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Strategies for crystallizing a chromatin protein in complex with the nucleosome core particle

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Abstract

The molecular details for how chromatin factors and enzymes interact with the nucleosome are critical to understand fundamental genetic processes including cell division and gene regulation. A structural understanding of such processes has been hindered by the difficulty producing diffraction quality crystals of chromatin proteins in complex with the nucleosome. We describe here the steps used to grow crystals of the 300 kDa RCC1 chromatin factor/nucleosome core particle complex which diffract to 2.9 Å resolution. These steps included both pre- and post-crystallization strategies potentially useful to other complexes. We screened multiple variant RCC1-nucleosome core particle complexes assembled using different RCC1 homologs and deletion variants, and nucleosomes containing nucleosomal DNA with different sequences and lengths as well as histone deletion variants. We found that using RCC1 from different species produced different crystal forms of the RCC1-nucleosome complex consistent with key crystal packing interactions mediated by RCC1. Optimization of post-crystallization soaks to dehydrate the crystals dramatically improved the diffraction quality of the RCC1/nucleosome crystal from 5.0 to 2.9 Å resolution.

Introduction

The nucleosome core particle (NCP) is a fundamental repeating unit of eukaryotic chromatin that contains 145 to 147 base pairs of DNA wound around a histone octamer protein core [1]. The nucleosome is the direct target of chromatin enzymes that regulates gene expression, nucleosome positioning and chromatin dynamics. For example, epigenetic chromatin modification enzymes install histone or DNA modifications that affect chromatin stability or the recruitment of other chromatin enzymes [2]. In addition, although the nucleosome is often believed to occlude binding of DNA-binding proteins, many transcription factors are now known to bind directly to nucleosomes [3]. A common feature of these cellular processes is the recognition of nucleosome by a chromatin factor. However, the molecular mechanism for this important class of interactions was confounded by the absence of any high resolution crystal structure for a chromatin protein in complex with the nucleosome core particle.

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The RCC1 (regulator of chromosome condensation 1) -propeller protein binds directly to nucleosomes and localizes the small GTPase Ran protein in its GTP bound state around the chromosomes. This concentration gradient of Ran-GTP around the chromosomes provides positional information for nuclear processes including mitotic spindle assembly and nuclear envelope synthesis during mitosis and nucleocytoplasmic transport during interphase [4]. Although biochemical studies had shown that RCC1 interacted with the H2A/H2B histone dimer [5], the exact interactions between RCC1 and the nucleosome were not known.

In this paper, we report our strategy to obtain high-resolution diffracting crystals of the RCC1 chromatin factor-NCP complex, resulting in the first crystal structure of a chromatin protein interacting with the nucleosome [6]. Successful crystallization of the complex required experimenting with multiple RCC1/NCP complexes reconstituted from several RCC1 orthologs and different versions of the nucleosome core particle. The best crystal form produced diffraction to 5 Å resolution, but optimization of post-crystallization dehydration soaks improved order in the crystal and permitted diffraction data collection to 2.9 Å resolution. This crystal form differs significantly from previous NCP crystals in both crystal packing (no end-to-end DNA packing) and crystallization conditions (no divalent ions present). Examination of the crystal lattices and crystal contacts for high and low resolution structures of the RCC1/NCP complex provide a molecular understanding for how these post-crystallization treatments improved the diffraction quality of the crystals.

Materials and Methods

Preparation of RCC1 proteins, nucleosome core particles and RCC1-nucleosome core particle complexes

The human RCC1 (hRCC1) coding region was amplified from HeLa cDNA, while *Saccharomyes cerevisiae* RCC1 (Srm1, referred to here as yRCC1) and *Ashbya gossypii* (agRCC1) were amplified from genomic DNA. The *Xenopus* (xRCC1) clone was a gift from Todd Stukenberg (University of Virginia), and *Drosophila* (dRCC1) and zebrafish (zRCC1) genes were purchased from the *Drosophila* Genomics Resource Center and Open Biosystems respectively. Full length and deletion variants of human RCC1 were expressed without affinity tags using pST50Tr, a T7-promoter based expression vector [7]. RCC1 from other species were expressed using pST50Tr with cleavable N-terminal Strept II – hexahistidine-TEV protease site (STRHISN) tags [7].

