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Cloning, expression, purification and preliminary X-ray crystallographic studies of *Escherichia coli* Hsp100 nucleotide-binding domain 2 (NBD2)

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Escherichia coli Hsp100 ClpB has been identified recently as playing critical roles in multi-chaperone systems. ClpB binds and disaggregates denatured polypeptides by employing ATP hydrolysis and allows other molecular chaperones such as Hsp70 DnaK and Hsp40 DnaJ to refold the non-native polypeptides. ClpB contains two nucleotide-binding domains (NBD1 and NBD2) in its primary sequence. Walker A and Walker B motifs exist in both nucleotide-binding domains. Therefore, ClpB belongs to the large ATPase family known as ATPase associated with various cellular activities (AAA). The mechanisms by which NBD1 and NBD2 function to support the ClpB molecular-chaperone activity are currently unknown. To investigate how NBD2 participates in ClpB function to disaggregate denatured proteins, ClpB NBD2 has been cloned and crystallized. The ClpB NBD2 crystals diffract X-rays to 2.5 Å using synchrotron X-ray sources. The crystals belong to space group $P2_12_12_1$, with unit-cell parameters a = 99.57, b = 149.34, c = 164.69 Å.

1. Introduction

The heat-shock protein 100 (Hsp100) family covers a wide range of proteins involved in various cellular functions such as degradation of misfolded proteins, refolding of aggregated polypeptides, transcription regulation and tolerance of high temperature by cells (reviewed in Schirmer *et al.*, 1996; Wawrzynow *et al.*, 1996). The Hsp100/Clp members have been divided into two subfamilies. The class I members have two ATPase domains, while the class II family contain only one ATPase domain (Schirmer *et al.*, 1996). *E. coli* ClpB belongs to the class I Hsp100 subfamily. Recently, members of this subfamily were identified as functioning as molecular chaperones to disaggregate denatured polypeptides (Glover & Lindquist, 1998; Goloubinoff *et al.*, 1999). ClpB can be induced *in vivo* by heat shock or other stress factors and plays essential roles at elevated temperature in *E. coli* (Schirmer *et al.*, 1996). It has been reported that ClpB can cooperate with the Hsp70 molecular-chaperone system (including DnaK, DnaJ and GrpE) in *E. coli* to form multi-chaperone systems to solubilize protein aggregates and refold them efficiently into active states (Zolkiewski, 1999; Goloubinoff *et al.*, 1999). ClpB binds denatured protein aggregates and unfolds them by catalyzing ATP hydrolysis. This may expose the hydrophobic side chains and allow subsequent DnaK binding and refolding of the polypeptides (Goloubinoff *et al.*, 1999).

Hsp100 proteins belong to a large family of Mg-dependent ATPases known as ATPase associated with various cellular activities (AAA; Patal & Latterich, 1998). The hallmark of the AAA protein family is an AAA domain (about 200–250 amino-acid residues) containing Walker A and Walker B motifs (Patal & Latterich, 1998). All Hsp100 proteins contain either one or two nucleotide-binding domains as predicted by sequence analysis (Schirmer *et al.*, 1996; Patal & Latterich, 1998). *E. coli* Hsp100 ClpB contains two AAA domains within its primary sequence: nucleotide-binding domains 1 and 2 (NBD1 and NBD2; Schirmer *et al.*, 1996; Barnett *et al.*, 2000). NBD1 and NBD2 presumably contribute to the Hsp100 protein ATPase activities at different levels, as suggested by mutagenesis in the Walker A and Walker B regions (Schirmer *et al.*, 1996). It remains unclear why ClpB needs two AAA domains to achieve ATPase activity for its molecular-chaperone activity.

The crystal structure of ClpB NBD1 has been recently determined in our laboratory to 1.8 Å by the MAD method (Li & Sha, 2002). The NBD1 structure contains one domain comprising 11 α-helices and six β-strands. When compared with the typical AAA structures, the crystal structure of ClpB NBD1 reveals a novel AAA topology with a six-stranded β-sheet as its core. The N-terminal portion of the NBD1 structure has an extra β-strand flanked by two extra α-helices that are not present in other AAA structures. Moreover, the NBD1 structure does not have a C-terminal helical domain as other AAA proteins do. No nucleotide molecule is bound with ClpB NBD1 in the crystal structure,

probably owing to the lack of the C-terminal helix domain in the structure. Based on our structure and structure-based mutagenesis data, we proposed a 'seesaw' model to illustrate the mechanism by which ClpB performs its ATPase activities for chaperone function. In this model, the ClpB C-terminal fragment may function as a 'seesaw' to toggle the NBD1 and NBD2 domains between the ATP-binding state and the ADP-binding state by undergoing two distinct conformations. To test our proposed model for ClpB functions, we have purified and crystallized ClpB NBD2.

2. Experiments

2.1. Cloning, expression and purification of *E. coli* ClpB N-terminal domain

The ClpB NBD2 domain has been determined to be within amino-acid residues 552–770 of the full-length ClpB by limited proteolysis (data not shown). The NBD2 gene was then cloned into vector pet28b (Novagen). The PCR products were digested by using restriction endonucleases *Nde*I and *Hin*dIII following the manufacturer's instructions (New England BioLabs). The inserts were then ligated into the digested pet28b vector by T4 ligase. The nucleotide sequence was confirmed by DNA sequencing. The plasmid encoding the NBD2 was transformed into *E. coli* strain BL21(DE3) for protein expression. The calculated molecular weight is 24 710 Da for the NBD2 monomer.

