Combining dehydration, construct optimization and improved data collection to solve the crystal structure of a CRM1–RanGTP–SPN1–Nup214 quaternary nuclear export complex

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High conformational flexibility is an intrinsic and indispensable property of nuclear transport receptors, which makes crystallization and structure determination of macromolecular complexes containing exportins or importins particularly challenging. Here, the crystallization and structure determination of a quaternary nuclear export complex consisting of the exportin CRM1, the small GTPase Ran in its GTP-bound form, the export cargo SPN1 and an FG repeat-containing fragment of the nuclear pore complex component nucleoporin Nup214 fused to maltose-binding protein is reported. Optimization of constructs, seeding and the development of a sophisticated protocol including successive PEG-mediated crystal dehydration as well as additional post-mounting steps were essential to obtain well diffracting crystals.

1. Introduction

The growth of well diffracting crystals is the bottleneck in macromolecular X-ray crystallography. Since protein crystals are characterized by a fairly high solvent content of 40–70%, they can be dehydrated and rehydrated, which may be associated with spatial changes within the crystal lattice or the space group. Post-crystallization techniques, which consider these properties of macromolecular protein crystals, have been widely used to improve the diffraction quality of previously obtained crystals (Heras & Martin, 2005; Newman, 2006). In recent decades, a number of crystal structures have been published that report the positive effect of humidity-controlled or precipitant-controlled crystal dehydration on the diffraction quality of such treated crystals (Weiss & Hilgenfeld, 1999; Kiefersauer *et al.*, 2000; Echalié *et al.*, 2004; Sanchez-Weatherby *et al.*, 2009; Russi *et al.*, 2011; Deng *et al.*, 2012; Awad *et al.*, 2013; Bowler *et al.*, 2015). In addition, truncation and optimization of protein constructs used for crystallization is known to improve the diffraction quality of the resulting crystals. Since structural flexibility is an intrinsic and essential feature of nuclear transport receptors (Conti *et al.*, 2006; Zachariae & Grubmüller, 2008; Monecke *et al.*, 2009, 2013; Roman *et al.*, 2009; Dölker *et al.*, 2013; Grünwald *et al.*, 2013),

Table 1

X-ray data-set statistics and properties of non-dehydrated and dehydrated crystals of CRM1–Ran–SPN1–(MBP)Nup214.

Values in parentheses are for the highest resolution shell.

Crystal	1	2	3	4
Data collection				
Beamline	ID23-2, ESRF	ID23-1, ESRF	14.1, BESSY II	14.1, BESSY II
Space group	<i>C</i> 222 ₁	<i>C</i> 222 ₁	<i>C</i> 222 ₁	<i>C</i> 222 ₁
No. of complexes in asymmetric unit	1	1	1	1
Wavelength (Å)	0.8726	0.9840	0.9184	0.9184
Unit-cell parameters				
<i>a</i> (Å)	127.38	108.45	111.57	112.33
<i>b</i> (Å)	264.31	245.49	247.38	248.97
<i>c</i> (Å)	229.83	210.34	209.98	210.57
$\alpha = \beta = \gamma$ (°)	90.0	90.0	90.0	90.0
Resolution (Å)	50.00–4.50 (4.60–4.50)	50.00–3.20 (3.34–3.20)	50.00–3.02 (3.12–3.02)	50.00–2.85 (2.95–2.85)
Unique reflections	23384	43727	56387	67922
<i>R</i> _{merge}	0.126 (1.095)	0.050 (0.735)	0.058 (0.793)	0.055 (0.571)
<i>CC</i> _{1/2}	99.8 (47.8)	99.9 (76.8)	99.9 (85.2)	99.9 (87.3)
<i>I</i> / σ (<i>I</i>)	8.15 (1.33)	14.44 (1.91)	15.34 (1.78)	15.52 (2.01)
Completeness (%)	99.7 (99.9)	93.7 (72.5)	98.4 (98.8)	98.2 (91.4)
Multiplicity	4.5 (4.5)	4.4 (4.6)	5.1 (5.2)	4.6 (4.1)
Crystal properties				
Dehydration state	Non-dehydrated	50% PEG 8000	45% PEG 8000	45% PEG 8000
SPN1 construct	Full length, 1–360	Full length, 1–360	Δ C, 1–291	Δ C, 1–291
Measurement	Loop, 20% glycerol	Loop, 50% PEG 8000	MicroMesh, no solvent	MicroMesh, no solvent
Matthews coefficient (Å ³ Da ^{−1})	3.95	2.86	2.96	3.00
Solvent content (%)	68.9	57.0	58.4	59.1
Unit-cell volume (Å ³)	7737872 [100%]	5599963 [72.4%]	5795487 [74.9%]	5888969 [76.1%]
Wilson <i>B</i> (Å ²)	182.17	115.81	96.10	80.00
<i>B</i> factor (Å ²)	286.87	195.72	128.33	125.00
Crystal contact area (Å ²)	797.4	5798.4	5088.6	4959.3

crystallization and structure determination of such transport receptors is particularly challenging.

