EVIDENCE FROM $^{31}$P NMR AND PULSED EPR SPECTROSCOPY THAT HEME AND PLP COFACTORS ARE NOT PROXIMAL IN THE HUMAN ENZYME

Received for publication, January 2, 2001, and in revised form, February 13, 2001
Published, JBC Papers in Press, February 26, 2001, DOI 10.1074/jbc.M100029200

Ömer Kabil‡, Shinichi Toaka‡, Russell LoBrutto§, Richard Shoemaker¶, and Ruma Banerjee‡‡

From the ‡Department of Biochemistry and the §Department of Plant Biology, Arizona State University, Tempe, Arizona 85287-1601

Two classes of cystathionine $\beta$-synthases have been identified in eukaryotes, the heme-independent enzyme found in yeast and the heme-dependent form found in mammals. Both classes of enzymes catalyze a pyridoxal phosphate (PLP)-dependent condensation of serine and homocysteine to produce cystathionine. The role of the heme in the human enzyme and its location relative to the PLP in the active site are unknown. $^{31}$P NMR spectroscopy revealed that spin-lattice relaxation rates of the phosphorus nucleus in PLP are similar in both the paramagnetic ferric ($T_1 = 6.34 \pm 0.01$ s) and the diamagnetic ferrous ($T_1 = 5.04 \pm 0.06$ s) enzyme, suggesting that the two cofactors are not proximal to each other. This is also supported by pulsed EPR studies that do not provide any evidence for strong or weak coupling between the phosphorus nucleus and the ferric iron. However, the $^{31}$P signal in the reduced enzyme moved from 5.4 to 2.2 ppm, and the line width decreased from 73 to 16 Hz, providing the first structural evidence for transmission to the active site of an oxidation state change in the heme pocket. These results are consistent with a regulatory role for the heme as suggested by previous biochemical studies from our laboratory. The $^{31}$P chemical shifts of the resting forms of the yeast and human enzymes are similar, suggesting that despite the difference in their heme content, the microenvironment of the PLP is similar in the two enzymes. The addition of the substrate, serine, resulted in an upfield shift of the phosphorus resonance in both enzymes, signaling formation of reaction intermediates. The resting enzyme spectra were recorded following addition of excess homocysteine, indicating that both enzymes retained catalytic activity during the course of the NMR experiment.

Cystathionine $\beta$-synthase catalyzes the condensation of serine and homocysteine to form cystathionine in a pyridoxal phosphate (PLP)-dependent reaction. The mammalian enzyme is novel in its dependence on a second cofactor, heme, which distinguishes it from all other members of the PLP family of enzymes in which this combination of cofactors is not seen (1). In addition, the mammalian enzyme is allosterically activated by S-adenosylmethionine (2), a regulatory feature that is lacking in the related yeast enzyme (3). The reaction is postulated to involve a series of PLP-bound intermediates that are analogous to other PLP enzymes that catalyze $\beta$-replacement reactions (Fig. 1). Thus, the addition of serine results in a transaldimination reaction in which the Schiff base-forming lysine in the internal aldime is replaced by serine to form the external aldime. Binding of the second substrate, homocysteine, is followed by abstraction of the $\alpha$-proton of serine and $\beta$-elimination of water to form the $\alpha$-aminoacrylate intermediate, which is poised for nucleophilic addition by the thiolate of homocysteine. A second transaldimination reaction results in product release and regeneration of the resting enzyme. Fluorescence spectroscopy provides evidence for this scheme (4, 5). Formation of the aminoacrylate species in the ternary rather than the binary complex is supported by a very low level of tritium washout from the $\alpha$-carbon of serine in the absence of homocysteine and a significant enhancement of this exchange in the presence of homocysteine (6). In contrast, the yeast enzyme appears to utilize a ping-pong mechanism, and the $\alpha$-aminoacrylate intermediate is observed in the presence of serine (7).

