Crystallization and preliminary X-ray study of recombinant betaine–homocysteine S-methyltransferase from rat liver

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Betaine–homocysteine S-methyltransferase is one of the three enzymes involved in homocysteine catabolism. It uses betaine as the methyl donor to convert homocysteine into methionine, also producing dimethylglycine. Recombinant BHMT from rat liver was crystallized by the vapour-diffusion method in both native and selenomethionyl-labelled forms. Crystals belong to space group P21, with unit-cell parameters a = 57.8, b = 149.3, c = 96.2 Å, β = 92.9°. Data from native, selenomethionine-labelled and two heavy-atom derivatives were collected using synchrotron sources. Self-rotation function and sedimentation-velocity experiments suggest that the enzyme is tetrameric with 222 symmetry.

1. Introduction

Betaine homocysteine methyltransferase (BHMT; EC 2.1.1.5) is one of the three enzymes involved in homocysteine catabolism (Mato et al., 1997). The catalyzed reaction serves to recover one of the three methylation equivalents used to synthesize phosphatidylcholine through choline oxidation to betaine. This last compound is the methyl donor that BHMT uses to convert homocysteine in methionine, leading also to the production of dimethylglycine (Barak & Tuma, 1979). In vitro experiments have shown that both homocysteine and dimethylglycine are necessary to stabilize BHMT and that dimethylglycine, isovalerate and 3,3-dimethylbutirate act as inhibitors (Garro, 1996). This reaction has been detected to occur almost exclusively in liver and kidney (Delgado-Reyes et al., 2001). Decreases in betaine levels have been observed in choline deficiencies, along with decreases in BHMT activity (Finkelstein & Martin, 1986). In methionine deficiencies, the enzyme acquires a key role in the maintenance of the levels of this essential amino acid for mammalian growth and development (Finkelstein et al., 1982). As for the kidney, betaine has been proposed to have a role in the protection of proteins against denaturation arising from the high level of urea accumulated in the cells (Coelho-Sampaio et al., 1994). A similar role has been ascribed to BHMT in Rhesus monkey lens (Rao et al., 1998). Using vitamin B12-deficient diets. a decrease in enzyme activity has been observed (Williams et al., 1953), while increases have been detected in low-protein diets combined with alcohol, alcohol liquid diets, high-protein diets and diets supplemented with methionine (Finkelstein et al., 1974). Studies in developing rats have shown that foetal liver activity is lower than in the adult and that a peak is observed in the first 10 d of the neonate (Klee et al., 1961). Moreover, hydrocortisone and tyroxine treatments also affect BHMT activity (Klee et al., 1961). Recently, it has been shown that BHMT regulates the expression of apolipoprotein B and hence an increase in related lipoproteins is observed (Sowden et al., 1999); it has been located bound to tubulin in liver extracts (Sandu et al., 2000).

BHMT from rat liver is an oligomer of identical subunits, each subunit being composed of 406 amino acids (Forestier et al., 1996; Garro, 1996). The protein has been shown to contain Zn2+ (Millian & Garro, 1998) linked to three cysteine residues (217, 299 and 300 according to the human sequence; Breksa & Garro, 1999). One of the mutations detected so far in patients with moderate hyperhomocysteinaemia (G199S) has been suggested to have an important role in the secondary structure of the enzyme (Heil et al., 2000). Gly214 has also been shown to be essential for activity by random mutagenesis (Breksa & Garro, 2002). Recently, crystallization of recombinant human BHMT has been reported (Bose & Momamy, 2001), but the three-dimensional structure of this enzyme remains unknown. Structural studies on BHMT will help to elucidate the catalytic mechanism and the function of this enzyme at the atomic level. This will provide new insights.
into the methionine metabolism and, therefore, in the search for drugs to control the diseases in which the enzyme is involved.

