Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Preliminary crystallographic studies of yeast mitochondrial peripheral membrane protein Tim44p

Ratnakar Josyula,[a] Zhongmin Jin,[b] Deborah McCombs,[c] Lawrence DeLucas[c] and Bingdong Sha[a]*

[a]Department of Cell Biology, Center for Biophysical Sciences and Engineering, University of Alabama at Birmingham, USA, [b]SER-CAT, APS, Argonne National Laboratory, 9700 South Cass Avenue, Argonne, IL 60439, USA, and [c]Center for Biophysical Sciences and Engineering, University of Alabama at Birmingham, USA

Correspondence e-mail: bdsha@uab.edu

Received 23 November 2005
Accepted 17 January 2006

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. Tim44p is an essential mitochondrial peripheral membrane protein and a major component of the TIM23 translocon. To investigate the mechanism by which Tim44p functions in the TIM23 translocon to deliver the mitochondrial protein precursors, the yeast Tim44p was crystallized. The crystals diffract to 3.2 Å using a synchrotron X-ray source and belong to space group $P6_322$, with unit-cell parameters a = 124.25, c = 77.83 Å. There is one Tim44p molecule in one asymmetric unit, which corresponds to a solvent content of approximately 43%. 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 an 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 few proteins: between three and 32, depending on the organism (Gray *et al.*, 1999). Therefore, most mitochondrial proteins are translated 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. However, 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). Imported protein precursors pass through the TOM complex to the intermembrane space, where the precursors can take one of the following two routes. Most precursors destined for the mitochondrial matrix typically contain an N-terminal targeting peptide signal that is recognized by the TIM23 complex (Neupert, 1997; Neupert & Brunner, 2002). Mitochondrial protein precursors that do not contain the N-terminal targeting signals will be trans-

Table 1
The statistics of the data set from the native Tim44p crystals.

Resolution shells (Å)	$I/\sigma(I)$	R_{sym}	Completeness (%)	Redundancy
50.00–6.46	67.7	0.031	93.2	12.0
6.46–5.13	71.2	0.053	92.1	11.9
5.13–4.48	34.7	0.070	92.0	12.0
4.48–4.07	22.1	0.098	90.8	11.1
4.07–3.78	11.8	0.130	90.1	8.8
3.78–3.56	8.3	0.148	88.9	5.9
3.56–3.38	7.7	0.213	90.9	4.1
3.38–3.20	3.8	0.363	82.2	2.8
Overall	15.7	0.062	90.3	9.7

ported by the TIM22 complex, the second and functionally distinct complex in the inner membrane (Kaldi & Neupert, 1998; Pfanner, 1998).

The yeast essential protein Tim23p forms the protein-translocation channel in the TIM23 translocon. The N-terminal half of Tim23p (residues 1–98) forms a soluble domain in the intermembrane space, while the C-terminal half of Tim23p forms the transmembrane channel (Truscott *et al.*, 2001). The C-terminal domain of Tim23p may interact with Tim44p at the matrix side to recruit mHsp70 to facilitate the protein translocation (Krimmer *et al.*, 2000; Moro *et al.*, 2002; Strub *et al.*, 2002).

Tim44p is an essential yeast protein and a major component of the TIM23 translocon. Tim44p can interact and form a complex with Tim23p *in vivo*. Initiation of translocation across the inner membrane depends on the membrane potential ψ, which is negative on the matrix side. For the completion of translocation, Tim44p recruits a fraction of the mHsp70 to the import channel and mHsp70 binds to the precursor in transit (Krimmer *et al.*, 2000; Moro *et al.*, 2002; Strub *et al.*, 2002). The binding of mHsp70 may promote the import of the mitochondrial proteins (Krimmer *et al.*, 2000; Liu *et al.*, 2003; Moro *et al.*, 2002; Neupert & Brunner, 2002; Strub *et al.*, 2002). Tim44p may function as the adaptor for the TIM23 translocon to recruit mHsp70. More importantly, Tim44p may play an important regulatory role in concerting the conformational changes between the ATPase domain and the peptide-binding domain within mHsp70. The mHsp70 conformational changes may provide the driving force for the protein translocations (D'Silva *et al.*, 2004; Liu *et al.*, 2003).

