

Functional Properties of the Active Core of Human Cystathionine β -Synthase Crystals*

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Stefano Bruno \ddagger , Francesca Schiaretti \ddagger ,
Peter Burkhard \S , Jan P. Kraus \parallel ,
Miroslav Janosik \parallel , and Andrea Mozzarelli \ddagger ||**

From the \ddagger Institute of Biochemical Sciences and the \parallel National Institute for the Physics of Matter, University of Parma, 43100 Parma, Italy; \S M. E. Müller Institute for Structural Biology, Biozentrum University of Basel, CH-4056, Basel, Switzerland; and the \parallel Department of Pediatrics, University of Colorado School of Medicine, Denver, Colorado 80262

Human cystathionine β -synthase is a pyridoxal 5'-phosphate enzyme containing a heme binding domain and an S-adenosyl-L-methionine regulatory site. We have investigated by single crystal microspectrophotometry the functional properties of a mutant lacking the S-adenosylmethionine binding domain. Polarized absorption spectra indicate that oxidized and reduced hemes are reversibly formed. Exposure of the reduced form of enzyme crystals to carbon monoxide led to the complete release of the heme moiety. This process, which takes place reversibly and without apparent crystal damage, facilitates the preparation of a heme-free human enzyme. The heme-free enzyme crystals exhibited polarized absorption spectra typical of a pyridoxal 5'-phosphate-dependent protein. The exposure of these crystals to increasing concentrations of the natural substrate L-serine readily led to the formation of the key catalytic intermediate α -aminoacrylate. The dissociation constant of L-serine was found to be 6 mM, close to that determined in solution. The amount of the α -aminoacrylate Schiff base formed in the presence of L-serine was pH independent between 6 and 9. However, the rate of the disappearance of the α -aminoacrylate, likely forming pyruvate and ammonia, was found to increase at pH values higher than 8. Finally, in the presence of homocysteine the α -aminoacrylate-enzyme absorption band readily disappears with the concomitant formation of the absorption band of the internal aldimine, indicating that cystathionine β -synthase crystals catalyze both β -elimination and β -replacement reactions. Taken together, these findings demonstrate that the heme moiety is not directly involved in the condensation reaction catalyzed by cystathionine β -synthase.

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** To whom correspondence should be addressed. Tel.: 39-0521-905138; Fax: 39-0521-905151; E-mail: biochim@unipr.it.

High plasmatic levels of homocysteine have recently been associated with an increased risk of cardiovascular disease (1). Homocysteine is formed from S-adenosylhomocysteine and is either removed by cystathionine β -synthase (EC 4.2.1.22, CBS)¹ in the trans-sulfuration pathway or remethylated to methionine in the methionine cycle. Deficiency of CBS is the major cause of inherited homocystinuria. CBS is a pyridoxal 5'-phosphate (PLP)-dependent enzyme catalyzing the synthesis of cystathionine from homocysteine and L-serine. The reaction proceeds via a β -replacement mechanism, similar to that of tryptophan synthase and O-acetylserine sulfhydrylase (2). These enzymes belong to the β -family and fold II type within the PLP-dependent enzymes classification (3, 4). Other members of the β -family are serine and threonine dehydratases. The human CBS is a 63-kDa homotetramer containing one PLP and one heme per subunit (5). Whereas the functional role of PLP is known, the role of the heme is less clear. It was demonstrated that the heme redox state affects the affinity of the enzyme for the substrates (6). Moreover, the catalytic activity of CBS is controlled by S-adenosylmethionine, which specifically binds to a C-terminal site. The trypsinolysis of a 18 kDa C-terminal fragment leads to a dimeric form, 2-fold more active than the native species and no longer regulated by S-adenosylmethionine (7). Similar results have been obtained on other truncated forms of CBS, obtained by insertion of nonsense mutations (8). Investigation of the catalytic reaction brought about by PLP is complicated by the overlapping chromophoric properties of the heme. The recent investigation of the yeast enzyme, which does not contain heme groups, permitted a better characterization of the PLP role in the catalytic steps (9, 10). An α -aminoacrylate species, absorbing at 470 and 320 nm, was observed upon reaction with L-serine. The nucleophilic attack of homocysteine on the α -aminoacrylate led to the formation of the product cystathionine. However, it is not yet known how closely the functional properties of the yeast enzyme resemble those of the human source.