Nucleocytoplasmic transport of protein and ribonucleo-protein (RNP) cargoes proceeds through nuclear pore complexes (NPC) and is largely mediated by soluble nuclear transport receptors of the karyopherin- β superfamily termed importins or exportins (Görlich & Kutay, 1999; Cook *et al.*, 2007). Commonly, members of the karyopherin- β superfamily are composed of repetitive elements named HEAT repeats [huntingtin, elongation factor 3 (EF3), protein phosphatase 2A and yeast P3 kinase Tor]. The major nuclear export receptor CRM1 (chromosome region maintenance 1) consists of 21 tandem HEAT repeats and exports a multitude of protein and RNP cargoes (Fu *et al.*, 2011; Xu *et al.*, 2012). Proteins carrying leucine-rich nuclear export signals (NES; *i.e.* the cargo) assemble with CRM1 and the small GTPase Ran in its GTP-bound form (RanGTP) in the nucleus to form a transport-competent export complex (Fornerod *et al.*, 1997; Fukuda *et al.*, 1997; Ossareh-Nazari *et al.*, 1997; Stade *et al.*, 1997). The crystal structures of an export-competent complex comprising CRM1, RanGTP and the cargo Snurportin1 (SPN1) and of a binary CRM1–SPN1 complex have been determined, unravelling the details of CRM1–cargo–RanGTP interaction (Dong *et al.*, 2009; Monecke *et al.*, 2009). In these crystal structures, CRM1 adopts an overall toroid-like conformation with the N- and C-terminal regions forming intimate contacts. The NES of SPN1 docks into a hydrophobic cleft (NES-binding cleft) on the outer surface of CRM1, while RanGTP is bound to the interior of the toroid. In addition, crystal structures of free CRM1 have been determined at

decent resolution and have unravelled the details of CRM1 allosteric regulation (Saito & Matsuura, 2013; Monecke *et al.*, 2013).

A key feature of all nuclear transport receptors is the ability to interact with the phenylalanine–glycine (FG) repeats of nucleoporins (FG-Nups), the proteins forming the permeability barrier of the NPC and the NPC framework itself (Görlich & Kutay, 1999; Macara, 2001). Generally, proteins exceeding a molecular mass of ~30–40 kDa are restricted from passage across the NPC permeability barrier, whereas nuclear transport receptors are able to overcome this size limit by transient binding to FG-Nups (Görlich & Kutay, 1999; Weis, 2002; Pemberton & Paschal, 2005). FG-Nups residing either at the cytoplasmic or the nuclear side of the NPC have been suggested as the initial or terminal binding sites for import and export complexes in general (Yokoyama *et al.*, 1995; Kehlenbach *et al.*, 1999). In particular, nucleoporin 214 (Nup214), which is located at the cytoplasmic filaments of the NPC, was shown to interact tightly with CRM1 export complexes exiting the nucleus (Kehlenbach *et al.*, 1999; Hutten & Kehlenbach, 2006). Owing to this high-affinity interaction, fragments of Nup214 containing FG repeats seemed to be a particularly suitable target for structural studies of the CRM1–FG repeat interaction. We crystallized a complex composed of CRM1–RanGTP–SPN1 (*i.e.* a ternary export-competent complex) and a 117-amino-acid C-terminal fragment of Nup214 fused to maltose-binding protein (MBP). Since the initial crystals diffracted X-rays poorly, a protocol was established including successive PEG-mediated crystal dehydration, crystal mounting using MicroMeshes and additional

post-mounting steps, which included the removal of the liquid surrounding the crystals in a humidity-controlled air stream and subsequent flash-cooling in liquid nitrogen. Treatment of crystals using this combined protocol not only led to a significant increase in the resolution from 7 to 2.85 Å but also enabled structure solution owing to significantly improved data statistics.