Tim44p is a peripheral membrane protein and does not contain any transmembrane domain (Weiss *et al.*, 1999; Strub *et al.*, 2002). Tim44p is stably associated with the inner membrane and is mainly exposed at the matrix side (Strub *et al.*, 2002). Tim44p contains a stable C-terminal domain which is responsible for penetrating the monolayer of the membrane. Tim44p prefers to bind negatively charged synthetic phospholipid vesicles (Weiss *et al.*, 1999). However, high salt concentrations do not affect the interactions between Tim44p and the membranes. These observations demonstrated that Tim44p may utilize hydrophobic interactions as well as charge–charge interactions to bind membranes (Weiss *et al.*, 1999). Tim44p does not have any sequence homology with other peripheral membrane proteins, which indicates that the mechanisms by which Tim44p interacts with the membrane may be novel.

2. Experimental and discussion

2.1. Cloning, expression and purification of Tim44p

The gene encoding *Saccharomyces cerevisiae* Tim44p was amplified by PCR using the yeast genome as the template. The PCR product was digested by the restriction enzymes *Nde*I and *Hin*dIII. The digested PCR product was then ligated into pET28b by T4 ligase. The Tim44p sequence was confirmed by DNA sequencing. The plasmid was then transformed into *Escherichia coli* strain BL21(DE3) for protein expression.

10 ml LB medium with 30 µg ml^{-1} kanamycin was inoculated using the transformed *E. coli* stock. The cells were allowed to grow at 310 K in a shaker for 12 h. The 10 ml LB medium was then used to inoculate 1 l LB medium with 30 µg ml^{-1} kanamycin. 0.5 ml 1 *M* IPTG was added to 1 l medium to induce protein expression when the OD_{600} of the medium reached 0.6. The cells were harvested 3 h after induction. The *E. coli* cells from 1 l of medium were pelleted by centrifugation and resuspended in 100 ml 100 m*M* Tris buffer pH 7.9, 0.5 *M* NaCl. The cells were lysed by sonication at 277 K. Since the Tim44p was histidine-tagged, it could be relatively easily purified using a metal-chelating column. After nickel-column purification, the N-terminal histidine tag of Tim44p was then digested by thrombin treatment. One unit of thrombin (Sigma) was utilized per milligram of Tim44p protein. Digestion took place for 12 h at room temperature and was stopped by the addition of 0.2 m*M* PMSF. The recombinant Tim44p 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 the Tim44p was shown to be about 40 kDa based on the protein elution time from the gel-filtration column, indicating that the Tim44p forms a monomer in solution. The typical yield of soluble Tim44p (∼95% pure from SDS–PAGE analysis) from 1 l of culture is ∼15 mg.

2.2. Crystallization, data collection and processing

The Tim44p protein was concentrated to 15 mg ml^{-1} in 10 m*M* HEPES buffer pH 7.2, 150 m*M* NaCl 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. We started our crystallization screening by using Hampton Crystal Screens I and II and screening kits from Molecular Dimensions Inc. (Structure Screens 1 and 2). Small hexagonal-shaped crystals were observed from condition No. 36 of Hampton Crystal Screen II, which contains 0.1 *M* HEPES pH 7.5, 4.3 *M* NaCl. No other conditions produced crystals. After crystallization optimization, large hexagonal-shaped crystals (0.5 × 0.5 × 0.1 mm) were obtained. The well solution consisted of 1 ml 100 m*M* Tris buffer pH 7.5, 4.1 *M* NaCl. The Tim44p crystals grew to full size within 2 d. The mass spectrum of the dissolved crystals indicated that the crystals contained full-length Tim44p protein.

The Tim44p crystals were then taken to beamline SER-CAT at APS for data collection. The crystals were sensitive to X-ray radiation and so were frozen. The crystal was flash-cooled at 100 K in a nitrogen-gas stream in a cryoprotectant consisting of 100 m*M* Tris buffer pH 7.5, 4.1 *M* NaCl and 20% ethylene glycol. The crystals were soaked in the cryoprotectant for about 30 s before being transferred to the cold stream. The crystals decayed quickly even after flash-cooling. Typically, about 50 images could be collected from one single crystal.

The Tim44p crystals diffracted X-rays to 3.2 Å using beamline 22-ID at SER-CAT. The wavelength was set at 1.0 Å. The data were collected by use of a MAR300 CCD detector. During data collection, the crystal-to-detector distance was kept at 380 mm. The oscillation angle for the crystal was 1.0°. 50 images were collected and processed using *HKL*2000 (Otwinowski & Minor, 1997). The data set revealed that the crystals belong to space group $P6_322$, with unit-cell parameters $a = 124.25$, $c = 77.83$ Å. The R_{sym} of the data set was 6.2%. The details of the data set are shown in Table 1. Crystal analysis shows

Table 2
Statistics from MAD data collection of SeMet Tim44p crystals (using three different crystals).

