Yeast Cystathionine β-Synthase Is a Pyridoxal Phosphate Enzyme but, Unlike the Human Enzyme, Is Not a Heme Protein*

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Our studies of cystathionine β-synthase from Saccharomyces cerevisiae (yeast) are aimed at (1) clarifying the cofactor dependence and catalytic mechanism and (2) obtaining a system for future investigations of the effects of mutations that cause human disease (homocystinuria or coronary heart disease). We report methods that yielded high expression of the yeast enzyme in Escherichia coli and of purified yeast cystathionine β-synthase. The absorption and circular dichroism spectra of the homogeneous enzyme were characteristic of a pyridoxal phosphate enzyme and showed the absence of heme, which is found in human and rat cystathionine β-synthase. The absence of heme in the yeast enzyme facilitates spectroscopic studies to probe the catalytic mechanism. The reaction of the enzyme with L-serine in the absence of L-homocysteine produced the aldimine of aminoaacrylate, which absorbed at 460 nm and had a strong negative circular dichroism band at 460 nm. The formation of this intermediate from the product, L-cystathionine, demonstrates the partial reversibility of the reaction. Our results establish the overall catalytic mechanism of yeast cystathionine β-synthase and provide a useful system for future studies of structure and function. The absence of heme in the functional yeast enzyme suggests that heme does not play an essential catalytic role in the rat and human enzymes. The results are consistent with the absence of heme in the closely related enzymes O-acetylserylserine sulfhydrylase, threonine deaminase, and tryptophan synthase.

Elevated plasma homocysteine is an important risk factor in coronary heart disease and other human diseases (1–3). One of the two major routes for detoxication of homocysteine is the pyridoxal phosphate (PLP)1-dependent βreplacement reaction with L-serine catalyzed by cystathionine β-synthase (CBS; EC 2.1.2.2).

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\text{L-homocysteine + L-serine \rightarrow L-cystathionine + H}_2\text{O} \quad \text{(Eq. 1)}
\]

The deduced sequences of human (4,5), rat (6), and Saccharomyces cerevisiae (yeast) (7,8) CBS are similar. The finding that human CBS complements the cysteine auxotrophy of a yeast strain lacking CBS (5) demonstrates the functional conservation of the human and yeast genes.

The remarkable observation that the sequence of rat CBS (6) is identical to the sequence of rat hemoprotein H-450 (9) led to the discovery that rat and human CBS contain both PLP and heme (10). Heme may play a role in redox regulation of the human enzyme and in binding homocysteine (11,12). Although yeast CBS has been purified to homogeneity (13), the absorption spectrum and cofactor content have not been reported.2 Here, we demonstrate that purified yeast CBS contains PLP but not heme. Because the absence of heme facilitates spectroscopic studies of the PLP and of enzyme-substrate intermediates, we are able to demonstrate directly that CBS converts L-serine to an aminoaacrylate intermediate, as expected for a PLP enzyme that catalyzes a β-replacement reaction (14,15).

EXPERIMENTAL PROCEDURES

Chemicals—L-Cystathionine and L-serine were from Fluka. δ-Aminolevulinic acid, L-homocysteine thiolactone, aprotinin, pepstatin A, leupeptin, benzamidine hydrochloride, TPCK, TLCK, and PMSF were from Sigma. Gigapite was from Seikagaku, Japan. L-Homocysteine was prepared from L-homocysteine thiolactone as described (16, 17). L-[U-14C]Serine (160 mCi/mmol) was from NEN Life Science Products.

Construction of pSEC, a Vector for Overexpression of CBS—To overexpress CBS from S. cerevisiae, we ligated the 1.5-kb PCR product containing the CBS gene from PSTR4-2 (cys4)1 (18) and the restricted pTRE 99A vector (Amersham Pharmacia Biotech) to yield the expression vector pSEC (Fig. 1). The PCR product of CBS was designed using an upstream PCR primer (5'-pATGACTAAATCTGAGCAGCAGCAAG-3') and a downstream primer (5'-pATGACTAAATCTGAGCAGCAGCAAG-3'), which ex- tends from 8 to 31 bases after the termination codon for the CBS open reading frame. The boldface bases are changes that introduce a PstI restriction site. PCR reactions were carried out in 20 μL with 2 min denaturation at 94 °C followed by 35 cycles of 1-min denaturation at 94 °C, 30-s annealing at 50 °C, and 1-min extension at 72 °C using Pfu DNA polymerase and the reaction conditions described in the Stratagene catalog. The ethanol-purificated PCR products were solubilized with TE buffer, pH 8.0 (10 mTris·HCl containing 1 mM EDTA), restricted by PstI (Amersham Pharmacia Biotech), and isolated by 7% agarose (Life Technologies, Inc., Ultra Pure) gel electrophoresis. The putative 1.5 kb CBS gene was cut out of the gel and extracted using a GeneClean II kit (Bio 101, Inc.).

