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Crystallization and preliminary X-ray diffraction analysis of the active core of human recombinant cystathionine β-synthase: an enzyme involved in vascular disease

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Cystathionine β-synthase (CBS) is a unique heme enzyme that catalyzes a PLP-dependent condensation of serine and homocysteine to give cystathionine. Deficiency of CBS leads to homocystinuria, an autosomal recessively inherited disease of sulfur metabolism. A truncated form of CBS in which the C-terminal amino-acid residues have been deleted has been prepared. The truncated CBS subunits form a dimer, in contrast to the full-length subunits which form tetramers and higher oligomers. The truncated CBS yielded crystals diffracting to 2.6 Å which belong to space group $P3_1$ or $P3_2$. This is the first comprehensive structural investigation of a PLP and heme-containing enzyme.

1. Introduction

Cystathionine β-synthase (CBS, L-serine hydrolyase; E.C. 4.2.1.22) is the first enzyme of the transsulfuration pathway in which the toxic homocysteine is converted to cysteine. A deficiency of CBS activity is the most common cause of homocystinuria, an inherited metabolic disease characterized by dislocated eye lenses, skeletal problems, vascular disease and mental retardation (Mudd *et al.*, 1995). There have been now over 100 mutations described in this gene (Kraus *et al.*, 1999). Hyperhomocysteinemia, a condition characterized by small increases in plasma concentrations of homocysteine, represents an independent risk for vascular disease.

The human CBS is a homotetramer consisting of 63 kDa subunits (Skovby *et al.*, 1984). Each CBS subunit of 551 amino-acid residues binds two substrates (homocysteine and serine) and three additional ligands: pyridoxal 5′-phosphate (PLP), *S*-adenosylmethionine (AdoMet) and heme (Kery *et al.*, 1994). While the role of heme in CBS is unknown, catalysis by CBS can be explained solely by participation of PLP in the reaction mechanism (Kery *et al.*, 1999). In fact, yeast CBS catalyzes the same reaction and does not contain heme (Jhee *et al.*, 2000). Limited proteolysis of the full-length enzyme yields the 'active core' of CBS (amino-acid residues 40–413). The reduction in size is accompanied by a significant increase in the specific activity of the enzyme and a change from a tetramer to a dimer. The dimer is about twice as active as the tetramer. It binds both PLP and heme cofactors, but is no longer activated by AdoMet (Kery *et al.*, 1998). This 45 kDa active core is the portion of CBS most homologous with the evolutionarily related enzymes isolated from plants or bacteria (Swaroop *et al.*, 1992; Kraus, 1994).

2. Material and methods

2.1. Cloning of truncated human CBS cDNA expression vector

In order to simplify expression and subsequent purification of the human CBS protein, we generated a human CBS expression construct that allowed us to express truncated human CBS in *Escherichia coli* as a fusion protein with glutathione S-transferase (GST). This fusion protein could subsequently be cleaved with Xa protease to generate human CBS active-core protein. This construct, designated pGEX-5X-1hCBSΔ414–551, was prepared by a three-piece ligation. Firstly, two primers were designed: 337, sense 5′-GATCCCGAGCGAAACCCCGCAGGCGGAAGTGGGGCC-3′, and 338, antisense 5′-CCACTTCCGCCTGCGGGGTTTCGCTCGG-3′. These two primers encode the first ten amino acids of human CBS and respect the codon preference of *E. coli*. Under favorable conditions, primers 337 and 338 will hybridize together to form a short double-stranded insert with sticky overhangs, which enables cloning of this insert as a *Bam*HI/*Apa*I fragment (Kraus *et al.*, 1988). Secondly, a portion of the human CBS cDNA coding for amino acids 11–413 was cut out of the previously described pAX5-HCBS construct (Bukovska *et al.*, 1994) using *Apa*I and *Sal*I restriction endonucleases. Thirdly, both annealed primers 337/338 and the gel-purified *Apa*I/*Sal*I fragment were cloned into *Bam*HI and *Sal*I sites of the pGEX-5X-1 expression vector (Pharmacia). Three of the *Bam*HI/*Apa*I cassettes were cloned into the final construct so that the CBS polypeptide

Table 1
Data-collection statistics.