Structural studies of catalytic intermediates of tryptophan synthase and O-acetylserine sulfhydrylase (11–18) have unveiled the nature of enzyme action. A common feature is an open to closed conformational transition of the active site taking place along the catalytic pathway. To fully exploit the structural information as well as to determine the structure of as many as possible catalytic intermediates, it is of paramount relevance to investigate the functional properties of the enzyme in the crystalline state by polarized absorption microspectrophotometry (19). This approach was pioneered in the late sixties by Rossi and Bernhard (20). In the case of tryptophan synthase (17, 21–23) and O-acetylserine sulfhydrylase (24), several catalytic intermediates were isolated and characterized in the crystalline state, opening the way to their structural determination. We have recently expressed and purified to near homogeneity recombinant human CBS comprising amino acid residues 2–413. This enzyme, missing 138 C-terminal residues, forms dimers, is not activated by S-adenosyl-L-methionine, and does not exhibit the aggregating properties of the full-length enzyme. In addition, the recombinant CBS polypeptide contains a 23-amino acid spacer at its N terminus. The

¹ The abbreviations used are: CBS, cystathionine β -synthase; PLP, pyridoxal 5'-phosphate; PEG, polyethylene glycol.

truncated enzyme still binds PLP and heme and is about 2 times more active than the full-length CBS (25). Crystals of the recombinant active core of CBS have been obtained, and the three-dimensional structure is being determined (26). Here, we have studied the reactivity of these crystals by polarized absorption microspectrophotometry as an essential prerequisite to the crystallographic analysis of the enzyme and the structure to function correlation.

MATERIALS AND METHODS

Crystallization—The recombinant truncated form of CBS was purified as described previously (25) and concentrated to 26 mg/ml in 20 mM HEPES, pH 7.4. Crystals of CBS were obtained using the vapor diffusion hanging drop method (26). 2 μ l of mother liquor containing 30% w/v PEG M_r 1000, 80 mM HEPES, pH 7.5, and 0.4 mM FeCl₃ were mixed with 2 μ l of protein solution and equilibrated against 1 ml of reservoir solution at room temperature. Given the dimeric nature of the truncated CBS enzyme, the crystals contained either two or three dimers per asymmetric unit corresponding to a solvent content of 64 or 46% and a calculated Matthews volume VM of 3.4 or 2.3 $\text{\AA}^3/\text{Da}$, respectively. The crystals belong to the trigonal space group P3₁ or P3₂ with unit cell dimensions $a = b = 144.46 \text{ \AA}$ and $c = 108.21 \text{ \AA}$ (25). Crystals were stored in 25% PEG M_r 1000, 80 mM HEPES, 2 mM ferric chloride, pH 8.0.

Chemicals—All chemicals were of the best commercial quality and were used without further purification. L-Homocysteine was prepared from L-homocysteine thiolactone and titrated with 5,5'-dithiobis-2-nitrobenzoic acid (27).

Microspectrophotometric Measurements—Single crystals of CBS were resuspended at least six times in a solution containing 35% PEG M_r 1000, 80 mM HEPES, pH 8.0 and mounted in a quartz flow cell. Replacement of the suspending medium was carried out by passing solutions through the cell. The cell was placed on the thermostatted stage of a Zeiss MPM03 microspectrophotometer, equipped with a $\times 10$ Zeiss UV-visible ultrafluar objective (19, 22). Polarized absorption spectra were collected between 300 and 700 nm with the electric vector of the linearly polarized light parallel and perpendicular to the c axis of the trigonal crystals. All the experiments were carried out at 15 $^\circ\text{C}$.

Oxidized and Reduced CBS Crystals—The crystals of oxidized CBS were suspended in a deoxygenated solution containing 35% PEG M_r 1000, 80 mM HEPES, pH 8.0 and then in a solution of the same composition plus 5 mM sodium dithionite. When sodium dithionite was removed, the crystals re-oxidized completely within the time required for the solution exchange. The spectrum of the oxidized form did not change when the crystals were treated with 5 mM potassium ferricyanide.