The overexpression vector pTRE 99A was restricted by NeoI, mung bean nuclease (19), and then by PstI. After each restriction step, the methylsulfonyl fluoride; TLCK, N-tosyl-L-phenylalanine chloromethyl ketone; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone; PCR, polymerase chain reaction; kb, kilobase pair(s).

After this work was completed and reported in abstract and poster form at the 10th International Symposium of Vitamin B6 and Carbonyl Catalysis and 4th Meeting on PQP and Quinoproteins, Santa Fe, New Mexico, October 31–November 5, 1999, we learned that another group had purified CBS from S. cerevisiae and found that the enzyme is not dependent on heme (K. N. Maclean, M. Janosik, J. Oliveriusova, V. Kerjan, and J. P. Kraus, submitted for publication).

3 PSTR4-2 (cys4) was a generous gift from Dr. Yolande Surdin-Kerjan.

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‡ The abbreviations used are: PLP, pyridoxal phosphate; CBS, cystathionine β-synthase; IPTG, isopropyl thiogalactoside; PMSF, phenyl-
4.2-kb product was purified by agarose gel electrophoresis and extraction as described above. The restricted pTrc99A vector was ligated with the amplified CBS PCR product using T4 DNA ligase and transformed into E. coli DH5α (Life Technologies, Inc.). The seven-base distance between the AGGA site (E. coli ribosome binding site) of pTrc99A and the CBS start codon gives a high yield of overexpression. Recombinant pSEC was isolated using a QIA plasmid kit (Qiagen), and the total DNA sequence of the 1.5-kb insert was confirmed by DNA sequence analysis (Biopolymer Core Facility, University of Maryland at Baltimore). Finally, pSEC was transformed into E. coli XL1-blue (Stratagene) for overexpression.

**Determination of CBS Activity and Protein Concentration—**Protein concentrations were determined by the Coomassie Blue protein assay reagent (Pierce) using bovine serum albumin as a standard or from the specific absorbance of purified CBS at 280 nm (A280 0.1% = 0.94). CBS activity was determined by a modification of a standard method (20). The reaction mixture, which contained 200 mM Tris-HCl, pH 8.6, 20 μM PLP, 0.25 mg/ml bovine serum albumin, 5 mM l-[1-14C]serine (800 cpm/nmol), and CBS (0.02–0.1 μg) in 18 μl, was preincubated for 5 min at 37 °C. The reaction was initiated by adding 2 μl of 50 mM homocysteine to 5 μl and was terminated after 10–15 min by adding 5 μl of 50% trichloroacetic acid. After the mixture was centrifuged for 5 min, 5 μl of the supernatant was applied to a cellulose thin layer chromatography plate (Kodak). The product, l-[14C]cystathionine, was separated from l-[14C]serine by ascending thin layer chromatography in 2-propanol/formic acid/H2O (80:60/20 v/v). Radioactivity of the product was determined by PhosphorImager (Molecular Dynamics). One unit of activity is defined as the production of 1 μmol of l-cystathionine/h at 37 °C.

**Overexpression and Purification of CBS—**A 1-liter culture of E. coli XL1-blue transformed with pSEC was grown at 37 °C in Super Broth (BioWhittaker or KD Medical) containing tryptophan (12 μg/liter), yeast extract (24 μg/liter), glycerol (6.3 μg/liter), K_HPO4 (12.5 μg/liter), KH2PO4 (3.8 μg/liter), d-aminolevulinic acid (50 μg/liter), ampicillin (100 μg/ liter), and 20 ml of 50-fold concentrated Vogel and Bonner minimal medium (21). A 10% inoculum was added to the medium, and growth proceeded for 3–4 h until the OD660 reached 2.5. IPTG was added to 0.1 mM and growth continued at 30 °C for 14–18 h. We found that adding IPTG to cells at high density (OD660 = 2.5) gave a higher yield of enzyme than adding IPTG to cells at lower density. Growth in triple-indented Tunair flasks with loose fitting plastic caps (Shelton Scientific Manufacturing, Inc.) (22) gave the highest yield of cells. Cells were harvested by centrifugation, washed with 0.85% NaCl containing 1 mM dithiothreitol, resuspended in Buffer BP (50 mM sodium/bicine, pH 7.8, containing 10 mM EDTA, 10 mM β-mercaptoethanol, 0.1 mM PLP, 1 mM PMSF, 0.1 mM TLCK, 0.1 mM TPCK, 1 mM/liter aproplatin, 2 mg/liter leupeptin, 2 mg/liter pepstatin, and 1 mM benzamidine-HCl) at pH 7.8), and the dialyzed CBS fractions had a specific activity of 438 units/mg (yield = 64%).

