines is a ligand to heme, the C244S, C272A, and C275S mutants were constructed. The UV−visible absorption properties of these three variants were indistinguishable from those of the wild-type enzyme in both the ferric and ferrous states (Table 1), and their activities were comparable.

While this work was in progress, it was reported that the yeast cystathionine β-synthase, in contrast to the human

[2] Reported at the cystathionine β-synthase missense mutation database site (www.uchsc.edu/sm/cbs/cbsdata/miss.htm).

Table 1: UV−Visible Absorption Maxima of Wild-Type and Mutant Cystathionine β-Synthases

mutation	ferric (nm)	ferrous (nm)
wild type	428, broad 548	449, 559, 571
C52S	415, 538, 573, 648	423, 559, 631
C52A	417, 538, 573, 648	423, 559, 631
C244S	428, broad 548	449, 559, 571
C272A	428, broad 548	449, 559, 571
C275S	428, broad 548	449, 559, 571
H65R	424, broad 548	421, 528, 559

enzyme, lacks heme (*27*). The two proteins share 38% identity and 72% similarity (*28*) and are of approximately the same length. However, a major difference between the two is the presence of a 66-residue N-terminal extension in the human enzyme, which suggested that this region may represent the heme binding domain (*21*). Of the two cysteines present in this region, C15 appeared unlikely to be important, since removal of ~40 residues from the N-terminus by partial tryptic digestion was reported to not affect the heme spectrum associated with the protein (*29*). We therefore introduced a conservative (C52S) and a nonconservative (C52A) change at the second cysteine residue and evaluated the properties of the resulting proteins.

Purification of C52A and C52S Mutants of Cystathionine β-Synthase. Both variants of cystathionine β-synthase proved to be relatively unstable and were present at lower levels in the cell extract compared to the wild-type enzyme. Recombinant human cystathionine β-synthase is expressed as a fusion protein with an N-terminal GST tag (*22*, *23*). During partial proteolytic digestion with thrombin, required to release GST, we observed extensive degradation of both C52 mutant proteins. Varying the concentration of thrombin, the duration of or temperature for limited proteolysis did not alleviate this problem, and did not yield pure enzyme in sufficient yield in our hands. In contrast, fusion proteins of all three mutants, C52S, C52A, and H65R, were readily obtained in >80% purity, and, therefore, their spectroscopic properties and activities were compared to those of the wild-type fusion enzyme in this study. Increased lability resulting from mutation of a ligand residue has been noted previously in other hemeproteins (*6*, *30*, *31*).

UV−Visible Absorption Properties of C52 and H65 Mutants. The electronic absorption spectra of wild-type fusion cystathionine β-synthase are indistinguishable from those reported previously for the cleaved enzyme (*23*). In the ferric state, the Soret peak is at 428 nm, while the α and β bands give rise to a broad absorption peak at 548 nm. In the ferrous state, the Soret peak red-shifts to 449 nm and is accompanied by a sharpening of the α and β bands at 571 and 559 nm, respectively. In contrast, significant changes were observed in the absorption spectra of both C52 mutants (Table 1). The Soret peaks of the ferric C52S and C52A variants were blue-shifted to 415 and 417 nm, respectively (Figure 1A,B). In the ferrous state, both the C52S and C52A mutants showed absorption maxima at 423, 559, and 631 nm. Addition of DTT to dilute enzyme solutions did not affect the absorption properties of the mutant enzymes. The ferrous CO forms of both mutants exhibited absorption maxima at 420 nm. Since the properties of the two mutants were very similar, detailed characterization of only the C52S variant is reported below.

