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Crystallization and preliminary X-ray crystallographic studies of RepDC, a hybrid rolling-circle plasmid replication initiator protein

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The hybrid plasmid-replication initiator protein RepDC, which is a fusion of the catalytic fragment of the RepD protein and the DNA-binding fragment of the RepC protein from *Staphylococcus aureus*, has been successfully crystallized and X-ray data to 3.5 Å have been collected on a synchrotron radiation source. Crystals belong to space group $I4_132$ with unit-cell dimensions $a = b = c = 165.1$ Å. The crystals are estimated to contain one protein monomer per asymmetric unit, with 55% solvent content.

1. Introduction

Small multicopy antibiotic resistance plasmids of the pT181 family from *Staphylococcus aureus* replicate *via* a rolling-circle mechanism mediated by plasmid-encoded Rep proteins (Koepsel *et al.*, 1985*a*; Majumder & Novick, 1988; Novick, 1989; Thomas, Balson & Shaw, 1990; Del Solar *et al.*, 1998). Rep proteins of the pT181 family possess sequence-specific DNA-binding, nicking/closing (topoisomerase I-like) and replication-initiation activities (Koepsel *et al.*, 1985*a*,*b*, 1986; Thomas, Balson & Shaw, 1990). They initiate replication only from their cognate origins, and are able to relax negatively supercoiled plasmids containing any replication origin of the pT181 family *in vitro* (Thomas, Balson & Shaw, 1990; Zock *et al.*, 1990). Rep proteins initiate replication by introducing a site-specific nick in the leading strand at the unique origin site (*ori*), which gives rise to 3′ extension and displacement of the leading strand, while the Rep protein remains covalently attached to the 5′ nick terminus *via* the active-site tyrosine (Thomas *et al.*, 1988; Thomas, Balson & Shaw, 1990). When the nascent strand is complete, the Rep protein religates both the displaced strand and the new strand.

The *ori* sequences of the pT181 family consist of three adjacent inverted complementary repeats, referred to as ICRI, ICRII and ICRIII (Projan *et al.*, 1985; Gennaro *et al.*, 1989). The nick site is located within the loop of a potential stem-loop structure formed by ICRII (Noirot *et al.*, 1990; Jin *et al.*, 1997). The interaction between the Rep protein and the replication origin spans the 3′ side of ICRII (down from the nick site) and ICRIII. ICRIII is the site of specific recognition of *ori* by Rep and the determinant of plasmid specificity (Koepsel *et al.*, 1986; Thomas *et al.*, 1995). It is Rep/ICRIII recognition which allows interaction of the protein with ICRII, resulting in unwinding of the DNA and further extrusion of the ICRII cruciform (Noirot *et al.*, 1990).

The two best known Rep proteins of the pT181 family are RepC and RepD, which are specified by tetracycline-resistant plasmid pT181 and chloramphenicol-resistant plasmid pC221, respectively (Khan & Novick, 1983; Brenner & Shaw, 1985; Projan *et al.*, 1985). RepC and RepD contain 314 and 311 amino acids (about 37.5 kDa), respectively, and share 84% sequence identity. Proteolysis studies of the RepD protein suggested that Rep proteins can be divided into three major fragments (Thomas, Balson, Wigley *et al.*, 1990): (i) a short (∼3.5 kDa) N-terminal fragment, which was shown to be dispensable for protein function *in vitro*; (ii) a central conserved 21 kDa fragment, involved in interaction with the PcrA helicase (Iordanescu, 1993) and containing the active-site tyrosine, and (iii) a C-terminal 'DNA-binding' 14 kDa fragment, the determinant of specific recognition of the replication origin (Thomas *et al.*, 1995). The proteins exist as homodimers in solution (Thomas, Balson & Shaw, 1990; Rasooly *et al.*, 1994).

No three-dimensional structure of any Rep protein of the pT181 family is known. To determine the Rep protein structure by X-ray crystallography, a number of variants of the RepD protein have been tested in crystallization trials, the most successful being the RepDC 34 kDa hybrid protein. This is a fusion of the N-terminal 21 kDa fragment of RepD and C-terminal 14 kDa fragment of RepC, lacking the short N-terminal tail (the positions of the 21 kDa:14 kDa breakpoint are as given in Thomas, Balson, Wigley *et al.*, 1990). The cloning and function of this protein, including the expected topoisomerase activity specific for pT181, will be described elsewhere (Thomas *et al.*, in preparation).

