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Crystallization and preliminary X-ray analysis of the *Escherichia coli* adaptor protein ClpS, free and in complex with the N-terminal domain of ClpA

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Protein degradation in *Escherichia coli* is accomplished by a handful of large oligomeric complexes. In most cases, these proteolytic machines are comprised of a chaperone (*e.g.* ClpA) that is required to prepare the substrate for degradation by the peptidase (*e.g.* ClpP). Recently, it was shown that the substrate recognition of the chaperone ClpA could be modified by the adaptor protein ClpS. To investigate the structural implications of this change in substrate specificity, ClpS was crystallized alone and in complex with the N-terminal domain of ClpA ($ClpA_N$). Crystals of ClpS diffract to 2.9 Å resolution and belong to space group $P2_12_12_1$, with unit-cell parameters $a = 82.63$, $b = 145.67$, $c = 152.31$ Å. Two different crystal forms of the $ClpA_N$–ClpS complex were characterized. Crystal form I (CFI) belongs to the orthorhombic space group $P2_12_12$, with unit-cell parameters $a = 91.63$, $b = 112.47$, $c = 38.47$ Å; data to 1.92 Å resolution were collected. Crystals of form II (CFII) belong to space group $P4_{1/3}2_12$, with unit-cell parameters $a = b = 93.57$, $c = 78.77$ Å, and diffract to 1.85 Å resolution. Data sets collected from heavy-atom derivatives of CFI indicated the incorporation of Pt and Hg atoms. Structure solution using MIR and MAD methods is currently under way.

1. Introduction

Changes in the environment of a bacterial cell (*i.e.* heat shock) can induce a range of intracellular responses such as the aggregation of thermolabile cellular proteins (Mogk *et al.*, 1999) and the stalling of protein synthesis. In order to recycle these stalled ribosomes, the incomplete protein must be released from the ribosome. This is achieved by a tmRNA (ssrA), which adds an 11-residue peptide to the C-terminus of the premature protein (for a review, see Karzai *et al.*, 2000). This tag also serves as a mailing address for several proteolytic machines (such as ClpAP) which unfold (Weber-Ban *et al.*, 1999) and ultimately degrade the tagged proteins (Gottesman *et al.*, 1998). In bacteria, the recognition of SsrA-tagged proteins is performed not only by ClpA, but also by other members of the Clp/Hsp100 family.

The Clp/Hsp100 family has been divided into class I, with two ATPase domains, and class II, with one ATPase domain. ClpA, a ring-shaped hexameric protein, is a class I member of the Clp/Hsp100 family that functions as an unfolding and remodelling machine. Although ATP binding is sufficient to support ring assembly, hydrolysis is required for substrate unfolding (Maurizi, 1998). Consequently, ClpA can act either alone as an unfoldase of substrate proteins or in complex with the peptidase ClpP to drive proteolysis. The chaperone ring of ClpA may interact with either one or both ends of ClpP, forming structures resembling the 26S proteasome (Wickner *et al.*, 1999).

ClpA consists of four domains: a conserved N-terminal region of approximately 150 residues, two ATPase domains and a C-terminal domain. The isolated C-terminal domain, also known as the sensor and substrate-discrimination (SSD) domain, has been implicated in substrate recognition (Smith *et al.*, 1999); however, since a conserved tripeptide in close proximity to the SSD was shown to be essential for ClpP binding (Kim *et al.*, 2001), it is unclear whether a substrate could indeed bind to the SSD in the intact protein. Nevertheless, it appears that substrate recognition is not limited to the C-terminal domain, as the N-terminal domain has also recently been implicated in modulating substrate binding (Lo *et al.*, 2001). The regulation of substrate recognition of a ClpP-associated ATPase was first described for the chaperone ClpX (Levchenko *et al.*, 1999). A ribosome-associated protein SspB was found to bind SsrA-tagged proteins specifically (Flynn *et al.*, 2001), thereby enhancing degradation of these

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proteins by ClpXP. Recently, a novel adaptor protein, ClpS, was shown to modulate the substrate specificity of the chaperone ClpA, thereby altering ClpAP activity. Upon binding to the N-domain of ClpA, ClpS inhibits the association of SsrA-tagged proteins and thereby prevents their degradation, while potentially redirecting ClpAP-mediated degradation towards model aggregated proteins (Dougan *et al.*, 2002).

