

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

Beatriz González,^a María A. Pajares,^b Heng-Phon Too,^c Francisco Garrido,^b T. L. Blundell^d and Julia Sanz-Aparicio^{a*}

^aGrupo de Cristalografía Macromolecular y Biología Estructural, Instituto de Química-Física 'Rocasolano', CSIC, Serrano 119, 28006 Madrid, Spain, ^bInstituto de Investigaciones Biomédicas 'Alberto Sols', CSIC-UAM, Arturo Duperier 4, 28029 Madrid, Spain, ^cDepartment of Biochemistry, National University of Singapore, Lower Kent Ridge Crescent, 119260 Singapore, Singapore, and ^dDepartment of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, England

Correspondence e-mail: xjulia@iqfr.csic.es

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 seleniomethionyl-labelled forms. Crystals belong to space group $P2_1$, with unit-cell parameters $a = 57.8$, $b = 149.3$, $c = 96.2$ Å, $\beta = 92.9^\circ$. Data from native, seleniomethionine-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.

Received 11 April 2002

Accepted 14 July 2002

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 (Garrow, 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; Garrow, 1996). The protein has been shown to contain Zn²⁺ (Millian & Garrow, 1998) linked to three cysteine residues (217, 299 and 300 according to the human sequence; Breksa & Garrow, 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 & Garrow, 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 *Nde*I (5'-AGTAGTCAGCA-TATGGCACCGATTGCC-3') and *Bam*HI (5'-AGTCAGGGATCCCTACTGTGCGG-ATTGAATTTT-3') restriction sites were designed. The amplified sequence was treated with the above-mentioned restriction enzymes and cloned in the pET11a plasmid (Stratagene, 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 3 l 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 pepstatin 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 × 30 s pulses at 30 s intervals, output power level 20 in a Branson 250 sonifier). The soluble fraction was obtained by centrifugation at 100 000g for 1 h and used for further chromatographic steps. Purification was obtained by using DEAE-Sepharcel (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.

Table 1
Data-collection statistics.

The space group is *P*2₁ for all crystals. Values in parentheses are for the highest resolution shell.

	Native 1	Native 2	SeMetBHMT (peak)	Hg-BHMT	Au-BHMT
Crystallization conditions	6 mg ml ⁻¹ protein, 16% PEG 1500 pH 8.5	6 mg ml ⁻¹ protein, 14% PEG 2K MME pH 8.5	6 mg ml ⁻¹ protein, 14% PEG 2K MME pH 7.5	6 mg ml ⁻¹ protein, 16% PEG 1500 pH 8.5	6 mg ml ⁻¹ protein, 16% PEG 1500 pH 8.5
Crystal size (mm)	0.10 × 0.05 × 0.01	0.20 × 0.10 × 0.03	0.20 × 0.10 × 0.01	0.20 × 0.10 × 0.02	0.20 × 0.10 × 0.02
Beamline	SRS, Daresbury	BM14, ESRF	ID14-4, ESRF	BM14, ESRF	BM14, ESRF
Wavelength (Å)	1.244	1.004	0.979	1.004	1.004
Unit-cell parameters					
<i>a</i> (Å)	57.95	57.83	58.25	57.98	57.79
<i>b</i> (Å)	149.87	149.27	152.68	143.20	150.20
<i>c</i> (Å)	96.37	96.25	96.32	95.82	96.10
β (°)	93.45	92.92	93.33	91.25	93.12
Resolution (Å)	3.2	2.7	3.0	2.8	2.8
Measurements	205332	375917	235069	370419	463031
Unique data	25079	48142	32340	36669	39591
<i>I</i> /σ(<i>I</i>)	4.5 (1.8)	6.0 (1.8)	5.0 (1.7)	4.6 (1.4)	3.5 (1.4)
Completeness (%)	98.3 (97.9)	99.8 (99.1)	97.0 (97.0)	100.0 (100.0)	97.9 (97.4)
Multiplicity	3.7 (3.7)	4.0 (4.0)	3.2 (2.6)	3.7 (3.7)	3.9 (3.9)
<i>R</i> _{merge} † (%)	12.4 (42.0)	8.0 (40.0)	11.9 (41.7)	9.7 (33.2)	10.4 (51.3)

$$\dagger R_{\text{merge}} = \sum(I - \langle I \rangle) / \sum(I).$$

Absorbance scans (0.005 cm step size) were taken at 280 nm. The sedimentation-velocity data were analysed with the program *SVEDBERG* (Philo, 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 (Jancarik & 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).