Sequence analysis of the N-terminal domain of cystathionine $\beta$-synthase revealed a phylogenetic relationship to a number of other PLP-dependent enzymes, notably O-acetylserine sulfhydrylase, tryptophan synthase, and threonine deaminase, and led to the assignment of the PLP binding pocket to the region extending between residues 68 and 209 in the human sequence (5). The alignment of the yeast and human cystathionine $\beta$-synthases with conserved active site residues that interact with PLP in O-acetyl serine sulfhydrylase indicates that the region of homology is in fact more extensive and extends between residues 85 and 350 in the human sequence (Fig. 2). This assignment is confirmed by the identification of Lys-119 as the residue involved in Schiff base formation with the PLP in the resting enzyme (8). Weak sequence similarity between a few hemeproteins and the central region of cystathionine $\beta$-synthase extending between residues 241 and 341 suggested that this region may be important in heme binding (5). However, the recent recognition that the closely related yeast enzyme lacks the ace-1romethyl ketone; TPCZ, 1-tosylamido-2-phenylethyl chloromethyl ketone; HYSCORE, hyperfine sublevel correlation spectroscopy.

* This work was supported in part by National Institutes of Health Grant HL68984. The costs of publication of this article were defrayed in part by the payment of page charges. This article therefore must be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Established Investigator of the American Heart Association. To whom correspondence should be addressed: Dept. of Biochemistry, N133 Beadle Center, University of Nebraska, Lincoln, NE 68588-0664. Tel.: 402-472-2941; Fax: 402-472-7842; E-mail: rbannerjee1@unl.edu.

‡ The abbreviations used are: PLP, pyridoxal phosphate; ESEEM, electron spin-echo envelope modulation spectroscopy; ENDOR, electron nuclear double resonance spectroscopy; TLCK, N′-p-tosyl-l-lysine chloromethyl ketone; TPCK, 1-tosylamido-2-phenylethyl chloromethyl ketone; HYSCORE, hyperfine sublevel correlation spectroscopy.
Heme and PLP Not Proximal in Human Cystathionine $\beta$-Synthase

Heme as well as a 66-residue N-terminal extension suggests that the N terminus of the human enzyme is involved in heme binding. Resolution of the question of whether or not the central region is also involved in the architecture of the heme pocket in the tertiary structure awaits solution of the crystal structure of the protein.

Regardless of the location of the heme binding domain along the primary sequence, the function of the heme in the reaction catalyzed by human cystathionine $\beta$-synthase is unknown. A catalytic function for heme had been proposed in which the thiolate of homocysteine is activated for nucleophilic attack by direct coordination with the heme replacing the endogenous cysteinate ligand (9). However, resonance Raman spectroscopy failed to provide evidence for this hypothesis (10). More recently, the generation of a heme-free enzyme in the crystalline state of the protein has permitted detection of PLP-bound intermediates that are formed in the presence of the two substrates (11). The heme-free enzyme is able to form the $\alpha$-aminoisocrylate species in the presence of serine, and the internal aldimine was reformed when homocysteine was added, providing evidence for the ability of the enzyme to support catalysis in the absence of heme. These studies, along with the observation that the related heme-independent yeast enzyme catalyzes the same overall reaction with similar kinetic parameters, argue against an essential catalytic role for this cofactor in the human enzyme.

Studies in our laboratory have revealed that perturbations in the heme environment influence the reaction catalyzed by human cystathionine $\beta$-synthase (4). Thus, reduction of the heme from the ferric to ferrous state is correlated with an approximately 2-fold diminution in enzyme activity, whereas binding of CO to the ferrous enzyme results in complete inhibition of enzyme activity, whereas binding of CO to the ferric heme as well as a 66-residue N-terminal extension suggests that the N terminus of the human enzyme is involved in heme binding. Resolution of the question of whether or not the central region is also involved in the architecture of the heme pocket in the tertiary structure awaits solution of the crystal structure of the protein.