Although the atomic structure of the bacterial peptidase ClpP from *E. coli* has been solved by X-ray crystallography (Wang *et al.*, 1997), structures of both regulatory ATPases and associated adaptor proteins are notably lacking. Here, we report the crystallization and preliminary X-ray analysis of crystals of the newly identified adaptor protein ClpS and the $ClpA_N$–ClpS complex using synchrotron radiation.

2. Materials and methods

2.1. Expression and purification

Both ClpS (H66A) and the N-domain (1–161) of ClpA ($ClpA_N$) were overexpressed from an IPTG-inducible plasmid (pUHE21-2fdΔ12). ClpS was purified as described by Dougan *et al.* (2002). The N-terminal domain of ClpA was expressed as a C-terminal His_6-fusion protein and purified by Ni-NTA affinity chromatography under non-denaturing conditions according to the manufacturer's guidelines.

2.2. Crystallization

All crystallization trials were carried out by the hanging-drop vapour-diffusion method in 24-well Linbro plates using the commercially available crystal screens I, II and Lite (CSI, CSII and CSL; Hampton Research, Laguna, CA, USA; Jancarik & Kim, 1991).

ClpS protein solution was freshly prepared by dialysis against 20 m*M* NaCl, 20 m*M* KCl and 10 m*M* HEPES buffer pH 7.5 at 278 K. The protein concentration was determined with the bicinchoninic acid protein assay (Brown *et al.*, 1989) and was adjusted to 10 mg ml^{-1}. 2 µl of the protein solution and 1 µl of the screening solution were carefully mixed on a siliconized cover slip. This was placed over a reservoir containing 500 µl of the screening solution and was incubated at 293 K. ClpS crystals were mounted in glass capillaries and analyzed using Cu $K\alpha$ radiation produced by a rotating-anode generator. For data collection using cryoconditions, the concentration of glycerol in the protein drop was increased stepwise to a final concentration of 20% in condition CSL-33 and 30% in condition CSL-44 and the crystals were flash-frozen in liquid nitrogen.

Protein solutions for crystallization of the complex between ClpS and the N-domain of ClpA were prepared as follows. Both proteins were dialyzed at 278 K against 20 m*M* NaCl, 20 m*M* KCl, 5 m*M* 2-mercaptoethanol, 10 m*M* HEPES pH 7.5. The protein concentration was carefully determined as mentioned for ClpS and adjusted to 10 mg ml^{-1} for ClpS and $ClpA_N$. The solutions were mixed in a 1:1 ratio to yield a final protein concentration of 5 mg ml^{-1} for each protein. Crystallization trials of the complex were performed as described for ClpS using CSI and CSII. To determine the composition of the crystals, a few crystals from a drop were collected, washed several times in crystallization buffer and subjected to 20% SDS–PAGE.

2.3. X-ray data collection

Initial characterization of ClpS crystals mounted in glass capillaries (Mueller, Berlin, Germany) was performed on a Rigaku copper-anode generator; the diffraction data were collected on a MAR345 image-plate detector system (MAR Research). Native data from frozen crystals were collected at beamline ID14-EH2 at the European Synchrotron Radiation Facility (ESRF), Grenoble, France. Flash-frozen crystals were transferred into supercooled N_2 gas supplied by an Oxford Cryosystems Cryostream and were maintained at 100 K during data collection. The diffraction patterns were collected on a Quantum Q4 CCD detector using exposure times of 5–10 s; the crystal was rotated 1° per frame. Indexing and integration of the diffraction images as well as merging and scaling of the reflections were performed using the *XDS* program (Kabsch, 1988).

X-ray diffraction analysis of $ClpA_N$–ClpS crystals was carried out exclusively at the ID14-EH2 beamline, ESRF, Grenoble, France. Diffraction experiments on flash-frozen crystals were performed on both crystal forms (CFI and CFII). Data collection from the crystals was performed at the fixed wavelength of 0.933 Å. Crystals were flash-frozen in liquid nitrogen and transferred to the goniometer base. Needle-shaped crystals (CFI) were rotated around the needle axis and 1° frames were collected, whereas CFII crystals were rotated by 0.8° per image. Autoindexing, data processing and scaling were carried out using the *XDS* program.

