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Purification, crystallization and preliminary X-ray diffraction analysis of a variant of the ColE1 Rop protein

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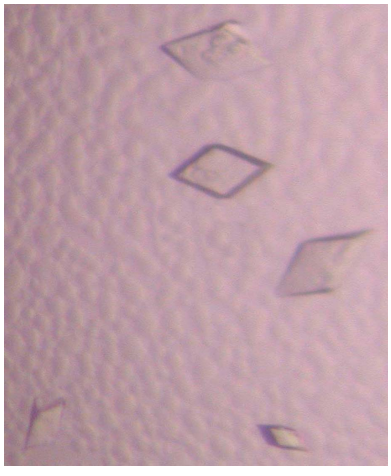
Rop is the paradigm of a canonical four-α-helical bundle. Its loop region has attracted considerable interest because a single alanine-to-proline substitution (A31P) in the loop is sufficient to change the topology of this small protein. In order to further analyse the loop region as a possible folding-control element, the double mutant D30P/A31G (RopPG) was produced, purified and crystallized. The crystals belonged to space group $P2_1$, with unit-cell parameters a = 26.7, b = 38.8, c = 56.6 Å, β = 100.9° and two molecules in the asymmetric unit. A complete data set was collected at 100 K to a resolution of 1.4 Å using synchrotron radiation.

1. Introduction

Rop (repressor of primer) is a small homodimeric RNA-binding protein that is involved in the regulation of copy number of the ColE1 plasmids of *Escherichia coli*, in which it is encoded (Polisky, 1988). Its structure has been studied using both X-ray crystallography (Banner *et al.*, 1987) and NMR (Eberle *et al.*, 1991). The Rop monomer consists of 63 residues (7223 Da) and forms two amphipathic antiparallel α-helices connected by a short hairpin loop in the region of residue Ala31. In the complete Rop molecule (*i.e.* the dimer), the two subunits are related by a twofold symmetry axis, with their four α-helices forming a tightly packed four-α-helical bundle. The Rop sequence displays a repeating pattern of hydrophobic and hydrophilic amino acids of the type $(abcdefg)_n$, which is typical for helical bundles (heptad repeat). Positions *a* and *d* are generally hydrophobic and form the core of the dimer with their side chains (Paliakasis & Kokkinidis, 1992). The heptad periodicity is only disrupted at the loop residue Ala31, which has a special position in the structure of Rop as it is the only residue which simultaneously forms hydrogen bonds to both helices (Banner *et al.*, 1987).

Rop is the paradigm of a canonical four-α-helical bundle. Its structural simplicity has rendered it a model system for the investigation of the sequence–structure relationships in the folding and dynamics of the tertiary motif of helical bundles, including the frequently debated question concerning the role of loops in protein folding. Numerous mutations in the loop region of Rop have been produced in order to study sequence–structure relationships (Castagnoli *et al.*, 1994; Nagi *et al.*, 1999; Vlassi *et al.*, 1994; Glykos *et al.*, 2006). With the exception of one mutant, which restores the heptad periodicity with the deletion of five loop residues and thereby totally alters the folding and assembly of the protein (Glykos *et al.*, 2006), the only mutant which results in drastic structural changes is the replacement of Ala31 by Pro (A31P), a conformationally constrained amino acid. This mutant exhibits a complete reorganization of the whole protein, which is converted from the canonical left-handed all-antiparallel form to a right-handed mixed parallel and antiparallel four-α-helical bundle (Glykos *et al.*, 1999; Glykos & Kokkinidis, 2001). Some properties of A31P are consistent with a molten globule state and analysis of A31P suggests that the role of



the loop is not to determine the fold, but to actively exclude some of the otherwise possible folding pathways (Glykos & Kokkinidis, 2004). However, the role of the loop sequence as a potential folding-control element of the helical bundle remains poorly understood.

To further explore this issue, we produced and initiated the characterization of the double mutant D30P/A31G (RopPG). The analysis of this mutant will offer insights into the interplay between highly flexible (glycine) and conformationally constrained (proline) loop residues and their implications for the properties and folding of the four-α-helical bundle.