2. Materials and methods

2.1. Macromolecule production

Full-length CRM1, RanGTP (amino acids 1–180, Q69L), SPN1 (amino acids 1–360 and 1–291) and a C-terminal fragment of Nup214 (1916–2033) fused to the C-terminus of MBP with a six-residue linker (amino-acid sequence NAAAEF)

were expressed and purified as described previously (Guan *et al.*, 2000; Strasser *et al.*, 2004; Dölker *et al.*, 2013; Port *et al.*, 2015).

In order to ensure stoichiometric complex formation, an excess of SPN1, RanGTP and (MBP)Nup214 was added to CRM1. The required relative amounts of the individual proteins were determined by initial gel-filtration experiments and are based on experience. The CRM1–SPN1–RanGTP–(MBP)Nup214 complex was assembled by mixing the individual components in a 1:3:5:5 molar ratio and was subsequently purified using a Superdex 200 gel-filtration column (GE Healthcare) in a buffer consisting of 50 mM NaCl, 20 mM Tris–HCl pH 7.5, 2 mM magnesium acetate, 2 mM DTT. The purified complex was concentrated to 5 mg ml^{−1}, cooled in liquid nitrogen and stored at −80°C.

2.2. Crystallization

The CRM1–SPN1–RanGTP–(MBP)Nup214_{1916–2033} export complex was crystallized by mixing 2 µl of a 5 mg ml^{−1} protein solution with 1 µl reservoir solution consisting of 5% (w/v) polyethylene glycol (PEG) 8000, 0.2 M L-proline, 0.1 M Tris–HCl pH 7.5, 4 mM D-maltose, 180 mM LiCl. Small initial crystals were crushed and diluted 1:100 000 (v/v) with the reservoir solution. Protein and reservoir solution were mixed and, after a pre-incubation time of 20 min, 0.2 µl seed solution was added to the drops. Crystals belonging to the

Figure 1
Crystal image (a) and diffraction pattern (b) of CRM1–RanGTP–SPN1–(MBP)Nup214 complex crystals treated with a combination of PEG-mediated dehydration and additional post-mounting steps. (c) Typical diffraction pattern of non-dehydrated crystals.

orthorhombic space group $C222_1$ grew at 20°C after 5 d to typical dimensions of $150 \times 150 \times 80 \mu\text{m}$.

2.3. Crystal dehydration and preparation

After size optimization, the crystals diffracted X-rays to a maximum resolution of 7 Å. The diffraction quality could be significantly improved by successive crystal dehydration. For this purpose, crystals were transferred stepwise to conditions with increasing PEG 8000 concentrations (from 5 to 45 or 50% PEG 8000 in 5% steps with 15 min incubation between each step). Notably, this treatment not only improved the diffraction quality of the crystals but additionally resulted in a significant reduction in the unit-cell dimensions [the a axis decreased from 126 to 112 Å (13%), the b axis from 263 to 248 Å (6%) and the c axis from 229 to 210 Å (8%)]. This corresponds to a remarkable decrease in the crystal solvent content from 69 to 59%.

To further improve the data quality and to reduce the scattering contribution from the surrounding liquid (the background noise) during data collection, the crystals were mounted using MicroMeshes (MiTeGen) on beamline 14.3 at BESSY II, Berlin equipped with an HC1c crystal humidifier (Mueller *et al.*, 2012, 2015). After complete removal of liquid using a paper wick, the crystals were flash-cooled in liquid nitrogen.

2.4. Data collection and processing

Flash-cooled crystals were transferred to beamline 14.1 operated by the Helmholtz-Zentrum Berlin (HZB) at the BESSY II electron-storage ring, Berlin-Adlershof, Germany equipped with a PILATUS 6M detector. Complete data sets were collected from two such treated crystals on beamline 14.1. Data were processed using *XDS* and *XSCALE* (Kabsch, 2010).

Figure 2

Molecular arrangement of the CRM1-RanGTP-SPN1-(MBP)Nup214 complexes from untreated or treated crystals. The unit-cell axes are labelled. The original molecule in the asymmetric unit is shown in yellow, while symmetry-related molecules in the structures of untreated and treated crystals are shown in red and green, respectively.

