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Crystallization and preliminary X-ray diffraction analysis of brefeldin A-ADP ribosylated substrate (BARS)

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Brefeldin A-ADP ribosylated substrate (BARS) is a newly discovered enzyme involved in membrane fission, catalyzing the formation of phosphatidic acid by transfer of an acyl group from acyl-CoA to lysophosphatidic acid. A truncated form of BARS, lacking the C-terminal segment expected to interact with the Golgi membrane, has been expressed in soluble form in *Escherichia coli*, purified and crystallized. BARS crystals diffract up to 2.5 Å resolution using synchrotron radiation and belong to space group $P6_222/P6_422$, with unit-cell parameters $a = b = 89.2$, $c = 162.6$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$ and one molecule (39.5 kDa) per asymmetric unit. SeMet-substituted BARS has been crystallized under growth conditions very similar to those of the native protein.

1. Introduction

BARS (brefeldin A-ADP ribosylated substrate; a 50 kDa cytosolic protein) was initially identified as the substrate of brefeldin A (BFA) dependent ADP-ribosylation (De Matteis *et al.*, 1994; Di Girolamo *et al.*, 1995). BFA, a fungal toxin, was recognized as a potent inhibitor of membrane traffic, causing rapid tranformation of the Golgi stack into tubular structures. BARS was later shown to counteract the Golgi tubulation induced by BFA in permeabilized cells, indicating, together with other lines of evidence, its likely role as a regulator of the Golgi complex structure and functionality (Mironov *et al.*, 1997; Spanò *et al.*, 1999). Indeed, more recently, BARS was identified as an essential component of the Golgi tubule fission-inducing activity normally present in the cytosol (Weigert *et al.*, 1999). When pure BARS is added to isolated Golgi membranes together with palmitoyl-CoA, it induces the formation of strongly constricted segments in Golgi tubules, referred to as fission intermediates. Constriction is then followed by fragmentation of the tubules. BARS fission-inducing activity is most likely to be related to the enzymatic conversion of lysophosphatidic acid into phosphatidic acid using acyl-CoA as acyl-group donor. Such activity leads to accumulation/localization of phosphatidic acid in the membrane, a process expected to promote a local deformation of the phospholipid bilayer, thus facilitating membrane fission. BARS lysophosphatidic acid acyl transferase activity was thus proposed to be essential in mediating fission of Golgi tubules, a crucial step in intracellular traffic (Weigert *et al.*, 1999).

BARS shows a striking sequence identity (>75%) to members of the C-terminal binding protein (CtBP) family (CtBP1 and CtBP2, Spanò *et al.*, 1999), which have been shown to act as transcription regulators (Schaeper *et al.*, 1995; Turner & Crossley, 1998; Criqui-Filipe *et al.*, 1999). Thus, BARS, alternatively referred to as CtBP3/BARS, might play a dual role, interconnecting entirely different cellular functions.

In view of a crystallographic investigation shedding first light on BARS structure and its activity towards membrane lipids, we present here the expression, purification, crystallization and preliminary X-ray diffraction analysis of the rat extra-membrane soluble BARS fragment (t-BARS, residues 1–350) in its native and selenomethionyl forms.

2. Materials and methods

2.1. Expression and purification

t-BARS, bearing a poly-His tag at its N-terminus, was expressed in *E. coli*. The region of BARS cDNA coding for amino acids 1–350 (Spanò *et al.*, 1999; Genbank accession number AF067795) was amplified by PCR with the following primers: 5′-GCTCATATGTCAGGCGTCCGACCTC-3′ (forward primer) and 5′-GCGTGATCACTAGTGGGTGGCAGCTGTC-3′ (reverse primer). It was then subcloned into a *Nde*I/*Bcl*I-digested pET11d-His vector generated from the pET11d vector (Novagen) by insertion of a sequence encoding

the initiator Met, a Gly and six His residues just upstream of the *Nde*I restriction site (Nuoffer *et al.*, 1994). The resulting pET11d-His-t-BARS plasmid was transformed in *E. coli* strain BL21(DE3)pLysS; the bacteria were grown in Luria–Bertani medium until they reached an OD_{600nm} of 0.6 and were induced by the addition of 0.4 m*M* IPTG for 2 h at 310 K. The protein was then purified on an Ni–NTA matrix (Quiagen) according to the manufacturer's instructions, with slight modification of the lysis, washing and elution buffers. The eluted protein was collected in vials containing EDTA (1 m*M* final concentration), concentrated in a Microcon device (Amicon) to 10 mg ml^{-1} and dialyzed twice against 1000× volumes of 5 m*M* Tris–HCl, 300 m*M* NaCl, 20% glycerol, 10 m*M* β-mercaptoethanol, 1 m*M* EDTA, 250 m*M* imidazole pH 8.0.