Table 1
Data collection and processing statistics.

Values in parentheses refer to the last resolution shell (3.69–3.50 Å).

Space group	$I4_132$
Unit-cell parameters (Å, °)	$a = b = c = 165.1$, $\alpha = \beta = \gamma = 90$
Resolution (Å)	3.5
Total observations	56768
Unique reflections	5091
Redundancy	11.1 (11.4)
Completeness (%)	100.0 (100.0)
$I/\sigma(I)$	4.2 (2.4)
$R_{\rm merge}$† (%)	13.5 (32.1)

† $R_{\rm merge} = \sum_h \sum_i |I_{hi} - \langle I_h \rangle| / \sum_h \sum_i I_h$, where I_{hi} is the ith observation of the reflection h and $\langle I_h \rangle$ is the average intensity of the ith reflection.

2. Results and discussion

2.1. Protein preparation

The RepDC protein was overexpressed and purified following the procedure established for RepD protein (Thomas, Balson & Shaw, 1990). For crystallization trials, the protein was dialysed against 700 m*M* KCl, 10 m*M* Tris–HCl buffer pH 7.5 and concentrated using Centricon 10 concentrators (Amicon Inc., USA). The final protein concentration was estimated based on the results of a dye-binding assay (Bradford, 1976) using bovine serum albumin as standard.

2.2. Crystallization

Crystallization trials were set up using 1–4 mg ml^{-1} protein solution. Crystals were grown by the sitting-drop method of vapour diffusion (McPherson, 1982) at room temperature using Linbro multiwell tissue-culture plates. Crystallization was achieved by reduction of ionic strength, known as 'salting-in' (McPherson, 1982), as RepDC appears to be poorly soluble at low ionic strength and is insoluble in water (Thomas & Klimenko, unpublished results). Since the initial crystals did not grow to sizes suitable for X-ray diffraction measurements, the crystal size was increased stepwise by repeated macroseeding (McPherson, 1982).

The protein solution was mixed with 100 m*M* sodium citrate buffer pH 5.5 in the proportions 1:1.8 to achieve a starting KCl concentration of 250 m*M*, and 7–10 µl droplets were placed over wells containing 500 µl water. Crystals were visible after about one week and grew slowly over one month to a maximum dimension of about 0.05 mm. For seeding, crystals were washed in 450 m*M* KCl, 10 m*M* sodium citrate buffer pH 5.5 until signs of surface deterioration were seen, upon which they were transferred to fresh drops at the initial conditions (as crystal growth was very slow, no alternation of the initial conditions was necessary). A stabilizing solution of 10 m*M* sodium citrate buffer pH 5.5 was used to handle crystals. The crystals were seeded three to five times, approximately once a month, before they reached a size of 0.20–0.25 mm, which was sufficient for X-ray diffraction experiments.

2.3. Data collection and processing

A complete data set was collected on a MAR Research image plate at the European Synchrotron Radiation Facility (Grenoble, France) on beamline ID14/3 (λ = 0.933 Å) from one crystal. The crystal was frozen at 100 K in a nitrogen gas stream, using 10 m*M* sodium citrate buffer pH 5.5, 35% glycerol as a cryoprotectant solution. 1° oscillations were used and diffraction to 3.5 Å was observed. There was no evidence for radiation damage or for anisotropy. A typical diffraction pattern is shown in Fig. 1.

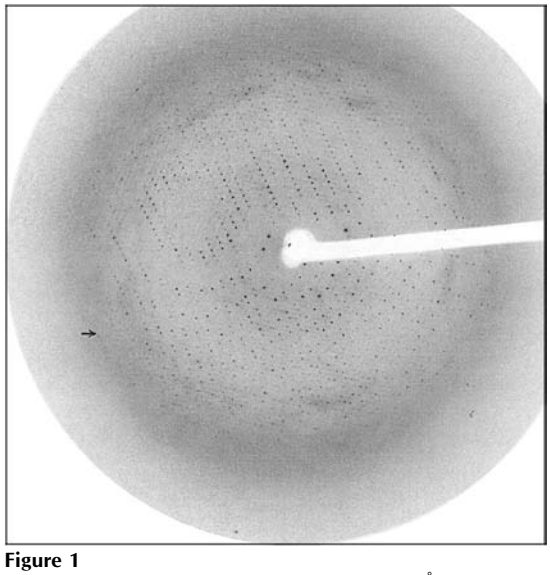

Figure 1
1° oscillation image. The arrow corresponds to 3.5 Å resolution.