3. Results

3.1. Crystallization and preliminary X-ray analysis of ClpS

Crystals were obtained under five crystallization conditions, namely CSL-2, CSL-15, CSL-29, CSL-33 and CSL-44, where the letters refer to the screen and the numbers refer to the respective reservoir solution. Conditions CSL-33 (2 *M* ammonium formate) and CSL-44 (0.1 *M* magnesium acetate) were further refined to yield crystals of dimensions 200 × 300 × 300 µm that appeared after 2–4 d (Fig. 1*a*) and were suitable for X-ray analysis. Starting with crystals mounted in glass capillaries, diffraction to 3.8 Å resolution was observed using X-rays from a Cu $K\alpha$-emitting rotating-anode generator.

The space group of the ClpS crystals was determined to be orthorhombic $P2_12_12_1$, with unit-cell parameters a = 82.63, b = 145.67, c = 152.31 Å. The mosaicity was estimated to be 0.7°. Unfortunately, the crystals were not stable in the strong synchrotron X-ray beam and merging of independent data sets resulted in a high $R_{\rm merge}$. We therefore collected only an 85% complete data set to 2.7 Å resolution (for further details, see Table 1). Assuming ClpS to be a homodimeric protein complex of 24 kDa, as determined by size-exclusion chromatography, four dimers in the asymmetric unit yield a rather high Matthews coefficient of 4.77 Å^3 Da^{-1}, whereas eight dimers would give a coefficient of 2.38 Å^3 Da^{-1}, which is in the typical range for protein crystals.

3.2. Crystallization and X-ray analysis of the $ClpA_N$–ClpS complex

Crystals initially appeared after 4–7 d under six different conditions, namely CSI-36, CSI-40, CSI-41, CS2-7, CS2-23 and CS2-30, all of which exhibited a bush-like appearance. Importantly, crystals of ClpS or the N-domain alone did not appear under any of the above conditions. However, in order to determine the protein composition of the crystals, the crystals were isolated, washed and analyzed by SDS–PAGE. Staining of the gel with colloidal silver revealed that both proteins were present in CFI (data not shown).

The crystallization condition CSI-41 (20% 2-propanol, 20% PEG 4000, 0.1 *M* sodium citrate buffer pH 5.6) was further refined by changing the pH, adding either 10 m*M* spermine or 10 m*M* 1,8-diaminooctane to the crystallization drop. Under the refined conditions, the crystals changed their

morphology to single crystals that could be analyzed by X-ray crystallography. The crystals of the CFI complex proved to be orthorhombic, space group $P2_12_12$, with unit-cell parameters a = 91.63, b = 112.47, c = 38.47 Å and one heterodimer in the asymmetric unit (V_M = 3.18 Å^3 Da^{-1}; Matthews, 1968). CFI diffracted to 1.88 Å resolution, yielding a 92.1% complete data set to this resolution and an overall R_{merge} of 7.3%.

The rhomboid-shaped $ClpA_N$–ClpS complex crystals (CFII) were grown under almost identical conditions as those for CFI. The typical dimensions of the crystals were 0.1 × 0.15 × 0.15 mm and they belonged to space group $P4_12_12$ or $P4_32_12$ with unit-cell parameters a = b = 93.89, c = 79.40 Å. Diffraction was observed to 1.92 Å and data collection yielded a 91.9% complete data set. The V_M for this crystal form was calculated to be 2.76 Å^3 Da^{-1}, with one molecule in the asymmetric unit.

In order to overcome the crystallographic phase problem, CFI and CFII crystals were soaked with different concentrations of platinum, mercury, gold and uranyl salts. The CFII crystals maintained their diffraction quality, but the unit-cell parameters changed by 3% compared with the native crystals and hence could not be used for isomorphous phasing. Most of the heavy-atom salts caused small isomorphous changes to the CFI crystals, but two compounds could be used for MIR phasing at lower resolution. Data obtained from crystals soaked with 1 mM K_2PtCl_4 and 0.5 mM thiomersal gave phasing power to only 4 Å and a FOM of 0.5 to 4 Å using *SHARP* for heavy-atom refinement (de La Fortelle & Bricogne, 1997).

