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Received 24 August 2013
Accepted 21 January 2014

Purification, crystallization and preliminary crystallographic analysis of the catalytic core of cystathionine β -synthase from *Saccharomyces cerevisiae*

Cystathionine β -synthase (CBS; EC 4.2.1.22) catalyzes the condensation of homocysteine and serine to form cystathionine, with the release of water. In humans, deficiency in CBS activity is the most common cause of hyperhomocysteinaemia and homocystinuria. More than 160 pathogenic mutations in the human CBS gene have been described to date. Here, the purification and preliminary crystallographic analysis of the catalytic core of CBS from *Saccharomyces cerevisiae* (ScCBS) is described which, in contrast to other eukaryotic CBSs, lacks the N-terminal haem-binding domain and is considered to be a useful model for investigation of the pyridoxal-5'-phosphate-mediated reactions of human CBS (*h*CBS). The purified protein yielded two different crystal forms belonging to space groups $P4_12_12$ and $P2_12_12_1$, with unit-cell parameters $a = b = 72.390$, $c = 386.794$ Å and $a = 58.156$, $b = 89.988$, $c = 121.687$ Å, respectively. Diffraction data were collected to 2.7 and 3.1 Å resolution, respectively, using synchrotron radiation. Preliminary analysis of the X-ray data suggests the presence of ScCBS homodimers in both types of crystals.

1. Introduction

Cystathionine β -synthase (CBS; EC 4.2.1.22) is a pyridoxal 5'-phosphate (PLP)-dependent enzyme that catalyzes the condensation of homocysteine (Hcy), a toxic metabolite of the methionine cycle, with serine to form cystathionine, with the release of water (Mudd *et al.*, 2001; Fig. 1). This reaction makes CBS a key regulator of plasma levels of Hcy. CBS deficiency leads to homocystinuria, an inherited metabolic disorder clinically characterized by dislocated optic lenses, cardiovascular and neurovascular diseases, skeletal deformities, connective tissue defects and mental retardation (Mudd *et al.*, 1982, 2001; Welch & Loscalzo, 1998; Seshadri *et al.*, 2002; Folin *et al.*, 2005). Additionally, CBS is also able to synthesize hydrogen sulfide (H_2S) via the desulfurization of cysteine, a task that can also be performed by two other enzymes: cystathionine γ -lyase (CSE; EC 4.4.1.1) and mercaptopyruvate sulfurtransferase (MST; EC 2.8.1.2). Among these three enzymes, CBS contributes the most to endogenous H_2S production and is thought to be the main H_2S -forming enzyme in the central nervous system (Yadav & Banerjee, 2012; Paul & Snyder, 2012). In addition to the reverse transsulfuration pathway found in mammals, yeast also possesses a forward transsulfuration route that enables the formation of methionine from cysteine (Fig. 1). Thus, yeast can utilize either methionine or cysteine as a sulfur source.

The canonical architecture, degree of oligomerization and regulatory mechanism of CBS enzymes vary among species (Fig. 2). In higher eukaryotes, such as insects, rodents and mammals (Meier *et al.*, 2001; Koutmos *et al.*, 2010), the N-terminal region includes a haem-binding domain that is thought to function in redox sensing and/or enzyme folding (Janosík, Oliveriusová *et al.*, 2001; Singh *et al.*, 2007; Majtan *et al.*, 2010). This region is absent in CBS enzymes from lower eukaryotes, such as *Saccharomyces cerevisiae*, *Trypanosoma cruzi* and *Caenorhabditis elegans* (Jhee *et al.*, 2000; Maclean *et al.*, 2000; Nozaki *et al.*, 2001; Vozdek *et al.*, 2012). The haem-binding domain is followed by a conserved catalytic core with the fold of the type II family PLP-dependent enzymes (Christen & Mehta, 2001; Meier *et al.*, 2001). Finally, the C-terminal region consists of a tandem pair of CBS motifs (commonly known as a 'Bateman module') that exhibits the highest degree of sequence variability (Vozdek *et al.*, 2012). This

