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Purification, crystallization and preliminary X-ray analysis of *Caenorhabditis elegans* ubiquitin-conjugation enzyme M7.1

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M7.1 is a class IV ubiquitin-conjugation enzyme (UBC) that belongs to the ubiquitination cascade in *Caenorhabditis elegans*. The clone for this UBC has been overexpressed in *Escherichia coli* and the 16.7 kDa protein was purified from the soluble fraction. M7.1 was crystallized by sitting-drop vapor diffusion in 10% ethanol, 1.5 *M* NaCl at 277.5 K. Crystals diffracted to 1.75 Å and belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 44.3$, $b = 54.3$, $c = 60.2$ Å. The asymmetric unit contains a single monomer. A molecular-replacement model has been determined and refinement is in progress.

1. Introduction

Damaged, misfolded or misassembled proteins and a host of normal proteins are targeted for degradation by ubiquitin (Ub) conjugation. Through Ub-dependent protein degradation, the abundance of critical regulatory proteins can be controlled; this process is now recognized as an essential pathway in all eukaryotes and is a key aspect of cell growth and differentiation (Laney & Hochstrasser, 1999). The general ubiquitination pathway begins with the formation of a high-energy thioester linkage between the C-terminus of Ub and the Ub-activating enzyme (E1) in an ATP-dependent manner. Next, Ub is transferred and covalently attached through another thioester linkage to a Ub-conjugating enzyme (UBC/E2). A Ub–protein ligase (E3) recruits a target protein and forms a functional ubiquitination complex with Ub–E2. Finally, E3 catalyzes the transfer of Ub from E2 to the target protein. Multiple cycles of ubiquitin transfer results in a poly-ubiquitinated target protein, which is detected and degraded by the 26S proteasome.

The Ub cascade is hierarchal: most eukaryotes have only one E1 enzyme, multiple E2s and a host of E3s (Pickart, 2001). Specificity for a given target protein arises primarily from interactions with the E3, although accessory factors may also participate in target recognition. A second level of specificity within the Ub cascade resides between E2s and E3s; generally, a given E2 serves a specific subset of E3s. From the E2 crystal structures solved to date and the high sequence homology of this family of proteins, it is likely that all E2s share a common well defined fold. However, the molecular details that give rise to specificity between E2s and their cognate E3s remains poorly understood. Additional E2 structures, along with structural investigations into E2–E3 complexes, will be of great importance to understanding specificity within the Ub cascade.

The M7.1 protein of *Caenorhabditis elegans* (also known as UBC-2 and LET-70) is a ubiquitin-conjugation enzyme (Zhen *et al.*, 1993, 1996). Genome-sequence analysis has identified 20 such UBCs in *C. elegans*, including M7.1, all sharing the UBC protein motif, which contains an active-site cysteine residue, a well conserved HPN sequence and an invariant proline and tryptophan (Jones *et al.*, 2002). Phylogenic analysis categorizes M7.1 as a class IV UBC (Jones *et al.*, 2002) and it is functionally homologous to yeast UBC4/5 (Zhen *et al.*, 1993). In humans, the ortholog of yeast UBC4, UBE2D2, has been implicated in the ubiquitination of IκBα (Chen *et al.*, 1996), among other short-lived proteins, and may associate with the SCF complex (Strack *et al.*, 2000). M7.1 is a 16.7 kDa protein of 147 amino acids and is 79% identical and 88% homologous to the previously solved yeast UBC-4 (PDB code 1qcq; Cook *et al.*, 1993). Here, we report the cloning, expression, purification and crystallization as well as the preliminary X-ray analysis of M7.1. Our goal is to solve the structure of this protein and eventually begin to characterize the surface features of UBCs that give rise to specificity within the *C. elegans* Ub cascade.

2. Materials and methods

PfuTurbo DNA polymerase enzyme and reagents for PCR were purchased from Stratagene. QIAquick PCR Purification and Gel Extraction kits were purchased from Qiagen. Research Genetics provided PCR primers and oligonucleotide-sequencing services. Kanamycin was purchased from Sigma. Restriction

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enzymes, T4 ligase required for subcloning and IPTG were purchased from Promega. The pET28b vector, BL21(DE3) *Escherichia coli* cells and thrombin protease were purchased from Novagen. 5 ml HiTrap Chelating Sepharose HP and SP Sepharose HP columns were purchased from Amersham Pharmacia Biotech. All other chemicals and reagents were purchased from Fisher.