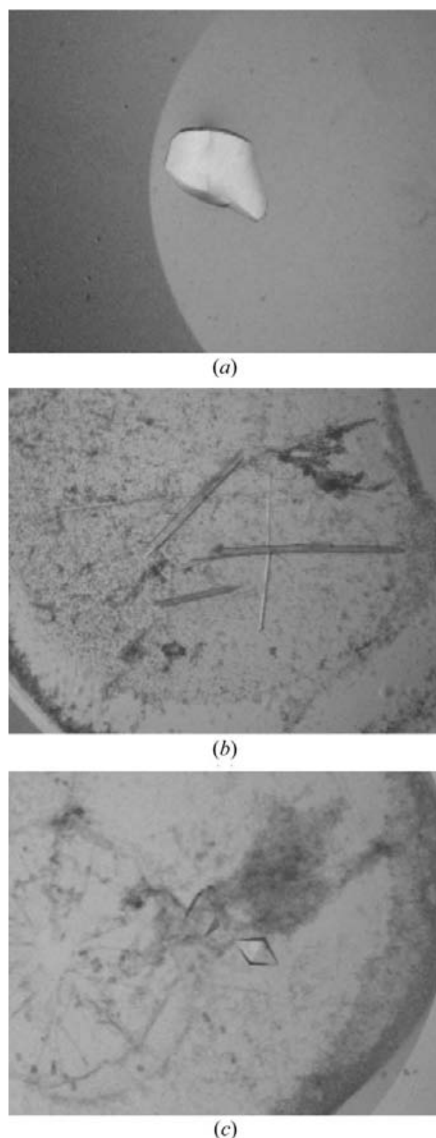

Figure 1
ClpS and $ClpA_N$–ClpS crystals were obtained by the vapour-diffusion method. (*a*) Crystals of ClpS appeared after 1–4 d and were approximately 200 × 200 × 300 µm in size. (*b*) Needle-shaped $ClpA_N$–ClpS crystals (CFI). These crystals have approximate dimensions of 60 × 60 × 400 µm and appeared after 2–10 d. (*c*) $ClpA_N$–ClpS CFII crystals were of rhombohedral shape and grew to 100 × 100 × 150 µm in size within 4–10 d.

Table 1
Data-collection parameters and statistics of ClpS and $ClpA_N$–ClpS crystals.

Values in parentheses refer to the highest resolution shell.

		$ClpA_N$–ClpS	
	ClpS	CFI	CFII
Wavelength (Å)	0.933	0.933	0.933
Crystal-to-detector distance (mm)	240	170	170
Oscillation range (°)	1	1	0.8
Resolution (Å)	40–2.9	20–1.88	20–1.92
Observations observed	81171	153693	216332
Unique observations	69200	57613	50008
Completeness (%)	85.3 (62.7)	92.1 (81.1)	91.9 (74.9)
$I/\sigma(I)$	3.51 (1.63)	7.76 (1.8)	9.50 (1.89)
Space group	$P2_12_12_1$	$P2_12_12$	$P4_12_12$ or $P4_32_12$
Unit-cell parameters			
a (Å)	82.63	91.63	93.89
b (Å)	145.67	112.47	93.89
c (Å)	152.31	38.47	79.40
$\alpha = \beta = \gamma$ (°)	90	90	90
R_{merge} (%)	15.7 (59)	7.3 (48)	7.9 (53)
Mosaicity (°)	0.7	0.2	0.35
No. of subunits per AU	8 dimers	1 heterodimer	2 heterodimers

4. Discussion

We have crystallized the recently described ClpA adaptor protein from *E. coli*, ClpS, both free and in complex with the N-terminal domain of ClpA. Two distinct crystal forms of the $ClpA_N$–ClpS complex diffracted to better than 2 Å resolution, while the ClpS crystal showed diffraction to 2.7 Å. We identified derivative salts that, although not sufficient for a straightforward structure solution of the $ClpA_N$–ClpS complex, will be used for MAD structure determination.