In this study we employed a combination of NMR and pulsed EPR methods to evaluate the interaction, if any, between the heme and PLP cofactors in the human enzyme. Interestingly, whereas these studies do not provide evidence for coupling between the paramagnetic ferric ion and the phosphorus nucleus, they reveal that the heme oxidation state influences the microenvironment of the PLP and provides the first structural evidence for communication between the two cofactor binding sites. We have also compared the $^{31}$P NMR spectra of the heme-independent yeast and heme-dependent magnetic effects on the spin-lattice ($T_1$) and spin-spin ($T_2$) relaxation rates can be evaluated by subtracting the relaxation rate measured in the presence of a diamagnetic center (e.g. ferrous heme in cystathionine $\beta$-synthase) and the corresponding paramagnetic state (ferric heme). This approach has been applied to map distances between magnetic nuclei and paramagnetic centers in a number of enzymatic systems (13, 14).

A second approach to determining distances between electronic and nuclear spins involves measurement of the hyperfine coupling between the two magnetic moments. Specifically the dipolar component of the coupling, which is anisotropic, varies in inverse proportion to the third power of the interspin distance. In cases where the overall hyperfine coupling is relatively large, it may be detected as a resolved splitting in the continuous wave EPR spectrum. More often, however, these splittings are obscured by inhomogeneous line-broadening effects, and one must resort to high resolution techniques such as ESEEM or ENDOR to obtain coupling constants. In both techniques a spectrum of transition energies of hyperfine-coupled nuclear spins is produced, and the orientation dependence of the hyperfine coupling yields the desired distance. Because the $g$-factor for low spin Fe$^{3+}$ is anisotropic, determination of hyperfine couplings to magnetic nuclei of ligand atoms usually requires the use of high resolution EPR methods.

In this study we employed a combination of NMR and pulsed EPR spectroscopic methods to evaluate the interaction, if any, between the heme and PLP cofactors in the human enzyme.
human enzymes that lead us to conclude that the PLP environment is similar in the two proteins.

**EXPERIMENTAL PROCEDURES**

**Materials**—Serine, ampicillin, TLCK, TPCK, leupeptin, pepstatin, aprotinin, benzamidine and isopropyl-1-thio-β-D-galactopyranoside were purchased from Sigma. D₂O (99%) was from Cambridge Isotope Laboratories. Homocysteine was generated from homocysteine thiolacetic acid (Sigma) as described (15), and the concentration was determined spectrophotometrically using Ellman’s reagent (16).

**Purification of Human Cystathionine β-Synthase**—Truncated human cystathionine β-synthase representing the active catalytic core of the enzyme was purified as described previously (5) using an expression vector provided by Warren Kruger (Fox Chase Cancer Center, Philadelphia).

**Purification of Yeast Cystathionine β-Synthase**—Full-length yeast cystathionine β-synthase was purified using a recombinant expression vector, pSEC, provided by Edith Miles (National Institutes of Health). _Escherichia coli_ containing the pSEC vector were cultured at 37 °C in 1 liter of super broth supplemented with 1 × Vogel and Bonner medium containing ampicillin (100 mg/liter) and induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside at an OD₆₅₀ of 2.1 as described previously (3, 17). Cells were harvested after 20 h and collected by centrifugation. The cell paste was suspended in 80 ml of buffer containing 50 mM Tris (pH 8.0), 10 mM EDTA, 1 mM benzamidine–HCl, 10 mM β-mercaptoethanol, 0.1 mM TLCK, 0.1 mM TPCK, 1 mg/liter leupeptin, 1 mg/liter aprotinin, and 1 mg/liter pepstatin. The cells (~12 g wet weight) were disrupted with a Heat Systems ultrasonic processor XL, operated at an output setting of 7 for 7 × 0.5 min with 3-min breaks between cycles to prevent overheating. The suspension was stirred for 1 h at 4 °C in the presence of 6 mg of lysozyme, 2 mg of DNase I, and 10 mM MgCl₂ and then centrifuged at ~12,000 × g for 30 min to remove cell wall debris and unbroken cells. The enzyme was purified by a modification of the reported procedure as described below. The supernatant was diluted 3-fold with distilled water and loaded onto a 5 × 7 cm fast flow hydroxylapatite column (from Calbiochem) equilibrated with buffer A containing 50 mM potassium phosphate (pH 7.8), 10 mM EDTA, 10 mM β-mercaptoethanol, 0.1 mM PLP, 1 mM benzamidine, 0.1 mM TLCK, 0.1 mM TPCK, 1 mM aprotinin, 2 mg/liter leupeptin, and 2 mg/liter pepstatin A. The column was washed with ~300 ml of buffer A. Proteins were eluted with a 2-liter linear gradient ranging from 0–4 mM KCl at a flow rate of 4 ml/min. The enzyme was collected in two pools eluting between 0.05 and 0.12 M KCl and 0.12 and 0.2 M KCl. The second fraction was ~95% pure and was concentrated and stored at −80 °C. The first pool of enzyme was concentrated and loaded onto a second hydroxylapatite column (5 × 7 cm) equilibrated with buffer B, which differed from buffer A by containing 12.5 mM (instead of 50 mM) potassium phosphate, pH 7.8. The column was washed with 300 ml of buffer B and eluted with a 2-liter linear gradient of potassium phosphate ranging from 12.5 mM to 250 mM at a flow rate of 4 ml/min. Highly pure enzyme eluted between 150 and 250 mM potassium phosphate and was concentrated and frozen at −80 °C. A total of 286 mg of yeast cystathionine β-synthase was obtained from a 1-liter culture and had a specific activity of 495 μmol/mg/h at 37 °C, which is comparable with the reported value (17).