	MAD	Native
Resolution (Å)	3.15	2.6
Unique reflections	83338	63997
Multiplicity	4.5	2.7
Completeness (%)		
Overall	94.4 (50.00–3.15 Å)	82.4 (50.00–2.60 Å)
Outermost shell	67.1 (3.26–3.15 Å)	63.9 (2.69–2.60 Å)
R_{sym} (%)		
Overall	12.2 (50.00–3.15 Å)	7.6 (50.00–2.60 Å)
Outermost shell	38.5 (3.26–3.15 Å)	30.9 (2.69–2.60 Å)

contains an extra 23 amino acids at its N-terminus. If only two residues separated the Xa cleavage site and the N-terminus of CBS, the fusion protein had no activity and did not bind to the glutathione-Sepharose column. The vector-insert junctions and the entire coding sequence were verified by DNA sequencing using the Thermo Sequenase Cy5.5 dye terminator cycle-sequencing kit and the Long-Read Tower System V 3.1 automated DNA sequencer (Visible Genetics, Toronto, Ontario, Canada).

2.2. Purification of recombinant human CBSΔ414–551

E. coli XL1-Blue MRF′ bearing the pGEX-5X-1-hCBSΔ414–551 construct were grown in 8 l batch cultures of NZCYM medium supplemented with 0.1 mg ml^{-1} ampicillin, 0.3 m*M* δ-aminoleuvalinate (δ-ALA) and 0.001%(*w*/*v*) thiamine. Cells were grown to an OD_{550} of ∼0.4 and then induced with 0.1 m*M* IPTG for 14 h. After the incubation period, the cells were chilled on ice and all subsequent steps were performed at 277 K using pre-chilled solutions at the same temperature. Cells were collected by centrifugation and washed twice with 1×PBS buffer. Cell lysis was performed by osmotic shock according to a previously described method (Witholt *et al.*, 1976). After a 1 h incubation on ice and a 5 min heat shock at 310 K, crude extract fractions were separated by ultracentrifugation (96 000*g* at 283 K for 1 h). The crude extract was saturated with PLP (1 m*M* final concentration), diluted 1:1 with the washing buffer (1xPBS containing 0.3 *M* NaCl and 1 m*M* DTT). This fraction was loaded onto a GST Sepharose 4B column (BioRad, 2 × 15 cm) equilibrated with 10 bed volumes of the washing buffer. The column was washed with 8–10 volumes of the washing buffer. The GST Sepharose 4B resin with the bound GST-CBS fusion protein was transferred into a 50 ml Falcon tube and the buffer was changed to 1×TBS with 1 m*M* DTT and 2 m*M* $CaCl_2$. The GST–CBS fusion protein was cleaved by digestion with Xa protease, which was used at a final concentration of 7.5 µg per milligram of fusion protein. The digest was performed at 277 K overnight. The mixture was centrifuged at 5000*g* for 5 min at 277 K. To further purify the cleaved CBS from any undigested product and from GST, we applied the supernatant to a secondary affinity column (GST Sepharose 4B) equilibrated with 1×PBS. The flowthrough fraction containing CBS was collected and concentrated using an Amicon 50 or Centricon 50-spin column. Prior to crystallization, the enzyme was stored in 20 m*M* HEPES pH 7.4 at 188 K.

2.3. Enzyme characterization

All enzyme preparations were further characterized for protein concentration (Lowry *et al.*, 1951), CBS specific activity (Kraus, 1987) and cofactor saturation. The PLP saturation of the purified enzyme was determined by a previously published fluorimetric method (Adams, 1979). The heme saturation was established using the pyridine–hemochromogen method (Morrison & Horie, 1965). For crystallization purposes we used only preparations with a PLP and heme content over 90%.

Figure 1
Crystals of human cystathionine β-synthase.

3. Results

The CBS tetramer has a strong tendency to aggregate, making physical studies of the enzyme very difficult. We have recently expressed and purified to near-homogeneity recombinant human CBS comprising amino-acid residues 1–413. This enzyme, missing ∼140 C-terminal residues and similar to the abovementioned active core, forms dimers and does not exhibit the aggregating properties of the full-length enzyme. Using this enzyme, we were able to obtain the first diffracting crystals (Fig. 1).