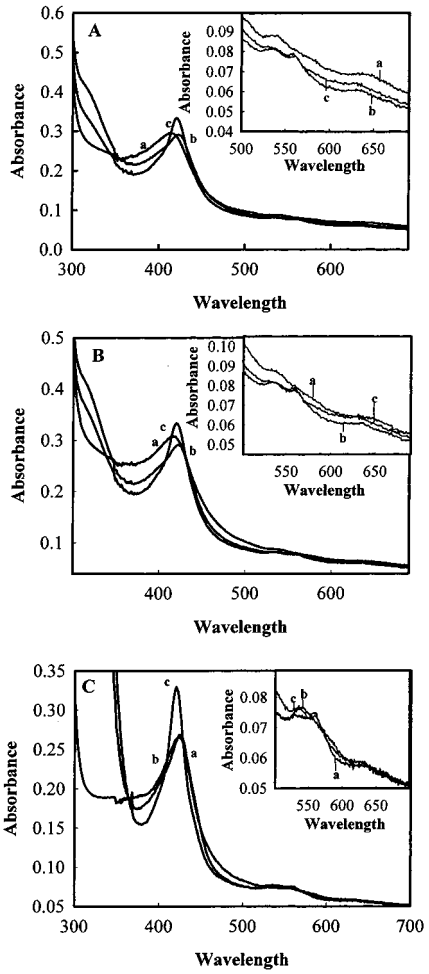

FIGURE 1: UV−visible absorption spectra of C52 and H65 mutants of human cystathionine β-synthase. (A) Electronic absorption spectra of C52S (1 mg/mL) in the ferric (a), ferrous (b) (reduced with 1 mM dithionite), and ferrous-CO (c) states. (B) Electronic absorption spectra of C52A (1.3 mg/mL) in the ferric (a), ferrous (b), and ferrous-CO (c) states. (C) Electronic absorption spectra of H65R (0.86 mg/mL) in the ferric (a), ferrous (b), and ferrous-CO (c) states.

In contrast to the cysteine mutants, the spectrum of the H65R variant was similar to that of wild-type enzyme in the ferric state, with the Soret peak at 424 nm showing a 4 nm blue shift with respect to the wild-type enzyme (Figure 1C). The similarity in the ferric spectra of wild-type and H65R forms of cystathionine β-synthase suggests that the H65R mutant also exists in the low-spin six-coordinate state despite substitution of the axial histidine ligand with arginine, with a positively charged side chain. H65 is one of three tandem histidine residues, of which H67 is also directed toward the heme in addition to the adjacent residue, P64 (Figure 2). Based on the UV−visible and EPR (see below) properties of the H65R mutant, we propose that either H67 or (less likely) P64 serves as an axial ligand in this mutant. The ferrous and ferrous CO forms of the enzyme display Soret maxima at 421 and 420 nm, respectively. The large difference in the position of the ferrous Soret peak (from 450 nm in wild type to 421 nm), but not in the ferric peak, indicates loss of the cysteinate ligand.[3] A similar spectrum has been noted previously for $HgCl_2$-treated ferric cystathionine β-synthase in the ferrous state (*14*). The similarity in the ferrous CO Soret peak for the H65R, C52S, and C52A

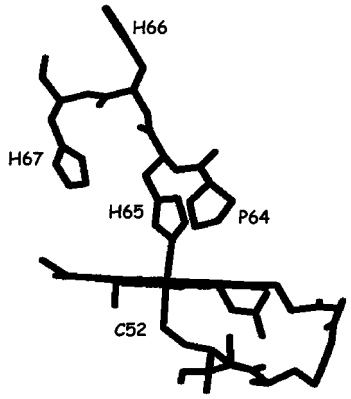

FIGURE 2: Structure of the heme binding domain of wild-type cystathionine β-synthase showing the axial heme ligands C52 and H65. P64 or H67 could potentially substitute as an axial ligand to the heme in the H65R mutant, explaining the observation of a six-coordinate low-spin heme. The structure was generated from the PDB file 1JBQ.

variants is consistent with retention of the sixth ligand in each case and loss of the cysteine ligand upon CO binding.

Cofactor Content of Mutant Enzymes and Correlation with Activity. The heme and PLP content of the C52S mutant and its correlation with enzyme activity were examined in detail (Table 2). Compared to the wild-type enzyme, the mutant had 19% heme saturation as determined by the pyridine hemochrome assay. When PLP was omitted from the sonication buffer, the final PLP concentration in the fusion protein was only 3.5% that of the wild-type enzyme, and the mutant displayed very low activity. In contrast, addition of PLP to the sonication buffer resulted in complete saturation of the purified enzyme with PLP, and an increase in the specific activity to ~14% that of wild-type fusion enzyme. Surprisingly, attempts to increase PLP saturation by dialyzing the purified enzyme against buffer containing 0.5 mM PLP for 12 h at 4 °C resulted in a net loss of the cofactor, and a corresponding decrease in activity. The observation that the PLP content could be increased in the mutants when it was added to the cell extract but not to the purified enzyme was unexpected and unexplained at this time.

Comparison of the heme versus PLP content of the C52S mutant obtained under different conditions is interesting to note since it has been suggested that the heme content of cystathionine β-synthase dictates its PLP content (*5*). Clearly, in the C52S mutant this is not the case, since the PLP content can vary from 3.5 to 100% while the heme content remains constant at 19%. Thus, at least in this variant, full saturation of the enzyme with PLP is possible even in the absence of stoichiometric heme.