**NMR Measurements**—Fourier transform 31P NMR spectra were collected at 202.4 MHz on a GE-Omega 500-MHz spectrometer using a 10-mm multinuclear probe head with broadband H emission decoupling using a standard WALTZ-16 decoupler phase modulation scheme. The NMR tube contained the sample (1.8 ml) and D₂O (0.2 ml) as field frequency lock and was maintained at 10 °C using a thermostated liquid nitrogen flow. A spectral width of 8298 Hz was acquired in 4096 complex data points with a pulse angle of 60°. Positive chemical shifts in ppm were reported versus 85% H₃PO₄. Chemical shift referencing was achieved by inserting a capillary containing 85% H₃PO₄ into the sample and adding 4–8 additional transients to the accumulated spectrum. Spin-spin relaxation times (T₂) were determined via progressive saturation using 90° excitation pulses with progressively shorter pulse-receive delays until the signal was eliminated by saturation and the data were fit to the appropriate exponential decay function. The progressive saturation recovery method was used rather than the more common inversion-recovery technique because of the sensitivity limitations of these samples. The spectrum of the reduced enzyme (Fig. 3A) was acquired on a GE-Omega 300 instrument operating at 121.65 MHz for 31P observation with 60° pulses. The substrates, serine and homocysteine, were added at the concentrations described in the figure legends.

**Pulsed EPR Measurements**—Pulsed ENDOR, ESEEM, and HYSCORE spectra were obtained using a Bruker ELEXSYS E580 spectrometer. A Bruker Flexline variable-Q dielectric resonator with an integral radiofrequency coil for ENDOR was employed for pulsed measurements. Radiofrequency power was delivered by an ENI model A-500 amplifier with a 500-watt nominal full output. An Oxford Instruments CF935 liquid helium cryostat maintained samples at a constant temperature for measurement.

All ESEEM data were obtained by the use of a stimulated echo pulse sequence (18). ESEEM experimental conditions were: sample temperature, 14 K; microwave frequency, 9.695 GHz; τ (delay between first and second microwave pulses), 200 ns; increment of T (delay between second and third microwave pulses), 16 ns; points per spectrum, 512; τ/2 pulse duration, 16 ns; each point is the average of ~5000 individual spin echoes. Each time domain ESEEM scan was fitted to a fourth-order polynomial function that was subtracted from the data set. Apodization with a trapezoid function, zero-filling to 1024 points, and calculation of the absolute-value Fourier transform yielded the final frequency domain spectrum.

**HYSCORE** is a two-dimensional ESEEM method that helps to deconvolute complex spectra by revealing correlations between nuclear spin transition frequencies. For HYSCORE, the standard four-pulse sequence was used (19). The 128 × 128 point data sets were base line...
corrected for ESEEM and processed by applying a two-dimensional fast Fourier transform.