10 ml of LB medium with 30 µg ml^{-1} kanamycin was inoculated using transformed *E. coli* stocks. The cells were allowed to grow at 310 K in a shaker for 12 h. The 10 ml of LB medium was then used to inoculate 1 l of LB medium with 30 µg ml^{-1} kanamycin. When the OD_{600} of the medium reached 0.6, 0.5 ml 1 *M* IPTG was added to the 1 l medium to induce protein expression. The cells were harvested 3 h after induction. The *E. coli* cells from 1 l of medium were pelleted down by centrifugation and resuspended in 100 ml 100 m*M* Tris buffer pH 7.9, 150 m*M* NaCl. The cells were lysed by sonication at 277 K. The debris and insoluble materials were pelleted at 15 000 rev min^{-1} using a Beckman JA20 rotor. Because the recombinant NBD2 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. The eluted protein was dialyzed against 2 l of 10 m*M* HEPES buffer pH 7.2, 50 m*M* NaCl. The typical yield of soluble NBD2 (∼95% pure from SDS–PAGE analysis) from 1 l culture is ∼50 mg. The N-terminal histidine tag of NBD2 was then removed by thrombin treatment. One unit of thrombin (Sigma) was utilized to digest the histidine tag per milligram of NBD2 protein. The digestion was allowed to continue for 12 h at room temperature and stopped by the addition of PMSF to a concentration of 0.2 m*M*. The protein 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 NBD2 is shown to be ∼25 kDa based on the elution time of the protein peak, indicating that the NBD2 forms a monomer in solution.

2.2. Crystallization, data collection and processing of the NBD2

The NBD2 protein was concentrated to 30 mg ml^{-1} in 10 m*M* HEPES buffer pH 7.5, 10 m*M* $MgCl_2$, 50 m*M* KCl and subjected to crystallization trials. Needle-shaped crystals (0.5 × 0.05 × 0.05 mm) were obtained by hanging-drop vapor-diffusion method using Linbro plates at room temperature. The well solution consisted of 1 ml 100 m*M* Tris buffer pH 8.5–9.0, 45% saturated Li_2SO_4. The hanging drops contained 2 µl protein solution plus 2 µl well solution. To examine whether the crystals are composed of NBD2, several crystals were collected, washed extensively and then dissolved in water. The sample was then subjected to SDS–PAGE analysis. The resulting gel showed that the crystals contained only NBD2.

Diffraction data for the NBD2 crystal were collected at BioCars beamline IDB14 and SBC beamline BM19 at the APS. The crystal was flash-frozen at 100 K in a nitrogen-gas stream using cryoprotectant consisting of 100 m*M* Tris buffer pH 8.5, 15% ethylene glycol and 75% Li_2SO_4. The data were collected using a Quantum 4 CCD detector (ADSC). 130 images covering an oscillation range of 130° were collected and processed using *DENZO* and *SCALEPACK* (Minor, 1993; Otwinowski, 1993). The ClpB NBD2 crystals diffracted X-rays to 2.5 Å. The crystals belong to space group $P2_12_12_1$, with unit-cell parameters a = 99.57, b = 149.34, c = 164.69 Å. The statistical results show a mosaicity value of 0.163 for the NBD2 crystal. The data set is 98.3% (87.7%) complete, with an $I/\sigma(I)$ of 33.1 (1.9). R_{sym} for the data set is 6.3% (54.4%). (Values in parentheses are for the outer resolution shell.) The V_M value is 3.1 Å^3 Da^{-1} assuming there to be eight molecules in one asymmetric unit.

Several AAA protein structures homologous to NBD2 have been determined (Li & Sha, 2002; Zhang *et al.*, 2000; Lenzen *et al.*, 1998). We are currently trying to determine the ClpB NBD2 crystal structure using the molecular-replacement method. If molecular replacement fails, we will utilize the MIR or MAD method to determine the structure. The ClpB NBD2 crystal structure may provide more evidence to support our proposed 'seesaw' mechanisms by which ClpB performs its ATPase activity in order to function as a molecular chaperone.

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References

Barnett, M. E., Zolkiewska, A. & Zolkiewski, M. (2000). *J. Biol. Chem.* **275**, 37565–37571.
Glover, J. R. & Lindquist, S. (1998). *Cell*, **94**, 73–82.
Goloubinoff, P., Mogk, A., Zvi, A. P. B., Tomoyasu, T. & Bukau, B. (1999). *Proc. Natl Acad. Sci.* **96**, 13732–13737.
Lenzen, C. U., Steinmann, D., Whiteheart, S. W. & Weis, W. I. (1998). *Cell*, **94**, 525–536.
Li, J. & Sha, B. D. (2002). Submitted.
Minor, W. (1993). *XdisplayF*. Purdue University, West Lafayette, USA.
Otwinowski, Z. (1993). *Proceedings of the CCP4 Study Weekend. Data Collection and Processing*, edited by L. Sawyer, N. Isaacs & S. Bailey, pp. 56–62. Warrington: Daresbury Laboratory.
Patal, S. & Latterich, M. (1998). *Trends Cell Biol.* **8**, 65–71.
Schirmer, E. C., Glover, J. R., Singer, M. A. & Lindquist, S. (1996). *Trends Biochem. Sci.* **21**, 289–296.
Wawrzynow, A., Banecki, B. & Zylicz, M. (1996). *Mol. Microbiol.* **21**, 895–899.
Zhang, X., Shaw, A., Bates, P. A., Newman, R. H., Gowen, B., Orlova, E., Gorman, M. A., Kondo, H., Dokurno, P., Lally, J., Leonard, G., Meyer, H., van Heel, M. & Freemont, P. S. (2000). *Mol. Cell*, **6**, 1473–1484.
Zolkiewski, M. (1999). *J. Biol. Chem.* **274**, 28083–28086.