Selenomethionyl His-t-BARS was obtained by transforming pET11d-His-t-BARS plasmid in the *met*$^-$ *E. coli* strain B834(DE3) (Ramakrishnan *et al.*, 1993), growing bacteria in M9 minimal medium supplemented with amino acids and vitamins (18.7 m*M* NH_4Cl, 22 m*M* KH_2PO_4, 48.8 m*M* Na_2HPO_4, 2 m*M* $MgSO_4$, 0.1 m*M* $FeSO_4$, 0.4% glucose, 0.04 mg ml^{-1} of each amino acid except methionine, 0.04 mg ml^{-1} Se-L-Met and 1 µg ml^{-1} of each of the following vitamins: riboflavin, niacinamide, pyridoxine monohydrochloride and thiamine). Expression, purification and concentration were performed as described for the methionyl protein. The incorporation of Se-L-Met was checked by mass-spectrometry analysis performed on a Kratos Compact MALDI 4 (Manchester, England) mass spectrometer, in a mass range including the expected molecular masses.

2.2. Crystal growth

Crystallization experiments on native t-BARS were performed using the hanging-drop vapour-diffusion method. Preliminary crystallization conditions were established using Hampton Research Crystal Screen kits I and II (Jancarik & Kim, 1991) at 294 K. Each drop was composed of equal volumes (1.5 µl) of protein solution and reservoir solution. The protein concentration was 10 mg ml^{-1} before mixing. The most promising initial crystallization conditions were refined through variation of the protein concentration, precipitant, temperature, pH, drop and reservoir volume. Crystals of SeMet-substituted protein were subsequently grown by the hanging-drop vapour-diffusion method under similar conditions to the native protein.

Native t-BARS crystals were also grown under microgravity conditions on the International Space Station (ISS mission 6A, April 19–August 22, 2001) using a vapour-diffusion crystal-growth apparatus (high-density protein crystal-growth system; HDPCG), stored in a modified commercial incubator/refrigerator module (CRIM-M) at 295 K, where crystallization experiments were activated one week after arrival on the ISS.

2.3. Data collection and processing

All diffraction data were collected from crystals cooled to 100 K in a cold nitrogen stream. Two full data sets from t-BARS crystals grown on Earth and on the ISS were collected at the XRD1 beamline of the Elettra synchrotron source, Trieste, Italy, using a MAR CCD detector. SeMet-substituted t-BARS crystals were tested on the BM14 beamline at ESRF, Grenoble, France, using a MAR CCD detector. All data were processed using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997) and programs from the *CCP*4 package (Collaborative Computational Program, Number 4, 1994).

3. Results and discussion

t-BARS, lacking 80 C-terminal residues, was expressed in *E. coli*, purified and crystallized. The site delimiting the mostly hydrophobic C-terminal segment was estimated based on the sequence homology (∼22% residue identity) found between BARS and D-3-phosphoglycerate dehydrogenase (Schuller *et al.*, 1995). The construct yielded a soluble truncated protein of 39.5 kDa (including the Met-Gly-His_6 N-terminal tag), suitable for crystallization purposes.

Small preliminary t-BARS crystals were obtained using reagents 32 and 33 from Hampton Research Crystal Screen kit II (Jancarik & Kim, 1991). After refinement, the best crystallization condition was established as 1.8–2.1 *M* ammonium formate, 100 m*M* HEPES buffer pH 7.5; crystals of bipyramidal shape and typical dimensions of 0.2 × 0.1 × 0.1 mm grew in about one week (Fig. 1). The crystals were stored in a stabilizing solution containing 2.0–2.3 *M* ammonium formate, 100 m*M* HEPES buffer pH 7.5 at room temperature and were transferred to the same solution supplemented with 30%(*v*/*v*) glycerol immediately prior to data collection at 100 K.