The *E. coli* BL21(DE3)pLysS strain was used to overexpress RCC1 proteins. Expression was induced using 0.2 mM IPTG for 3–5 hours at 37°C for hRCC1 and 14–18 hours at 18°C for other RCC1 proteins. Recombinant hRCC1 was purified from the soluble cell lysate using SP-Sepharose cation-exchanger followed by SourceQ anion-exchange chromatography. The tagged RCC1 proteins were purified from soluble lysates using Talon cobalt metal affinity chromatography, followed by TEV (tobacco etch virus) protease cleavage, and further purification over SourceS or SourceQ chromatography. With the exception of xRCC1, RCC1 from the different species could be concentrated to at least 10 mg/ml with polydispersities of <20% using a Protein Solutions DynaPro dynamic light scattering device. Recombinant *Xenopus* core histones and nucleosome core particles were prepared as described previously [8].

RCC1-nucleosome core particle complexes were prepared by mixing 20–30 nmol of RCC1 protein with 10–15 nmol of nucleosome core particles (~2.2:1 molar ratio) in a volume of 300–400 µl and incubating for 10–30 minutes at room temperature. The RCC1-nucleosome complex was purified by Superdex 200 HR size exclusion chromatography in T50Ac buffer (5 mM Tris-Cl pH 7.5, 50 mM sodium acetate, 1 mM DTT). The complex was concentrated to 10–20 mg/ml in the same buffer.

Crystallization of the complex

Initial crystallization screening and subsequent optimizations were performed using microbatch under oil procedure in 96 U-bottom microtiter plates [9]. 1 μ l of 10–20 mg/ml RCC1/nucleosome complex in T50Ac buffer was mixed with 1 μ l of crystallization solution, and overlaid with Al's oil. Crystals were also grown using hanging or sitting drop vapor diffusion techniques. Microseeding was used in hanging or sitting drop trials to enhance yields of crystals.

Our initial crystal cryoprotection soaking conditions used a base solution with 24% glycerol, 24% glycerol + 10% MPD (2-methyl-2,4-pentanediol), or 24% MPD, where the base solution was the crystallization mother liquor with 1.1–1.2 fold excess of precipitant concentration used in the crystallization drop. Crystals were transferred to the final cryoprotection soaking condition in 3 to 4 steps of increasing cryoprotectant concentrations for 30–60 seconds each. After flash-cooling by plunging into liquid nitrogen or exposure to a nitrogen cryostream, the crystals were exposed to X-rays at 100 K using a laboratory source (Rigaku Microfocus Micromax 007 X-ray generator with a Saturn 944 CCD detector) or at a synchrotron source (CHESS A1 and F1 beamlines, APS NE-CAT 24-ID-E beamline).

Crystallographic analysis and refinement of models

The 2.9 Å high resolution structure of the dRCC1/nucleosome complex (PDB 3MVD) was solved as described previously [6]. The 5.2 Å low resolution structure of the same complex was determined using data collected from crystals prior to the post-crystallization soaking improvements. Diffraction data were integrated, scaled and merged using the HKL2000 software package. Data collection statistics are shown in Table 1. The structure solution was obtained by Phaser molecular replacement using the Drosophila RCC1 and nucleosome core particle structures from the 2.9 Å structure as two rigid bodies. The structure was partially refined using PHENIX [10] allowing for rigid body, group B-factor and TLS refinements to a $R_{\text{working}}/R_{\text{free}}$ of 22/24%. No manual rebuilding of the low resolution structure was performed. Analysis of crystal packing and graphics output were performed using the molecular graphics programs Coot and PyMOL [11,12].

