Resonance Raman Characterization of the Heme Cofactor in Cystathionine β-Synthase. Identification of the Fe−S(Cys) Vibration in the Six-Coordinate Low-Spin Heme[†]

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Received May 12, 2000; Revised Manuscript Received November 6, 2000

ABSTRACT: Human cystathionine β-synthase (CBS) is an essential enzyme for the removal of the toxic metabolite homocysteine. Heme and pyridoxal phosphate (PLP) cofactors are necessary to catalyze the condensation of homocysteine and serine to generate cystathionine. While the role for the PLP cofactor is thought to be similar to that in other PLP-dependent enzymes that catalyze β-replacement reactions, the exact role for the heme remains unclear. In this study, we have characterized the heme cofactor of CBS in both the ferric and ferrous states using resonance Raman spectroscopy. Positive identification of a cysteine ligand was achieved by global ^{34}S isotopic substitution which allowed us to assign the ν(Fe−S) for the six-coordinate low-spin ferric heme at 312 cm^{-1}. In addition, the CO adduct of ferrous CBS has vibrational frequencies characteristic of a histidine−heme−CO complex in a hydrophobic environment, and indicates that the Fe−S(Cys) bond is labile. We have also found that addition of $HgCl_2$ to the ferric heme causes conversion of the low-spin heme to a five-coordinate high-spin heme with loss of the cysteine ligand. The present spectroscopic studies do not support a reaction mechanism in which homocysteine binds directly to the heme via displacement of the Cys ligand in the binary enzyme complex, as had been previously proposed.

Human cystathionine β-synthase (CBS)[1] is a unique heme protein that catalyzes a pyridoxal phosphate dependent condensation of serine and homocysteine to generate cystathionine. This enzyme is clinically important since it represents one of two major metabolic avenues for the removal of homocysteine in humans. Elevated levels of homocysteine are correlated with cardiovascular diseases (*1*), neural tube defects (*2*), and Alzheimer's disease (*3*). Mutations in CBS are the single most common cause of homocystinuria, an inborn error of metabolism that results in very high levels of circulating homocysteine with attendant cardiovascular complications (*4*).

The sequences of the human, rat, and yeast cystathionine β-synthase reveal that they are quite similar. However, the yeast enzyme is devoid of heme (*5*). This suggests that evolutionarily, the heme is a relatively recent acquisition, and argues against an essential catalytic role for this cofactor. However, the activity of human CBS is modulated by oxidation (*6*), ligation (*7*), and coordination state (Taoka and Banerjee, manuscript in preparation) changes in the heme, suggesting a possible regulatory role for the heme cofactor.

To define the role of the heme in this enzyme, experiments are underway to characterize its properties. Recent EPR and X-ray absorption spectroscopic studies of the human enzyme implicated histidine and cysteine as the axial heme ligands in both the ferric and ferrous states (*8*). In the oxidized enzyme, the heme is low spin and six-coordinate and exhibits a Soret peak at 428 nm (*6*). Treatment of the enzyme with the thiol chelator $HgCl_2$ results in conversion of the low-spin heme to a high-spin species (*8*). Reduction of the heme results in a large red shift of the Soret peak to 450 nm, consistent with retention of the thiolate ligand in the six-coordinate heme (*9*).

In this study, we have used resonance Raman spectroscopy to characterize the heme in human cystathionine β-synthase. The Fe−S(Cys) vibration in the ferric enzyme has been observed at 312 cm^{-1} and verified by global ^{34}S labeling of the enzyme. This is the first detection of ν(Fe−S) in a 6cLS heme. This identification has permitted us to evaluate directly whether the thiolate substrate, homocysteine, coordinates to the heme in CBS by displacement of the active site cysteine ligand, as had been postulated (*6*). Our results indicate that homocysteine does *not* replace cysteine as a ligand in the binary complex. Further studies of the ferric and ferrous enzymes in the presence of $HgCl_2$ and of the ferrous−CO

[†] This work was supported by grants from the National Institutes of Health (GM 34468 to T.M.L. and HL58984 to R.B.). R.B. is an Established Investigator of the American Heart Association. The Raman spectroscopy facility at OGI was purchased by funds from an NSF shared instrumentation award (BIR 9216592) and matching funds from OGI.