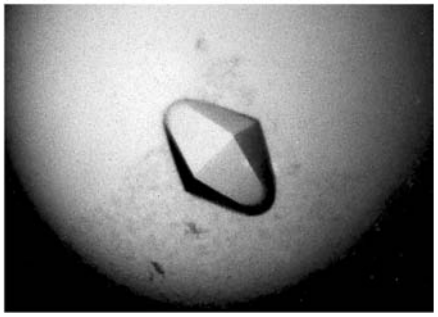

Figure 1
Crystal of recombinant t-BARS from rat grown under Earth gravity. The crystal dimensions are approximately 0.2 × 0.1 × 0.1 mm.

Table 1
Data-collection statistics for t-BARS crystals.

Values in parentheses are for the highest resolution shell (2.54–2.50 Å).

	Earth native crystal	ISS native crystal
X-ray source	ELETTRA	ELETTRA
Wavelength (Å)	1.00	1.00
Sample temperature (K)	100	100
Detector	MAR CCD	MAR CCD
Resolution range (Å)	40–2.5	40–2.5
Unit-cell parameters		
$a = b$ (Å)	89.2	89.5
c (Å)	162.6	162.2
$\alpha = \beta$ (°)	90	90
γ (°)	120	120
Space group	$P6_222/P6_422$	$P6_222/P6_422$
No. of observed reflections	108329	112880
No. of unique reflections	13942	13971
Mosaicity (°)	0.74	0.71
Completeness (%)	99.3 (100)	99.0 (99.1)
Multiplicity	7.8	8.1
Average $I/\sigma(I)$	37.0 (5.5)	39.7 (5.9)
R_{merge}† (%)	4.1 (39.7)	3.9 (31.0)

† $R_{merge} = \sum_h \sum_i |I_{hi} - \langle I_h \rangle| / \sum_h \sum_i I_{hi}$.

The crystals diffracted to 2.5 Å resolution using synchrotron radiation (XRD1 beamline, Elettra synchrotron, Trieste, Italy). Inspection of the diffraction pattern and systematic absences allowed the assignment of the t-BARS crystals to the hexagonal space group $P6_222/P6_422$, with unit-cell parameters $a = b = 89.2$, $c = 162.6$ Å (Table 1). Evaluation of the crystal-packing parameter (Matthews, 1968) indicates that the lattice can accommodate one molecule per asymmetric unit ($V_M = 2.37$ Å^3 Da^{-1}), with an estimated solvent content of 48%.

Improvement of the t-BARS crystal quality was attempted through crystallization under microgravity conditions on the ISS using the HDPCG apparatus. Out of 12 crystallization trials (1.6–2.7 *M* ammonium formate in 0.1 *M* steps, 100 m*M* HEPES buffer pH 7.5), three wells showed crystals, with one drop (2.7 *M* ammonium formate, 100 m*M* HEPES buffer pH 7.5) providing crystals of size comparable to those grown on Earth. Microgravity-grown crystals diffract to 2.5 Å resolution using

synchrotron radiation (XRD1 beamline, Elettra synchrotron, Trieste, Italy) and are isomorphous with those grown in gravity (Table 1). Comparison of diffraction data collected under identical experimental setups on crystals of almost identical size (0.2 × 0.1 × 0.1 mm) shows a modest decrease of mosaicity for the microgravity crystals. Although they do not diffract to higher resolution, they display slightly better $I/\sigma(I)$ and $R_{\rm merge}$ values in almost all resolution shells relative to Earth-grown crystals (Table 1).

Low sequence homology with proteins of known three-dimensional structure and the presence of seven Met residues suggested multiwavelength anomalous diffraction (MAD) on Se atoms as the method for t-BARS structure analysis. Expression and purification of the SeMet-substituted t-BARS were performed as described for the native protein. According to the mass-spectrometric analysis, the molecular mass of the SeMet-substituted t-BARS is 334 Da higher than that of native t-BARS, close to the expected value of 328 Da corresponding to seven S→Se atom substitutions.

Crystals of the SeMet-substituted protein could be grown under conditions similar to those of native t-BARS crystals, substituting the ammonium formate precipitant with sodium formate and adding 10 m*M* β-mercaptoethanol. A preliminary diffraction test using synchrotron radiation at the BM14 beamline, ESRF, Grenoble, France, showed that SeMet-substituted t-BARS crystals diffract to 3.2 Å resolution. They belong to space group $P6_222/P6_422$, with unit-cell parameters $a = b = 86.9$, $c = 163.0$ Å and one molecule per asymmetric unit.

The crystal structure determination of t-BARS based on MAD (SeMet) methods is currently under way.

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