Removal of Heme from CBS Crystals—Crystals of CBS were anaerobically suspended in 35% PEG, 80 mM HEPES, pH 8.0, 5 mM sodium dithionite, saturated with CO at 1 atm. Polarized absorption spectra were recorded as a function of time on crystals stored at 4 $^\circ\text{C}$.

Re-binding of Heme to Heme-free Crystals—Heme-free crystals were anaerobically suspended in a deoxygenated solution containing 35% PEG M_r 1000, 80 mM HEPES, pH 8.0, 5 mM sodium dithionite, and increasing concentrations of hemin. Polarized absorption spectra were recorded as a function of time on crystals stored at 4 $^\circ\text{C}$.

Measurements on Heme-free CBS Crystals—Heme-free CBS crystals were washed and stored in a CO-free solution of 35% PEG M_r 1000, 80 mM HEPES, pH 8.0. Individual crystals were mounted in the flow cell and resuspended in reagent before collecting spectra.

RESULTS AND DISCUSSION

Redox States of CBS Crystals—Polarized absorption spectra of CBS crystals under nonreducing conditions (Fig. 1a) exhibited peaks at 428 and 550 nm, similar to solutions for the oxidized form of the enzyme. A shoulder at 590 nm was more evident when spectra were collected with light polarized parallel to the c crystallographic axis, indicating an unequally polarized x, y electronic transition of the heme (28). This was also reflected in the variation of the polarization ratio, *i.e.* the ratio of the absorption intensity parallel and normal to the c axis of the crystal (Fig. 1a). The isotropic spectrum, calculated from the equation $\epsilon = 1/3(\epsilon_{\parallel c} + 2\epsilon_{\perp c})$ exhibited the same ratio of absorbance intensity at 428 and 550 nm as in solution (Fig. 1a, inset). Oxidized CBS crystals, titrated between pH 6.0 and 8.0 (data not shown), did not exhibit any significant spectral

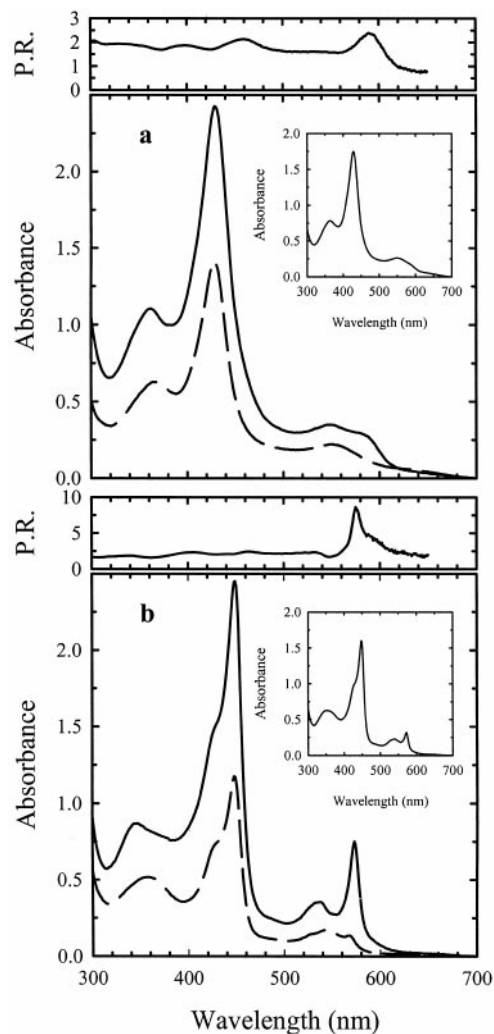


FIG. 1. Polarized absorption spectra of 45-kDa CBS crystals in the ferric (a) and ferrous (b) state. A single crystal of CBS was suspended in a solution containing 35% (w/v) PEG M_r 1000, 80 mM HEPES, pH 8.0. The ferrous state was obtained by resuspending the crystal in a deoxygenated solution containing 2 mM sodium dithionite. Spectra were recorded with light linearly polarized parallel (*solid line*) and normal (*dashed line*) to the c crystal axis at 15 $^\circ\text{C}$. The polarization ratio (*P.R.*) is reported in the top of each panel. *Inset*, calculated isotropic spectrum (see “Results and Discussion”).

changes, suggesting that the heme iron is not coordinated to a water molecule, which is different from what was observed for metmyoglobin and methemoglobin (29).

