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Crystallization and diffraction to ultrahigh resolution (0.8 Å) of a designed variant of the Rop protein

Aspasia Spyridaki,[a] Nicholas M. Glykos,[a] Dina Kotsifaki,[a] Vasiliki E. Fadouloglou[a,b] and Michael Kokkinidis[a,b]*

[a]Institute of Molecular Biology and Biotechnology (IMBB), PO Box 1527, GR-71110 Heraklion, Crete, Greece, and [b]University of Crete, Department of Biology, PO Box 2208, GR-71409 Heraklion, Crete, Greece

Correspondence e-mail: kokkinid@imbb.forth.gr

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The Rop protein is the paradigm of a highly regular four-α-helix bundle and as such has been subject to numerous structural and mutagenesis studies. Crystals of a designed Rop variant which establishes a continuous heptad pattern through the bend region have been obtained by a combination of vapour-diffusion and seeding techniques. The crystals diffract to ultrahigh (0.8 Å) resolution using synchrotron radiation and cryogenic conditions.

1. Introduction

In spite of the rapidly increasing number of protein structures determined by X-ray crystallography, there is still only a small number of structures solved at atomic resolution as defined by Sheldrick (1990). According to this definition, atomic resolution data should extend to at least 1.2 Å, with 50% or more intensities higher than 2σ in the outer resolution shell.

In this report, we present the diffraction to ultrahigh resolution from crystals of a designed variant of the Rop protein (referred to as Rop$_{<2aa>}$). Rop (repressor of primer) is a small dimeric protein. The 63 amino-acid monomer consists almost entirely of two antiparallel α-helices joined by a short hairpin bend (Banner *et al.*, 1987). A highly regular four-α-helical bundle structure (Cohen & Parry, 1986) is formed by the Rop dimer. Four-α-helical bundles consist of four amphipathic α-helices that cross at an angle of approximately 20° and pack in an antiparallel fashion. They display a specific pattern of hydrophobic and hydrophilic residues consisting of seven-residue repeats (heptads) of the type $(a,b,c,d,e,f,g)_n$. Positions *a* and *d* are generally hydrophobic. The heptad pattern in Rop shows a discontinuity corresponding to a deletion of two residues in the region of the hairpin bend (Banner *et al.*, 1987). Such discontinuities in regular heptad patterns have been frequently associated with bend formation (Cohen & Parry, 1986). The bend region of Rop has attracted considerable interest, not least because of the ongoing debate about the role of loops in the folding and stability of bundles and proteins in general (Predki *et al.*, 1996; Chou *et al.*, 1992; Nagi & Regan, 1997), and as such it has been subject to numerous mutagenesis experiments (Castagnoli *et al.*, 1989, 1994). We designed the Rop$_{<2aa>}$ mutant in order to test the hypothesis that bend formation in four-α-helical bundles is induced by discontinuities in the heptad pattern. In Rop$_{<2aa>}$ the discontinuity is eliminated with the insertion of Ala on both sides of bend residue Asp30. The two Ala residues inserted establish a continuous heptad pattern.

2. Experimental procedures

2.1. Purification

The oligonucleotide-directed mutagenesis has been described previously (Castagnoli *et al.*, 1989). Bacteria (*Escherichia coli* strain K38) were cultured in a 30 l fermenter using standard nutritients and conditions for *E. coli* growth. For a typical preparation, 50 g of cell paste was thawed in 100 ml buffer containing 50 m*M* Tris–HCl pH 7.5, 150 m*M* NaCl, 0.5 m*M* DTT, 1 m*M* EDTA and 1 m*M* phenylmethyl sulfonyl fluoride (PMSF) as protease inhibitor. The cells were disrupted at 273 K by pulsed sonication for 7 min. After centrifugation (14 000*g* for 1 h) and adjustment of the pH to 8.5, the supernatant was loaded onto an 80 ml Q-Sepharose column equilibrated in 25 m*M* Tris buffer pH 8.5. The column was washed with three bed volumes of 25 m*M* Tris buffer pH 8.5 and 20–30 bed volumes of 20 m*M* Tris buffer pH 7.5; a linear gradient of 0–600 m*M* NaCl (ten bed volumes) was then applied. Protein-containing fractions were localized by SDS–polyacrylamide gels and loaded onto an 80 ml hydroxylapatite column equilibrated in 20 m*M* Tris pH 7.5. The column was initially washed with two bed volumes of 20 m*M* Tris pH 7.5 and then with ten bed volumes of 10 m*M* phosphate buffer pH 6.8. A linear gradient of 10–300 m*M* phosphate (ten bed volumes) was applied and protein-containing fractions were concentrated to 3 ml. The sample was loaded onto a 300 ml S-100 Sephacryl gel-filtration column, from which the protein eluted as a dimer with a purity of at least 95% (as judged from SDS–polyacrylamide gels). After buffer exchange

Table 1
Summary of data collection and statistics.

Data collection.

	Ultrahigh resolution	High resolution	Medium resolution	Low resolution
Crystal-to-plate distance (mm)	85	85	250	340
Minimum resolution (Å)	1.7	1.7	30	30
Maximum resolution (Å)	0.8	1.0	1.55	1.7
Oscillation per image (°)	1.0	1.0	1.9	1.8
Number of images	180	180	128	100

Statistics of merged data.