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[1] Abbreviations: CBS, cystathionine β-synthase; EPR, electron paramagnetic resonance; ν(Fe−S), iron−sulfur stretching frequency; 6cLS, six-coordinate low spin; 5cHS, five-coordinate high spin; MCD, magnetic circular dichroism; RR, resonance Raman; PLP, pyridoxal phosphate.

10.1021/bi0010874 CCC: $20.00
Published on Web 12/15/2000

complex confirm the presence of cysteine and histidine as the axial ligands to the heme in human cystathionine β-synthase.

MATERIALS AND METHODS

Chemicals. The following materials were obtained from Sigma Chemical Co.: L-homocysteine thiolactone, δ-aminolevulinic acid, sodium dithionite, IPTG, and $HgCl_2$. Homocysteine was prepared from L-homocysteine thiolactone as described previously (*10*). Sodium [^{34}S]sulfate (93% ^{34}S) was from ICON (Marion, NY).

Enzyme Labeling and Purification. The truncated and highly active form of human CBS lacking 143 amino acids at the C-terminus was purified from *E. coli* containing the expression vector pGEXCBSN (*11*) provided by Warren Kruger (Fox Chase Cancer Center, Philadelphia). The cells were cultured, and the protein was purified as described previously (*12*). For global ^{34}S-labeling of CBS, 10 mL of an overnight culture (in LB medium at 25 °C) was used to inoculate 1 L of M9 minimal medium containing 75 mg of ampicillin. The minimal medium was supplemented with 10 mL of modified mineral salt solution (in which the sulfate salts were replaced with chloride salts in the original recipe) (*13*), 4 mL of a nitrogen stock solution (*13*), 284 mg of sodium [^{34}S]sulfate, and 40 mg of each of the amino acids except methionine. The cells were grown at 25 °C, and at an OD_{600} of 0.5, 75 mg of δ-aminolevulinic acid and 0.1 mM IPTG (final concentration) were added. The cells were harvested after 21 h when the OD_{600} was ~1.8. Approximately 6 g of cell paste (wet weight) was obtained from a 1 L culture and yielded 13 mg of ^{34}S-labeled cystathionine β-synthase after purification.

Resonance Raman Samples. Stock solutions of oxidized CBS (native unlabeled and globally ^{34}S-labeled) were in 100 mM Tris buffer (pH 8.6) at a concentration of ~200 μM ferric heme. For RR measurements, these solutions were diluted 2-fold with 50 mM Tris buffer (pH 8.6), and aliquots of these ~100 μM heme solutions were transferred to glass capillaries. Samples of reduced CBS were prepared in a Coy anaerobic glovebox. First, oxidized CBS was equilibrated with an anoxic atmosphere (N_2, CO_2, H_2O, and H_2) over several hours to exchange the dissolved oxygen. Two microliters of freshly prepared 40 mM sodium dithionite was then added to a 15 μL aliquot of the deaerated solution to give a final concentration of ~90 μM ferrous heme. The reduced CBS solution was transferred to sealed Pasteur pipets, and capped with rubber septa to maintain anaerobicity. Reduction of the heme was monitored by UV/Vis spectroscopy on samples in the Pasteur pipets (*14*), with the shift of the Soret peak from 428 to 450 nm indicating the extent of reduction (Figure 1). Samples of the oxidized enzyme were also treated with $HgCl_2$ to give final concentrations of ~90 μM heme and 1 mM $HgCl_2$, resulting in a shift in the sharp Soret band at 428 nm to a broad band at 390 nm. The $HgCl_2$-treated sample was then made anaerobic by placing the solution in the glovebox for several hours and reduced with dithionite to give a final concentration of ~80 μM heme and ~0.9 mM $HgCl_2$. CO binding to reduced cystathionine β-synthase is indicated by a shift of the Soret peak from 450 to 422 nm (Figure 1). CO adducts of CBS were prepared by injection of 2 mL of gas (^{12}CO, CP grade, Air Products; or ^{13}CO, 99% ^{13}C, Cambridge Isotope Laboratories) into sealed Pasteur pipets containing the ferrous enzyme at a concentration of ~90 μM heme. All RR experiments were performed at least in duplicate on separate days to confirm all vibrational frequencies and intensities. Optical absorbance spectra were collected on a Perkin-Elmer Lambda 9 UV/Vis spectrophotometer.