When CBS crystals were suspended in a solution containing sodium dithionite, polarized absorption spectra exhibited bands at 450, 538, and 576 nm (Fig. 1b). In solution these peaks are indicative of the ferrous state of the heme iron. The shoulder at about 430 nm was more prominent in the isotropic spectrum calculated from crystal polarized absorption spectra (Fig. 1b, *inset*) with respect to solution (5, 6). This finding might suggest an incomplete reduction. However, in the isotropic spectrum (Fig. 1b, *inset*), the ratio of the peaks at 450 and 576 nm was the same as in solution (5, 6), indicating that the crystalline enzyme was fully reduced. Heme reduction takes place without any apparent crystal damage. Furthermore, when reduced enzyme crystals were resuspended in a dithionite-free solution, the fully oxidized form of the enzyme was readily obtained (data not shown). Therefore, it is possible to prepare oxidized and reduced forms of CBS crystals suitable for crystallographic analysis. This study may allow us to determine the conformational changes associated with different

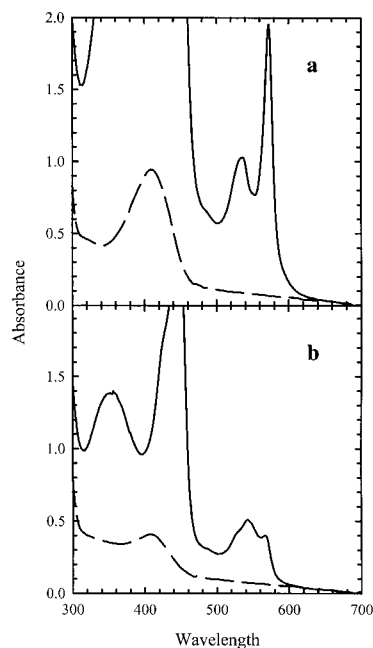


FIG. 2. Formation of heme-free crystals in the presence of carbon monoxide under reducing conditions. Crystals of CBS were suspended in a solution containing 35% (w/v) PEG M_r 1000, 80 mM HEPES, 2 mM sodium dithionite, pH 8.0. Crystal size does not permit collecting reliable spectra of the Soret peak parallel (*a*, solid line) and normal (*b*, solid line) to the *c* crystal axis. The crystal was then anaerobically suspended and stored at 4 °C in a solution of the same composition, previously equilibrated with carbon monoxide at 1 atm. The spectra of the heme-free CBS crystal were collected after 48 h (*a* and *b*, dashed lines).

heme redox states controlling catalytic properties of PLP catalysis (6).

Formation of Heme-free CBS Crystals—Crystals of reduced CBS were suspended in a solution containing 5 mM sodium dithionite, saturated with carbon monoxide at 1 atm. The spectra collected immediately after exposure to CO showed a progressive decrease of absorbance intensity without any change of the spectral shape (Fig. 2). Prolonged incubation of CBS crystals under these experimental conditions resulted in the complete loss of the absorption intensity of the heme (Fig. 2). This process takes place in 2–3 days depending on the crystal size. The resulting polarized absorption spectra (Fig. 2) were characterized by an absorption peak at 412 nm, typical of a PLP Schiff base, as observed in other PLP-dependent enzyme crystals (22, 24). Some spectral differences among CBS crystals were observed in the 300–340 nm region likely because of crystal aging, as previously observed in other PLP-enzymes.² These findings indicate that under these conditions the heme groups that were bound to CBS had been completely released. When the oxidized form of the heme-containing enzyme was exposed to carbon monoxide, no release of heme was observed (data not shown). Furthermore, reduction of the iron or other protein groups, *i.e.* disulfide bridges, by dithionite did not trigger heme release (data not shown). The presence of either dithiothreitol or nitric oxide was also ineffective in heme release. These results indicate that carbon monoxide binds only to the reduced form of CBS, as observed in solution (30), and the weakening of heme affinity to the enzyme is very specific. Carbon monoxide likely replaces a protein residue bound as an axial ligand to the iron, decreasing significantly the heme affinity to the protein. Alternatively, carbon monoxide-bound heme triggers displacement of iron into the heme plane, which

² A. Mozzarelli, unpublished observations.