Recently, the crystal structures of the related Clp/Hsp100 protein HslU (ClpY) from two different organisms, *E. coli* and *Haemophilus influenzae*, were determined (Bochtler *et al.*, 2000; Sousa *et al.*, 2000). From these data, it was proposed that the I-domain was responsible for substrate binding. Interestingly, this I-domain is absent from all other members of the Clp/Hsp100 family; in ClpA it appears that the N-domain plays an important role not only in recognition of ClpS, but also in the recognition of some substrates. Moreover, since there is little sequence similarity between the N-domains of ClpA and HslU, there is still a great deal of interest in determining the structure of the N-domain of ClpA. Similarly, there is also considerable interest in the structure of ClpB, a close relative of ClpA. Recently, Li & Sha (2001*a*,*b*) reported the crystallization of two different ClpB fragments; the isolated N-domain and the first ATPase domain (D1) lacking the N-domain. Although ClpA and ClpB have homologous N-domains, these proteins clearly interact with different target substrates; hence, a structural comparison of the two N-domains may provide some insights into the substrate specificity of different Clp/Hsp100 proteins. Moreover, although electron microscopy has been used extensively to study the hexameric assembly of both ClpA and ClpX with ClpP and translocation of substrates through the ClpP chamber, examination of the interaction of a chaperone with its adaptor protein has not yet been studied in molecular detail. Therefore, this structure will provide a first glimpse at atomic level of the interaction between a chaperone (ClpA) and its adaptor protein (ClpS). These details will make a significant contribution to the understanding of how ClpA-mediated substrate binding is modified by ClpS.

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References

Bochtler, M., Hartmann, C., Song, H. K., Bourenkov, G. P., Bartunik, H. D. & Huber, R. (2000). *Nature (London)*, **403**, 800–805.

Brown, R. E., Jarvis, K. L. & Hyland, K. J. (1989). *Anal. Biochem.* **180**, 136–139.

Dougan, D. A., Reid, B. G., Horwich, A. L. & Bukau, B. (2002). *Mol. Cell*, **9**, 673–683.

Flynn, J. M., Levchenko, I., Seidel, M., Wickner, S. H., Sauer, R. T. & Baker, T. A. (2001). *Proc. Natl Acad. Sci. USA*, **98**, 10584–10589.

Gottesman, S., Roche, E., Zhou, Y. N. & Sauer, R. T. (1998). *Genes Dev.* **12**, 1338–1347.

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

Kabsch, W. (1988). *J. Appl. Cryst.* **21**, 67–71.

Karzai, A. W., Roche, E. D. & Sauer, R. T. (2000). *Nature Struct. Biol.* **7**, 449–455.

Kim, Y. I., Levchenko, I., Fraczkowska, K., Woodruff, R. V., Sauer, R. T. & Baker, T. A. (2001). *Nature Struct. Biol.* **8**, 230–233.

La Fortelle, E. de & Bricogne, G. (1997). *Methods Enzymol.* **276**, 590–620.

Levchenko, I., Seidel, M., Sauer, R. T. & Baker, T. A. (1999). *Science*, **289**, 2354–2356.

Li, J. & Sha, B. (2001*a*). *Acta Cryst.* D**57**, 909–911.

Li, J. & Sha, B. (2001*b*). *Acta Cryst.* D**57**, 1933–1935.

Lo, J. H., Baker, T. A. & Sauer, R. T. (2001). *Protein Sci.* **10**, 551–559.

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

Maurizi, M. R. (1998). *Curr. Biol.* **8**, 453–456.

Mogk, A., Tomoyasu, T., Goloubinoff, P., Rudiger, S., Roder, D., Langen, H. & Bukau, B. (1999). *EMBO J.* **18**, 6934–6949.

Smith, C. K., Baker, T. A. & Sauer, R. T. (1999). *Proc. Natl Acad. Sci. USA*, **96**, 6678–6682.

Sousa, M. C., Trame, C. B., Tsuruta, H., Wilbanks, S. M., Reddy, V. S. & McKay, D. B. (2000). *Cell*, **103**, 633–643.

Wang, J. M., Hartling, J. A. & Flanagan, J. M. (1997). *Cell*, **91**, 447–456.

Weber-Ban, E. U., Reid, B. G., Miranker, A. D. & Horwich, A. L. (1999). *Nature (London)*, **401**, 90–93.

Wickner, S., Maurizi, M. R. & Gottesman, S. (1999). *Science*, **286**, 1888–1893.