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Isolation, purification and preliminary X-ray characterization of Cpn60-2 (65 kDa heat-shock protein) from *Mycobacterium tuberculosis*

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Cpn60-2 is a member of a unique family of putative molecular chaperones homologous to GroEL (Cpn60) but of unknown function and found only in *Mycobacterium tuberculosis* and closely related species. Cpn60-2 has mainly been studied for its strong immunogenity. Here, the purification, crystallization and preliminary crystallographic analysis of *M. tuberculosis* Cpn60-2 are reported. The crystals belong to space group *P*2, with unit-cell parameters $a = 57$, $b = 115.5$, $c = 81.5$ Å, $\beta = 95.5^\circ$, and contain a dimer in the asymmetric unit. The crystals diffract to 4.0 Å using a Cu rotating-anode X-ray generator.

1. Introduction

One of the most common responses to stress in all organisms is the appearance or amplification of molecular chaperones, proteins that are involved in different aspects of protein folding. A number of such proteins from a variety of biological systems have been cloned, expressed, isolated and in some cases the three-dimensional structure has been determined. These structures have led to proposals on the mechanisms of chaperone activity.

The molecular-chaperone system for which the highest degree of structural information exists is the GroEL/ES (or Cpn60/Cpn10) system from *Escherichia coli* (Boisvert *et al.*, 1996; Grallert & Buchner, 2001; Hemmingsen *et al.*, 1988). This complex has been crystallized in a number of operational states, which has led to the elucidation of its mode of activity. It has been shown that GroEL/ES chaperone function is dependent on formation of large oligomeric complexes. In the presence of ATP, proteins can be folded within the complex interior as a result of large conformational changes to the GroEL subunit.

In bacteria, the two genes that encode GroEL/ES are in the same operon (Kong *et al.*, 1993). *M. tuberculosis* (*Mt*) expresses a second GroEL homologue, denoted Cpn60-2, which has 61% homology to the *Mt* homologue of GroEL (Cpn60-1) (Kong *et al.*, 1993). The Cpn60-2 gene is located ~3.3 Mbp upstream of the Cpn10/Cpn60-1 operon. The function of Cpn60-2 is not known and its oligomeric state is also unclear. It has been suggested that it may be able to form heterocomplexes with Cpn60-1 in a GroEL/ES-type complex (Kong *et al.*, 1993).

In addition to its putative role as a molecular chaperone, the Cpn60-2 protein has been shown to be of medical interest owing to its strong immunogenity and the ever-increasing level of tuberculosis infection in humans (Lewthwaite *et al.*, 2001).

In certain strains of rats, injection of heat-killed *Mt* in the presence of Freunds adjuvant elicits an autoimmune response resulting in arthritic inflammation in limbs (Handley *et al.*, 1996; Pearson, 1956; Taurog *et al.*, 1988). This response has been found to be elicited specifically by the presence of the Cpn60-2 protein. In rat strains immune to this response and in susceptible rats that have recovered, anti-Cpn60-2 antibodies have been found. These antibodies can prevent onset of the arthritic inflammation when injected into young susceptible rats. Further analysis has identified a single peptide of the Cpn60-2 protein which can induce the immune response (Ulmansky *et al.*, 2002). In order to try to further ascertain the structural basis for the phenomenon associated with Cpn60-2, we have overproduced, purified and crystallized this protein. Here, we describe the preliminary crystallographic analysis of the resulting crystals.

2. Materials and methods

2.1. Protein expression and purification

The gene encoding the *Mt*Cpn60-2 protein was cloned into a pCYTEXP1 vector (a kind gift from Dr M. Singh, WHO Recombinant Protein Bank, Germany). Expression was performed in MZ-1 cell by growth to an OD_{600nm} of 0.6 at 303 K in LB medium (with 100 μg ml^{-1} ampicillin), followed by a 3 h incubation at 315 K. The bacterial cells were collected by centrifugation and frozen at 193 K.