The recombinant CBS enzyme was purified as described above and concentrated to 26 mg ml^{-1} in 20 m*M* HEPES pH 7.4. Small crystals of CBS were obtained in sparse-matrix screens (Hampton Research, Laguna Hills, CA; Jancarik & Kim, 1991) using the vapor-diffusion hanging-drop method (McPherson, 1982) with 4 µl drops consisting of 2 µl protein and 2 µl mother liquor equilibrated against 1 ml of reservoir solution at room temperature. Subsequent optimization resulted in the following crystallization procedure: CBS was mixed with an equal volume of reservoir solution containing 30% PEG 1000, 80 m*M* HEPES pH 7.5 and 0.4 m*M* $FeCl_3$. Crystals appeared

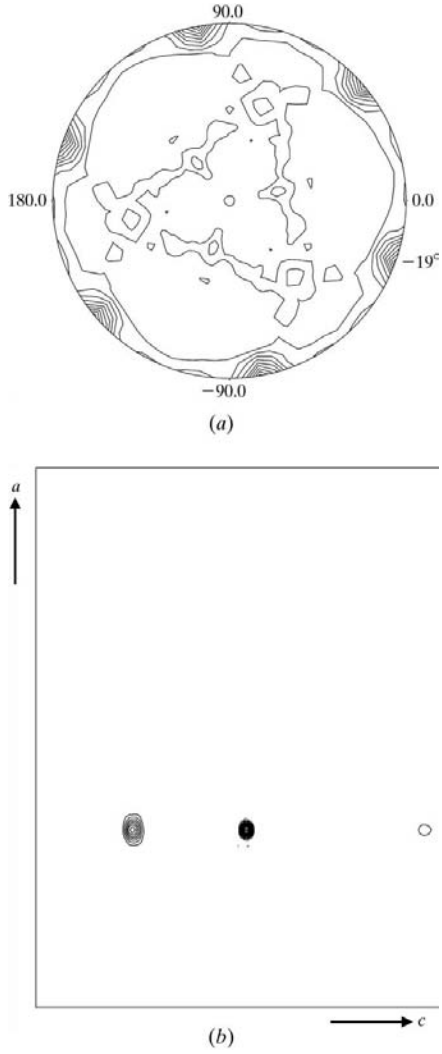

Figure 2
(*a*) Plot of the self-rotation function of cystathionine β-synthase at κ = 180°. The orientation of the dimer twofold axes are perpendicular to the crystallographic threefold axis indicating that all dimers in the asymmetric unit have the same orientation. (*b*) Plot of the section *b* = 1/3 of the native Patterson function of cystathionine β-synthase. The fact that there is more than one native Patterson peak indicates that there are more than two molecules with the same orientation in the crystal packing.

within 7 d and grew to a final size of ~0.5 × 0.2 × 0.2 mm after 30 d. The addition of $FeCl_3$ helped to prevent the formation of small satellite crystals on the surface of the crystals.

The X-ray diffraction data of the native crystal were collected at beamline BM1A of the SNBL at the ESRF in Grenoble at a temperature of 100 K. Furthermore, a MAD data set at three wavelengths around the absorption edge of the iron ion (λ = 1.74 Å) was collected at the BW7A beamline (EMBL, DESY Hamburg). All data were processed and integrated using *DENZO* and scaled using *SCALEPACK* (Otwinowski & Minor, 1997; Table 1). The crystals belong to a trigonal space group, with unit-cell parameters $a = b = 144.46$, $c = 108.21$ Å; systematic absences (00l except for $l = 3n$) indicate that the space group is either $P3_1$ or $P3_2$. Given the dimeric nature of the truncated CBS enzyme, the crystals contain either two or three dimers per asymmetric unit, corresponding to a solvent content of 64 or 46% and a calculated Matthews volume V_M of 3.4 or 2.3 Å^3 Da^{-1}, respectively, with both values being within the range typically observed in protein crystals (Matthews, 1968).

Analysis of the self-rotation function (Fig. 2*a*) showed strong peaks at $\kappa = 180^\circ$ corresponding to an orientation of the dimer twofold axis perpendicular to the crystallographic threefold axis. The presence of these strong peaks indicates that all dimers in the asymmetric unit have the same orientation. This is confirmed by the native Patterson function (Fig. 2*b*), which shows two strong peaks. The fact that there is more than one native Patterson peak indicates that there are more than two molecules with the same orientation in the crystal packing. Hence, the crystal contains three dimers in the asymmetric unit, all having the same orientation, namely, with their twofold axis being perpendicular to the threefold crystallographic axis. The quality of the anomalous data sets is rather low (high R_{sym}) owing to the high mosaicity of the crystals (Table 1) and the anomalous Patterson map does not show any peaks. Using the preliminary phase information obtained from molecular replacement with the structure of *O*-acetylserine sulfhydrylase from *Salmonella typhimurium* (Burkhard *et al.*, 1998), the Fe atoms could unambiguously be identified. Combining the phase information obtained from molecular replacement and MAD significantly improved the quality of the electron density (M. Meier, manuscript in preparation).

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