The dialyzed enzyme solution (4 ml) was applied to a Gigapite column (3.1 × 27 cm) equilibrated with Buffer KP. Gigapite is a modified form of hydroxyapatite that has large particles and gives a high flow rate. The column was washed with 400 ml of Buffer KP followed by 300 ml of Buffer KP that contained 50 mM potassium phosphate. The enzyme was eluted with a 1.8-liter linear gradient ranging from 50 to 400 mM potassium phosphate in Buffer KP. The active fractions, which eluted at 150–200 mM potassium phosphate, were concentrated to 10–30 mg/ml, dialyzed against Buffer K (50 mM potassium phosphate, pH 7.5, containing 1 mM EDTA, 1 mM dithiothreitol, and 0.02 mM PLP), and stored at −85 °C. The Gigapite fractions had a specific activity of 470 units/mg (yield = 49%) and were >95% pure by the criterion of SDS-polyacrylamide gel electrophoresis. Approximately 600 mg of homogeneous CBS was obtained from a 1-liter culture. All procedures were completed within 72 h to limit proteolysis.

**Spectroscopic Methods—**Absorption spectra of CBS were made using a Hewlett Packard 8452-diode array spectrophotometer thermostatted at 25 °C by a Peltier junction temperature-controlled cuvette holder. CD measurements (mean residue ellipticity in degree cm2/dmol) were made at 25 °C in a Jasco J-715 spectrophotometer interfaced with a personal computer (Japan Spectroscopic Co., Easton, MD).

**RESULTS AND DISCUSSION—**Our new expression vector, pSEC (Fig. 1), gave a very high level of expression of the yeast CBS gene in E. coli (see “Experimental Procedures”). A 2-fold purification of yeast CBS by DEAE-Sepharose and Gigapite chromatography yielded homogeneous CBS in approximately 50% yield. Although yeast CBS has been purified previously (13), the absorption spectrum was not reported.2 The absorption spectrum of our purified yeast CBS (Fig. 2A) exhibited major peaks at 280 and 412 nm in a ratio of 1:0.16, typical of a PLP enzyme (15). Removal of the PLP resulted in an apoenzyme having no absorbance in the visible range.4

In contrast, the absorption spectra of rat and human CBS (10–12, 23–26) exhibit a visible absorption band at 428 nm, which is approximately equal in intensity to the 280 nm absorption band. The 428 nm band in rat and human CBS is attributed to the presence of both heme and PLP, which have overlapping visible absorption spectra in the 410–430 nm region. The absence of heme in yeast CBS is not due to expression in E. coli; human CBS expressed in E. coli does contain heme. We added d-aminolevulinate to the E. coli growth medium because the presence of this precursor of heme has been shown to increase the heme content of human CBS (27).

The presence of heme in rat and human CBS is surprising because no other PLP enzyme has been reported to contain heme. Several PLP enzymes that catalyze β-elimination and β-replacement reactions exhibit a sequence similarity (10, rat, human, and yeast CBS). Three of these related enzymes, O-acetylserine sulfhydrylase (29), the β-subunit of tryptophan synthase (30), and threonine deaminase (31), have been analyzed by x-ray crystallography and shown to exhibit structural similarity.

The spectroscopic properties of PLP provide a sensitive probe for detecting chemical intermediates in PLP-dependent β-replacement reactions (Scheme 1) (15) and in other PLP-dependent reactions. The absence of heme in yeast CBS facilitates spectroscopic studies to detect intermediates in the reactions of CBS and to probe the reaction mechanism. The addition of

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4 K.-H. Jhee, P. McPhie, and E. W. Miles, manuscript in preparation.
L-serine to yeast CBS resulted in the disappearance of the 412 nm band attributed to the internal aldimine (E in Scheme 1) and the appearance of a new spectroscopic species with a major band centered at 460 nm and a shoulder at 330 nm (Fig. 2A). The 460 nm band is likely due to the aldimine of aminoacrylate (E-AA in Scheme 1), which has been detected in the reaction of O-acetylserine sulphydrylase with O-acetyl-L-serine (32–34) and of D-serine dehydratase with D-serine (35). The 330 nm shoulder may be due to a different tautomer of E-AA, which is the predominant intermediate in the reaction of the closely related tryptophan synthase with L-serine (36). Our results (Fig. 2A) demonstrate the E-AA intermediate by direct absorption spectroscopy for the first time. Previous studies of the reaction of truncated or full-length human CBS with L-serine detected a putative aminoacrylate intermediate by difference absorption spectroscopy (12) or by fluorescence spectroscopy (fluorescence emission at 400 nm with excitation at 330 nm) (11). Rapid scanning spectroscopy may be needed to detect the external aldimine intermediate (E-Ser in Scheme 1) in the reaction of CBS with L-serine or intermediates in the reaction of CBS with L-serine and L-homocysteine. The addition of L-homocysteine to CBS in the presence of L-serine under the conditions shown in Fig. 2A resulted in a transient decrease in absorbance at 460 nm (data not shown), providing additional evidence that the 460 nm band is due to the E-AA intermediate. The reaction of the substrate analog L-alanine with yeast CBS yielded a band at 420 nm, which is a wavelength characteristic of the expected external aldimine, E-Ala (15). E-Ala is analogous to E-Ser in Scheme 1.