	Peak	Edge	Remote
Wavelength (Å)	0.9789	0.9793	0.9743
Resolution (Å)	3.2	3.2	3.2
R_{sym}	0.059 (0.330)	0.065 (0.372)	0.066 (0.355)
$I/\sigma(I)$	18.2 (4.2)	15.5 (2.9)	16.7 (2.8)
Completeness (%)	92.1 (83.2)	88.9 (73.5)	91.6 (81.2)
Redundancy	10.1 (2.9)	9.2 (2.3)	9.6 (2.6)

that one asymmetric unit contains one Tim44p molecule, which corresponds to a solvent content of 43% (V_M = 2.16 Å^3 Da^{-1}; Matthews, 1968).

We propose to determine the Tim44p crystal structure by the MAD or SAD method. SeMet Tim44p has been produced. The incorporation of Se was confirmed by mass-spectrometric studies of the SeMet Tim44p protein. The SeMet protein can be crystallized using similar conditions to those for the native proteins. SeMet Tim44p crystals were taken to beamline 22-ID at SER-CAT for MAD data collection and diffracted X-rays to 3.2 Å. Analysis of the MAD data set using the program *SOLVE* clearly revealed three of the possible four Se atoms in the protein molecule (Terwilliger & Berendzen, 1999). The SeMet Tim44p crystals decayed as quickly as the native Tim44p crystals and we could only collect a single-wavelength data set from a single crystal. The statistics of a MAD data set collected from three different crystals are listed in Table 2. We are currently trying to determine the crystal structure by the SAD method using the peak data set or by the MAD method using the three data sets collected at peak, edge and remote wavelengths.

We are grateful to the staff scientists at the APS SER-CAT beamline for their help in data collection. This work was supported by grants from NIH (R01 DK56203 and R01 GM65959) and NASA.

References

D'Silva, P., Liu, Q., Walter, W. & Craig, E. A. (2004). *Nature Struct. Mol. Biol.* **11**, 1084–1091.
Gray, M. W., Burger, G. & Lang, B. F. (1999). *Science*, **283**, 1476–1481.
Kaldi, K. & Neupert, W. (1998). *Biofactors*, **8**, 221–224.
Koehler, C. M. (2004). *Annu. Rev. Cell Dev. Biol.* **20**, 309–335.
Krimmer, T., Rassow, J., Kunau, W. H., Voos, W. & Pfanner, N. (2000). *Mol. Cell Biol.* **20**, 5879–5887.
Liu, Q., D'Silva, P., Walter, W., Marszalek, J. & Craig, E. A. (2003). *Science*, **300**, 139–141.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Moro, F., Okamoto, K., Donzeau, M., Neupert, W. & Brunner, M. (2002). *J. Biol. Chem.* **277**, 6874–6880.
Neupert, W. (1997). *Annu. Rev. Biochem.* **66**, 863–917.
Neupert, W. & Brunner, M. (2002). *Nature Rev. Mol. Cell Biol.* **3**, 555–565.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Pfanner, N. (1998). *Curr. Biol.* **8**, R262–R265.
Pfanner, N. (2000). *Curr. Biol.* **10**, R412–R415.
Rehling, P., Model, K., Brandner, K., Kovermann, P., Sickmann, A., Meyer, H. E., Kuhlbrandt, W., Wagner, R., Truscott, K. N. & Pfanner, N. (2003). *Science*, **299**, 1747–1751.
Sickmann, A., Reinders, J., Wagner, Y., Joppich, C., Zahedi, R., Meyer, H. E., Schonfisch, B., Perschil, I., Chacinska, A., Guiard, B., Rehling, P., Pfanner, N. & Meisinger, C. (2003). *Proc. Natl Acad. Sci. USA*, **100**, 13207–13212.
Strub, A., Rottgers, K. & Voos, W. (2002). *EMBO J.* **21**, 2626–2635.
Terwilliger, T. C. & Berendzen, J. (1999). *Acta Cryst.* D**55**, 849–861.
Truscott, K. N., Kovermann, P., Geissler, A., Merlin, A., Meijer, M., Driessen, A. J., Rassow, J., Pfanner, N. & Wagner, R. (2001). *Nature Struct. Biol.* **8**, 1074–1082.
Weiss, C., Oppliger, W., Vergeres, G., Demel, R., Jeno, P., Horst, M., de Kruijff, B., Schatz, G. & Azem, A. (1999). *Proc. Natl Acad. Sci. USA*, **96**, 8890–8894.