2.1. Protein expression

The M7.1 gene was amplified from pENTR-M7.1 by PCR with the following primers: 5′-GGTATCTCCATATGGCTCT-CAAAAGAATCCAGAAGG-3′ (forward primer) and 5′-CCGGAATTCTTACATA-GCGTACTTTTGCGTCCATTC-3′ (reverse primer). The PCR product was purified, *Nde*I/*Eco*RI-digested and gel purified. It was then subcloned into an *Nde*I/*Eco*RI-digested pET28b vector; this vector expresses M7.1 as a thrombin-cleavable His_6-fusion protein. DNA sequencing confirmed the integrity of the M7.1 insert [there was one silent base difference from ACA at codon 36 reported by Zhen *et al.* (1993) to ACG in our construct]. The resulting pET28-M7.1 plasmid was then used to transform *E. coli* strain BL21(DE3). The transformed bacteria were then grown at 310 K with shaking in Miller LB broth containing 35 µg ml^{-1} kanamycin to an OD_{600nm} of ∼1.0 and induced with 1 m*M* IPTG; the cells were then incubated for a further 2 h at 310 K with shaking before harvesting.

2.2. Purification

The induced *E. coli* BL21(DE3)-(pET28-M7.1) cell mass was resuspended at 10 ml per gram of wet weight in Ni buffer *A* (20 m*M* Tris–HCl pH 8.0, 500 m*M* NaCl, 5 m*M* imidazole) and sonicated on ice. After centrifugation at 20 000*g* for 30 min at 277 K, the supernatant was filtered through a 0.45 µm filter and applied to Chelating Sepharose which had been previously charged with 50 m*M* $NiSO_4$ and equilibrated with Ni buffer *A*. The column was then washed with Ni wash buffer (20 m*M* Tris–HCl pH 8.0, 500 m*M* NaCl, 55 m*M* imidazole) and the His_6-M7.1 fusion protein was eluted with Ni elution buffer (20 m*M* Tris–HCl pH 8.0, 500 m*M* NaCl, 350 m*M* imidazole). The His_6-M7.1 fusion protein was then dialyzed against *hst* buffer (20 m*M* Tris–HCl pH 8.4, 500 m*M* NaCl, 2.5 m*M* $CaCl_2$), 0.1 units of thrombin were added per milligram of fusion protein and the His tag was removed by digestion for 6 h at 298 K (thrombin cleavage of the His_6-M7.1 fusion protein results in an M7.1 protein with an N-terminal GSH extension). The thrombin-cleavage reaction mixture was then filtered through a 0.45 µm filter, diluted 1:9(*v*/*v*) with 20 m*M* sodium citrate pH 5.0 and applied to SP Sepharose which had been previously equilibrated with *SP* buffer *A* (20 m*M* sodium citrate pH 5.0, 50 m*M* NaCl). Native M7.1 was eluted from SP Sepharose with a 50–500 m*M* NaCl linear gradient in sodium citrate pH 5.0 and fractions containing native M7.1 were identified by denaturing gel electrophoresis, pooled and dialyzed against 2 m*M* sodium citrate pH 5.0; the protein was judged to be >99% pure on the basis of denaturing gel electrophoresis.

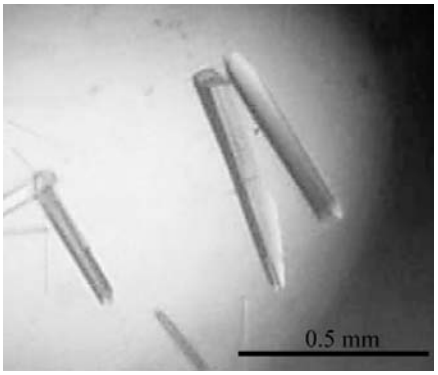

Figure 1
Crystals of M7.1.

2.3. Crystallization

Purified M7.1 was concentrated by ultrafiltration to 7 mg ml^{-1} in 2 m*M* sodium citrate pH 5.0. Crystal screening was performed by the sparse-matrix system (Jancarik & Kim, 1991) using Hampton Research Crystal Screens I and II with the sitting-drop vapor-diffusion method. 10 µl drops containing 1:1(*v*/*v*) protein to reservoir solution were placed into the depression of sitting-drop plates, sealed with clear tape and allowed to equilibrate against 0.5 ml of reservoir solution at room temperature (295.5 K) and at 277.5 K.

