Transsulfuration Depends on Heme in Addition to Pyridoxal 5’-Phosphate

CYSTATHIONINE β-SYNTHASE IS A HEME PROTEIN*

(Vladimir Kery, Gabriela Bukovska†, and Jan P. Kraus§)

From the Departments of Pediatrics and Cellular and Structural Biology, University of Colorado School of Medicine, Denver, Colorado 80262

The first committed step of transsulfuration is catalyzed by cystathionine β-synthase (CBS), a known pyridoxal 5’-phosphate (PLP)-dependent enzyme. The inferred amino acid sequences of rat liver CBS and rat liver hemoprotein H-450 are identical. We now confirm the presence of heme b in rat and human liver CBS. Heme almost entirely accounts for the visible spectrum of CBS rather than PLP. Human CBS, expressed in *Escherichia coli*, acquires heme b from the host bacteria. δ-Aminolevulinic acid supplementation during bacterial growth increases both the heme saturation and the specific activity of the homogeneous enzyme more than 3-fold. 1 mol of the 63-kDa CBS subunit binds 1 mol of each (heme and PLP). The presence of heme is required for PLP binding, and the amount of PLP bound is limited by the heme content. Removal of PLP, but not heme, from CBS is reversible. These findings suggest that heme is functionally incorporated into CBS only during protein folding. This report describes the first instance of an enzyme that depends upon both heme and PLP for its function.

Transsulfuration of homocysteine to cysteine in eukaryotes is catalyzed by two pyridoxal 5’-phosphate (PLP)-dependent enzymes (Mudd et al., 1989). The first, cystathionine β-synthase (CBS) (γ-serine hydro-lyase (adding homocysteine), EC 4.2.1.22), catalyzes the condensation of homocysteine and serine to cystathionine. The second, cystathionine γ-lyase (EC 4.4.1.1), hydrolyzes cystathionine to cysteine and α-ketobutyrate.

The sulfur flux through the transsulfuration pathway is regulated by AdoMet concentrations (Finkelstein et al., 1975; Finkelstein and Martin, 1984). AdoMet is another ligand of CBS and activates 2–4-fold the rat (Roper and Kraus, 1992) and human (Kozich and Kraus, 1992; Bukovska et al., 1994) enzymes.

Purification-Expression and Purification—Cloning of human liver CBS cDNA in the expression vector pAX5 (U. S. Biochemical Corp.), transformation of *E. coli* XLI-Blue MR (Stratagene) with the construct, and purification of the enzyme to homogeneity was described elsewhere (Bukovska et al., 1994). Bacteria were grown in NZCYMT media (Life Technologies, Inc.). In some cultures, heme synthesis was induced by 0.3 mM 6-aminolevulinic acid (Sigma) added to the medium at the time of inoculation.

**Experimental Procedures**—PLP-free enzyme—PLP was removed from the enzyme by dialyzing 100 μl of CBS solution for 6 h against 250 ml of 5 mM hydroxylamine in TBS and then twice against TBS for 6 h at 4 °C. The efficiency of PLP removal was checked by CBS assay. The fraction of the remaining PLP-saturated enzyme was calculated as a ratio of CBS activity in the absence or presence of 0.5 mM PLP. The dialysis did not change the heme saturation of CBS.

Heme Removal—Heme was removed from the enzyme by a treatment with 10 mM sodium dithionite in TBS, pH 8.6, for 10 min at room temperature and subsequent dialysis against TBS overnight at 4 °C. More heme was removed by dithionite treatment in the presence of freshly prepared nitric oxide under the same experimental conditions.

CBS Assay—CBS activity was determined by a previously described radiotope assay (Kraus et al., 1978). 1 unit of activity is the amount of enzyme that catalyzes the formation of 1 μmol of cystathionine in 1 h at 37 °C. Enzyme concentrations were determined by a modified Bradford method (Sedmak and Grossberg, 1977).

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Present address: Institute of Molecular Biology, Slovak Academy of Sciences, Bratislava, Slovakia.

§ To whom correspondence and reprint requests should be addressed: Dept. of Pediatrics, Box C325, University of Colorado School of Medicine, 4200 E. 9th Ave., Denver, CO 80262. Tel.: 303-270-7888; Fax: 303-270-8080; E-mail: kraus@esex.hsc.colorado.edu.