A Mims pulse sequence was used for all ENDOR measurements (20). ENDOR experimental conditions were: sample temperature, 14 K; $\tau$, 140–240 ns; $T_1$, 12 $\mu$s; radiofrequency pulse duration, 6.0 $\mu$s; microwave frequency, 8.695 GHz; magnetic field, 278.0 mT; pulse train repetition rate, 1.0 kHz; each point is the average of ~2000 individual spin echoes. Spin-echo-detected EPR spectra were obtained by monitoring a two-pulse electron spin echo ($\tau = 140$ ns) during a magnetic field scan.

RESULTS AND DISCUSSION

The PLPBinding Sites in Human and Yeast Cystathionine $\beta$-Synthases Are Similar—$^{31}$P NMR spectroscopy has been a useful tool for studying the environment of PLP bound in the active sites of enzymes. The chemical shift for PLP free in solution ranges from 0.75 to 4.3 ppm as the pH is increased from a low to high value (21). The low field resonances observed in the $^{31}$P NMR spectra of human (5.4 ppm) and yeast (5.17 ppm) cystathionine $\beta$-synthase suggests that the phosphate group of PLP is bound as a dianion in the active sites of both enzymes (Fig. 3). A similar chemical shift has been reported in the PLP enzyme, O-acetylserine sulphydrylase (5.2 ppm) (22). A correlation between the $^{31}$P chemical shift and the smallest O–P–O bond angle has been reported by Gorenstein (23). For free PLP in solution, the observed $^{31}$P chemical shift is presumed to arise from the minimal energy conformation of the phosphate. In human and yeast cystathionine $\beta$-synthases, the magnitude of the downfield chemical shifts for the bound PLP is greater than that for the free cofactor (4.3 ppm for the dianion) and suggests that the conformation of the phosphate moiety is strained. A resonance at an even lower field has been observed for PLP bound to ornithine decarboxylase (6.2 ppm) (24). The broadness of the $^{31}$P signals in both yeast and human enzymes indicates that the PLP is bound tightly. The signal associated with the yeast enzyme is significantly broader than that of the human enzyme (73 Hz line width) because of the larger size of the protein (224 kDa versus 96 kDa for the truncated human protein), and its line width could not be estimated accurately.

The similarity in the $^{31}$P NMR spectra of the yeast and oxidized human enzyme is consistent with the high degree of conservation in the PLP binding domains between the two enzymes at the primary sequence level (Fig. 2). To guide interpretation of our data, we have used the crystal structure of the Salmonella typhimurium O-acetylserine sulphydrylase to develop a model for human cystathionine $\beta$-synthase by employing a molecular replacement strategy “in silico.” All the active site residues that interact with the PLP in the O-acetylserine sulphydrylase (25) are completely conserved in both yeast and human cystathionine $\beta$-synthase (Fig. 2). Thus, the glycine-rich loop that is involved in electrostatic stabilization of the phosphate oxygen atoms, the serine that hydrogen-bonds with N1 of the PLP ring, and the asparagine that hydrogen-bonds to the 3'-hydroxyl group are present in all three enzymes (Fig. 4).

The 5'-phosphate is likely to be dianionic in all three enzymes based on their respective chemical shifts. In O-acetylserine sulphydrylase, His-152 is within hydrogen-bonding distance to a water molecule that in turn hydrogen-bonds to the 5'-phosphate. It has been postulated that the histidine may be positively charged, resulting in near neutrality around the dianionic phosphate moiety (25). A similarly positioned histidine residue is observed in the modeled structure of cystathionine $\beta$-synthase (His-232 in the human sequence). Thus, the sequence alignments of the human and yeast cystathionine $\beta$-synthase and the O-acetylserine sulphydrylase provide strong evidence for the conservation of the PLP binding site in the three enzymes and explain the similar $^{31}$P NMR chemical shifts observed in the resting forms of these enzymes. More importantly, these results indicate that the phosphorus group in PLP is shielded from the paramagnetic ferric heme in the human enzyme and that the two cofactors are not proximal to each other.