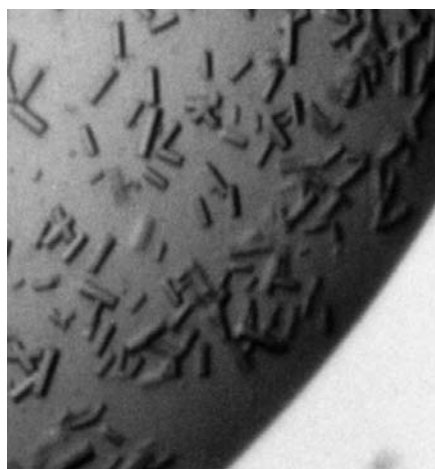
2.5. Data collection and processing

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-

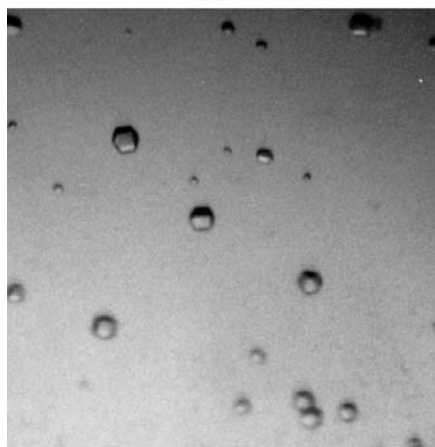
borative Computational Project, Number 4, 1994).



Figure 1
Plate-like crystals of recombinant rat-liver BHMT (habit 1). The maximum dimensions are $0.20 \times 0.10 \times 0.03$ mm.



(a)



(b)

Figure 2
Effect of the addition of ATP or spermine on SeMetBHMT crystals: (a) habit 2 with no additive and (b) habit 3, grown in the same conditions and 50 mM ATP.

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 $P2_1$ 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 $2.2 \text{ \AA}^3 \text{ Da}^{-1}$. 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 \AA , with Patterson vectors up to a radius of integration of 20 \AA . Analysis of self-rotation peaks revealed the presence of non-crystallographic twofold symmetry almost along the *a* and *c* axes. The stereographic projection ($\kappa = 180^\circ$ section) of the self-rotation is

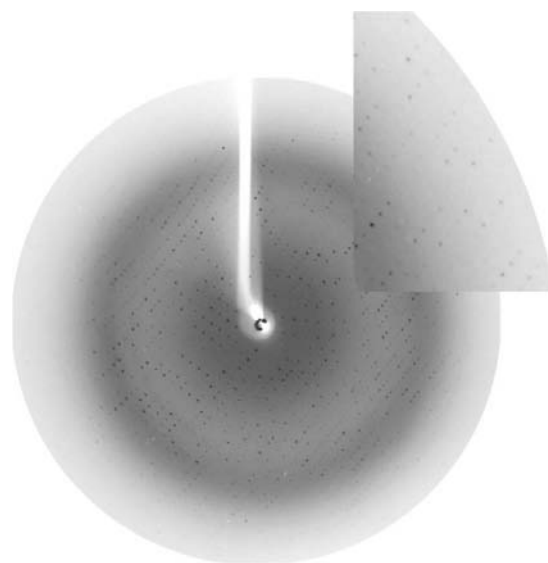


Figure 3
X-ray diffraction pattern from a crystal of native BHMT using a synchrotron source. The outer resolution shell is 2.5 \AA .

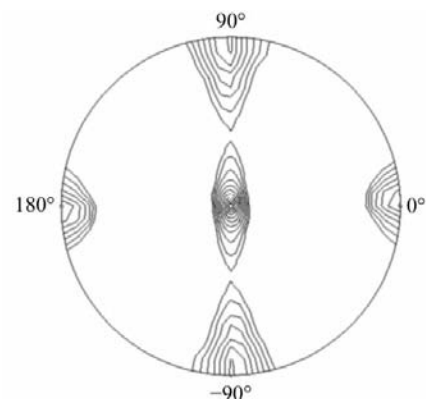


Figure 4
Plot of the self-rotation function of BHMT native crystals using data in the range 15–3 \AA and a 20 \AA radius of integration in the $\kappa = 180^\circ$ section. The view is down the *b* axis. $\varphi = 0$ and 90° correspond to the *a* and *c*^{*} axes, respectively.