1 The abbreviations used are: PLP, pyridoxal 5’-phosphate; AdoMet, S-adenosylmethionine; CBS, cystathionine β-synthase; TBS, Tris-buffered saline.
Cystathionine β-Synthase Is a Heme Protein

**FIG. 1.** Rat liver CBS is a heme protein. Visible spectra of immunoprecipitated rat liver CBS in sodium hydroxide-pyridine solution were measured before (a) and after (b) reduction with sodium dithionite. A 50-μl aliquot of the resuspended immunoprecipitate in TBS, containing approximately 3 μg of enzyme, was dissolved in 200 μl of 50 mM sodium hydroxide, and then 100 μl of pyridine was added. The spectra were recorded immediately. Thereafter, 1 mg of solid sodium dithionite was added, and the spectra were recorded after 5 min.

**Heme Identification and Determination**—Spectral changes at 500–600 nm characteristic of heme proteins were measured in alkaline pyridine solutions after reduction with sodium dithionite (Morrison and Horie, 1965). Heme identification was performed by comparison of the wavelengths and peak heights of CBS alkaline pyridine hemichromes and hemochromes with those derived for heme standards (Berry and Trumpower, 1987). Heme content was calculated by comparing the absorbance of CBS hemochrome at 556 nm with that of of heme b under the same conditions. The hemochrome was prepared by mixing 90 μl of the heme-containing sample with 10 μl of 0.1 M NaOH, 40 μl of pyridine, and 10 μl of 1 M freshly prepared sodium dithionite. The spectra were measured immediately after the reduction. All measurements were made at the room temperature.

**PLP Determination**—PLP in CBS was spectrofluorometrically measured (Adams, 1979) after reaction with cyanide. The precise molar concentration of the PLP stock solution was determined in 0.1 N HCl using the extinction coefficient 5.1 mM⁻¹cm⁻¹ measured at 295 nm. Inner filter effect of heme absorption on the PLP-cyanide complex fluorescence was excluded by measuring the calibration curves for PLP in the presence and absence of hemin.

**Spectral Measurements**—Measurements were carried out in Beckman DU-50 or Gilford 2400 spectrophotometers at 25 °C in 150 μl of 0.1 M Tris-HCl buffer, pH 8.6, in 1-cm quartz cuvettes. Bovine hemin from Sigma was used for heme spectral titrations. The spectrum of this substance in alkaline pyridine solution was identical to that of heme b from horse heart myoglobin (M1882, Sigma).
Cystathionine β-Synthase Is a Heme Protein

RESULTS

Rat CBS Is a Heme Protein—To investigate the possibility that CBS is a heme protein, we compared the spectral characteristics of immunoprecipitated rat liver CBS with the published characteristics of "hemoprotein H-450" (Kim and Deal, 1976; Kim, 1982; Omura et al., 1984) and other heme proteins (Berry and Trumpower, 1987). The immunoprecipitated CBS was free of contaminating proteins because a single polypeptide band, corresponding to the 63-kDa CBS subunit, was detected by SDS-polyacrylamide gel electrophoresis (results not shown).

Absorption spectra of oxidized and reduced pyridine hemochromogen complexes provide unambiguous evidence for the presence of heme in a protein. Fig. 1a shows the visible spectrum of pyridine hemichrome freshly prepared from rat CBS. A strong absorption maximum is evident at 417 nm with a smaller one at 556 nm, consistent with the characteristic pyridine-hemichromogen absorption maxima (Morrison and Horie, 1965). Absorbance increased at both wavelengths following reduction with sodium dithionite (Fig. 1b).

The α- and β-hemochrome peaks at 525 and 556 nm, respectively, are in complete agreement with those observed for the sodium dithionite-reduced heme b (Berry and Trumpower, 1987). The extinction ratio of reduced/oxidized CBS at 556 and 550 nm was 3.50, which agrees with the established ratio of hemochrome/hemichrome b, 3.56 (Berry and Trumpower, 1987). These data indicate that the native rat CBS is a heme protein and that the heme moiety is heme b, which is present in the isolated native enzyme in its oxidized form.

