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The purification and crystallization of mDia1 in complex with RhoC

Rolf Rose, Alfred Wittinghofer* and Michael Weyand

Max-Planck-Institut für Molekulare Physiologie, Department of Structural Biology, Otto-Hahn-Strasse 11, 44227 Dortmund, Germany

Correspondence e-mail: alfred.wittinghofer@mpi-dortmund.mpg.de

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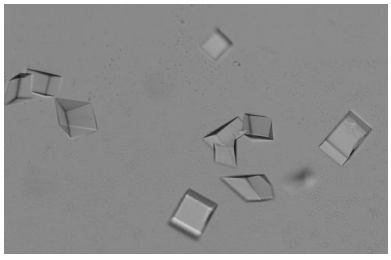

An N-terminal construct of mouse mDia1 was recombinantly expressed in *Escherichia coli*, purified and crystallized in complex with truncated human RhoC using the hanging-drop vapour-diffusion method. Crystals were obtained using PEG 2K MME and $MgSO_4$ as a precipitating agent and belong to the orthorhombic space group $P2_12_12$, with unit-cell parameters a = 148.4, b = 85.2, c = 123.2 Å. Complete native and SeMet-derivative data sets were collected at 100 K to 3.0 and 3.4 Å resolution, respectively, using synchrotron radiation.

1. Introduction

Rho proteins, members of the Ras superfamily of small GTPases, act as molecular switches which are toggled by guanine nucleotide-exchange factors (GEFs) and GTPase-activating proteins (GAPs) between the inactive GDP-bound form and the active GTP-bound form (for a review, see Etienne-Manneville & Hall, 2002). The downstream effects are mediated by effector proteins such as mDia1 which preferentially bind to the GTP-bound conformation of the small GTPases (Vetter & Wittinghofer, 2001).

The protein mDia1 belongs to the family of diaphanous-related formins (DRF), which are involved in actin cytoskeleton reorganization and are characterized by a GTPase-binding domain (GBD) and a C-terminal diaphanous autoregulatory domain (DAD) (Alberts, 2001; Watanabe *et al.*, 1997). All formins, including the DRFs, also feature a formin-homology 2 (FH2) domain, which is the active element for actin polymerization and is in most cases sufficient to mediate the nucleation of actin filaments *in vitro* (Shimada *et al.*, 2004; Xu *et al.*, 2004).

Two protein structures of the FH2 domain from yeast (Bni1p) and mouse (mDia1) have recently been solved (Shimada *et al.*, 2004; Xu *et al.*, 2004). It was shown that an additional short N-terminal segment in the Bni1p construct is sufficient to cause homodimerization and the enhancement of actin polymerization at nanomolar concentrations, while the monomeric FH2 construct lacking this stretch blocked actin polymerization at micromolar concentrations (Shimada *et al.*, 2004).

It has been proposed that the activity of the DRFs is regulated by two mechanisms. An intramolecular inhibition is mediated by interaction of the N-terminal GBD with the C-terminal DAD that causes a 'closed conformation' of the protein. This closed conformation blocks the catalytic activity of the FH2 domain. Relief of this inhibition by the binding of Rho proteins in their active GTP-bound form enables the protein to enter an active 'open conformation' (Alberts, 2001). Pull-down assays from mammalian cells also showed that the GBD alone is not sufficient for binding of the DAD and that a formin-homology 3 (FH3) domain is also needed to mediate this interaction (Krebs *et al.*, 2001).

To obtain further insight into the regulation of mDia1 by Rho GTPases and the DAD, we crystallized the regulatory N-terminus of mDia1 encompassing the GBD and the FH3 domains in complex with truncated human RhoC.

2. Experimental

2.1. Overexpression and purification

The N-terminal construct of mDia1 comprising residues 69–451 ($mDia_N$) was generated by PCR and cloned in the pGEX4-T1 vector

(Amersham Biosciences). The protein was expressed in BL21(DE3) cells, which were grown to an OD_{600} of 0.8 at 310 K. After induction with 0.1 m*M* isopropyl β-D-thiogalactopyranoside (IPTG), overexpression was carried out overnight at 293 K and cells were harvested by centrifugation. The bacterial pellet was usually resuspended in 10 ml buffer 1 [50 m*M* Tris–HCl pH 7.5, 100 m*M* NaCl, 5 m*M* dithioerythritol (DTE)] per litre of culture and lysed by sonic disruption. The resulting slurry was subjected to ultracentrifugation for 45 min at 100 000*g* and the supernatant was applied onto a GSH-Sepharose column equilibrated with buffer 1. After washing the column with at least ten column volumes of buffer 2 (buffer 1 containing 300 m*M* NaCl instead of 100 m*M*) the GST-fusion protein was eluted with buffer 2 containing 40 m*M* glutathione. The protein-containing fractions were concentrated using centrifugation concentrators (Amicon) and the GST was cleaved off with 5 U thrombin (Serva) per milligram of protein at 277 K overnight.

The $mDia_N$ construct could then be separated from the GST by gel filtration on a Superdex 200 column (Amersham Biosciences) equilibrated with low-salt buffer 3 (20 m*M* Tris–HCl pH 7.5, 20 m*M* NaCl, 5 m*M* DTE) for crystallization, as the salt concentration had no effect on the behaviour of the protein during gel filtration. After a final concentration step with centrifugation concentrators, the protein was frozen in liquid nitrogen and stored at 193 K. Truncated human RhoC (1–181) (carrying the mutation F25N for stability reasons; Ahmadian, unpublished work) was prepared and loaded with guanosine-5′-[(β,γ)-imido]triphosphate (GppNHp) as described in Ahmadian *et al.* (2002) and John *et al.* (1993).