2. Materials and methods

2.1. Expression and purification

Rat-liver RNA was isolated using the procedure described by Too & Maggio (1995) and used to obtain the BHMT cDNA by PCR. For this purpose, oligonucleotides including the NdeI (5'-AGTAGTCAGCATATTGGCCAGATGCCC-3') and BamHI (5'-AGTCAGGATCTTACTGTCGGATATTGATTTTT-3') restriction sites were designed. The amplified sequence was treated with the above-mentioned restriction enzymes and cloned in the pET11a plasmid (Strategene, Santa Clara, CA, USA). This plasmid was used to transform competent Escherichia coli BL21(DE3) cells. Overnight cultures prepared from a single colony were used to inoculate 31 of LB medium plus ampicillin (100 μg ml^{-1}). The cells were grown until absorbance (λ = 600 nm) reached 0.6; a 3 h induction was then started with 0.5 mM IPTG (Ambion, Austin, TX, USA). The bacteria overexpressing BHMT were harvested by centrifugation and kept frozen at 203 K until use.

Cell pellets were dissolved in 30 mM potassium phosphate pH 7.6, 5 mM 2-mercaptoethanol, 2 mM EDTA, 2 μg ml^{-1} aprotinin, 1 μg ml^{-1} peptatin A, 0.5 μg ml^{-1} leupeptin, 2.5 μg ml^{-1} antipain, 0.5 mM benzamidine, 0.1 mM PMSF (Sigma Chemical Co., St Louis, MO, USA). Disruption of the cells was carried out by sonication at 277 K (15 soniﬁer). The soluble fraction was obtained by centrifugation at 100 000 g for 1 h and used for further chromatographic steps. Purification was obtained by using DEAE-Sephacel (Pharmacia, Uppsala, Sweden) and Biogel HTP (Bio-Rad, Hercules, CA, USA) columns as described by Millian & Garrow (1998) with minor modifications. The purified protein was stored in 50% glycerol at 203 K until use.

2.2. Determination of the oligomeric state

Samples of purified BHMT in the concentration range 0.2–0.6 mg ml^{-1} were used for sedimentation-velocity experiments, which were performed at 50 000 rev min^{-1} and 283 K in a Beckman Optima XL-A analytical ultracentrifuge (Beckman Instruments, Inc.) equipped with absorbance optics, using an An50Ti rotor.

Absorbance scans (0.005 cm step size) were taken at 280 nm. The sedimentation-velocity data were analysed with the program SVEDBERG (Phil, 1997). Sedimentation-velocity coefficients were corrected for solvent composition and temperature to obtain s_{20,w} (van Holde, 1986).

2.3. Crystallization

The purified protein was initially concentrated to 6 mg ml^{-1} by ultrafiltration using YM-30 membranes (Amicon, Millipore Corporation, Bedford, MA, USA) in a solution containing 10 mM Tris–HCl pH 8.2, 0.5 mM EDTA and 1 mM 2-mercaptoethanol. All crystallization experiments were carried out at 291 K on Linbro tissue-culture plates using the hanging-drop vapour-diffusion technique. Hanging drops consisted of 1 μl of protein solution and 1 μl of reservoir solution. Initial crystallization conditions were identified using the commercially available Hampton Research Crystal Screens I and II (Janecarik & Kim, 1991). Plate-like crystals were observed in two test solutions: 30% PEG 1500 and 20% PEG 2000 monomethylether pH 8.5, 0.01 M NiCl₂. These conditions were later refined to the final values indicated in Table 1. For heavy-atom derivatization, native crystals were soaked with saturated phenylmercury acetate (PMA) for 24 h or with 10 mM AuK(CN)₂ for 12 h.

2.4. Preparation and analysis of SeMetBHMT crystals

To solve the structure by the MAD method, selenomethionyl BHMT was produced using the same vector and host strain under modified conditions. As E. coli BL21(DE3) is not a methionine auxotroph, the conditions were chosen to exclude methionine from the medium and to provide ample amounts of other amino acids known to inhibit methionine biosynthesis (van Duyne et al., 1993). The purification of the protein was performed following the same protocol as that described for the native enzyme. The incorporation of selenium into selenomethionyl-derivatized protein was assayed by comparison of the mass of this form with that of the native protein using ESI spectrometry. The preparation of BHMT seleno-l-methionine crystals was similar to that used to grow the native crystals.