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 ($V_M = 2.2 \text{ \AA}^3 \text{ Da}^{-1}$). 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' (inflection point) and an f'' (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 $3\text{--}4$ Å resolution shell. As an alternative, data from the two heavy-atom derivatives are also under study.

References

- Barak, A. J. & Tuma, D. J. (1979). *Lipids*, **14**, 860–863.
- Bose, N. & Momamy, C. (2001). *Acta Cryst.* **D57**, 431–433.
- Breksa, A. P. III & Garrow, T. A. (1999). *Biochemistry*, **38**, 13991–13998.
- Breksa, A. P. III & Garrow, T. A. (2002). *Arch. Biochem. Biophys.* **399**, 73–80.
- Coelho-Sampaio, T., Ferreira, S. T., Castro, E. J. Jr & Vieyra, A. (1994). *Eur. J. Biochem.* **221**, 1103–1110.
- Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* **D50**, 760–763.
- Delgado-Reyes, C. V., Wallig, M. A. & Garrow, T. A. (2001). *Arch. Biochem. Biophys.* **393**, 184–186.
- Duyne, G. D. van, Standaert, R. F., Karplus, P. A., Schreiber, S. L. & Clardy, J. (1993). *J. Mol. Biol.* **276**, 523–530.
- Evans, G. & Pettifer, R. F. (2001). *J. Appl. Cryst.* **34**, 82–86.
- Finkelstein, J. D., Cello, J. P. & Kyle, W. E. (1974). *Biochem. Biophys. Res. Commun.* **61**, 525–531.
- Finkelstein, J. D., Harris, B. J., Martin, J. J. & Kyle, W. E. (1982). *Biochem. Biophys. Res. Commun.* **108**, 344–348.
- Finkelstein, J. D. & Martin, J. J. (1986). *J. Biol. Chem.* **261**, 1582–1587.
- Forestier, M., Reichen, J. & Solioz, M. (1996). *Biochem. Biophys. Res. Commun.* **225**, 377–383.
- Garrow, T. A. (1996). *J. Biol. Chem.* **271**, 22831–22838.
- Heil, S. G., Lievers, K. J., Boers, G. H., Verhoef, P., den Heijer, M., Trijbels, F. J. & Blom, H. J. (2000). *Mol. Genet. Metab.* **71**, 511–519.
- Holde, K. E. van (1986). *Physical Biochemistry*, 2nd ed., pp. 110–136. New Jersey, USA: Prentice-Hall.
- Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.
- Kabsch, W. (1976). *Acta Cryst.* **A32**, 922–923.
- Klee, W. A., Richards, H. H. & Cantoni, G. L. (1961). *Biochim. Biophys. Acta*, **54**, 157–164.
- Leslie, A. G. W. (1990). *Crystallographic Computing*. Oxford University Press.
- Mato, J. M., Alvarez, L., Ortiz, P. & Pajares, M. A. (1997). *Pharmacol. Ther.* **73**, 265–280.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
- Millian, N. S. & Garrow, T. A. (1998). *Arch. Biochem. Biophys.* **356**, 93–98.
- Philo, J. S. (1997). *Biophys. J.* **72**, 435–444.
- Rao, P. V., Garrow, T. A., John, F., Garland, D., Millian, N. S. & Zigler, J. S. Jr (1998). *J. Biol. Chem.* **273**, 30669–30674.
- Sandu, C., Nick, P., Hess, D., Schiltz, E., Garrow, T. A. & Brandsch, R. (2000). *J. Biol. Chem.* **381**, 619–622.
- Skiba, W. E., Taylor, M. P., Wells, M. S., Mangum, J. H. & Awad, W. M. Jr (1982). *J. Biol. Chem.* **257**, 14944–14948.
- Sowden, M. P., Collins, H. L., Smith, H. C., Garrow, T. A., Sparks, J. D. & Sparks, C. E. (1999). *Biochem. J.* **341**, 639–645.
- Too, H. P. & Maggio, J. E. (1995). *Peptides*, **16**, 45–53.
- Williams, J. N. Jr, Monson, W. J., Sreenivasan, A., Dietrich, L. S., Harper, A. E. & Elvehjem, C. A. (1953). *J. Biol. Chem.* **202**, 151–156.