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Purification, crystallization and preliminary crystallographic analysis of the full-length cystathionine β -synthase from *Apis mellifera*

Cystathionine β -synthase (CBS) is a pyridoxal-5'-phosphate-dependent enzyme that catalyzes the first step of the transsulfuration pathway, namely the condensation of serine with homocysteine to form cystathionine. Mutations in the CBS gene are the single most common cause of hereditary homocystinuria, a multisystemic disease affecting to various extents the vasculature, connective tissues and central nervous system. At present, the crystal structure of CBS from *Drosophila melanogaster* is the only available structure of the full-length enzyme. Here we describe a cloning, overexpression, purification and preliminary crystallographic analysis of a full-length CBS from *Apis mellifera* (*AmCBS*) which maintains 51 and 46% sequence identity with its *Drosophila* and human homologs, respectively. The *AmCBS* yielded crystals belonging to space group $P2_12_12_1$, with unit-cell parameters $a = 85.90$, $b = 95.87$, $c = 180.33$ Å. Diffraction data were collected to a resolution of 3.0 Å. The crystal structure contained two molecules in the asymmetric unit which presumably correspond to the dimeric species observed in solution.

1. Introduction

Cystathionine β -synthase (CBS, L-serine hydrolyase, EC 4.2.1.22) is the first enzyme of the transsulfuration pathway, which links methionine metabolism to the biosynthesis of cellular redox-controlling molecules like cysteine, glutathione and taurine (Meier *et al.*, 2003). CBS catalyzes the pyridoxal-5'-phosphate (PLP)-dependent condensation of serine and homocysteine to form cystathionine (Banerjee *et al.*, 2003; Miles & Kraus, 2004), which is then converted to cysteine by another PLP-dependent enzyme, cystathionine gamma lyase. Besides maintaining cellular homocysteine homeostasis, CBS also gates the flow of sulfur into glutathione, and contributes to the biogenesis of H_2S , a gaseous signaling molecule that activates adenylyl cyclase and leads to the formation of cyclic AMP (cAMP) (Kimura, 2000; Watanabe *et al.*, 2003). Deficiency of CBS activity (over 160 mutations have been described in patients; <http://cbs.lf1.cuni.cz/cbsdata/cbsmain.htm>) (Kraus *et al.*, 1999; Kozich *et al.*, 2010) is the most common cause of homocystinuria, an inherited metabolic disease characterized by dislocated eye lenses, neural tube defects, skeletal problems, vascular disease, mental retardation and Alzheimer's disease (Beyer *et al.*, 2004; Mudd, 2011). Interestingly, the levels of CBS are enriched in the brain of Down's syndrome patients (Ichinohe *et al.*, 2005). CBS malfunction induces the generation of reactive species of oxygen and halogens and promotes the synthesis of pro-inflammatory molecules by macrophages. This has led some authors to propose a potential link between the transsulfuration pathway and tumor development (Rosado *et al.*, 2007).

The canonical domain architecture, the oligomerization degree and the regulatory mechanisms of eukaryotic CBS enzymes are not conserved across phyla. The mammalian CBSs are tetrameric enzymes and each of their ~63 kDa polypeptide chains contains three different regions: (i) the N-terminal domain binds heme which is thought to act as a redox sensor (Kery *et al.*, 1994; Kabil *et al.*, 2011); (ii) the middle portion of the polypeptide chain forms a highly conserved catalytic core with the fold-type II of PLP-dependent

Figure 1

Sequence alignment of full-length cystathionine β -synthases from *Apis mellifera* (AmCBS), *Drosophila melanogaster* (DmCBS) and *Homo sapiens* (hCBS). The Uniprot codes of AmCBS, DmCBS and hCBS are Q2V0C9, Q9VRD9 and P35520, respectively. Based on the domain distribution of the human enzyme, the sequences are organized in three blocks: (i) heme-binding domain (residues 1–69) (marked with a red vertical line on the left side); (ii) catalytic core (residues 70–413) (blue vertical line); and (iii) regulatory domain (residues 414–551) (green vertical line). The regulatory domain contains two CBS motifs (CBS1: residues 455–505 and CBS2: residues 521–577; Pfam code PF00571). Most common pathological mutations are indicated in red along the sequence. Residues involved in heme and PLP binding are marked with red and blue asterisks, respectively. Sequence alignment was done with *ClustalW* (Larkin *et al.*, 2007) and represented with *CINEMA* (Parry-Smith *et al.*, 1998).