Pulsed EPR Studies Indicate That the Heme and PLP Binding Sites Are Distant from Each Other—We have employed ESEEM and pulsed ENDOR spectroscopy to determine whether or not the $^{31}$P nucleus of PLP is in the immediate vicinity of the ferric heme iron. Measurements were performed on three states of the enzyme, (i) the resting enzyme, (ii) the enzyme and homocysteine, and (iii) the enzyme, homocysteine, and serine.

The ENDOR spectrum of the resting enzyme, obtained using the pulse sequence of Mims, exhibits a four-line pattern that is characteristic of heme nitrogen hyperfine couplings (Fig. 5a). None of the four lines tracked by magnetic field scan changed at the rate expected for $^{31}$P, and none was altered upon addition of one or both substrates (Fig. 5b). A weak $^{31}$P coupling should produce a pair of transitions positioned symmetrically about the Larmor frequency for that nucleus and positioned close to that frequency. In no case was a transition observed at or above the Larmor frequency of $^{31}$P, except for $^1$H features well above 10 MHz. Thus, the ENDOR spectra show no evidence of the proximity of the $^{31}$P nucleus to the heme and
Heme and PLP Not Proximal in Human Cystathionine β-Synthase

ESEEM spectra of truncated human cystathionine β-synthase in the resting state. Spectra obtained at magnetic field (B) = 345.0 mT (dotted line), 350.0 mT (solid line), and 355.0 mT (dashed line).

Oxidation State Changes in the Heme Modulate the $^{31}\text{P}$ NMR Spectrum of PLP—A change in the oxidation state of the heme from ferric to ferrous diminishes the activity of cystathionine β-synthase by a factor of −2 (9). Binding of CO to ferrous heme results in complete inhibition of cystathionine β-synthase activity, indicating that changes in the heme binding site are communicated to the active site containing PLP, and that the heme may play a regulatory role (4). To provide structural evidence for this allosteric communication, we examined the $^{31}\text{P}$ NMR spectrum of the ferrous enzyme. Reduction of cystathionine β-synthase results in conversion of the heme from a low spin paramagnetic ferric to a low spin diamagnetic ferrous state and is accompanied by significant changes in the $^{31}\text{P}$ NMR spectrum of the bound PLP (Fig. 3). The phosphorus resonance shifts to higher field (from 5.4 to 2.2 ppm), and the signal is substantially narrowed (from 73 to 16 Hz). To exclude the possibility that the signal observed upon reduction of the heme is due to free PLP, the enzyme was subjected to filtration in a Centricon concentrator. Free PLP was not detected in the filtrate. In addition, the chemical shift of free PLP in 50 mM Tris, pH 8.3, is −4.3 ppm. The upfield shift in the resonance position is consistent with either a change in the ionization state of the phosphate group or a substantial change in the smallest O–P–O bond angle. The narrowing of the line width suggests that the phosphate group of PLP is more mobile and may be less tightly bound in the ferrous enzyme.

To evaluate whether or not the longitudinal relaxation of the phosphorus nucleus is influenced by the presence of the paramagnetic ferric heme, the relaxation rates were determined by saturation recovery experiments and found to be 6.34 ± 0.01 s and 5.04 ± 0.06 s for the oxidized and reduced enzymes, respectively. The limiting values for the transverse relaxation rate, $T_2$, estimated from the spectral line widths, are 4.4 ms and 21.1 ms for the oxidized and reduced enzymes, respectively (the measured $T_2$ value using the spin echo technique for the oxidized enzyme is 2.8 ms). Because the presence of the paramagnetic ferric heme in the vicinity of PLP is expected to measurably enhance the spin relaxation rate of the phosphorus nucleus primarily by dipolar interactions between the ferric ion and the $^{31}\text{P}$ nucleus, the absence of a paramagnetic effect on the longitudinal relaxation rate indicates that the two cofactors are not proximal.