2.5. Structure solution and refinement

The structure was solved by molecular replacement with *Phaser* (McCoy *et al.*, 2007) using the export complex CRM1-SPN1-RanGTP (PDB entry 3gix; Monecke *et al.*, 2009) as a starting model. Subsequently, MBP (PDB entry 1anf; Quiocho *et al.*, 1997) was fitted manually into positive $|F_o - F_c|$ difference density, since localization by the molecular-replacement routines in *Phaser* did not provide an unambiguous solution, most likely owing to elevated B factors and disorder of the C-terminal MBP lobe. After replacement and initial rigid-body refinement, positive $|F_o - F_c|$ difference electron density near the MBP C-terminus and CRM1 HEAT repeats 14–20 as well as at the CRM1 N-terminus (HEAT repeats 2–4) allowed us to build three regions of Nup214, each containing a number of canonical FG repeats. The structure was refined by iterative cycles of *CNS* (Brünger *et al.*, 1998) and manual model building in *Coot* (Emsley *et al.*, 2010). Figures were generated with *PyMOL* (v.1.5.0.4; Schrödinger).

3. Results and discussion

To obtain structural information on the interaction of CRM1 with the phenylalanine-glycine (FG) repeats of nucleoporins, we wanted to co-crystallize a ternary CRM1-RanGTP-SPN1 complex with fragments of Nup214. Since complexes comprising CRM1-RanGTP-SPN1 and various untagged C-terminal Nup214 fragments did not crystallize, Nup214 was fused to the C-terminus of maltose-binding protein (MBP) to facilitate crystallization.

The individual components of the CRM1-RanGTP-SPN1-(MBP)Nup214_{1916–2033} complex were mixed and the resulting complex was purified by gel filtration. This complex crystallized in a reservoir solution consisting of 10% (w/v) PEG 3350, 0.2 M L-proline, 0.1 M HEPES pH 7.5 (Index screen condition No. 66, Hampton Research). Initial crystals diffracted X-rays to a maximum resolution of 7 Å. After optimization of the crystallization condition with respect to precipitant (PEG 8000) and additives (D-maltose), larger crystals grew and a 4.5 Å resolution data set could be collected on beamline ID23-2 at ESRF, Grenoble, France from a crystal grown in 5% (w/v) PEG 8000, 0.2 M L-proline, 4 mM D-maltose, 100 mM Tris pH 7.5 with 20% glycerol as a cryoprotectant. The crystals belonged to space group $C222_1$, with unit-cell parameters $a = 127.38$, $b = 264.31$, $c = 229.83$ Å (Table 1). Since this data set was collected from an untreated (*i.e.* non-dehydrated) crystal, these unit-cell parameters will be used as a reference. Despite the limited resolution of the obtained data, the structure could be partially solved by means of molecular replacement using the crystal structure of CRM1-RanGTP-SPN1 (PDB entry 3gix). Weak density for MBP as well as traces of Nup214 at two distinct sites on the surface of CRM1 could be identified; however, the resolution was too low to unambiguously build a model including MBP and the Nup214 residues.

In order to increase the achievable resolution of previously obtained crystals, a PEG-mediated dehydration protocol was

established. Crystals were transferred stepwise from 5 to 50% PEG 8000 (in steps of 5%) with 15 min incubation between the steps. While no significant change in diffraction quality was observed at 30% PEG 8000, further dehydration steps to 50% PEG 8000 led to a significant increase in resolution to 3.5 Å using a rotating-anode generator. Such dehydrated crystals, in which the high amount of PEG served as cryoprotectant, yielded a 3.20 Å resolution data set on beamline ID23-1 at ESRF, Grenoble, France and are characterized by reduced unit-cell parameters of $a = 108.45$, $b = 245.49$, $c = 210.34$ Å (Table 1). This corresponds to a marked decrease of 15, 7 and 8% for the individual unit-cell axes a , b and c , resulting in a reduction of the unit-cell volume by 28% ($7\,737\,872$ Å³ for non-dehydrated crystals and $5\,599\,963$ Å³ for dehydrated crystals). Strikingly, while the sum of all of the contact areas

between symmetry-related complexes in the non-dehydrated crystals equals only 797.4 Å², there is a more than sevenfold increase in the crystal contact area for crystals that have been dehydrated to 50% PEG 8000 (total area of 5798.4 Å²; Table 1). In this specific case, the increase in crystal contact area obviously facilitated better order in the crystal lattice and thus led to improved diffraction and an overall higher data-set quality. Since PEG-mediated crystal dehydration was rather hard to reproduce, crystal dehydration using a humidity-control device (HC1c) was attempted (Bowler *et al.*, 2015). Unfortunately, the positive effect of PEG-mediated dehydration on the diffraction quality of the crystals could not be achieved using the HC1c.