The overall reaction catalyzed by cystathionine β-synthase involves the β-replacement of the hydroxyl group of serine by the thiolate of homocysteine to form cystathionine. This type of reaction is related to those catalyzed by other members of the Fold II (*32*) or β-family (*33*) of PLP enzymes such as tryptophan synthase and *O*-acetylserine synthase. In analogy with these other enzymes, the cystathionine β-synthase-catalyzed reaction is expected to involve a series of PLP-bound intermediates. It is therefore surprising that the activity of C52S is not correlated with its PLP content. Thus, full PLP saturation in the presence of substoichiometric heme does not result in a level of activity comparable to that of the wild-type enzyme and suggests that communication between the regulatory heme and catalytic PLP binding sites is important in controlling enzyme activity. Alternatively, the absence of heme in the majority of subunits could lead to a conformational change that affects activity. This is supported by the differences in proteolytic susceptibility of the wild-type and C52 variants. The H65R mutant has 0.4 and 0.75 equiv of heme and PLP, respectively, with respect to the wild-type fusion enzyme and exhibits an ~9-fold lower activity.

Table 2: Cofactor Content and Activity of the C52S and H65R Fusion Mutants after Different Isolation Procedures Relative to That of Wild-Type Fusion Enzyme

CBS	% heme saturation	addition of PLP to sonicate[a]	% PLP content	specific activity[b]
wild type	100	+	100	225
C52S	19	−	3.5	1.5
C52S	19	+ (dialyzed)[c]	22	12
C52S	19	+	100	27
H65R	40	+	75	26

[a] Indicates whether PLP was added to the sonication buffer. [b] Specific activity is reported in units of μmol of cystathionine formed h^{-1} (mg of protein)$^{-1}$ at 37 °C. [c] The purified enzyme was dialyzed against 50 mM Tris, pH 8.0, containing 0.5 mM PLP.

pH Dependence of C52 and H65 Mutants. The wild-type fusion protein exhibits a bell-shaped pH dependence with pK_{a1} and pK_{a2} of 7.4 ± 0.1 and 9.0 ± 0.1, respectively, similar to the values reported for the wild-type enzyme [7.9 ± 0.2 and 8.8 ± 0.2 (*23*)]. In contrast, both heme ligand mutants exhibited significantly altered pH dependencies with a single pK_a (Figure 3). C52S and C52A titrated with similar pK_a's of 8.96 ± 0.09 and 8.69 ± 0.16, respectively, while the H65R form exhibited a pK_a of 7.79 ± 0.05.

EPR Spectroscopic Analysis of Mutant Enzymes. The EPR spectra of the C52 mutants are shown in Figure 4A. At 10 K, the ferric forms of the mutants showed EPR signals in the $g = 6$ region, indicating the presence of high-spin heme. A similar EPR spectrum has been observed with wild-type cystathionine β-synthase in the presence of the thiol chelator $HgCl_2$ (*14*). Addition of DTT to the C52 mutant enzymes did not alter their EPR spectra (data not shown), in contrast to the behavior of the corresponding cysteine mutant (C75A) in CooA (*30*). Wild-type cystathionine β-synthase displays a rhombic EPR signal at 10 K with g values of 2.5, 2.3, and 1.86, characteristic of low-spin heme (*14*), whereas no signal was observed in the $g = 2$ region for the C52 mutant enzymes. Since the EPR spectra observed for the C52 mutants are clearly different from the rhombic EPR spectra seen for high-spin thiolate-ligated hemes, cysteinate ligation to these mutants can be ruled out.

The H65R mutant exhibits a complex spectrum in the $g = 1.8-3$ region, and a small signal at $g = 6$ revealing the predominance of a low-spin species (Figure 4B). The spectrum in the $g = 1.8-3$ region of the H65R mutant resembles more closely that of the rat rather than the wild-

[3] We have been unable to obtain independent confirmation of the heme spin and coordination states in the mutants by resonance Raman spectroscopy. The high background fluorescence and photolability associated with the full-length fusion enzyme precluded efforts to obtain high-quality spectra (Moenne-Loccoz, Ojha, and Banerjee, unpublished results).

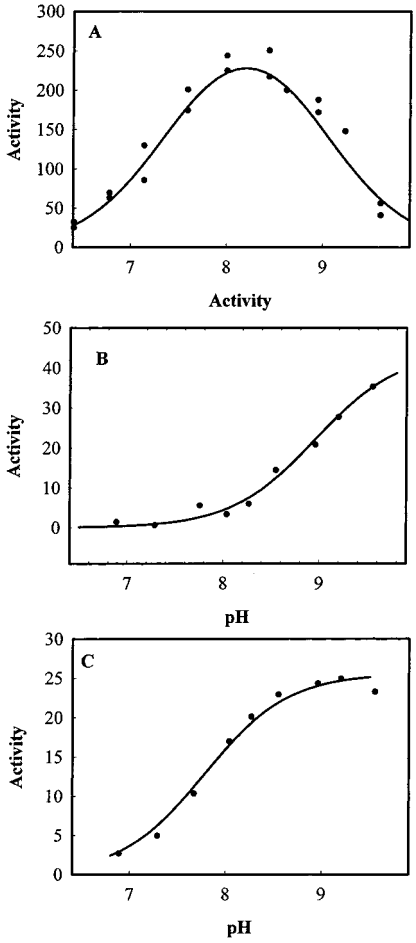

FIGURE 3: pH dependencies of the wild-type fusion protein (A) and C52S (B) and H65R (C) mutants of cystathionine β-synthase. The activity of the enzymes was monitored in 0.25 M Tris propane buffer in which the pH was varied from 6.9 to 9.6. The activity is reported in units of μmol of cystathionine formed h^{-1} (mg of protein)$^{-1}$ at 37 °C.