Cloned Human CBS Takes Heme from Host E. coli—To further evaluate the nature of the CBS-heme interactions, we examined the human enzyme expressed in E. coli. These bacteria synthesize heme (Schellhorn and Hassan, 1986). Electrophoretically homogeneous human CBS, purified from transformed bacteria (Bukovska et al., 1984), exhibits the characteristic features of a heme protein: a sharp Soret peak at 428 nm with a shoulder at 363 nm (Fig. 2a) and a broad band at 550 nm (Fig. 2a, inset). These absorption maxima in the expressed human enzyme are similar to those of purified porcine (Kim and Deal, 1976) and rat (Omura et al., 1984; Hasegawa et al., 1984) liver hemoproteins H-450.

The absorbance profile of the cloned human enzyme, dissolved in alkaline pyridine and reduced with sodium dithionite (Fig. 2b), was similar to the rat enzyme (Fig. 1b). In the human enzyme, a larger β-absorption maximum was observed at the same wavelength (556 nm) compared with that of the rat. Furthermore, a smaller α-absorption maximum was also observed at 525 nm. These results support the conclusion that both rat and human enzymes are hemoproteins that bind heme b regardless of its source.

Visible Spectrum of CBS Is Essentially Due to the Bound Heme—Heme and PLP-protein complexes have overlapping spectra in the 410–430 nm region. Well characterized heme b-containing proteins like cytochrome P-450 (Dawson et al., 1982) and myoglobin (Sono et al., 1982) exhibit Soret peaks between 414 and 427 nm, depending on the coordination of the oxidized iron and ligand bound. In PLP-dependent enzymes, the protonated PLP-Schiff base absorbs light between 414 and 428 nm (Morino and Nagashima, 1984). At least one of the chromophores must be selectively and reversibly removed from CBS to distinguish between their individual contributions to the visible spectrum. Heme removal is irreversible (see below); however, PLP can be reversibly removed from native CBS by hydroxylamine (Lipson et al., 1980).

Fig. 3 compares the spectra of the PLP-free and PLP-saturated enzyme at identical heme saturation. From the data in this figure, it may be concluded that enzyme-bound heme and PLP exhibit the same absorption maximum at 428 nm. The visible spectrum of CBS is mostly due to heme rather than PLP.

Fig. 2. Presence of heme in cloned human CBS purified from E. coli. a, absorption spectrum of 26.5 µg CBS, measured in 0.1 mM Tris-HCl buffer, pH 8.6; inset, enlarged region between 500 and 600 nm; b, spectrum in alkaline pyridine solution after reduction with 1 mg of solid sodium dithionite.

Fig. 3. CBS-visible spectrum is mostly due to heme rather than PLP. Enzyme spectra were measured in 10 mM TBS, pH 8.6, before (—) and after (——) dialysis against 5 mM hydroxylamine in TBS at 25 °C. The CBS concentration was 34 µg. The dialyzed enzyme retained only 7% of CBS activity; 90% of the original activity was restored by incubation in 0.5 mM PLP. Addition of PLP to the dialyzed enzyme caused an immediate increase of the absorbance at 428 nm (not shown). The enzyme used for these studies was 50% saturated with heme before and after PLP dialysis.
CBS spectra. Native PLP-saturated (---) and PLP-free (-----) enzymes (0.9 μM) were reduced with 10 mM sodium dithionite in TBS, pH 8.6, and the spectra were recorded immediately. The enzyme was pre-saturated with 0.5 mM PLP during the purification (Bukovska et al., 1994), and PLP-free enzyme was prepared as described in under "Experimental Procedures."

The Effect of PLP on Heme.—To determine the effect of PLP on the heme spectrum, we used PLP-free and PLP-saturated enzymes reduced with sodium dithionite. Dithionite induces a hypsochromic shift in the visible spectra of PLP-dependent enzymes (Adams, 1969), whereas it shifts the H-450 Soret peak from 428 to 448 nm (Omura et al., 1984; Hasegawa et al., 1984). These spectral differences allowed us to dissect the spectral contributions of heme from PLP.

The enzyme preparations shown in Fig. 3 were reduced. Dithionite immediately shifted the 428-nm Soret peak of the PLP-free enzyme to 448 nm with a 1.2-fold increase of the extinction coefficient of the PLP enzyme. These preparations were used to establish the correlation between the specific activity and the heme saturation of the enzyme (Fig. 5).