proteins (Mehta & Christen, 2000); and (iii) the C-terminal domain is an autoinhibitory module that possesses two defined CBS motifs in tandem referred to as the ‘Bateman domain’ (Fig. 1) (Bateman, 1997; Baykov *et al.*, 2011). While CBS1 is mainly a hydrophobic motif, CBS2 is less conserved among species. Together, these two CBS domains

bind *S*-adenosylmethionine (AdoMet), an allosteric activator of mammalian CBSs, which induces a conformational change that liberates the intrinsic inhibition formed by the Bateman domain (Kery *et al.*, 1998; Janosik, Kery *et al.*, 2001; Janosik, Oliveriusová *et al.*, 2001; Hnizda *et al.*, 2010). The C-terminal domain of mammalian

CBS is also thought to be responsible for multimerization of the enzyme into homotetramers and higher oligomeric forms (Kery *et al.*, 1998; Taoka *et al.*, 1999), since its removal yields an ~45 kDa truncated form (45CBS) which forms dimers and is more active than the full-length enzyme (Skovby *et al.*, 1984; Kery *et al.*, 1998; Watanabe *et al.*, 2003). The heme-binding domain is absent in yeast, protozoan and nematode CBS, in contrast to its presence in insects, such as fruit fly and honey-bee (Fig. 1) (Jhee *et al.*, 2000*a,b*; Nozaki *et al.*, 2001; Koutmos *et al.*, 2010; Vozdek *et al.*, 2012). The catalytic domain is conserved in the CBS enzymes of yeast, protozoa, fruit fly and honey-bee. The C-terminal portion exhibits the highest degree of variability. The yeast, fruit fly and honey-bee CBS proteins contain the Bateman domain but lack response to AdoMet. Interestingly, while the C-terminal portion of the yeast CBS inhibits the activity of the enzyme and supports the formation of tetramers and octamers (Jhee *et al.*, 2000*a,b*), the fruit fly and honey-bee CBS form only dimers (Koutmos *et al.*, 2010). In contrast, the protozoan and nematode CBS do not contain the Bateman domain and are not activated by AdoMet.

Despite the experimental effort of several laboratories, the crystallization of CBS has proved to be a very difficult task, probably owing to the intrinsic mechanisms of regulation of these enzymes involving conformational changes when binding ligands. Up until 2010, only two CBS structures of the catalytic core of human CBS had been deposited in the databases (Meier *et al.*, 2001; Taoka *et al.*, 2002). More recently, the structure of CBS from *Drosophila melanogaster* (*DmCBS*) was determined which represents the only structural framework of the full-length protein available so far (Koutmos *et al.*, 2010). The authors claim that the reported structure corresponds to the activated form of the enzyme (Koutmos *et al.*, 2010). Here we describe the crystallization and preliminary analysis of the full-length CBS from *Apis mellifera*. These data provide the basis for the crystallographic analysis of the full-length protein and should help us comprehend marked differences in the regulation of CBSs among species, the role played by the C-terminal domain and the effect of pathogenic mutations in the human enzyme.