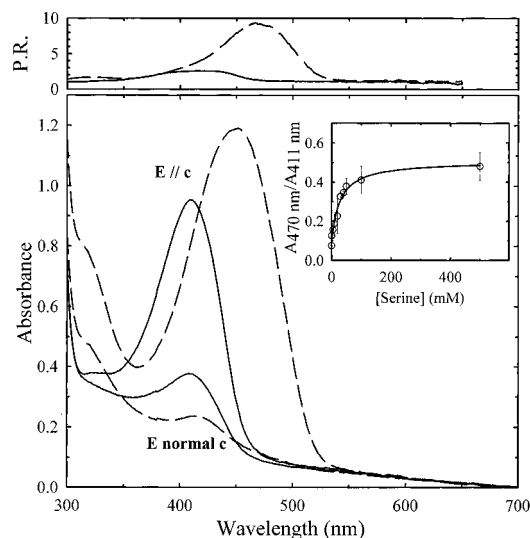


FIG. 3. Polarized absorption spectra of heme-free CBS crystals in the absence and presence of L-serine. The crystal was suspended in a solution containing 35% (w/v) PEG M_r 1000, 80 mM HEPES, pH 8.0. Polarized absorption spectra were recorded with the electric vector either parallel (*E*//*c*) or normal (*E*⊥*c*) to the *c* crystal axis, in the absence (—) and presence of 500 mM (---) L-serine. The corresponding polarization ratio (*P.R.*) is reported. *Inset*, changes of the ratio of absorbance at 470 and 411 nm as a function of L-serine concentration, recorded with light polarized parallel to the *c* crystal axis. The data points are the average of the values obtained for two crystals in different titration experiments. The curve through the points is the fitting to a binding isotherm with a dissociation constant of 6.4 mM.

in turn may be transmitted to the protein matrix by movement of the second heme axial ligand, similar to that observed in hemoglobin (31).

The heme release is compatible with the crystal integrity, as evidenced by the significant difference of absorption intensity of heme-free CBS crystals along the two optical directions (compare Fig. 2, *a* and *b*), a property of well ordered chromophoric crystals. The heme release is reversible, because incubation of heme-free CBS crystals with 0.3 mM reduced hemin led to the re-binding of hemes with the retention of the original polarization ratios (data not shown). On the basis of the spectra of the native and reconstituted enzyme, it was estimated that about 50% of heme content was recovered. Higher concentrations of hemin might be required to attain a stoichiometric recovery. These findings enable structural determination of the enzyme in the presence and absence of heme, and, thus, characterization of the conformational changes associated with heme binding and its regulation of PLP catalysis.

Reactivity of Heme-free CBS Crystals—In solution, CBS reacts with L-serine to form the α -aminoacrylate intermediate (9, 32). This species absorbs at about 460 and 330 nm (9). Crystals of heme-free CBS were titrated with increasing concentrations of L-serine. The spectra at high L-serine concentration (Fig. 3), recorded parallel to the *c* crystal axis, show the appearance of bands centered at about 450 and 320 nm, indicating the accumulation of the α -aminoacrylate Schiff base. Therefore, the enzyme is catalytically competent in the β -elimination reaction. The spectral changes observed for light polarized normal to the *c* crystal axis are limited. There is a small red shift of the peak and a decrease of absorption intensity. The high polarization ratio around 450 nm reflects the spectral differences along the two extinction directions (Fig. 3). This behavior might be explained by a reduced reactivity of one of the coenzymes, as suggested in solution for the dimeric CBS (30) and assuming that most of the inactive PLP is observed along the direction normal to the *c* crystal axis. This partial reactivity does not