CBS Specific Activity Increases with Its Heme Content.—The human CBS isolated and purified from E. coli XL1 to homogeneity (Bukovska et al., 1994) was saturated with variable amounts of heme (8–50%), 20% in an average purification. Heme production by the bacteria can be increased by supplementation with δ-aminolevulinate (Doss and Philip-Dormston, 1971), a precursor of heme. Addition of 0.3 mM δ-aminolevulinate to the growth medium increased heme content in the transformed bacteria up to 50-fold. Under these conditions, purified CBS was up to 75% saturated with heme.

Using media with and without δ-aminolevulinate allowed us to isolate a number of purified CBS enzymes distinct in their heme saturations. These preparations were used to establish the correlation between the specific activity and the heme saturation of the enzyme (Fig. 5).

The specific activity of CBS fully saturated with heme was estimated to be 242 units/mg. We normalized this activity to 1 mol of heme. The data further indicate that heme may need to be present at the time of protein folding to be properly incorporated into the enzyme molecule.

Home Is Essential for PLP Binding to CBS.—Having established that heme is present in CBS and necessary for its activity, we next turned our attention to the potential interaction of heme and PLP within the enzyme. Specifically, we asked two questions: does PLP bind to CBS regardless of its heme saturation, and is there an interdependence between heme and PLP binding to CBS?

We used several enzyme preparations differing in their heme content to prepare PLP-free CBS as described under "Experimental Procedures." We subtracted the spectrum of the PLP-free enzyme from that of the PLP-saturated one and calculated the δε428 as shown in Fig. 3. The contribution of PLP to ε428 increased from 3.1 to 18.1 μm⁻¹ cm⁻¹ for enzymes increasingly saturated with heme between 0.23 and 0.69 mol/mol subunit. This suggested that the enzyme proportionally binds PLP to its heme content.

To confirm this suggestion, we determined the molar PLP content in our enzyme preparations variably saturated with heme (Fig. 6). Indeed, CBS binds equimolar amounts of PLP with regard to heme.

Therefore, heme incorporation in CBS is prerequisite for PLP binding, and the amount of PLP bound to the enzyme is limited by its heme saturation. This strongly suggests a role of heme in the correct folding of CBS. However, it still does not exclude a contribution of the heme moiety to CBS catalysis.

DISCUSSION

CBS, purified from human, rat, and chicken liver (Kraus and Rosenberg, 1983; Kimura and Nakagawa, 1971; Braunstein and Goryachenkova, 1976), was reported to contain PLP; therefore, its nucleotide and peptide sequence (Swaroop et al., 1992) identity to hemoprotein H-450 (Ishihara et al., 1990) was unexpected. Our spectrophotometric analyses of rat and human

V. Kery and J. P. Kraus, unpublished results.

FIG. 4. PLP affects the intensity of reduced hemochromophore. The enzyme preparations shown in Fig. 3 were reduced. Dithionite immediately shifted the 428-nm Soret peak of the PLP-free enzyme to 448 nm with a 1.2-fold increase of the extinction coefficient of the PLP enzyme. These preparations were used to establish the correlation between the specific activity and the heme saturation of the enzyme (Fig. 5).

FIG. 5. Specific activity of CBS increases with its heme saturation. Cloned human enzyme was purified from E. coli to electrophoretic homogeneity (Bukovska et al., 1994). Samples less than 50% saturated with heme were purified from bacteria grown in the absence of heme precursor. Samples over 50% saturation were purified from bacteria supplemented with 0.3 mM δ-aminolevulinate. CBS activity, heme, and protein were measured as described under "Experimental Procedures." Inset, the relative activity was calculated using estimated specific activity of 242 units/mg (normalized to 1) for CBS fully saturated with heme.

"Experimental Procedures"
CBS in their native forms as well as of their pyridine hemochromophores confirmed that they are also heme proteins.

In this report, we confirmed the absolute dependence of CBS activity on PLP. The addition of PLP to the inactive PLP-free enzyme restored its activity. However, to our knowledge, no known PLP-containing enzyme possesses as high overall visible absorption as CBS (84.9 mM^(-1) cm^(-1) for CBS 69% saturated with both heme and PLP). We showed that PLP contribution to the absorption maximum at 428 nm was between 3.07 and 18.09 mM^(-1) cm^(-1), consistent with the reported values for other PLP-containing enzymes (Morino and Nagashima, 1984).