Yeast CBS exhibited a positive CD band centered in the visible absorption band of the cofactor at 412 nm (Fig. 1B), as reported for O-acetylserine sulphydrylase (37) and tryptophan synthase (38, 39). The addition of L-serine gave a negative CD band centered at 460 nm and a strong positive band at 280 nm. Negative visible CD bands for E-AA have been reported for O-acetylserine sulphydrylase (37) and tryptophan synthase (39, 40). The tryptophan synthase E-AA intermediate also has a strong positive band at 280 nm (40). The addition of L-alanine gave a negative CD band centered at 430 nm.

To probe the reversibility of the postulated CBS reaction in Scheme I, we measured the absorption spectra (Fig. 3A) and CD spectra (Fig. 3B) of yeast CBS in the presence of L-cystathionine. The initial (15 s) absorption spectrum showed a peak at 430 nm and a prominent shoulder at 460 nm. The absorbance at 460 nm decreased with time (inset, Fig. 3A). The CD spectrum (−6 min) exhibited a negative band at 460 nm and positive bands at 400 and 280 nm. Thus, L-cystathionine appears to undergo the reverse reaction to form E-AA and an aldimine, either the internal aldimine, E, or an external aldimine, E-Ser or E-Cyst (Scheme 1).

Our results provide the first direct, spectroscopic evidence that the reaction catalyzed by yeast CBS proceeds through the postulated intermediates in Scheme I and that the reaction is at least partially reversible. The absorption spectrum in Fig. 2A showed that L-serine was largely or completely converted to E-AA in the absence of L-homocysteine. This result demonstrates that the CBS reaction does not proceed by direct displacement of the OH of L-serine, as proposed by Braunstein and Goryachenkova (41). Our results, therefore, resolve an old controversy over the mechanism of CBS (14, 15, 41) and are consistent with the stereochemical data showing that displacement of the OH of L-serine proceeds with retention of configuration (14). The direct displacement mechanism of Braunstein and Goryachenkova (41) was proposed to explain the inability of CBS to catalyze the conversion of L-serine to pyruvate and NH₃ by a β-elimination reaction. Our data provide evidence that CBS, like the tryptophan synthase α₂β₂ complex and O-acetylserine sulphydrylase, forms a stable, enzyme-bound E-AA intermediate that

**FIG. 2. Absorption (panel A) and CD (panel B) spectra of yeast CBS alone (E) and in the presence of 50 mM L-serine (E-AA) or 50 mM L-alanine. The protein concentration was 1 mg/ml.**

**SCHEME 1.** Intermediates in the reaction of CBS, where E is the internal aldimine; E-Ser, E-AA, and E-Cyst are the external aldmines of PLP with L-serine, aminoacrylate, and L-cystathionine, respectively; and L-Hcys is L-homocysteine.
mixing.

enzymes consistent with the absence of heme in the closely related catalytic role in the rat and human enzymes. The results are the functional yeast enzyme shows that heme is not essential spectrum in the presence of L-cystathionine was recorded nucleophile specificity of yeast CBS.4 The absence of heme in domain composition, oligomeric structure, and substrate and mechanism. Work is in progress to investigate the reaction kinetics, spectroscopic studies that establish the overall catalytic mech-

anism. In conclusion, we have demonstrated that yeast CBS is a heme-independent, PLP enzyme and have carried out initial spectroscopic studies that establish the overall catalytic mechanism. Work is in progress to investigate the reaction kinetics, domain composition, oligomeric structure, and substrate and nucleophile specificity of yeast CBS.4 The absence of heme in the functional yeast enzyme shows that heme is not essential for catalysis and suggests that heme does not play an essential catalytic role in the rat and human enzymes. The results are consistent with the absence of heme in the closely related enzymes O-acetylseryne sulphydrylase, threonine deaminase, and tryptophan synthase.

FIG. 3. Absorption (panel A) and CD (panel B) spectra of yeast CBS alone (E) and at the indicated times after adding 4 mM L-cystathionine. The protein concentration was 1 mg/ml. Inset in A shows the absorbance at 460 nm of CBS alone (0 min) and at intervals (15 s–60 min) after the addition of 4 mM L-cystathionine. The CD spectrum in the presence of L-cystathionine was recorded ~6 min after mixing.

do not undergo hydrolysis or further reaction in the absence of an added nucleophile.

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