2. Materials and methods

2.1. Expression and purification

The *ropPG* gene encoding RopPG (mutations were obtained using the QuikChange II Site-Directed Mutagenesis Kit from Stratagene) was cloned, inserted into the pET-26b(+) vector (Novagen) containing a C-terminal 6×His tag and transformed into *E. coli* strain BL21 (DE3). A sufficient amount of soluble protein was obtained after expression using the following conditions. Cells were grown in 1 l LB medium containing 25 µg ml^{-1} kanamycin at 310 K until OD_{600} reached 0.6. The culture was induced with 1 m*M* isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 h at 310 K and harvested by centrifugation at 6370*g* for 30 min at 277 K. Approximately 8 g of cell paste was resuspended in 80 ml lysis buffer containing 50 m*M* Na_2HPO_4 pH 8.0, 300 m*M* NaCl, 15 m*M* β-mercaptoethanol, 5 m*M* imidazole, 1 m*M* PMSF, 150 µg ml^{-1} benzamidine and homogenized by sonication for 10 min, after which the precipitate was removed by centrifugation at 18 500*g* for 1 h at 277 K. Purification was performed *via* the His tag by affinity chromatography at 277 K on a 5 ml Ni–NTA chelating column (Qiagen) pre-equilibrated in lysis buffer and initially washed stepwise with 10 and 20 m*M* imidazole. With a subsequent increase in imidazole concentration, RopPG eluted at 250 m*M* imidazole. Fractions containing more than 90% homogeneous RopPG, as judged by 15% SDS–PAGE gels, were pooled, dialyzed extensively against 25 m*M* Tris–HCl pH 7.5 containing 150 m*M* NaCl and 15 m*M* β-mercaptoethanol in order to remove imidazole and concentrated using Amicon Ultra-15 filters. The protein was further purified using a calibrated 350 ml S-100 Sephacryl gel-filtration column pre-equilibrated with one bed volume of dialysis buffer. The fractions of interest were dialyzed against storage buffer containing 20 m*M* Tris–HCl pH 7.5, 20 m*M* NaCl and concentrated to approximately 15 mg ml^{-1} for subsequent crystallization experiments. Typical yields are 35 mg of RopPG from approximately 8 g of cell paste.

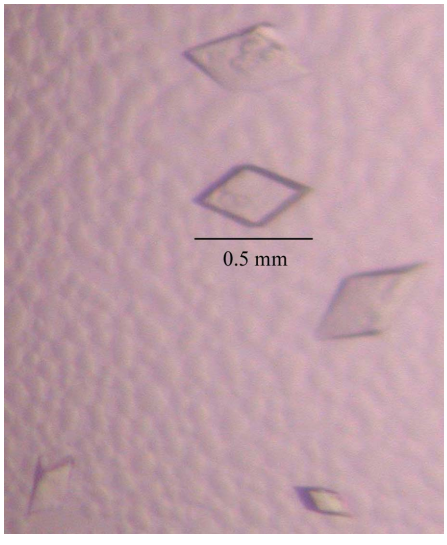


Figure 1
Crystals of the RopPG protein. The crystals are approximately 0.5 mm in their longest direction.

Table 1
Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell (1.45–1.40 Å).

Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 26.7, b = 38.8, c = 56.6, β = 100.9
Wavelength (Å)	0.817
Resolution (Å)	50.0–1.4
Observed reflections	413525
Unique reflections	22394
Redundancy	3.4 (3.1)
Data completeness (%)	99.5 (97.7)
R_{merge}† (%)	5.4 (39.1)
Average $I/\sigma(I)$	21.6 (2.3)
Mosaicity (°)	1.9

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the ith observation of reflection hkl, $\sum_{hkl}$ is the sum over all reflections and $\sum_i$ is the sum over i measurements of reflection hkl.

2.2. Crystallization

Crystallization conditions for RopPG were screened using the hanging-drop vapour-diffusion method and 24-well Linbro cell-culture plates. The drops were made up of 2 µl protein solution mixed with 2 µl reservoir solution and were equilibrated against 1000 µl reservoir solution at 291 K. Data-quality crystals were obtained with 45%(*v*/*v*) methanol, 50 m*M* HEPES pH 6.4 and 100 m*M* Li_2SO_4; the final crystal size was reached in 3 d (Fig. 1). These crystals were used for data collection. Crystals of comparable quality were also obtained with 45%(*v*/*v*) methanol, 50 m*M* MES pH 6.0 and 100 m*M* NaCl; however, the crystal-growth time increased to three months.

2.3. Data collection and processing

X-ray diffraction data to a resolution of 1.4 Å were collected from a single crystal using synchrotron radiation at the EMBL X11 beamline at the DORIS storage ring, DESY, Hamburg. The crystal was flash-cooled to 100 K in a nitrogen-gas cold stream using an Oxford Cryosystems device and 35% PEG 400 as cryoprotectant, which was added to the mother liquor. 240 images with 0.75° rotation each were collected. The diffraction data were recorded on a MAR CCD detector with a diameter of 165 mm. X-ray diffraction data were indexed, integrated and scaled with *DENZO* and *SCALEPACK* from the *HKL* program suite (Otwinowski & Minor, 1997). The *TRUNCATE* program from the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994) was used to convert intensities to amplitudes.

3. Results and discussion

The diffraction data of Rop PG are consistent with the monoclinic space group $P2_1$. Data-collection and processing statistics are given in Table 1. Assuming the presence of two molecules in the asymmetric unit, the Matthews coefficient (Matthews, 1968) is 2.06 Å^3 Da^{-1}, corresponding to a solvent content of 40.25%. We are pursuing structure determination of the RopPG protein using molecular

replacement with the native structure of Rop (PDB code 1rop) as a model.

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