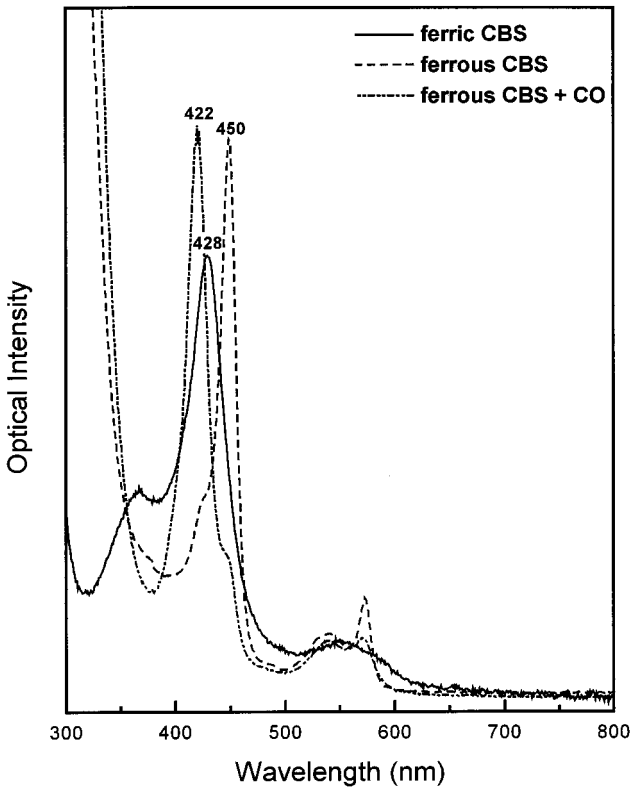

FIGURE 1: Electronic absorption spectra of cystathionine β-synthase in oxidized, reduced, and reduced + CO forms. Similar data have been previously reported (*6*).

Resonance Raman Spectroscopy. Resonance Raman spectra were collected on a custom McPherson 2061/207 spectrograph set to a focal length of 0.67 m, fitted with a 2400 groove/mm grating, a Kaiser Optical holographic supernotch filter, and a Princeton Instruments (LN-1100PB) liquid-N_2-cooled CCD detector. The excitation sources were an Innova 302 Kr laser (413.1 nm) and a LiConix 4240NB HeCd laser (441.6 nm). Spectra were collected from samples contained in glass capillary tubes or Pasteur pipets fitted with rubber septa, in a 90° scattering geometry, and a 4 cm^{-1} spectral resolution. Data were accumulated at 5−10 min intervals for all samples. Frequencies were calibrated relative to indene, CCl_4, CD_3CN, aspirin, and ^{14}NO as standards and are accurate to ± 1 cm^{-1}. Spectra of samples substituted with isotopes were obtained under identical instrumental conditions such that frequency shifts are accurate to ± 0.5 cm^{-1}.

RESULTS AND DISCUSSION

Resonance Raman of Oxidized Cystathionine β-Synthase. We have used resonance Raman spectroscopy to characterize the heme in human cystathionine β-synthase, and establish that the axial ligands of the heme cofactor are cysteine and histidine as had been previously suggested (*8*). Excitation of oxidized CBS within the Soret band at 428 nm produces a RR spectrum dominated by porphyrin modes (Figure 2).