Figure 1

The forward and reverse transsulfuration pathway in yeast. Transsulfuration is the metabolic pathway that allows the interconversion of cysteine and methionine through the common intermediates homocysteine and cystathionine. In yeast, both forward and reverse routes are present, which enables the use of either cysteine or methionine as a sulfur source. The forward transsulfuration pathway (dotted grey arrows), which is also found in bacteria and plants, catalyzes the formation of methionine from cysteine, while the reverse transsulfuration pathway of mammals (thick black arrows) leads to the formation of cysteine from methionine. Unlike human and other mammalian CBS enzymes, yeast CBS is haem-independent and its activity is not regulated by *S*-adenosyl-L-methionine (AdoMet). Abbreviations: CBS, cystathionine β -synthase; CBL, cystathionine β -lyase; CGL, cystathionine γ -lyase; CGS, cystathionine γ -synthase.

regulatory portion is present in the majority of CBS enzymes, with a few exceptions, such as *T. cruzi* and *C. elegans* CBS (Nozaki *et al.*, 2001; Vozdek *et al.*, 2012). Interestingly, the presence of missense mutations in the C-terminal portion, partial thermal denaturation of the enzyme or complete removal of the Bateman module alleviates the autoinhibitory effect imposed by this region in the human (Kery *et al.*, 1998; Janosik, Kery *et al.*, 2001; Jhee *et al.*, 2001; Maclean *et al.*, 2002) and yeast enzymes (Taoka & Banerjee, 2002). The majority of CBS enzymes ranging from yeast to humans form homotetramers. Moreover, the regulatory domain is essential for tetramerization in the human and yeast enzymes, since its removal yields dimers (Kery *et al.*, 1998; Jhee *et al.*, 2000; Taoka & Banerjee, 2002). In contrast, insect CBSs appear to exist as homodimers (Koutmos *et al.*, 2010). The molecular mechanisms regulating the activity of the CBS enzyme vary widely across phyla. Binding of the allosteric regulator *S*-adenosyl-L-methionine (AdoMet) to the Bateman module of human CBS alleviates the otherwise imposed autoinhibition of the C-terminal regulatory domain and leads to an increase in catalytic activity by up to fivefold (Bateman, 1997; Kery *et al.*, 1998; Ereño-Orbea *et al.*, 2013). In contrast, insect and yeast CBSs already are

constitutively activated and are not further regulated by AdoMet (Maclean *et al.*, 2000; Koutmos *et al.*, 2010).

The wide variability of CBSs and the scarce structural data, which are currently limited to the crystal structure of the human (Meier *et al.*, 2001; Taoka *et al.*, 2002; Ereño-Orbea *et al.*, 2013) and fruit fly enzymes (Koutmos *et al.*, 2010), make the current knowledge insufficient to comprehend the physiology of CBSs across phyla. To overcome this deficiency, we have addressed the crystallization and preliminary crystallographic analysis of a protein construct that contains the catalytic core of CBS from *S. cerevisiae* (ScCBS_{1–344}, residues 1–344). The overlapping absorbances of the PLP and haem cofactors of human CBS (*h*CBS) complicate the spectroscopic investigation of catalytic intermediates, precluding pre-steady-state kinetics as a tool for mechanistic studies of CBS. Since ScCBS catalyzes the same reaction as the human enzyme and does not contain haem, ScCBS has been considered to be a useful model for investigation of the PLP-associated reactions of *h*CBS (Jhee *et al.*, 2001; Taoka & Banerjee, 2002; Aitken & Kirsch, 2004; Lodha *et al.*, 2010).

The truncated ScCBS_{1–344} enzyme yielded two different crystal forms belonging to space groups *P*₄₁₂₁₂ and *P*₂₁₂₁₂ that diffracted X-rays to resolutions of 2.7 and 3.1 Å, respectively (Table 1). The crystal structures appear to contain three and two molecules in the asymmetric unit, respectively, that presumably correspond to a dimeric form of the enzyme consistent with the behaviour of the protein in solution. These data provide the first basis for a crystallographic analysis of the yeast CBS enzyme. Comparison with the equivalent region in the human and insect CBSs should aid understanding of the regulatory role played by the N-terminal domain as well as the effect of some of the pathogenic mutations.