Comparison of visible spectra of reduced PLP-free and PLP-saturated CBS suggested that PLP directly affects the heme moiety. Following dithionite reduction of CBS, we observed the Soret bandshift from 428 to 448 nm in agreement with the Soret bandshift from 428 to 448 nm in agreement with the reported value for rat H-450 (Omura et al., 1984; Hasegawa et al., 1984). We also showed that this shift is independent of the presence of PLP; however, the magnitude of all peaks significantly differed between the PLP-saturated and PLP-free enzymes. The higher absorption maxima of the PLP-saturated enzyme suggest that PLP binding alters the polypeptide environment of the heme moiety or directly interacts with it.

Excellent correlation between the specific activity of the enzyme and its heme saturation clearly demonstrated that heme is required for full CBS activity in addition to PLP. The stoichiometry of heme and CBS subunit fully agrees with the spectrophotometric estimates published for rat H-450 (Omura et al., 1984).

On the other hand, the inability of heme to bind correctly and activate the partially heme-saturated enzyme suggests that the heme moiety may be incorporated into CBS only at the time of the enzyme folding. We also demonstrated that the enzyme fraction containing heme is able to bind PLP and, consequently, is active. The data indicate that heme most likely allows the enzyme to fold properly and form a structural domain for PLP.

This major finding may change our understanding of the eukaryotic transsulfuration pathway and its relationship to the heme metabolism. It may also change our understanding of homocystinuria and other related genetic diseases. For instance, it is not clear why certain types of homocystinuria are PLP responsive while others are not (Mudd et al., 1989). Perhaps, certain CBS mutations primarily decrease the heme binding and thus indirectly affect the interaction of the enzyme with PLP.

The direct involvement of heme in CBS-catalyzed reactions is difficult to estimate since PLP-mediated catalysis sufficiently explains the observed experimental data. They suggest that CBS belongs to the family of PLP-dependent lyases, exclusively catalyzing β-replacement reactions of α-amino acids with electronegative substituents in the β-position (Braunstein and Goryachenkova, 1976). However, CBS differs from other PLP-mediated β-replacement reaction enzymes. No product release occurs from CBS during the catalysis until after the second substrate is bound (Borecos and Abeles, 1982).

It is conceivable that CBS is a multifunctional enzyme and that heme participates in a reaction not involving β-replacement. However, our results imply that heme is required for homocysteine condensation with serine. Based on our present state of knowledge, the following two catalytic mechanisms could explain the role of heme in β-replacement.

First, heme binds the leaving OH group of serine. This would allow a nucleophilic attack on serine carbanion intermediate by homocysteine with the retention of configuration at β-carbon of serine as postulated (Borecos and Abeles, 1982). Thus, CBS may represent another example of a system in which a redox cofactor participates in non-redox catalysis, such as the iron-sulfur clusters in some serine dehydratases. Serine dehydratases utilize either the iron-sulfur cluster or PLP in the β-elimination of water from serine (Grabowski et al., 1993). On the other hand, CBS, an enzyme with 21% sequence identity to the dehydratases (Swaroop et al., 1992), contains both PLP and iron, as heme iron, in the same molecule.

Second, heme could bind homocysteine in a site remote from that of serine. In this event, a specific conformational change of the enzyme could mediate the β-replacement. A similar mechanism has been shown for bacterial tryptophan synthase (Dunn et al., 1990), an enzyme with 28% identity to CBS (Swaroop et al., 1992).

In summary, we demonstrated that CBS is a heme protein; thus, heme is the fifth in a growing list of CBS-specific ligands, which includes its two substrates serine and homocysteine, the essential cofactor, PLP, and the allosteric activator, AdoMet. CBS is the first known enzyme binding both PLP and heme. We showed that CBS requires heme for activity. The presence of heme is an absolute requirement for correct folding of the enzyme and formation of the PLP binding domain. Consequently, both the porphyrin and PLP participate in the transsulfuration of homocysteine to cysteine.