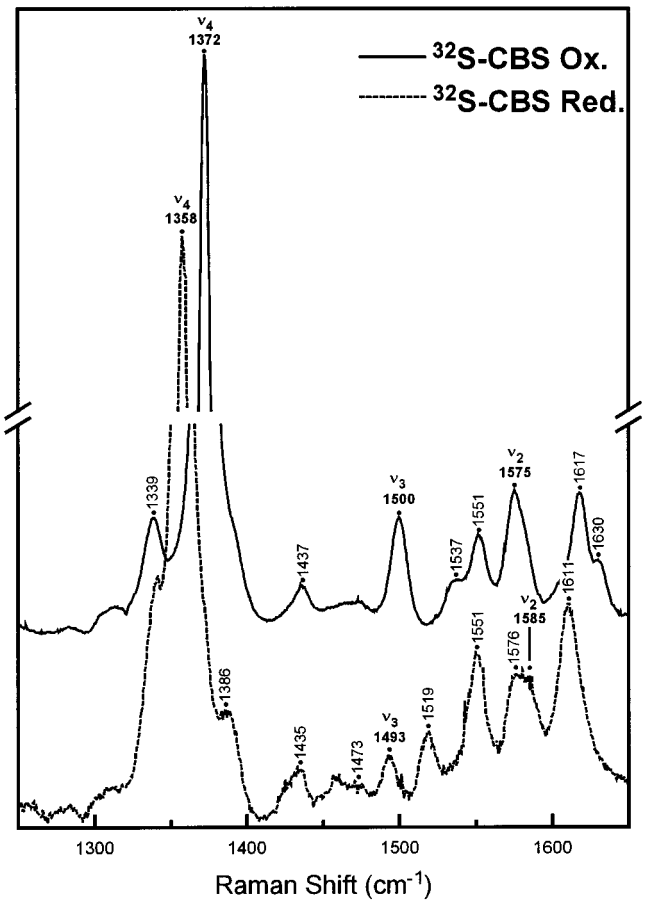

FIGURE 2: High-frequency Raman spectra of nonlabeled ferric cystathionine β-synthase (λ_{ex} = 413 nm) and nonlabeled ferrous cystathionine β-synthase (λ_{ex} = 442 nm). The heme cofactor is 6cLS for both oxidized and reduced cystathionine β-synthase as indicated by the heme marker bands ν_4, ν_3, and ν_2.

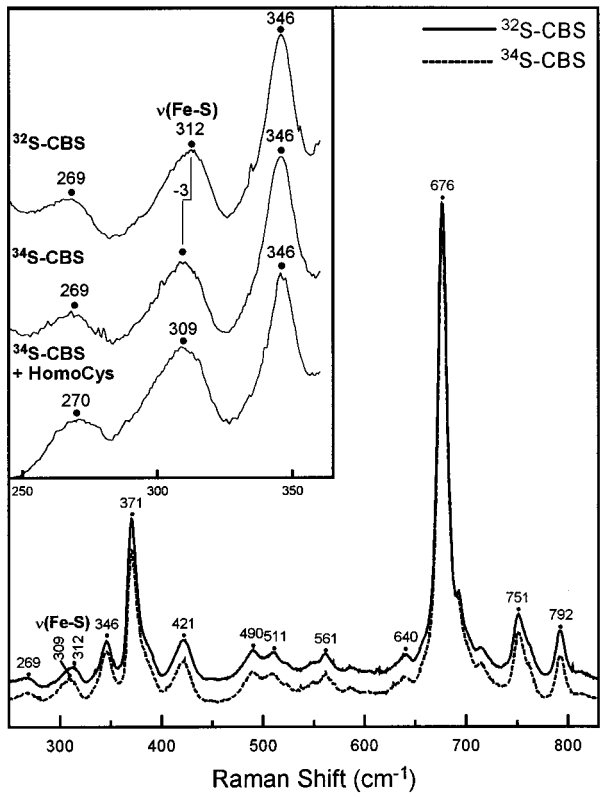

FIGURE 3: Low-frequency Raman spectra of unlabeled ferric cystathionine β-synthase and global ^{34}S-labeled ferric cystathionine β-synthase. Isotopic substitution allows for the identification of the Fe−S(Cys) vibrational peak at 312 cm^{-1} (λ_{ex} = 413 nm). Inset: Resonance Raman spectrum of unlabeled versus uniformly ^{34}S-labeled cystathionine β-synthase after addition of excess homocysteine substrate. The position of the Fe−S(Cys) vibrational peak is unchanged, indicating that the substrate does not bind directly to the heme cofactor by displacement of the cysteine ligand.

Bands in the high-frequency region (1200−1700 cm^{-1}) are sensitive indicators of the oxidation state (ν_4) and spin and coordination states (ν_2, ν_3, and ν_{10}) of the heme iron (*15*). The RR spectrum of oxidized CBS in Figure 2 is dominated by the oxidation state marker, ν_4, at 1372 cm^{-1}, while higher frequency porphyrin skeletal modes are at the positions expected for a 6cLS *b*-type heme. These frequencies are consistent with the heme in cystathionine β-synthase being coordinated to histidine and cysteine residues as suggested by EPR (*8*, *16*), X-ray absorption (*8*), and MCD (*17*) spectroscopic studies. There are no isotope-sensitive peaks that result from global ^{34}S substitution in this spectral region (data not shown).