The data were processed with the *MOSFLM* package (Leslie, 1996) and merged using the *CCP*4 package (Collaborative Computational Project, Number 4, 1994). The statistics are shown in Table 1. The self-rotation function calculated using the program *POLARRFN* (Collaborative Computational Project, Number 4, 1994) did not reveal any non-crystallographic symmetry, which together with the size of an asymmetric unit of the RepDC crystals suggests that there is one RepDC monomer per asymmetric unit. Based on this assumption, the Matthews formula (Matthews, 1968) gives a volume-to-mass ratio V_m of 2.76 Å^3 Da^{-1}, which is within the normal range of protein crystals, and a solvent content of 55%.

Structure determination by the multiple isomorphous replacement method is in progress.

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References

Bradford, M. M. (1976). *Anal. Biochem.* **72**, 248–254.
Brenner, D. G. & Shaw, W. V. (1985). *EMBO J.* **4**, 561–568.
Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.
Del Solar, G., Giraldo, R., Ruiz-Echevarría, M. J., Espinosa, M. & Díaz-Orejas, R. (1998). *Microbiol. Mol. Biol. Rev.* **62**, 434–464.
Gennaro, M. L., Iordanescu, S., Novick, R. P., Murray, R. W., Steck, T. R. & Khan, S. A. (1989). *J. Mol. Biol.* **205**, 355–362.
Iordanescu, S. (1993). *Mol. Gen. Genet.* **241**, 185–192.
Jin, R., Fernandez-Beros, M.-E. & Novick, R. P. (1997). *EMBO J.* **16**, 4456–4466.
Khan, S. A. & Novick, R. P. (1983). *Plasmid*, **10**, 251–259.
Koepsel, R. R., Murray, R. W. & Khan, S. A. (1986). *Proc. Natl Acad. Sci. USA*, **83**, 5484–5488.
Koepsel, R. R., Murray, R. W., Rosenblum, W. D. & Khan, S. A. (1985*a*). *Proc. Natl Acad. Sci. USA*, **82**, 6845–6849.
Koepsel, R. R., Murray, R. W., Rosenblum, W. D. & Khan, S. A. (1985*b*). *J. Biol. Chem.* **260**, 8571–8577.
Leslie, A. G. W. (1996). *Crystallographic Computing*, pp. 110–125. Oxford University Press.
McPherson, A. J. (1982). *Preparation and Analysis of Protein Crystals.* New York: John Wiley.
Majumder, S. & Novick, R. P. (1988). *Nucleic Acids Res.* **16**, 2897–2912.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Noirot, P., Bargonetti, J. & Novick, R. P. (1990). *Proc. Natl Acad. Sci. USA*, **87**, 8560–8564.
Novick, R. P. (1989). *Annu. Rev. Microbiol.* **43**, 537–565.
Projan, S. J., Kornblum, J., Moghazeh, S. L., Edelman, I., Gennaro, M. L. & Novick, R. P. (1985). *Mol. Gen. Genet.* **199**, 452–464.
Rasooly, A., Wang, P.-Z. & Novick, R. P. (1994). *EMBO J.* **13**, 5245–5251.

Thomas, C. D., Balson, D. F. & Shaw, W. V. (1988). *Biochem. Soc. Trans.* **16**, 758–759.
Thomas, C. D., Balson, D. F. & Shaw, W. V. (1990). *J. Biol. Chem.* 265, 5519–5530.
Thomas, C. D., Balson, D. F., Wigley, D. B. & Shaw, W. V. (1990). *Molecular Biology of the Staphylococci*, edited by R. P. Novick, pp. 211–218. New York: VCH.
Thomas, C. D., Nikiforov, T. T., Connolly, B. A. & Shaw, W. V. (1995). *J. Mol. Biol.* **254**, 381–391.
Zock, J. M., Birch, P. & Khan, S. A. (1990). *J. Biol. Chem.* **265**, 3484–3488.