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Preliminary X-ray crystallographic studies of yeast mitochondrial protein Tom70p

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Protein translocations across mitochondrial membranes play critical roles in mitochondrion biogenesis. Protein transport from the cell cytosol to the mitochondrial matrix is carried out by the translocase of the outer membrane (TOM) complex and the translocase of the inner membrane (TIM) complexes. Tom70p is an important TOM-complex member and a major surface receptor of the protein-translocation machinery in the outer mitochondrial membrane. To investigate the mechanism by which Tom70p functions to deliver the mitochondrial protein precursors, the cytosolic fragment of yeast Tom70p (cTom70p) was crystallized. The crystals diffract to 3.2 Å using a synchrotron X-ray source and belong to space group $P2_1$, with unit-cell parameters $a = 44.89$, $b = 168.78$, $c = 83.41$ Å, $\alpha = 90.00$, $\beta = 102.74$, $\gamma = 90.00^\circ$. There are two Tom70p molecules in one asymmetric unit, which corresponds to a solvent content of approximately 51%. Structure determination by MAD methods is under way.

1. Introduction

The mitochondrion plays critical roles in cell physiology. In addition to its exclusive role in cellular respiration, the mitochondrion is involved in a number of important cellular processes, including the synthesis of metabolites, lipid metabolism, free-radical production and metal-ion homeostasis. The mitochondrion is a double-membrane organelle with an outer and inner membrane with the intermembrane space (IMS) between them. The mitochondrion contains a large number of proteins, possibly between 600 and 1000 proteins (Sickmann *et al.*, 2003). The mitochondrion has its own small genome that codes for a small number of proteins depending on the organism (Gray *et al.*, 1999). Therefore, most of the mitochondrial proteins are synthesized in the cytosol and imported into the mitochondrion.

The mitochondrial protein precursors possess specific targeting and sorting information to reach the correct locations. Most mitochondrial protein precursors contain an N-terminal targeting sequence (Gray *et al.*, 1999; Neupert, 1997; Neupert & Brunner, 2002; Pfanner, 2000). The targeting sequence may form a short helix which is positively charged on one side and hydrophobic on the other side. In contrast, some mitochondrial membrane proteins of the inner and outer membrane lack the N-terminal targeting sequence, but instead contain sorting and targeting information within the mature protein (Rehling *et al.*, 2003).

The mitochondrion has developed an elaborate set of translocons for protein-precursor transport into the mitochondrial matrix. The outer mitochondrial membrane contains a single translocase of the outer membrane (TOM) for the passage of polypeptides, while the inner membrane contains two translocases of the inner membrane: TIM23 and TIM22 (Koehler, 2004; Neupert & Brunner, 2002; Pfanner, 2000). The translocase of the outer mitochondrial membrane (TOM) includes two surface receptors: Tom20p and Tom70p (Sollner *et al.*, 1989, 1990). Tom20 recognizes classical N-terminal mitochondrial targeting sequences from the preproteins. However, Tom70p binds to internal targeting sequences of the preproteins, such as those in the multi-transmembrane carrier proteins of the inner mitochondrial membrane (Brix *et al.*, 1997; Sollner *et al.*, 1989, 1990). Protein precursors are then transferred from the receptors to the common Tom22/Tom40 translocation pore

Table 1
Statistics of the data set from the cTom70p crystals.

Resolution shells (Å)	$I/\sigma(I)$	R_{sym}†	Completeness (%)	Redundancy
50.00–6.38	49.1	0.056	92.2	4.1
6.38–5.07	27.1	0.088	94.9	4.1
5.07–4.43	24.1	0.080	95.1	4.0
4.43–4.03	18.2	0.096	95.5	3.8
4.03–3.74	10.8	0.145	95.1	3.4
3.74–3.52	6.1	0.218	94.4	2.8
3.52–3.35	3.5	0.275	89.8	2.1
3.35–3.20	2.0	0.366	62.7	1.5
Overall	16.3	0.080	90.0	3.3

† $R_{sym} = (\sum_{hkl} \sum_i |I_i - \langle I \rangle|)/\sum_{hkl} \langle I \rangle$.

within the TOM complex and are further sorted by the translocases of the inner membrane (TIM; Pfanner & Geissler, 2001).

It has been suggested that Tom70p is not only a preprotein receptor but also a co-chaperone that facilitates Hsp70/Hsp90 in the targeting of the preproteins to mitochondria (Young *et al.*, 2003). Many mitochondrial protein precursors are unusually hydrophobic (Claros *et al.*, 1995). Molecular chaperones such as Hsp70 and Hsp90 can protect these preproteins from aggregation during the translocation from ribosomes to mitochondria (Beddoe & Lithgow, 2002). Direct cooperation has been implicated between the cytosolic chaperones Hsp70 (yeast and mammals), Hsp90 (mammals) and Tom70p tetratricopeptide repeats (TPR) (Young *et al.*, 2003). Therefore, Tom70p may function as a co-chaperone to assure the efficient preprotein transfer from the molecular chaperones to the TOM complex for translocation (Wiedemann *et al.*, 2001; Brix *et al.*, 2000).