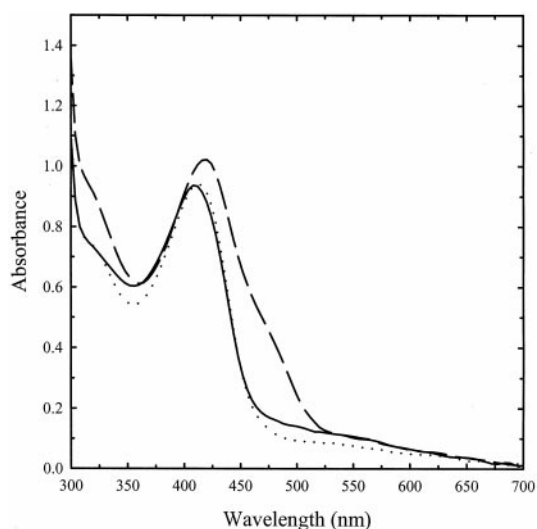


FIG. 4. Polarized absorption spectra of heme-free CBS crystals in the absence and presence of L-serine and homocysteine. Spectra were recorded for heme-free CBS crystals suspended in a solution containing 35% (w/v) PEG M_r 1000, 80 mM HEPES, pH 8.0, in the absence (solid line), presence of 30 mM serine (dashed line) and 30 mM L-serine and 34 mM homocysteine (dotted line). Spectra recorded with the electric vector parallel to the c direction are shown. Spectra of internal aldimines were scaled with respect to the absorbance intensity at 412 nm to account for different crystal thickness.

seem to be present in the yeast enzyme (9). The calculated dissociation constant of L-serine for CBS crystals is 6.4 mM (Fig. 3, inset). In solution, at pH 8.6, a K_m value of 3.0 mM was determined for the tetrameric CBS (7) and a K_m value of 2.7 mM for the dimeric CBS (7). The amount of α -aminoacrylate Schiff base that was accumulated in the presence of 500 mM L-serine was found to be pH-independent between 6 and 9 (data not shown). However, the rate of the α -aminoacrylate disappearance, likely to form pyruvate and ammonia (11, 12), increases at pH higher than 8. A bell-shape dependence on pH, centered at pH 7.2, was previously observed for the accumulation of the α -aminoacrylate-*O*-acetylserine sulfhydrylase crystals (24).

Finally, when α -aminoacrylate CBS crystals were suspended in a solution containing 30 mM L-serine and 34 mM homocysteine, the polarized absorption spectra of the internal aldimine species were readily recovered (Fig. 4). The exposure of CBS crystals to homocysteine alone did not cause any spectral changes, as observed in solution for the yeast enzyme (10), suggesting that also in the human enzyme homocysteine does not form the external aldimine.

Overall, these findings indicate that the heme-free enzyme is catalytically competent not only in the β -elimination reaction but also in the β -replacement reaction, in agreement with preliminary solution experiments.³ It is, therefore, very unlikely that the heme plays a catalytic role in the activation of homocysteine, as recently proposed (6). A quantitative evaluation of native *versus* heme-free enzyme activity either using a microcrystalline suspension (19) or the soluble form is planned.

CONCLUSION

Cryo-crystallography (33) and kinetic crystallography (34), making use of either slowly reacting substrates and substrate analogues (20) or slowly reacting mutant enzymes (35), have considerably expanded the capability to detect and characterize not only the native form of enzymes and proteins but also transiently accumulating species. Detailed functional studies of protein crystals by spectroscopic techniques have allowed us

to define the experimental conditions for the accumulation of catalytic intermediates, thus directing the crystallographic measurements. These conditions are not always similar to those derived by experiments in solution, because crystal lattice forces affect the relative stability of catalytic intermediates in unpredictable ways. Examples are the different effect of cations on the accumulation of the quinonoid species of tryptophan synthase in the crystal and in solution (22) and the different affinity of the natural substrate *O*-acetylserine to several crystal forms of *O*-acetylserine sulfhydrylase, where one form is 500-fold less active than the enzyme in solution and another is completely inactive (24).

The present investigation of the active core of human CBS crystals has allowed to prepare the oxidized and reduced forms of the enzyme, the heme-free protein, and the key catalytic intermediate α -aminoacrylate. For the first time, it has also been demonstrated that the heme does not participate in PLP-dependent catalysis of CBS. However, further investigations in solution are required to assess the fine-tuning of ligand binding and catalysis by the heme moiety.

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³ J. Kraus, unpublished observations.

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