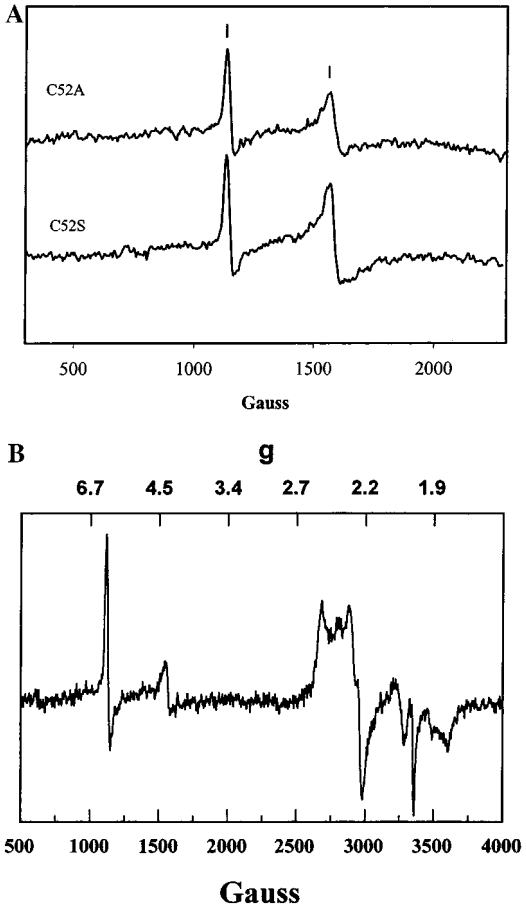

FIGURE 4: EPR spectra of the C52A and C52S (A) and H65R (B) mutants of human cystathionine β-synthase. The samples contained enzyme in 100 mM Tris, pH 8.5. For panel A, the spectra were recorded at 10 K temperature, 1 mW microwave power, 2×10^4 receiver gain, 12.78 G modulation amplitude, 9.47 GHz microwave frequency, and 100 kHz modulation frequency using 1024 data points. The line markers are at $g = 6$ and 4.3, respectively. For panel B, the spectra were recorded at 15 K temperature, 2 mW microwave power, 60 dB receiver gain, 15 G modulation amplitude, 9.42 GHz microwave frequency, and 100 kHz modulation frequency using 1024 data points. Scan rate was 11.9 G/s, with a receiver time constant of 82 ms. Panel B was obtained by subtraction of a background spectrum (obtained using a distilled water blank measured under identical conditions) from the H65R sample spectrum.

type human cystathionine β-synthase. Thus, the rat enzyme has been reported to display two sets of interconverting EPR spectra, with g values of 2.4, 2.28, and 1.91 (alkaline) and 2.5, 2.3, and 1.85 (acidic) which are pH-dependent (*15*). In contrast, the EPR spectrum of recombinant human cystathionine β-synthase is independent of pH between 6.5 and 9.0 (*14*). The predominance of low-spin six-coordinate heme revealed by EPR spectroscopy of the H65R mutant is consistent with the UV−visible absorption spectrum (Figure 1C) and supports the proposal that an alternative residue, viz., H67, replaces H65 in the mutant.

Summary. The heme ligand mutations, C52S, C52A, and H65R, are characterized by the presence of substoichiometric heme but an almost full complement of PLP with respect to wild-type enzyme and display low catalytic activity. These results are consistent with the growing body of biophysical evidence from our laboratory that the heme in cystathionine β-synthase serves a regulatory role and that changes in this cofactor binding domain are transmitted to the physically distant active site (*19*, *21*, *34*). The C52 mutants have five-coordinate high-spin heme, and display spectroscopic properties consistent with the loss of the thiolate ligand. In contrast, the H65R mutant, described in a homocystinuric patient, reveals the presence of low-spin six-coordinate heme in the ferric state and suggests substitution of H65 by an alternative ligand. This is the first characterization of a heme domain mutation associated with homocystinuria and demonstrates that perturbation of the heme environment, despite high saturation with PLP, is detrimental to the catalytic activity of cystathionine β-synthase.

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