While the improvement in resolution seemed promising at this stage, the density corresponding to the bound Nup214 and

Figure 3

Detailed comparison of crystal packing and crystal contacts between symmetry-related complexes of treated and untreated crystals of the CRM1-RanGTP-SPN1-(MBP)Nup214 complex. The contact area between symmetry-related molecules in the crystal lattice increases by a factor of six. The original molecules are coloured yellow. A symmetry-related molecule from the structure of untreated crystals is depicted in red (left panels). The same symmetry-related molecule is shown in green (right panels) for the structure obtained from crystals that had been treated with the described protocol and in which the SPN1 construct has been shortened (amino acids 1–291). Treatment included crystal dehydration prior to mounting and the removal of liquid before flash-cooling and data collection. The individual proteins involved in the crystal contacts are labelled.

MBP improved only slightly. In order to further improve the resolution limit and the data quality, the following changes were introduced. Firstly, the SPN1 residues that were disordered in the ternary export complex (PDB entry 3gix; *i.e.* residues 288–348) were removed for crystallization. Hence, an SPN1 construct lacking the C-terminal 69 residues (SPN1_{1–291}) was purified and the CRM1–RanGTP–(MBP)Nup214–SPN1_{1–291} complex was prepared and crystallized. Secondly, the resulting new crystals were dehydrated up to only 45% (*w/v*) PEG 8000, which was the optimum dehydration level for this complex as judged by analysis of test data sets on a home source. Crystals were mounted on a goniometer head equipped with a humidity-control device (HC1c) using MicroMesh supports (beamline 14.3, BESSY II, Berlin) in an air stream with a defined humidity. In order to minimize the scattering and diffraction caused by the liquid surrounding the crystal, the liquid was removed using a paper wick, the crystal was flash-cooled in liquid nitrogen and data were obtained at 100 K. This procedure, combining construct optimization, successive PEG-mediated crystal dehydration and complete removal of the surrounding liquid prior to flash-cooling, yielded a 3.02 Å resolution data set on beamline 14.1 at BESSY II (Table 1). Notably, for such crystals not only did the achievable resolution increase, but the Wilson *B* factor also decreased significantly. Simultaneously, electron density corresponding to the Nup214 FG repeats was clearer and allowed two stretches of Nup214 to be traced. After extensive testing and dehydration of a number of crystals using the described protocol, a 2.85 Å resolution data set allowed complete structure solution and modelling of the quaternary export complex (Fig. 1). Finally, three stretches of Nup214 could be traced on the surface of CRM1 corresponding to 64 of the 117 amino acids (Port *et al.*, 2015). For these crystals, unit-cell dimensions that were decreased by 12, 6 and 8% were observed for the *a*, *b* and *c* axes, respectively (Fig. 2). Inspection of the crystal lattice revealed that MBP mediates crystal contacts to a symmetry-related MBP molecule as well as to CRM1 and RanGTP of another symmetry-related complex. Interestingly, the shift of symmetry-related molecules towards the original molecule in the asymmetric unit causing the decrease in unit-cell dimensions is rather isotropic and equals 10–15 Å in all three dimensions. Consequently, the overall crystal packing was significantly tighter and the crystal contact area increased by a factor of six (Fig. 3). For example, the CRM1 molecules of two symmetry-related complexes are shifted by 11 Å towards each other and form intimate crystal contacts in the dehydrated state, while there are hardly any crystal contacts in the non-dehydrated structure (Fig. 3, middle panel). Likewise, an MBP molecule of a symmetry-related complex, which neatly fits into a convex surface generated by the interior of CRM1 together with RanGTP of a dehydrated crystal, is 10 Å further apart in the non-dehydrated crystal (Fig. 3, bottom panel). On the opposite side of the complex, two symmetry-related SPN1 molecules closely interact *via* a number of crystal contacts in the structure derived from dehydrated crystals but are 9 Å apart in the non-dehydrated crystals, forming only a few contacts.

Taken together, our results show that improvement of resolution and data quality can be achieved by a combination of pre-crystallization considerations, such as construct optimization, and post-crystallization treatment of crystals. The latter includes crystal dehydration, which may be controlled by an external humidity-control device or by gradually increasing precipitant concentrations and complete removal of the solution surrounding the crystal followed by flash-cooling and data measurement. The combination of the described individual changes led to a higher overall resolution and significantly improved data-set quality.

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