2.4. Data collection

For data collection, selected crystals were transferred to a cryoprotectant solution containing 25%(*v*/*v*) glycerol in the crystallization solution and flash-frozen with a soaking time of less than 60 s. Flash-frozen crystals were placed in a 100 K nitrogen-gas stream produced by an MSC X-Stream Cryogenic Crystal Cooler System (Molecular Structure Corporation, The Woodlands, Texas, USA) for data collection. X-ray diffraction data were collected on a Rigaku R-AXIS IV image-plate detector using Cu $K\alpha$ radiation from a Rigaku RU-H3R rotating-anode X-ray generator operating at 50 kV and 100 mA and focused with Osmic Blue Confocal Optics. A crystal-to-detector distance of 150 mm and 10 min exposure per frame were used for data collection. Data were indexed, integrated and scaled with the *HKL*2000 suite (Otwinowski & Minor, 1997) and further processing was preformed with *CNS* (Brünger *et al.*, 1998) and *XTALVIEW* (McRee, 1999).

Table 1
Summary of X-ray data statistics.

Values for the highest resolution shell (1.81–1.75 Å) are given in parentheses.

Wavelength (Å)	1.54
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 44.3, b = 54.3, c = 60.2
Resolution range (Å)	25–1.75
No. of observations	40653
No. of unique reflections	11278
Data completeness (%)	74.2 (33.1)
R_{merge}† (%)	5.6 (30.8)
$I/\sigma(I)$	19.0 (2.6)
Matthews coefficient (Å^3 Da^{-1})	2.18
Solvent content (%)	43.45

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

3. Results and discussion

Crystals formed using 10% PEG 6000, 2.0 *M* NaCl at room temperature and using 10% ethanol, 1.5 *M* NaCl at 277.5 K. In both cases, crystals grew in 2–3 d (Fig. 1). Individual crystals were selected and carefully separated. After refining the cryoconditions, a single crystal was grown that diffracted to 1.75 Å resolution. The minimum distortion index shows that the crystal belongs to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 44.3, b = 54.3, c = 60.2 Å. R_{merge} of the data set was 5.6 overall and 30.8% for the 1.81–1.75 Å shell. Data statistics are given in Table 1. Using the molecular weight of 16 986 Da and assuming one molecule per asymmetric unit, the Matthews coefficient (V_M) was calculated to be 2.18 Å^3 Da^{-1} (Matthews, 1968), corresponding to a solvent content of 43.45% (Westbrook, 1985).

The structure of yeast UBC-4 (88% homologous to M7.1; Cook *et al.*, 1993; PDB code 1qcq) was used as the search model for molecular replacement. The fast direct rotation algorithm from *CNS* was run using 25–4.0 Å data against the unmodified model. The top ten picks were used to search for the translation solution. The correlation function value for the best rotation–translation combination solution was 0.503, with the

fraction of the unit cell occupied by the model being 0.664. The initial model gave an R value of 0.45 and an $R_{\rm free}$ of 0.45. In order to reconstruct the model, a $2F_o - F_c$ composite omit map was calculated and visualized using *XTALVIEW*. Visual inspection shows electron density consistent with the protein sequence. After manual rebuilding of the model, cycles of refinement and minimization (rigid body, simulated annealing and grouped B value) resulted in an R value of 0.30 and an $R_{\rm free}$ of 0.36. Refinement is in progress.

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References

Brünger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T. & Warren, G. L. (1998). *Acta Cryst.* D**54**, 905–921.

Chen, Z. J., Parent, L. & Maniatis, T. (1996). *Cell*, **84**, 853–862.

Cook, W. J., Jeffrey, L. C., Xu, Y. & Chau, V. (1993). *Biochemistry*, **32**, 13809–13817.

Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.

Jones, D., Crowe, E., Stevens, T. A. & Candido, E. P. (2002). *Genome Biol.* **3**, Research 0002.1–0002.15.

Laney, J. D. & Hochstrasser, M. (1999). *Cell*, **97**, 427–430.

McRee, D. E. (1999). *Practical Protein Crystallography*. New York: Academic Press.

Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.

Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.

Pickart, C. M. (2001). *Annu. Rev. Biochem.* **70**, 503–533.

Strack, P., Caligiuri, M., Pelletier, M., Boisclair, M., Theodoras, A., Beer-Romero, P., Glass, S., Parsons, T., Copeland, R. A., Auger, K. R., Benfield, P., Brizuela, L. & Rolfe, M. (2000). *Oncogene*, **19**, 3529–3536.

Westbrook, E. M. (1985). *Methods Enzymol.* **114**, 187–196.

Zhen, M., Heinlein, R., Jones, D., Jentsch, S. & Candido, E. P. (1993). *Mol. Cell Biol.* **13**, 1371–1377.

Zhen, M., Schein, J. E., Baillie, D. L. & Candido, E. P. (1996). *EMBO J.* **15**, 3229–3237.