2. Materials and methods

2.1. Cloning of ScCBS_{1–344}

The coding sequence of a full-length *S. cerevisiae* CBS (ScCBS; UniProt P32582) was PCR-amplified from a previously prepared pGEX-5X-1yCBS plasmid (Maclean *et al.*, 2000) using the 656 (5'-CTAGGGGCCCCACTAAATCTGAGCAGCAAGCC) and 657 (5'-CTAGGCGGCGCGTTATGCTAAGTAGCTCAGTAAATCC) oligonucleotides. The *Apa*I- and *Not*I-digested, gel-extracted PCR product was ligated with a similarly prepared pGEX-6P1 vector using T4 DNA ligase (NEB). Prepared ScCBS construct, designated as pGEX-6P1-yCBS2extraAA, was transformed into *Escherichia coli* XL1-Blue cells (Agilent) and its authenticity was confirmed by DNA sequencing. Subsequently, the full-length ScCBS construct served as a template for the preparation of a truncated ScCBS L345* expression construct by introducing a translational STOP codon using the QuikChange II XL site-directed mutagenesis kit (Agilent) according to the manufacturer's recommendations. The primers 790 (5'-CAATTTGTGGGATGATGACGTGTAGGCCCGTTTGA) and 791 (5'-TCAAAACGGGCGCTACACGTCATCATCCACAAATTG) were used to mutate the codon for residue Leu345 into a STOP codon. The presence of the desired STOP codon in our construct, designated as pGEX-6P1-yCBS2extraAA L345*, was confirmed by DNA sequencing. The verified plasmid was finally transformed into *E. coli* Rosetta2 (DE3) expression host cells (Novagen).

2.2. Expression and purification of recombinant ScCBS_{1–344}

The preparation of recombinant ScCBS_{1–344} followed the protocol that we have developed for various CBS constructs (Majtan *et al.*, 2010; Majtan & Kraus, 2012; Su *et al.*, 2013) with a few modifications. Briefly, bacterial cells were grown (30°C, 275 rev min^{−1}) in six 2.8 l

Table 1
Data-processing statistics for ScCBS₁₋₃₄₄ crystals.
Values in parentheses are for the outer resolution shell.

Data set	ScCBS ₁₋₃₄₄	ScCBS ₁₋₃₄₄
Beamline	ID29, ESRF	ID29, ESRF
Wavelength (Å)	0.9790	0.9790
Resolution range (Å)	60.00 (2.70)	48.90 (3.08)
Total No. of reflections	592303 (94751)	277958 (12267)
No. of unique reflections	29703 (4623)	11736 (836)
Space group	<i>P</i> 4 ₁ 2 ₁ 2	<i>P</i> 2 ₁ 2 ₁ 2 ₁
Unit-cell parameters (Å)	<i>a</i> = <i>b</i> = 72.39, <i>c</i> = 386.79	<i>a</i> = 58.16, <i>b</i> = 89.99, <i>c</i> = 121.69
CC _{1/2} † (%)	99.8 (95.4)	99.9 (91.4)
Mosaicity (°)	0.1	0.2
Completeness (%)	99.9 (99.4)	95.1 (70.5)
Multiplicity	19.94 (20.49)	23.68 (14.67)
<i>R</i> _{meas} ‡ (%)	17.7 (75.6)	14.8 (78.1)
Mean <i>I</i> /σ(<i>I</i>)	15.96 (3.61)	20.14 (3.44)
Wilson <i>B</i> value (Å ²)	48.9	48.3

† CC_{1/2} indicates the percentage of correlation significant at the 0.1% level between intensities from random half data sets (Karplus & Diederichs, 2012). ‡ *R*_{meas} = $\sum_{hkl} \{N(hkl)/[N(hkl) - 1]\}^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where *I*_{*i*}(*hkl*) are the observed intensities, $\langle I(hkl) \rangle$ are the average intensities and *N*(*hkl*) is the multiplicity of reflection *hkl*.

baffled Fernbach flasks each containing 1 l Terrific Broth supplemented with 0.001% thiamine–HCl, 0.0025% pyridoxine–HCl and 100 µg ml^{−1} ampicillin. When the cell density reached an *A*₆₀₀ of ~0.8, expression of ScCBS₁₋₃₄₄ was induced by adding IPTG to a final concentration of 1 mM. A temperature of 30°C was maintained during the whole fermentation process, *i.e.* before as well as after induction. After induction, cell growth was continued overnight and the cells were then harvested by centrifugation at 9000g for 7 min at 4°C. A total of 52.9 g wet cells were obtained after overnight cultivation. The cell pellet was washed with 1× PBS and kept at −80°C before processing.