The low-frequency RR spectrum also exhibits signals characteristic of the heme cofactor, with intense peaks at 676 and 371 cm^{-1} (Figure 3). We have identified the peak at 312 cm^{-1} as the Fe−S(Cys) stretching frequency by its 3 cm^{-1} downshift that is observed in the RR spectrum of the globally ^{34}S-labeled enzyme (Figure 3, inset). No other bands in the RR spectrum were sensitive to the isotopic substitution. The observation of the Fe−S(Cys) stretch in CBS is unique in that it is the first detection of this vibration in a 6cLS heme species. Fe−S(Cys) vibrations have been seen previously only in 5cHS species. In cytochrome P450$_{cam}$ (*18*), chloroperoxidase (*19*), and the His25Cys mutant of heme oxygenase (*20*), ν(Fe−S) occurs at ∼350 cm^{-1}. The lower frequency in cystathionine β-synthase suggests that the Fe−S bond is weaker than in the 5cHS species found in the other heme enzymes, the difference being consistent with its 6-coordinate state.

Homocysteine Binding to Cystathionine β-Synthase. We have examined the effect of the thiolate substrate, homocysteine, on the RR spectrum of CBS. It has been postulated that the heme may function to activate the substrate via direct coordination to the heme (*6*). If homocysteine were to displace the coordinated cysteine residue, then the 309 cm^{-1} Fe−S peak of the ^{34}S-labeled enzyme would be expected to shift back to 312 cm^{-1} provided that the Fe−S(Cys) and Fe−S(homocysteine) bonds were of comparable strengths. However, addition of homocysteine, even in large excess over the enzyme, resulted in no significant changes in the Fe−S peak frequency and intensity in both the unlabeled and, more importantly, the ^{34}S-labeled protein (Figure 3, inset). Furthermore, no new peaks appeared in the low-frequency region that would be expected if Fe−S(homocysteine) ligation had occurred. In fact, no changes were detectable in any region of the RR spectrum in the presence of homocysteine. These data indicate that homocysteine does not displace the cysteine ligand of the heme cofactor in the binary enzyme−homocysteine complex.

Resonance Raman of Reduced Cystathionine β-Synthase. Formation of ferrous heme in CBS results in the Soret band shifting from 428 to ∼450 nm (*16*, *22*, and Figure 1). Excitation at 442 nm within the Soret band gives a RR

spectrum characteristic of a 6cLS ferrous heme, identified by the ν_4, ν_3, and ν_2 markers at 1358, 1493, and 1585 cm^{-1}, respectively (Figure 2). As with oxidized CBS, the ferrous heme spectrum also exhibits no observable shifts upon global ^{34}S substitution in this region (data not shown).

The low-frequency region of the resonance Raman spectrum displays several changes including the disappearance of the Fe−S(Cys) vibrational peak at 312 cm^{-1}. In the ferrous heme, the Fe−S(Cys) bond is expected to be weaker due to the lower positive charge on the iron atom, and, therefore, ν[Fe−S(Cys)] should be at a lower frequency than in the oxidized enzyme. However, no isotope-sensitive peaks were observed in the spectrum of the reduced enzyme (data not shown), suggesting that the Fe−S(Cys) vibration has fallen out of resonance in the ferrous heme complex. The high-frequency RR spectrum clearly shows that the ferrous heme is in a 6cLS state (Figure 2), a result consistent with histidine and cysteine residues still ligated in the reduced enzyme. The 450 nm Soret band also supports the presence of a thiolate ligand in a 6-coordinate ferrous heme (*9*). In contrast, in CooA, the CO-sensing transcriptional activator from *Rhodospirillum rubrum*, the ferric heme is also coordinated by a cysteine ligand, but reduction leads to replacement of the ligating residue Cys75 by His77 (*23, 24*).

Spectrum of Oxidized Cystathionine β-Synthase Treated with $HgCl_2$. Addition of $HgCl_2$ to oxidized enzyme results in loss of the sharp Soret absorbance at 428 nm and the appearance of a broad band at ∼390 nm (*8*). The RR spectrum of $HgCl_2$-treated CBS reveals the presence of a 5cHS ferric heme with ν_2 at 1570, ν_3 at 1492, and ν_4 at 1373 cm^{-1} (Figure 4). This finding is consistent with recent EPR data that have identified a spin-state conversion of the ferric heme on treatment of the enzyme with $HgCl_2$ (*8*).