For selenomethionine labelling, $mDia_N$ was expressed in LeMaster medium containing 0.1 g l^{-1} SeMet. Bacteria were grown to an OD_{600} of 0.6 at 310 K, induced with 0.1 μ*M* IPTG and the protein was overexpressed at 289 K for 24 h. Purification of the SeMet $mDia_N$ was performed as described for the native protein.

2.2. Crystallization

The $mDia_N$–RhoC complex was crystallized using the hanging-drop vapour-diffusion technique at 293 K. As the complex of the two proteins could not be separated from excess $mDia_N$ by gel filtration, $mDia_N$ and RhoC were mixed in an equimolar ratio directly before the crystallization experiments without a further purification step.

The final condition used for crystallization contained 14%(*w*/*v*) PEG 2K MME, 200 m*M* $MgSO_4$, 100 m*M* Tris–HCl pH 7.2, 5 m*M* DTE and both proteins were used at a concentration of 250 μ*M* in buffer 3 containing a final concentration of 5 m*M* GppNHp. The crystallization drop was made up of 0.7 µl protein solution with an equal amount of precipitant solution. The first crystals were observed after several hours and grew to final dimensions of 0.150 × 0.050 × 0.050 mm within 3 d (Fig. 1). The SeMet-derivative $mDia_N$–RhoC complex was crystallized as described for the native protein but at 285 K and in 16%(*w*/*v*) PEG 2K MME, 200 m*M* $MgSO_4$, 100 m*M* Tris–HCl pH 7.0, 5 m*M* DTE. Under these conditions the first crystals appeared after 2 d and reached final dimensions of 0.100 × 0.050 × 0.050 mm in about one week. Crystals were then frozen in liquid nitrogen using a cryosolution containing 30%(*w*/*v*) PEG 1500, 50 m*M* $MgSO_4$, 100 m*M* Tris–HCl pH 7.0 and 2.5%(*v*/*v*) glycerol.

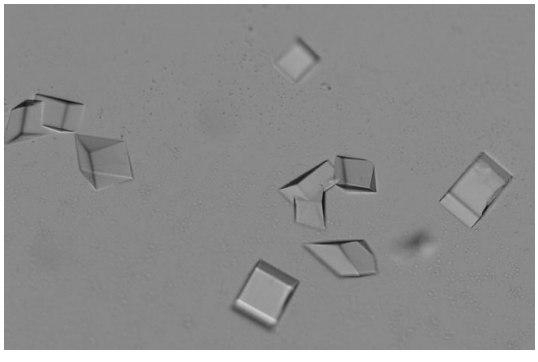

Figure 1
Photograph of a $mDia_N$–RhoC crystal (approximate dimensions 0.15 × 0.05 × 0.05 mm).

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

	Native	SeMet
Wavelength (Å)	0.933 (ID14-EH2)	0.9795 (BW7A)
Resolution (Å)	15–2.9 (2.972–2.9)	15–3.4 (3.5–3.4)
Space group	$P2_12_12$	$P2_12_12$
Unit-cell parameters (Å)	a = 148.4, b = 85.2, c = 123.2	a = 151.4, b = 85.6, c = 123.9
V_M (Å^3 Da^{-1})	3.1	3.4
Total measurements	165508	485533
Unique reflections	35204	42529†
Average redundancy	4.7 (4.7)	11.4 (11.3)
$I/\sigma(I)$	16.1 (3.2)	14.1 (3.1)
Completeness (%)	99.5 (99.4)	99.5 (100.0)
Wilson B factor (Å^2)	55	77
R_{sym}‡	8.1 (52.4)	8.5 (56.4)

† Friedel pairs are treated as separate reflections. ‡ $R_{sym} = \sum|I(h,i) - \langle I(h)\rangle|/\sum I(h,i)$, where $I(h, i)$ is the scaled observed intensity of the ith symmetry-related observation of reflection h and $\langle I(h)\rangle$ is the mean value.

2.3. Data collection

A native data set was collected from a $mDia_N$–RhoC crystal at 100 K at the European Synchrotron Radiation Facility (ESRF, Grenoble, France) beamline 14.2 at a wavelength of 0.933 Å using an ADSC Q105 CCD detector. The crystal-to-detector distance was 265 mm, the oscillation width per frame was 1.0° and 116 frames were collected.

A second data set was collected from selenomethionine-substituted $mDia_N$–RhoC at 100 K at the DESY synchrotron (Hamburg, Germany) beamline BW7A at a wavelength of 0.9795 Å using a MAR CCD detector in order to solve the phase problem. The crystal-to-detector distance was 230 mm, the oscillation width per frame was 0.75° and 700 frames were collected in order to achieve a very highly redundant data set.

Data were indexed, integrated and scaled with the *XDS* package (Kabsch, 1993). The native crystals diffracted to better than 3.0 Å resolution (Table 1) and systematic absences revealed that they belonged to space group $P2_12_12$.

3. Results and discussion

There are two heterodimers per asymmetric unit, corresponding to a V_M of 3.1 Å^3 Da^{-1} (Matthews, 1968) and a solvent content of 60%. Currently, we are trying to refine the selenium sites in order to solve the phase problem: as extensive molecular-replacement attempts to solve the complex using RhoA as a search template failed, structure determination will be attempted by experimental phasing using the SeMet derivative.

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