Interestingly, these results reveal that whereas the heme is not sufficiently proximal to the PLP to affect the $T_1$ relaxation properties of the phosphorus nucleus, a change in the oxidation state is transmitted to the active site resulting in changes in the microenvironment of the PLP cofactor. This is manifested...
by significant alterations in the spectral line width (and $T_2$) and resonance frequency of the phosphorus signal. These results provide the first structural evidence for communication between the heme and the PLP binding sites and are consistent with functional studies from our laboratory that reveal that perturbations in the heme pocket affect catalytic activity (4).

Changes in the $^{31}$P NMR Spectra Are Induced by the Addition of Substrates to Human Cystathionine β-Synthase—PLP is bound as a Schiff base to Lys-119 in the active site of human cystathionine β-synthase (Fig. 1). The addition of the substrate, serine, results in fluorescent changes that are consistent with the formation of an external aldimine and the reported absence of tritium washout from the α carbon of serine in the absence of homocysteine. In the related enzymes tryptophan synthase (26) and O-acetylserine sulfhydrylase (22), the corresponding external aldimines rather than the elimination product, α-aminoacrylate, are also observed in the presence of serine alone. This is in contrast to the reported formation of the α-aminoacrylate product in heme-free cystathionine β-synthase in the crystalline state (11). These observations suggest that the presence of heme in the human enzyme affects the equilibrium between intermediates along the reaction coordinate and/or that the properties of the enzyme observed in the crystalline state are different from those observed for human cystathionine β-synthase in solution.

The addition of 6 mM serine to 1.3 mM cystathionine β-synthase results in the appearance of a new signal at 4.7 ppm (Fig. 7). The smaller signal at 5.4 ppm corresponds to residual internal aldime. The addition of 8 mM homocysteine to the sample containing the external aldime results in reformation of the internal aldime (at 5.4 ppm). This is expected because homocysteine was added in excess, and it establishes that the enzyme used in the NMR experiment remained catalytically active.

Changes in the $^{31}$P NMR Spectrum Induced by the Addition of Substrates to Yeast Cystathionine β-Synthase—The addition of serine to yeast cystathionine β-synthase results in the disappearance of the 412-nm absorption band assigned as the internal aldime and in the appearance of a 460-nm band that is attributed to the α-aminoacrylate (7, 17). We have examined the $^{31}$P NMR spectra of the enzyme in the presence of the two substrates, serine and homocysteine (Fig. 8). Addition of 4.8 mM L-serine to the enzyme (1.2 mM in PLP) resulted in an upfield shift of the 5.15 ppm resonance to 4.07 ppm and was accompanied by a decrease in the line width. The latter suggests increased mobility, whereas the upfield shift in resonance frequency is consistent with an increase in the smallest O–P–O bond angle, resulting in increased shielding of the phosphorus nucleus (23). The addition of 9.6 mM DL-homocysteine resulted in recovery of the resting enzyme spectrum.

Conclusions—In this study, we employed a combination of NMR and pulsed EPR studies to detect possible interactions between the paramagnetic ferric iron and the phosphorus nucleus in PLP in human cystathionine β-synthase. The spectroscopic studies failed to show a direct coupling between the two nuclei and a paramagnetic effect on the relaxation rate of the phosphorus nucleus, thus supporting the conclusion that PLP and heme are not proximal in human cystathionine β-synthase. However, reduction of the heme in cystathionine β-synthase is accompanied by chemical shift and line width changes in the $^{31}$P signal, revealing that an oxidation state change in the heme pocket is transmitted to the active site, consistent with previous biochemical results from our laboratory that suggest a regulatory role for heme. The similarity in the $^{31}$P NMR spectra of the resting forms of yeast and human cystathionine β-synthase indicates that the PLP environment in the two enzymes are similar notwithstanding the absence of heme in the yeast enzyme. Furthermore, the addition of the substrate, serine, results in chemical shift changes that are consistent with the formation of the spectroscopically identified intermediates in the two enzymes.

Acknowledgment—We gratefully acknowledge the assistance of Bryan Lepore (Bersonstiel Center, Brandeis University) in generating the modeled structure of human cystathionine β-synthase and in preparing Fig. 4.