The low-frequency RR spectrum of the $HgCl_2$-treated sample displays several other changes compared to that of oxidized enzyme (Figures 3 and 4). The three peaks at 346, 371, and 421 cm^{-1} have greatly changed in relative intensities and, to a smaller degree, in frequencies and are now seen at 347, 379, and 415 cm^{-1}, respectively. The prominent peak at 676 cm^{-1} has also decreased in intensity. We propose that the cysteine ligand dissociates when cystathionine β-synthase is treated with $HgCl_2$. This is supported by the loss of the Fe−S(Cys) vibration at 312 cm^{-1}. There are no observable frequency shifts upon global ^{34}S isotopic substitution (data not shown).

Spectrum of Reduced Cystathionine β-Synthase Treated with $HgCl_2$. Very different results are obtained when oxidized CBS, treated with $HgCl_2$, is then reduced. The broad Soret band shifts from ∼390 to ∼422 nm. The RR spectrum of the $HgCl_2$-treated ferrous CBS indicates a 6cLS species, as identified by ν_4, ν_3, and ν_2 at 1359, 1493, and 1581 cm^{-1}, respectively (Figure 4), showing that the heme converts back to a 6cLS species upon reduction. The position of the absorbance band at ∼422 nm also supports a 6-coordinate ferrous heme in which the Cys ligand has been replaced (*25*). It is unclear as to which amino acid could replace the Cys ligand as there is no analogous His residue as seen in CooA (*23, 24*). Global ^{34}S-isotope substitution resulted in no observable shifts in either the low- or the high-frequency regions (data not shown).

CO Adduct of Cystathionine β-Synthase. Addition of CO to ferrous heme results in a blue shift of the Soret peak from 450 to 422 nm (*16* and Figure 1). The appearance of ν(CO) and ν(Fe−CO) modes in the RR spectrum confirms that CO binds to the heme by displacement of one of the original axial ligands. The vibrational modes of the CO complex were identified by $^{12}CO/^{13}CO$ isotopic substitution (Figure 5), with ν(Fe−CO) observed at 497 cm^{-1} (downshifts 4 cm^{-1} with ^{13}CO) and ν(C═O) at 1961 cm^{-1} (downshifts ∼45 cm^{-1} with ^{13}CO). These frequencies are similar to those seen for other 6cLS CO complexes having a hydrophobic distal pocket and, furthermore, are characteristic of a heme−CO complex with histidine as the sixth heme ligand (*26, 27*). Thus, the present RR data indicate that the cysteine ligand is displaced by CO, a result supporting the recent assignment of histidine as the sixth ligand in the ferrous−CO heme complex of cystathionine β-synthase by Taoka et al. (*7*). Carbon monoxide is a competitive inhibitor with respect to homocysteine, and CO binding results in complete loss of enzyme activity (*7*). Since our data indicate that homocysteine does not displace the cysteine ligand, they suggest that CO interferes with homocysteine binding by affecting the heme microenvironment resulting from displacement of the cysteine ligand.