2. Materials and methods

2.1. Cloning of *AmCBS*

The *AmCBS* coding sequence was sub-cloned into a pET-28a vector (Novagen) following a similar strategy to the one we used for the human CBS expression construct (Majtan & Kraus, 2012). The resulting recombinant *AmCBS* has an intact N-terminus compared to the annotated sequence (Uniprot: Q2V0C9) and carries a non-removable 6xHis tag preceded by Lys and Glu residues from the *XhoI* recognition site at the C-terminus, which enables us to employ an immobilized metal-affinity chromatography for the purification of only full-length *AmCBS* polypeptides. Briefly, *AmCBS* was amplified by PCR from the cDNA library (Lambda ZAP, Stratagene) prepared from the heads of nurse honey-bees (Klaudiny *et al.*, 1994) using a forward primer containing an *NcoI* site (5'-ctagCCATGGAatttaaacacctaac) and a reverse primer containing an *XhoI* site (5'-ctagCTCGAGatttattaataattattagatgtacc). After cleavage with *NcoI* and *XhoI* (NEB Biolabs), the *AmCBS* fragment was separated in 1% agarose gel, cut out and cleaned up using a QIAquick gel extraction kit (Qiagen). Subsequently, *AmCBS* was ligated into the *NcoI*–*XhoI* linearized pET-28a vector using T4 DNA ligase (NEB Biolabs). The construct pET28-C-AMCBS was transformed into *Escherichia coli* XL1-Blue cells (Stratagene) and its authenticity was confirmed by DNA sequencing. Verified plasmid was transformed into *E. coli* Rosetta2 (DE3) expression host cells (Novagen).

2.2. Expression and purification of recombinant *AmCBS*

The preparation of recombinant *AmCBS* followed a similar methodology to the one we used for various human CBS constructs (Majtan & Kraus, 2012). Briefly, bacterial cells were grown (303 K, 275 rev min⁻¹) in six 2.8 l baffled Fernbach flasks containing 1 l of LB medium supplemented with 0.001% thiamine–HCl, 0.0025% pyridoxine–HCl, 0.1 mM ferric chloride, 0.3 mM δ -aminolevulinic acid and 30 μ g ml⁻¹ kanamycin. When the cell density reached $A_{600} \approx 0.8$, *AmCBS* expression was induced by adding IPTG to a final concentration of 1 mM. After induction, the cells continued to grow overnight and were then harvested by centrifugation at 9000g for 7 min at 277 K. The cell pellet was washed with 1 $\times$ PBS and kept at 193 K before processing.

The cell pellets were resuspended in a lysis buffer [50 mM sodium phosphate pH 7.4, 300 mM NaCl, 1% Triton X-100, 0.1 mM PLP and a protease inhibitor cocktail VII (A.G. Scientific)] at a 1:5(w/v) ratio using a homogenizer. Resuspended cells were treated with 2 mg ml⁻¹ lysozyme for 1.25 h at 277 K prior to sonication (eight cycles of 2 min at 50% duty; Misonix S-3000, Qsonica). The cell lysate was clarified by subsequent centrifugation at 58 000g for 30 min at 277 K. The supernatant was loaded on a 50 ml TALON column (Clontech) equilibrated in 50 mM sodium phosphate, pH 7.4, and 300 mM NaCl. The bound protein was subsequently washed with at least five column volumes of wash buffer (50 mM sodium phosphate, pH 7.4, 300 mM NaCl, 10 mM imidazole) and then eluted with 200 mM imidazole in the wash buffer. Subsequently, the fraction was desalted on a Sephadex G-25 (GE Healthcare) column (3.2 cm inner diameter $\times$ 22 cm) and the buffer was exchanged with the DEAE Sepharose loading buffer (15 mM potassium phosphate pH 7.2, 1 mM EDTA, 1 mM dithiothreitol, 10% ethylene glycol). The next day, after keeping *AmCBS* at 277 K, the sample was loaded onto a DEAE Sepharose (GE Healthcare) column (2.5 cm inner diameter $\times$ 8.0 cm) and washed with three column volumes of the DEAE Sepharose loading buffer followed by 125 mM potassium phosphate in the DEAE Sepharose loading/wash buffer in order to elute *AmCBS*. The DEAE Sepharose eluate was formulated into a final buffer (20 mM HEPES pH 7.4, 1 mM TCEP [tris(2-carboxyethyl)phosphine], 0.01% Tween 20) on a Sephadex G-25 column and subsequently concentrated using an ultrafiltration device (Amicon) equipped with a YM-30 (Millipore) membrane. Finally, after 30 min spin at 21 000g, 277 K, the enzyme was aliquoted, flash-frozen in liquid nitrogen and stored at 193 K.