2. Experiments and discussion

2.1. Cloning, expression and purification of Tom70p

The gene encoding the *Saccharomyces cerevisia* Tom70p cytosolic fragment (residues 39–617; cTom70p) was amplified by PCR using the yeast genome as the template. The PCR product was digested by the restriction enzymes *Nde*I and *Bam*HI. The digested PCR product was then ligated into pET28b by T4 ligase. The cTom70 sequence was confirmed by DNA sequencing. The plasmid was then transformed into *Escherichia coli* strain BL21 (DE3) for protein expression.

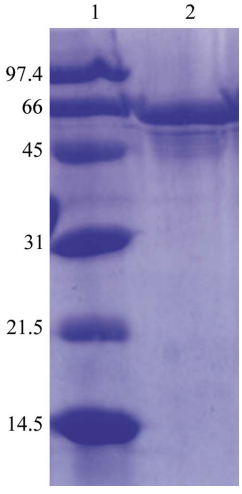

Figure 1
A 13% SDS–PAGE gel of purified cTom70p. Lane 1 contains the protein standards (molecular weights marked in kDa). Lane 2 contains purified yeast cTom70p.

The *E. coli* cells were harvested 20 h after induction at 291 K. Because the recombinant cTom70p was histidine-tagged, it could be relatively easily purified using a metal-chelating column. The supernatant was pumped through an Ni-charged column containing about 10 ml resin. The column was thoroughly washed with 50 m*M* Tris buffer pH 7.9, 0.5 *M* NaCl and 50 m*M* imidazole to remove contaminating proteins. The bound protein was then eluted with 50 m*M* Tris buffer pH 7.9, 0.5 *M* NaCl and 200 m*M* imidazole. After Ni-column purification, the N-terminal histidine tag of Tom70 was released by thrombin treatment. The recombinant cTom70p was further purified on a Superdex 200 gel-filtration column (Pharmacia) mounted on an AKTA HPLC system (Pharmacia) to remove thrombin and digested peptides. The apparent molecular weight of cTom70p was shown to be about 120 kDa based on the protein elution time from the gel-filtration column, indicating that cTom70p forms a dimer in solution. The typical yield of purified soluble cTom70p from 1 l of culture is ∼20 mg (Fig. 1).

2.2. Crystallization, data collection and processing

The cTom70p protein was concentrated to 20 mg ml^{-1} in 10 m*M* MES buffer pH 6.2, 150 m*M* NaCl, 1 m*M* DTT and subjected to crystallization trials. The hanging-drop vapor-diffusion method was used for the crystallization trials. 2 µl protein solution was mixed with 2 µl mother liquor to constitute the hanging drop. Large rod-shaped crystals (0.5 × 0.2 × 0.2 mm) were finally obtained by the hanging-drop vapor-diffusion method using Linbro plates at room temperature. The well solution consisted of 1 ml 100 m*M* MES buffer pH 6.0, 30% PEG 4K, 0.2 *M* ammonium acetate. The cTom70p crystals grew to full size within 2 d. The mass spectrum of the dissolved crystals indicated that the crystals contained the Tom70p cytosolic fragment (39–617) protein.

Diffraction data were collected on the SER-CAT beamline at the APS. The crystal was flash-frozen at 100 K in a nitrogen-gas stream in a cryoprotectant consisting of 100 m*M* MES buffer pH 6.0, 30% PEG 4K, 0.2 *M* ammonium acetate and 20% ethylene glycol. The crystals were soaked in the cryoprotectant for about 30 s before being transferred to the cold stream. The cTom70p crystals still decayed quickly even after freezing. Typically, about 100 images of data could be obtained from one single crystal.

The cTom70p crystals diffracted X-rays to 3.2 Å using SER-CAT beamline 22-ID. The wavelength was set to 1.0 Å. The data were collected using a MAR300 CCD detector. During data collection, the crystal-to-detector distance was kept at 380 mm. 90 images covering an oscillation range of 90° were collected and processed using *HKL*2000 (Otwinowski & Minor, 1997). The crystals belong to space group $P2_1$, with unit-cell parameters $a = 44.89$, $b = 168.78$, $c = 83.41$ Å, $\alpha = 90.00$, $\beta = 102.74$, $\gamma = 90.00°$. The R_{sym} of the data set is 8.0%. The details of the data set are shown in Table 1.

3. Discussion

Tom70p is an important mitochondrial protein for protein translocation and is one of the two major surface receptors of the translocase of the outer membrane (TOM) complex. The mechanisms by which Tom70p interacts with preproteins are currently unknown. The crystal structure of Tom70p is critically required in order to understand the molecular basis of the function of Tom70p. In this study, yeast cTom70p has been purified and crystallized. The cTom70p crystals diffracted X-rays to 3.2 Å at the SER-CAT beamline at the APS. We propose to determine the cTom70p crystal structure using the MAD method. SeMet cTom70p has been produced and the

incorporation of selenium was confirmed by mass-spectrometric studies. We are currently working on the crystallization of SeMet cTom70p.

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