The cell pellet was resuspended in lysis buffer (10 mM Tris–HCl pH 8.0, 150 mM NaCl, 20 mM DTT, 1% Triton X-100, 0.1 mM PLP)

and protease-inhibitor cocktail (Sigma P8465) 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 4°C prior to sonication (eight cycles of 2 min at 50% duty; Misonix S-3000, Qsonica). The cell lysate was clarified by centrifugation at 58 000g for 30 min at 4°C. The supernatant was loaded onto a 50 ml Glutathione Sepharose 4 Fast Flow column (GE Healthcare) equilibrated in 10 mM sodium phosphate pH 7.4, 500 mM NaCl, 1 mM DTT. The bound protein was subsequently washed with at least five column volumes of equilibration buffer and was then eluted with elution buffer (50 mM Tris–HCl pH 8.0, 1 mM DTT, 20 mM reduced glutathione). In order to remove the GST fusion protein, the eluate was adjusted with 10× cleavage buffer (0.5 M Tris–HCl pH 7.0, 1.5 M NaCl, 10 mM EDTA, 10 mM DTT, 1 mM PLP) to 1× and the fusion protein was cleaved overnight at 4°C with HRV3C protease (A.G. Scientific; 1 U per milligram of fusion protein). The next day, the mixture was buffer-exchanged on a Sephadex G-25 (GE Healthcare) column (3.2 cm internal diameter × 22 cm) with equilibration/wash buffer. The sample was loaded again onto an equilibrated Glutathione Sepharose 4 FF column and the flowthrough fraction was collected. The flowthrough was buffer-exchanged into final buffer (20 mM HEPES pH 7.4, 1 mM TCEP) on a Sephadex G-25 column and was subsequently concentrated using an ultrafiltration device (Amicon) equipped with a YM-30 (Millipore) membrane. Finally, after a 30 min spin at 58 000g and 4°C, the ScCBS₁₋₃₄₄ was aliquoted, flash-frozen in liquid nitrogen and stored at −80°C. A total of 180 mg pure protein concentrated to 65 mg ml^{−1} was obtained.

2.3. Crystallization

Crystallization trials were carried out by the vapour-diffusion technique in a sitting-drop format in 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), The JCSG+ Suite and PACT Premier HT-96 (Molecular

Figure 2
Domain architecture of CBS enzymes from different organisms: hCBS (*Homo sapiens*), RnCBS (*Rattus norvegicus*), MmCBS (*Mus musculus*), ScCBS (*Saccharomyces cerevisiae*), DmCBS (*Drosophila melanogaster*), AmCBS (*Apis mellifera*) and CeCBS (*Caenorhabditis elegans*). Regions corresponding to the catalytic core and regulatory domain are indicated. The region linking both domains is represented in turquoise. The degree of oligomerization for each species (tetramers or dimers marked with four or two red spheres, respectively) as well as the catalytic responsiveness to the allosteric regulator AdoMet are indicated on the right.

Dimensions)]. Screening plates were set up in the high-throughput crystallization facility at CIC bioGUNE and were incubated at a constant temperature of 20°C. Drops consisted of 200 nl protein solution mixed with 200 nl precipitant solution and the reservoir volume was 50 µl; the protein concentration was 60 mg ml⁻¹. Improved crystals of ScCBS₁₋₃₄₄ (Fig. 3) were subsequently obtained by scaling up the successful conditions in a hanging-drop format using 24-well VDX plates (Hampton Research) with drops consisting of 0.5 µl protein solution and 0.5 µl precipitant solution [crystals belonging to space group $P4_12_12$ were obtained using a precipitant solution consisting of 22% polyethylene glycol 4000, 0.1 M Tris-HCl pH 8.5, while crystals belonging to space group $P2_12_12_1$ were obtained using a precipitant solution consisting of 0.19 M calcium chloride dehydrate, 0.095 M HEPES sodium pH 7.5, 26.6% (v/v) polyethylene glycol 400, 5% (v/v) glycerol] equilibrated over a reservoir volume of 0.5 ml and incubated at a constant temperature of 20°C. The diffraction properties of the crystals were examined on beamline ID29 of the ESRF, Grenoble.