2.3. Crystallization

Crystallization trials were carried out by the vapor-diffusion technique in a sitting-drop format with 96-well MRC crystallization plates using a variety of commercially available screens [Crystal Screen HT, Index HT (Hampton Research), JCSG Core Suites I–IV (Qiagen), JCSG+ Suite, PACT Premier HT-96 (Molecular Dimensions)]. Screening plates were set up in the high-throughput crystallization facility at CIC bioGUNE and incubated at a constant temperature of 293 K. Drops consisted of 200 nl protein solution mixed with 200 nl precipitant solution and the reservoir volume was 50 μ l; the protein concentration was ~6 mg ml⁻¹. An initial crystallization hit yielding tiny red prism-shaped crystals was obtained using a precipitant solution containing 10% PEG 6000, 0.1 M HEPES pH 7.5, 5% (+/–)-2-methyl-2,4-pentanediol. Improved crystals were subsequently obtained in the same precipitant solution in a hanging-drop format using 24-well VDX plates (Hampton Research) with drops consisting of 0.5 μ l protein with 0.5 μ l precipitant solution

equilibrated over a reservoir volume of 0.5 ml and incubated at a constant temperature of 293 K (Fig. 2).

Crystal growth required high availability of sample and a significant experimental effort. Crystals with approximate dimensions $200 \times 50 \times 50 \mu\text{m}$ grew in 2–3 d in only ~10–15% of the drops. The nucleation ratio improved slightly to ~20–25% after using these crystals as seeds in further experiments. Among all crystals tested (~200), only 5% showed suitable diffraction properties at 293 K in our in-house equipment. A similar ratio was obtained from frozen crystals at the ESRF.

2.4. Preliminary crystallographic analysis

The diffraction quality of the crystals was firstly evaluated at room temperature in the absence of cryoprotectants. Then, and after testing a wide variety of cryoprotectants including alcohols, low-molecular-weight PEGs, sucrose, agarose, silicone oils or paratone, the crystals were transferred to crystallization buffer containing 25% (+/–)-2-methyl-2,4-pentanediol for a few seconds before being flash-cooled by directly immersing into liquid nitrogen at 93 K. Crystals were mounted for X-ray data collection using either Cryoloops (Hampton Research) or MicroMount loops (MiTeGen). Data sets were collected in-house using a MAR345 detector mounted on a Microstar-H rotating-anode X-ray generator (Bruker), operated at 60 kV and 100 mA, with optics Helios and a copper target (Cu $K\alpha$; $\lambda = 1.542 \text{ \AA}$). A total of 300 diffraction images over a range of 150° could be collected from the best crystal before severe crystal decay was observed. Diffraction data were processed using *HKL-2000* (Otwinowski & Minor, 1997) and *XDS* (Kabsch, 2010). Preliminary analysis of the data sets was performed using the *CCP4* program suite (Winn *et al.*, 2011). Native crystals of *AmCBS* diffracted to $3.0 \text{ \AA}$ resolution (Fig. 3, Table 1) and belonged to space group $P2_12_12_1$ (unit-cell parameters $a = 86.98$, $b = 96.01$, $c = 182.21 \text{ \AA}$). One, two or three molecules within the asymmetric unit gave a Matthews coefficient of 6.60, 3.30 and $2.20 \text{ \AA}^3 \text{ Da}^{-1}$, and a solvent content of 81, 63 and 44%, respectively (Matthews, 1968). Considering the fragility of the $P2_12_12_1$ crystals of *AmCBS* and their limited diffraction power, we estimated that two molecules per asymmetric unit (a dimeric species of CBS) was the most probable value. In support of this, the corresponding plot of the self-rotation function at $k = 180^\circ$ (Fig. 4) showed one twofold non-crystallographic axis at 20 and 30° of the Y and Z axes, respectively, suggesting that the two molecules are related

Figure 2
Crystals of full-length *AmCBS*. Crystals belong to space group $P2_12_12_1$ and diffracted X-rays to $3.0 \text{ \AA}$. The red color reveals the presence of a bound heme b group.

Table 1

Data-processing statistics for *AmCBS* crystals.