X-ray fluorescence spectra were measured on station ID14-4 at the ESRF (Grenoble, France) using a single plate-like crystal of SeMetBHMT. Calculation of anomalous scattering factors from the raw fluorescence spectrum was performed using the CHOOCH program (Evans & Pettifer 2001).

2.5. Data collection and processing

Native, SeMetBHMT and heavy-atom derivative crystals were soaked in cryo-protectant solutions containing the precipitant and 20% glycerol before being cooled to 100 K. Diffraction data were collected using synchrotron radiation at the beamlines given in Table 1. The different data sets were processed using the program MOSFLM (Leslie, 1990) and the CCP4 suite (Colla-
3. Results and discussion

Preliminary plate-like crystals were very thin and consequently the diffraction pattern was of very poor quality. Crystallization conditions were refined by varying several parameters, e.g. buffer content, pH, temperature, sample size and the type and concentration of the precipitant agent. Plates appeared after 5–15 d; most of them grew as twinned clusters and only a few drops yielded crystals appropriate for diffraction experiments (Fig. 1). Screening of many additives (Additive Screens, Hampton Research) was also tried but did not improve the crystal quality. Addition of several detergents (CYMAL-5 and nonyl-β-D-glucoside) gave very small needles that did not progress. In the case of SeMetBHMT, plate-like crystals were also obtained, but a new habit was also observed with the same precipitant agents and similar conditions as those used to grow the plate-like crystals (Fig. 2a). Addition of additives was again investigated, leading to small hexagonal crystals (Fig. 2b) with either ATP or spermine in the range 10–100 mM. These hexagonal crystals reach a maximum size of 0.04 mm and failed to diffract using a synchrotron X-ray source. MAD diffraction data were then collected from the plate-like crystals. A typical diffraction pattern for crystals of this habit is shown in Fig. 3. Data were always collected using synchrotron sources, as they showed very poor diffraction using rotating-anode-generated X-rays. Data-collection statistics for native, SeMetBHMT and two heavy-atom derivatives are summarized in Table 1. The space group is P2₁ for all forms. The molecular weight of the monomer is 45 kDa; therefore, assuming two, three or four molecules per asymmetric unit, the Matthews coefficient (Matthews, 1968) would be 4.5, 3.0 or 2.2 Å³ Da⁻¹. We have investigated the local symmetry relating the protomers in the asymmetric unit using POLARRFN (Kabsch, 1976) from the CCP4 package. Several self-rotation functions were computed in the resolution range 15–3 Å, with Patterson vectors up to a radius of integration of 20 Å. Analysis of self-rotation peaks revealed the presence of non-crystallographic twofold symmetry almost along the a and c axes. The stereographic projection (κ = 180° section) of the self-rotation is shown in Fig. 4. On the other hand, the sedimentation-velocity experiments give an estimated molecular weight of 160 kDa, which indicates a tetrameric aggregation state for BHMT. Taking into account these data and the local symmetry, we consider the possibility of the asymmetric unit containing a tetramer with point symmetry 222 and a solvent content of 44% in the cell (Vₐ = 2.2 Å³ Da⁻¹). This tetrameric aggregation of rat-liver BHMT is contradictory to previously reported data obtained by gel-filtration chromatography, where an apparent molecular weight of 270 kDa was calculated (Skiba et al., 1982). Those data were ascribed to the presence of the protein as an hexamer and differences could arise...
from the use of globular protein standards. This tetrameric association of recombinant human BHMT has been described previously (Bose & Momamy, 2001), but in that case the crystal was grown from the protein containing a polyhistidine tag at the N-terminus of the chain.

The X-ray fluorescence spectrum was measured at the selenium $K$ edge for a SeMetBHMT crystal and the anomalous scattering factors were calculated as a function of X-ray energy. This gives an $f^\prime$ (inflection point) and an $f^\prime\prime$ (peak) of $-13.2$ and $10.5$, respectively. Analysis of MAD data is under way using direct and Patterson techniques. The diffraction pattern is very anisotropic, this effect being particularly significant in the case of SeMetBHMT crystals. This leads to poor data in the 3–4 Å resolution shell. As an alternative, data from the two heavy-atom derivatives are also under study.

References