2.4. Preliminary crystallographic analysis

The diffraction quality of the crystals was initially evaluated at room temperature in the absence of cryoprotectants. The $P4_12_12$ crystals were then transferred to a cryoprotection buffer consisting of 22% polyethylene glycol 4000, 0.1 M Tris-HCl pH 8.5 and paraffin oil for a few seconds before being flash-cooled by direct immersion into liquid nitrogen at -180°C. The $P2_12_12_1$ crystals were directly flash-cooled by immersion into liquid nitrogen at -180°C. Crystals were mounted for X-ray data collection using either Cryo-Loops (Hampton Research) or MicroMount loops (MiTeGen). Data sets were collected on beamline ID29 of the ESRF, Grenoble ($\lambda = 0.9390$ Å). A total of 2800 and 3600 diffraction images over ranges of 280 and 720° could be collected from the best $P4_12_12$ and $P2_12_12_1$ crystals, respectively, before severe crystal decay was observed. Diffraction data were processed using *HKL-2000* (Otwinowski & Minor, 1997) or *XDS* (Kabsch, 2010). Preliminary analysis of the data sets was performed using the *CCP4* program suite (Winn *et al.*, 2011). A plot of the self-rotation function at $\kappa = 180^\circ$ was calculated with

MOLREP (Vagin & Teplyakov, 2010). The data-collection statistics are summarized in Table 1.

3. Results and discussion

Based on careful analyses of sequence alignments and structural data of ScCBS *versus* hCBS (Meier *et al.*, 2001; Taoka *et al.*, 2002; Ereño-Orbea *et al.*, 2013) and *Drosophila melanogaster* CBS (*DmCBS*; Koutmos *et al.*, 2010), we engineered a protein construct (ScCBS₁₋₃₄₄) that lacks the amino-acid residues 345–507 which correspond to the C-terminal regulatory domain (Fig. 2). Similarly to human CBS, truncation of the Bateman module of ScCBS results in a tetramer-to-dimer conversion of the enzyme (Jhee *et al.*, 2000; Taoka & Banerjee, 2002).

We initially explored potential crystallization conditions of the ScCBS₁₋₃₄₄ construct using all available commercial screens as well as in-house-developed recipes. After 3–4 d, microcrystalline precipitates and/or tiny crystals appeared in the conditions mentioned above. Obtaining nicely formed crystals which were suitable for crystallographic study just involved scaling up the initial conditions (Fig. 3). Native crystals of ScCBS₁₋₃₄₄ diffracted to 2.7 and 3.1 Å resolution (Fig. 4; Table 1) and belonged to space groups $P4_12_12$ and $P2_12_12_1$, respectively. Notably, although the $I/\sigma(I)$ values (and also the $CC_{1/2}$ parameters) suggested that the resolution limit of the presented data might be slightly higher than 2.7 and 3.1 Å, the inclusion of higher resolution data resulted in a dramatic worsening of the diffraction statistics. Accordingly, we allocated the maximum resolution of our crystals to the values shown in Table 1. The exact resolution limit of our crystals will have to be evaluated once the structure has been solved and refined. The presence of one, two and three molecules within the asymmetric unit gave Matthews coefficients of 6.67, 3.33 and 2.22 Å³ Da⁻¹, respectively, for the $P4_12_12$ crystals and of 4.19, 2.1 and 1.4 Å³ Da⁻¹, respectively, for the $P2_12_12_1$ crystals. The corresponding solvent content values are 82, 63 and 45%, respectively, for the $P4_12_12$ crystals and 71, 41 and 12%, respectively, for the $P2_12_12_1$ crystals (Matthews, 1968). Considering the limited diffraction power of the crystals, we estimated that three and two molecules in the