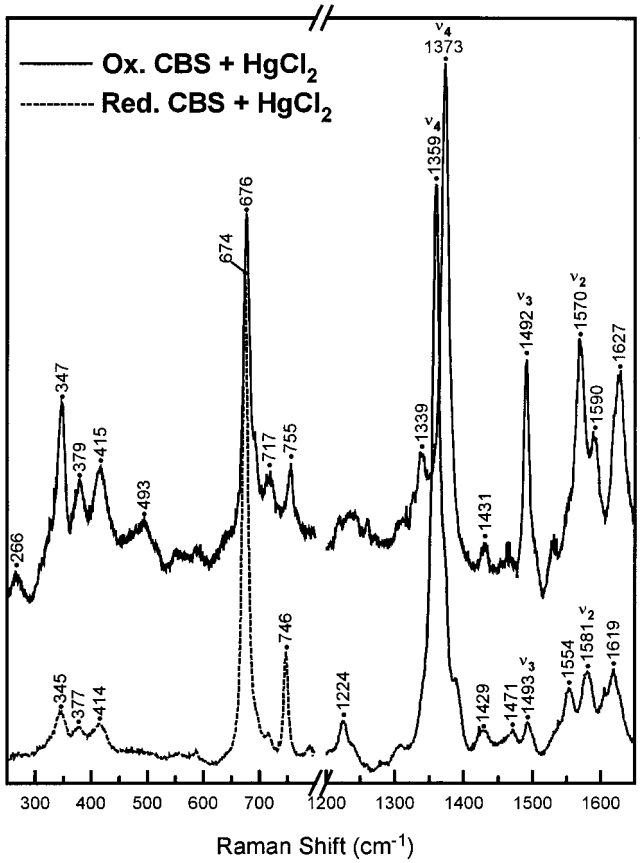

FIGURE 4: Resonance Raman spectra of unlabeled ferric and ferrous CBS after treatment with $HgCl_2$ (λ_{ex} = 413 nm). The spectra demonstrate conversion of 6cLS to 5cHS heme as indicated by the heme marker bands ν_4, ν_3, and ν_2 at 1373, 1492, and 1570 cm^{-1}, respectively, for the oxidized treated sample. Upon reduction of the oxidized treated sample, the heme marker bands clearly demonstrate a conversion back to 6cLS as identified by ν_4, ν_3, and ν_2 at 1359, 1493, and 1581 cm^{-1}, respectively.

Summary. In summary, characterization of human cystathionine β-synthase by resonance Raman spectroscopy has led to the first detection of the ν(Fe−S) mode in a 6cLS ferric heme and provides supporting evidence for the assignment of cysteine and histidine as the axial ligands.

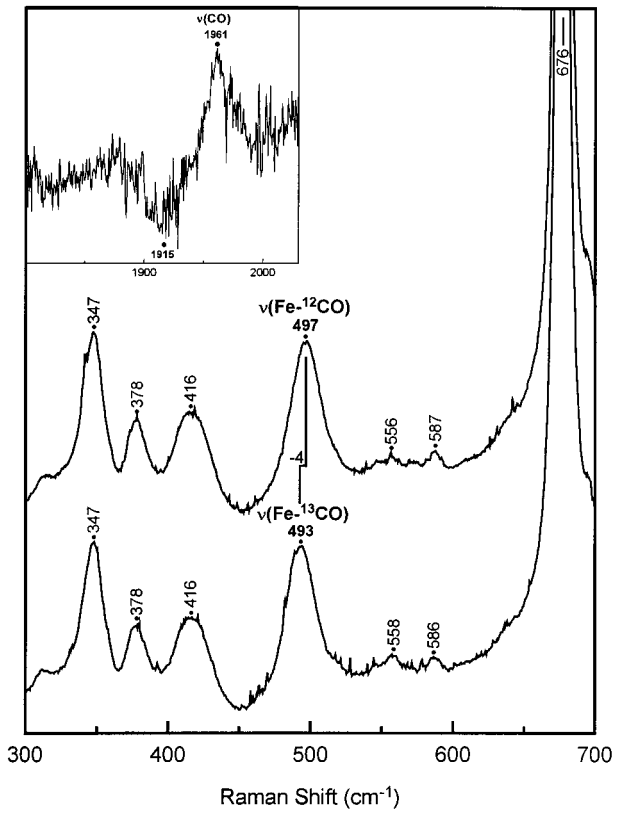

FIGURE 5: Resonance Raman spectrum of the CO adduct of ferrous cystathionine β-synthase (λ_{ex} = 413 nm). Isotopic substitution with ^{13}CO allows for the identification of the ν(Fe−CO) stretch at 497 cm^{-1} and the ν(CO) stretch at 1961 cm^{-1} (inset).

Addition of unlabeled homocysteine to the isotopically labeled enzyme does not perturb the heme spectrum and, in particular, the ν(Fe−S) peak, indicating that homocysteine does not replace cysteine as an axial ligand as had been proposed. In the ferrous state, the heme retains its 6cLS electronic configuration. CO binds to the ferrous heme by displacement of the cysteine ligand, and the ν(Fe−CO)/ν(CO) correlation supports a His/CO axial ligand set. Addition of the thiol chelator $HgCl_2$ to the ferric enzyme results in a spin-state conversion that is readily detected by RR spectroscopy. However, addition of $HgCl_2$ to the ferrous enzyme results in a 6cLS heme, indicating substitution of the coordinating cysteine by another, and as yet unidentified, residue in the active site.

ACKNOWLEDGMENT

We are grateful to Dr. Jingyuan Ai for preliminary RR experiments and Dr. Pierre Moënne-Loccoz for helpful discussions.

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BI0010874