Cells were thawed and resuspended in 50 m*M* Tris pH 7.5 with 1 m*M* DTT (buffer *A*) and treated for 15 min with lysozyme

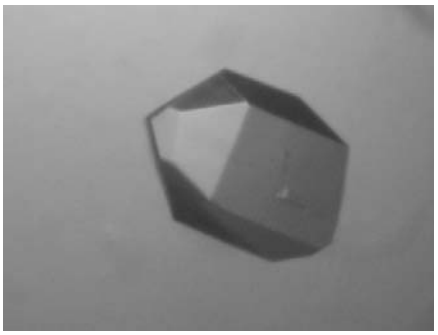

Figure 1
A monoclinic crystal of Cpn60-2. The crystal has dimensions of 0.3 × 0.2 × 0.1 mm.

(1 mg ml^{-1}) in the presence of a protease-inhibitor cocktail (Roche Molecular Biochemicals). The suspension was passed through a Yeda-type pressure cell operating at 1.5 MPa of nitrogen. The insoluble fraction was removed by centrifugation and the soluble fraction was treated with 1.6 *M* ammonium sulfate. Following centrifugation, the supernatant was discarded and the Cpn60-2-containing pellet was resuspended in buffer *A* and then further dialysed against buffer *A* to remove remnants of ammonium sulfate. The protein was applied to a low-pressure chromatography system with Sepharose Q Fast Flow (Pharmacia Biotech) as the stationary phase. Bound protein was eluted using a 0–600 m*M* NaCl gradient (in buffer *A*). The chromatographic procedure was repeated to obtain very highly purified Cpn60-2. Following dialysis against buffer *A*, the Cpn60-2 protein was concentrated to 20 mg ml^{-1} by ultrafiltration on Centricon-30 (Amicon).

2.2. Crystallization

Cpn60-2 protein crystallization condition screening was initially performed using the Crystal Screen I and II kits (Hampton Research, USA). Cpn60-2 protein crystallization was performed using the hanging-drop vapour-diffusion method. 4 µl of protein (14 mg ml^{-1}) was mixed with an equal volume of crystallization reservoir solution containing 10% propanol, 20% PEG 4000 and 0.1 *M* HEPES pH 7.5. Crystallization was performed at 297 K and crystals grew within 1–2 weeks.

3. Results and discussion

Cpn60-2 expressed in *E. coli* was purified by multiple rounds of ion-exchange chromatography. Analysis by size-exclusion HPLC of the isolated protein showed the exclusive presence of monomers (data not shown). Crystallization trials were performed with a large variety of conditions (Crystal Screens I and II, Hampton Research; Wizard I and II, Emerald Biostructures). Needles were obtained readily in more than one trial condition; however, formation of diffraction-quality crystals appeared to be inhibited by lack of homogeneity of the protein preparation, perhaps owing to binding of small peptides by Cpn60-2. Following further optimization of the purification protocol, diffraction-quality crystals were obtained in the presence of PEG 4000 and propanol (Fig. 1). Crystals appeared within two weeks and reached dimensions of 0.3 × 0.2 × 0.1 mm. The protein drops also contained a rather thick film of denatured protein and precipitate, suggesting that the crystallization solution may have provided a further purification step. The presence of a small organic solvent such as propanol or methanol was found to be an absolute prerequisite and may have the role of removing small peptides bound to the Cpn60-2 protein, as has been shown to be the case for the homologous *E. coli* GroEL (Todd & Lorimer, 1998).

Crystals were mounted in quartz capillary tubes and exposed to X-rays on a Rigaku R-AXIS IIc diffractometer mounted on a Rigaku RU-200 rotating-anode X-ray generator. The crystals diffracted to a maximum resolution of 4.0 Å. Analysis of the diffraction pattern using *DENZO* showed that the crystals belong to a monoclinic space group, with unit-cell parameters $a = 57$, $b = 115.5$, $c = 81.5$ Å, $\beta = 95.5^\circ$. Two molecules exist in the asymmetric unit and $V_M = 2$ Å^3 Da^{-1} (Matthews, 1968).

We are currently in the process of improving crystal quality by sequential microseeding and will attempt structure determination by molecular replacement utilizing the *E. coli* GroEL structure as the molecular model.

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