Figure 3

Crystals of truncated yeast CBS. ScCBS₁₋₃₄₄ yielded two different crystal forms belonging to two different space groups, (a) $P4_12_12$ and (b) $P2_12_12_1$, that diffracted X-rays to 2.7 and 3.1 Å resolution, respectively. In contrast to other CBSs, like human CBS or insect CBS which are reddish, the crystals of yeast CBS are colourless or pale yellowish owing to the absence of a bound haem group. The approximate dimensions of the crystals are 300 × 100 × 30 and 250 × 150 × 20 µm, respectively.

asymmetric unit were the most probable values for the $P4_12_12$ and $P2_12_12_1$ crystals, respectively.

As shown in Fig. 5, the plot of the self-rotation function at $\kappa = 180^\circ$ for the $P2_12_12_1$ data set reflects the Laue symmetry of an orthorhombic crystal, but does not show a noncrystallographic symmetry axis (NCS) relating the two molecules in the asymmetric unit. These data are consistent with the presence of dimers in the crystal if the twofold NCS axis that relates these two molecules runs parallel to the

x , y or z crystallographic axis. On the other hand, the $P4_12_12$ data set reflects the Laue symmetry of a tetragonal crystal, with two additional peaks corresponding to twofold NCS axes (marked 1 and 2 in

Figure 4
Representative X-ray diffraction data frame from the $P4_12_12$ (a) and the $P2_12_12_1$ (b) ScCBS_{1-344} crystals recorded on ESRF beamline ID29. The crystals were exposed for 1 s per image over a 1° rotation range. Resolution circles are included for clarity. The resolution at the edge is 3.0 Å in both images.

Figure 5
Plot of the self-rotation function at $\kappa = 180^\circ$ of the ScCBS_{1-344} crystals. (a) Besides the peaks expected from the Laue symmetry of a tetragonal crystal, the plot for the $P4_12_12$ data set shows two additional peaks (marked as 1 and 2) at approximately 20° and 50° from the z axis. (b) The $P2_12_12_1$ data set just reflects the symmetry expected for an orthorhombic crystal, with the twofold crystallographic axis parallel to the x , y and z axes. The self-rotation functions were calculated with *MOLREP* (Vagin & Teplyakov, 2010) using a radius of 16 Å, with data between 20 and 5.8 Å and between 20 and 5.0 Å for the $P4_12_12$ and $P2_12_12_1$ crystals, respectively.

Fig. 5). This fact is consistent with the presence of two different types of dimers in the crystals. The first dimer appears to be formed by two molecules which are related by one of the twofold NCS axes, whereas the second dimer may be formed by applying the space-group symmetry to the third molecule in the asymmetric unit. We should note, however, that the presence of dimeric species in either of the crystals can only be confirmed once the corresponding crystal structures have been solved and refined. Interestingly, the data presented here are consistent with the expected dimeric state of a truncated ScCBS protein which lacks the C-terminal regulatory domain (similarly to human CBS, truncation of the CBS motif pair of ScCBS results in a tetramer-to-dimer conversion of the enzyme; Taoka & Banerjee, 2002; Miles & Kraus, 2004). Accordingly, the ongoing structure determination will help to determine whether the traditionally proposed tetramer of ScCBS is in fact an association of two dimers related by a twofold symmetry axis.

We thank the staff of beamline ID29 of the ESRF for valuable support and excellent technical assistance during synchrotron data collection. We also thank Dr Adriana Rojas for maintenance of the in-house X-ray equipment. This work has been supported by Post-doctoral Fellowship 0920079G from the American Heart Association (to TM), by National Institutes of Health grant HL065217, by the American Heart Association Grant-In-Aid 09GRNT2110159, by a grant from the Jerome Lejeune Foundation (all to JPK) and by grants from Departamento de Educación, Universidades e Investigación del Gobierno Vasco (PI2010-17), Departamento de Industria, Innovación, Comercio y Turismo del Gobierno Vasco (ETORTEK IE05-147, IE07-202), Diputación Foral de Bizkaia (Exp. 7/13/08/2006/11 and 7/13/08/2005/14) and Ministerio Español de Ciencia y Tecnología (MICINN; BFU2010-17857) (all to LAMC).

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