Acknowledgements—We thank Dr. T. Omura for information about the identity of the "homoporphyrin H-450" and CBS cDNA sequences. We also thank Drs. F. Freeman, T. Keben, and M. Roper for review and criticism of the manuscript.

REFERENCES

Adams, E. (1969) Anal. Biochem. 31, 484-492
Adams, E. (1970) Methods Enzymol. 23, 407-410
Berry, B. A., and Trumpower, B. L. (1987) Anal. Biochem. 161, 1-15
Borecos, E., and Abeles, R. H. (1983) Arch. Biochem. Biophys. 213, 695-707
Braunstein, A. E., and Goryachenkova, E. V. (1976) Biochimie (Paris) 58, 5-17
Bukowska, G., Kery, V., and Kraus, J. P. (1994) Protein Expression Purif., in press
Dawson, J. H., Anderson, L. A., and Sono, K. (1982) J. Biol. Chem. 257, 3090-3097
Dosa, M., and Philip-Dormont, W. K. (1971) Hoppe-Seyler's Z. Physiol. Chem. 352, 725-733
Dunn, M. F., Augilar, V., Brzovic, P., Drew, F. W., Jr., Houben, K. F., Leja, C. A., and Roy, M. (1990) Biochemistry 29, 8955-8967
Finkelstein, J. D., and Martin, J. J. (1984) Biochem. Biophys. Res. Commun. 118, 14-19
Cystathionine β-Synthase Is a Heme Protein

Finkelstein, J., Kyle, W., Martin, J., and Pick, A. M. (1975) Biochem. Biophys. Res. Commun. 66, 81-87
Grabowski, R., Hofmeister, A. E. M., and Buckel (1993) Trends Biochem. Sci. 18, 297-300
Hasegawa, T., Sadano, H., and Omura, T. (1984) J. Biochem. (Tokyo) 96, 265-268
Ishihara, S., Morohashi, K., Sadano, H., Kawabata, S., Gotoh, O., and Omura, T. (1990) J. Biochem. (Tokyo) 108, 899-902
Kim, I. C. (1982) J. Biol. Chem. 257, 1063-1070
Kim, I. C., and Deal, W. C., Jr. (1976) Biochemistry 15, 4925-4930
Kimura, H., and Nakagawa, H. (1971) J. Biochem. (Tokyo) 68, 711-723
Kozich, V., and Kraus, J. P. (1992) Hum. Mutat. 1, 113-123
Kraus, J., and Rosenberg, L. E. (1983) Arch. Biochem. Biophys. 222, 44-52
Kraus, J., Packman, S., Fowler, E., and Rosenberg, L. E. (1978) J. Biol. Chem. 253, 6523-6528
Kraus, J. P., Le, K., Swaroop, M., Ohura, T., Tahara, T., Rosenberg, L. E., Roper, M. D., and Kozich, V. (1990) Hum. Mol. Genet. 2, 1633-1639
Lipson, M. H., Kraus, J. P., Solomon, L. R., and Rosenberg, L. E. (1980) Arch. Biochem. Biophys. 204, 486-489
Morrison, Y., and Nagashima, F. (1984) Methods Enzymol. 106, 116-137
Mudd, S. H., Levy, H. L., and Skovby, F. (1989) in The Metabolic Basis of Inherited Disease (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., eds), 6th Ed., pp. 603-754, McGraw-Hill, New York
Omura, T., Sadano, H., Hasegawa, T., Yoshida, Y., and Kominami, S. (1984) J. Biochem. (Tokyo) 96, 1491-1500
Roper, M. D., and Kraus, J. P. (1992) Arch. Biochem. Biophys. 298, 514-521
Schellhorn, H. E., and Hassan, H. M. (1988) J. Bacteriol. 170, 78-83
Sedmak, J. J., and Grossberg, S. E. (1977) Anal. Biochem. 78, 544-552
Skovby, F., Kraus, J. P., Rosenberg, L. E. (1984) J. Biol. Chem. 259, 588-593
Sono, M., Anderson, L. A., and Dawson, J. H. (1982) J. Biol. Chem. 257, 8308-8320
Swaroop, M., Bradley, K. B., Ohura, T., Tahara, T., Roper, M. D., Rosenberg, L. E., and Kraus, J. P. (1992) J. Biol. Chem. 267, 11455-11461