Values in parentheses are for the outer-resolution shell. Data sets were collected using the CIC bioGUNE in-house Microstar-H rotating-anode generator. The outermost resolution shell used to calculate parameters ranges from 3.16 to $2.98 \text{ \AA}$. $CC_{1/2}$ indicates the percentage of correlation significant at the 0.1% level between intensities from random half data sets (Karplus & Diederichs, 2012).

Beamline	Microstar-H, Bruker
Wavelength ($\text{\AA}$)	1.5418
Resolution range ($\text{\AA}$)	50–3.0
Total number of reflections	207245 (26070)
Number of unique reflections	30526 (4272)
Space group	$P2_12_12_1$
Unit-cell parameters ($\text{\AA}$)	$a = 86.98$, $b = 96.01$, $c = 182.21$
Cell volume ($\text{\AA}^3$)	1521626.56
$CC_{1/2}$ (%)	99.2 (65.8)
Mosaicity ($^\circ$)	0.25
Completeness (%)	97.5 (86.0)
Redundancy	6.8 (6.1)
$R_{\text{merge}}^\dagger$ (%)	18.6% (63.4)
Mean $I/\sigma(I)$	11.3 (3.1)
Wilson B value ($\text{\AA}^2$)	42.8

$^\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the i th observation of reflection hkl and $\langle I(hkl) \rangle$ is the weighted average intensity for all observations i of reflection hkl .

by a two-symmetry and probably form a dimer, coinciding with the oligomerization state observed *in vitro*. The plot of the self-rotation function at $k = 180^\circ$ was calculated with *MOLREP* (Fig. 4) (Vagin & Teplyakov, 2010). The data-collection statistics are summarized in Table 1.

3. Results and discussion

Growing X-ray-grade diffraction crystals of *AmCBS* required ~20 mg of pure protein and a significant experimental effort. More

Figure 3
Representative X-ray diffraction image from the *AmCBS* crystals. The crystals, in size of approximately $0.7 \times 0.5 \times 0.4 \text{ mm}$, were exposed for 20 min per image over a 0.5° oscillation range. Resolution circles are included for clarity.

than 5000 different conditions, which included all available commercial screens as well as in-house-developed recipes, were tested before promising crystallization hits could be obtained. Additionally, we found that, despite using a unique protein purification protocol, crystallization success and more importantly crystal quality depended significantly on the sample batch used for the crystallization assays. This suggested that minor impurities and/or partially unfolded *AmCBS* present in the sample seriously impeded nuclei formation, which took place in a small portion of the drops (~2–3%). Besides that, formation of non-merohedral twinned crystals was a permanent difficulty.

We found *AmCBS* sensitive to slow thawing on ice, forming aggregates and precipitate. To overcome this obstacle, we defrosted protein aliquots stored at 193 K by submerging the bottom half of a screw-cap tube in a 310 K water bath with optional gentle mixing while partially submerged. After the whole aliquot became liquid, we briefly spun the protein down (20 000g, 277 K, 2 min) and then kept it on ice. Protein aggregation represents a constant challenge among CBS enzymes, particularly observed for human CBS (Kraus & Rosenberg, 1983; Majtan & Kraus, 2012), and is mainly attributed to the hydrophobic CBS domains. Therefore, we follow this protocol with all of our different CBS constructs and CBSs from various species.

Among all crystals tested (~200), only 5% showed suitable diffraction properties (~3–3.5 Å resolution) at our in-house X-ray equipment or at the ESRF (Table 1). The high similarity maintained between *AmCBS* and its *Drosophila melanogaster* homolog (51% sequence identity) (Fig. 1) offers an excellent scenario for the use of molecular replacement methods to phase the actual data. This work is now being carried out with the *D. melanogaster* CBS monomer serving as the search model (PDB codes 3pc2, 3pc3, 3pc4; Koutmos *et al.*, 2010).

Figure 4
Plot of the self-rotation function at $k = 180^\circ$ of the $P2_12_1$ *AmCBS* crystals. A twofold non-crystallographic symmetry axis at 20 and 30° of the Y and Z axes, respectively, suggests that the two molecules in the asymmetric unit form a dimer.

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