No. of unique reflections	55686
Total No. of reflections	500615
R_{merge} (%)	
Overall	4.1
0.83–0.80 Å resolution shell	19.5
Completeness (%)	
Overall	92.5
0.83–0.80 Å resolution shell	82.9
$I/\sigma(I)$ for the 0.83–0.80 Å resolution shell	3.1

with 2 m*M* Tris–HCl pH 7.5, 1 m*M* EDTA, 1 m*M* DDT, $Rop_{<2aa>}$ was concentrated to ∼30 mg ml^{-1}. ∼60 mg of pure protein was the final yield from 50 g cell paste.

2.2. Crystallization

Crystals of $Rop_{<2aa>}$ were obtained by a combination of the hanging-drop vapour-diffusion and seeding techniques (Ducruix & Giegé, 1992). 10 µl drops of protein solution containing 20 mg ml^{-1} protein, 50 m*M* NaCl, 100 m*M* acetate buffer (pH 4.8–5.2) and 0.4–0.6 *M* ammonium sulfate were equilibrated against 1 ml of reservoir solution consisting of 200 m*M* acetate buffer (pH 4.8–5.2) and 0.8–1.2 *M* ammonium sulfate. Small crystals appeared within 1 d and served as a seed stock for subsequent macroseeding steps. The best seeds were washed in reservoir solution and transferred to 10 µl hanging drops prepared as previously described, where they continued to grow for 2–4 d. The procedure was repeated until the transferred crystals reached a sufficient size for crystallographic analysis.

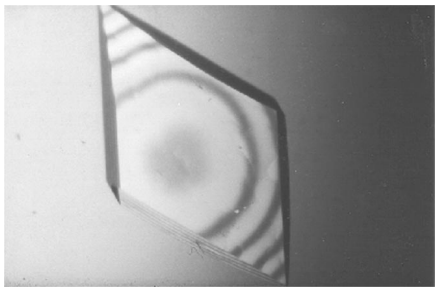

Figure 1
Crystal of the Rop mutant $Rop_{<2aa>}$. The edge of the crystal corresponds to 1.0 mm.

2.3. X-ray diffraction analysis

Native data extending to a resolution of 0.8 Å were collected at 100 K from a single cryoprotected crystal (using an aqueous solution of 40% PEG 400 as a cryoprotectant agent) of dimensions 0.5 × 0.4 × 0.3 mm. Data collection was performed at the EMBL/DESY synchrotron-radiation beamline BW7B using a MAR Research 345 imaging-plate detector. The wavelength of the X-ray beam was 0.8445 Å. Because of extensive overload effects, four sets of data were collected at overlapping resolution limits. Intensities were integrated with *DENZO* and scaled with *SCALEPACK* (Otwinowski & Minor, 1997).

3. Results and discussion

Crystals of $Rop_{<2aa>}$ (Fig. 1) belong to the monoclinic space group *C*2, with unit-cell parameters a = 46.37, b = 37.37, c = 31.56 Å, α = 100.62°; their dimensions frequently exceed 1.0 mm. Assuming one monomer per asymmetric unit, a Matthews coefficient (Matthews, 1968) V_M of 1.82 Å^3 Da^{-1} is obtained; this is in the range found for proteins.

Fig. 2 shows a diffraction pattern of the $Rop_{<2aa>}$ crystal recorded at the closest possible crystal-to-detector distance (85 mm) for the BW7B beamline. Diffraction extends to the edge of the detector (resolution of 0.8 Å). Crystallographic and data-collection parameters are given in Table 1.

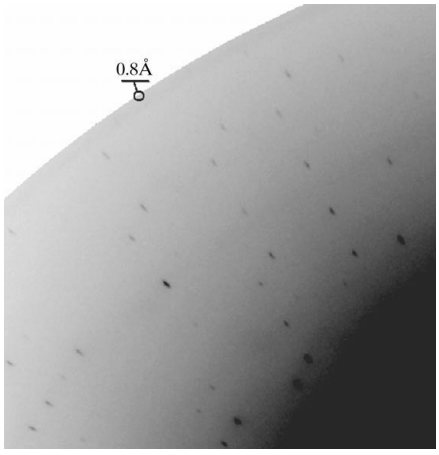

Figure 2
Detail from the diffraction pattern of $Rop_{<2aa>}$ obtained with synchrotron radiation and cryogenic (100 K) conditions. The detector edge corresponds to 0.8 Å resolution.

For the collection of ultrahigh-resolution data, technological advances in data collection, including synchrotron sources, cryogenic cooling and two-dimensional detectors, were essential. Owing to those advances, the rate of macromolecular determinations at atomic resolution has recently accelerated considerably (Dauter *et al.*, 1997; Merritt, 1999). The great advantage of atomic resolution lies in the large number of experimental observations, *i.e.* measured Bragg intensities, which allows anisotropic description of the position of each atom in the structure. Additionally, atomic resolution data enable modelling of flexible regions with confidence and analysis of solvent structures, yielding high-accuracy models which can be used to improve the target libraries of stereochemical parameters used in macromolecular refinement programs.

The structure of the $Rop_{<2aa>}$ protein determined at an ultrahigh resolution will provide valuable information about the folding of four-α-helical bundle proteins, particularly about the role of discontinuities in a regular heptad pattern.

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