REFERENCES

1. Kery, V., Bukovska, G., and Kraus, J. P. (1994) J. Biol. Chem. 269, 25283–25288
2. Finkelstein, J. D., Kyle, W. E., Martin, J. J., and Pick, A.-M. (1975) Biochem. Biophys. Res. Commun. 66, 81–87
3. Jhee, K. H., McPhie, P., and Miles, E. W. (2000) J. Biol. Chem. 275, 11541–11545
4. Taoka, S., West, M., and Banerjee, R. (1999) Biochemistry 38, 2738–2744
5. Taoka, S., Widjaja, L., and Banerjee, R. (1999) Biochemistry 38, 13155–13161
6. Borosok, E., and Abeles, R. H. (1982) Arch. Biochem. Biophys. 213, 695–707
7. Jhee, K. H., McPhie, P., and Miles, E. W. (2000) Biochemistry 39, 10544–10556
8. Kery, V., Poneleit, L., Meyer, J. D., Manning, M. C., and Kraus, J. P. (1999) Biochemistry 38, 2710–2724
9. Taoka, S., Ohja, S., Shan, X., Kruger, W. D., and Banerjee, R. (1998) J. Biol. Chem. 273, 25179–25184
10. Green, E. L., Taoka, S., Banerjee, R., and Loehr, T. M. (2000) Biochemistry 40, 459–463
11. Bruno, S., Schiarettti, F., Burkhard, P., Kraus, J. P., and Mozzarelli, A. (2000) J. Biol. Chem. 276, 16–19
12. Mildvan, A. S., and Gupta, R. K. (1978) Methods Enzymol. 49, 322–359
13. Fang, C. H., Feldmann, R. J., and Mildvan, A. S. (1976) Biochemistry 15, 75–84
14. Makinen, A. L., and Nowak, T. (1989) J. Biol. Chem. 264, 12148–12157
15. Drummond, J. T., Jarrett, J., Gonzalez, J. C., Huang, S., and Matthews, R. G. (1995) Anal. Chem. 67, 323–329
16. Ellman, G. L. (1959) Arch. Biochem. Biophys. 82, 70–77
17. Jhee, K. H., McPhie, P., Miles, E. W. (2000) Biochemistry 39, 10548–10556
18. Mims, W. B. (1972) Physiol. Rev. 52, 2409–2419
19. Hofer, P., Grupp, A., Nebenführ, H., and Mehring, M. (1996) Chem. Phys. Lett. 132, 279–282
20. Mims, W. B. (1986) Proc. Roy. Soc. Lond. A 283, 452–457
21. Schnackertz, K. D., Wabler, G., Vincent, M. G., and Jansonius, J. N. (1989) Eur. J. Biochem. 185, 525–531
22. Schnackertz, K. D., Tai, C.-H., Simmons, J. W., Rao, G. S. J., and Cook, P. F. (1995) Biochemistry 34, 12152–12160
23. Gorenstein, D. G. (1975) Biochemistry 14, 4558–4567
24. Osterman, A. L., Brooks, H. B., Rizo, J., and Phillips, M. A. (1997) J. Biochem. 2409–2419
25. Burkhard, P., Rao, G. S., Hohenester, E., Schnackertz, K. D., Cook, P. F., and Jansonius, J. N. (1998) J. Biol. Chem. 273, 121–133
26. Schnackertz, K. D., and Mozzarelli, A. (1998) J. Biol. Chem. 325247–32525
Pyridoxal Phosphate Binding Sites Are Similar in Human Heme-dependent and Yeast Heme-independent Cystathionine β-Synthases: EVIDENCE FROM 31P NMR AND PULSED EPR SPECTROSCOPY THAT HEME AND PLP COFACTORS ARE NOT PROXIMAL IN THE HUMAN ENZYME

Ömer Kabil, Shinichi Toaka, Russell LoBrutto, Richard Shoemaker and Ruma Banerjee

J. Biol. Chem. 2001, 276:19350-19355.
doi: 10.1074/jbc.M100029200 originally published online February 26, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M100029200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 26 references, 6 of which can be accessed free at http://www.jbc.org/content/276/22/19350.full.html#ref-list-1