Results

Reconstitution and purification of RCC1-nucleosome core complexes

To produce RCC1-nucleosomes core particles complexes, we prepared recombinant nucleosome core particles as described previously and we cloned and expressed RCC1 from multiple species. In particular, we reconstituted nucleosome core particles containing *Xenopus* core histones H2A, H2B, H3 and H4 and a 147 bp Widom 601 sequence and we expressed RCC1 orthologs from human, *Xenopus*, zebrafish, *Drosophila* and two different yeasts (*Saccharomyces cerevisiae* and *Ashbya gossypii*). Each of the purified RCC1 orthologs could be concentrated as monodisperse proteins except for *Xenopus* RCC1 which formed large soluble aggregates.

The complex of RCC1 bound to the nucleosome could be prepared by simply mixing purified RCC1 protein with recombinant nucleosome core particles in a low ionic strength buffer. Addition of RCC1 protein to nucleosome core particle solutions produced a white precipitate that typically cleared within 10 seconds, a convenient visual indicator for the formation of the RCC1/nucleosome complex. Size exclusion chromatography over a Superdex 200-HR column was used to purify the RCC1/nucleosomes complex from free nucleosomes and excess RCC1 protein (Fig. 1). The RCC1/nucleosome eluted sooner than would be anticipated for the expected molecular weight of 300 kDa, suggesting that the complex was elongated and not globular.

Crystallization of the human RCC1/nucleosome complex

Following our unsuccessful attempts to prepare monodisperse *Xenopus* RCC1, we turned to human RCC1 for which the crystal structure had been determined [13]. For these initial studies, we used human RCC1 lacking its N-terminal 20 residues (hRCC1^{Δ1} = hRCC1(21–421)) to reconstitute RCC1/nucleosome complexes. The N-terminal 20 amino acids are disordered in the human RCC1 and human RCC1-Ran complex crystal structures, and we had shown that hRCC1^{Δ1} bound to nucleosome core particles *in vitro* [13–15]. The γ -propeller structured hRCC1^{Δ1} forms a stable complex with recombinant nucleosome core particles as determined by size exclusion chromatography (Fig. 1) and the purified complex could be concentrated to at least 10 mg/ml without aggregating. Crystallization trials produced crystals under multiple crystallization conditions at different pH and salt concentrations. One initial crystallization condition (25 mM HEPES pH 7.5, 50 mM sodium acetate, 1 mM DTT and 10 % PEG2000 MME) was optimized to grow larger crystals (0.2 × 0.2 × 0.5 mm). These crystals grew as hollow tubes in clusters (Fig. 2a). Analysis of washed crystals by SDS-PAGE confirmed the crystals contained the expected polypeptide components of the RCC1-nucleosome complex (Fig. 2b). However, such crystals produced smeary diffraction to only 20 Å on a home X-ray source. Attempts to improve the crystals by exploring other crystallization conditions including varying pH, monovalent and divalent salts, precipitants, crystallization geometries (sitting versus hanging drops) or by seeding were not successful.

We interpreted the hollow crystal morphology as a two-dimensional sheet folded into a tube and reasoned that this might reflect weaker crystal contacts in a third dimensions compared to the two dimensions of the sheet. To promote new or alternative crystal contacts, we next focused on manipulating the macromolecular components of the complex. We reconstituted full length human RCC1, i.e. restoring the 20 residues N-terminal extension to the RCC1 γ -propeller, with nucleosome core particles but this complex crystallized with similar crystal morphology growing in clusters as with hRCC1^{Δ1}/nucleosomes.

In crystals of the nucleosome core particle on its own, critical crystal contacts are mediated by the nucleosomal DNA ends [1]. In the 1.9 Å high resolution nucleosome core particle crystals, additional crystal contacts are made by the histone tails which extend beyond the core histone-fold motif [16]. We therefore reconstituted RCC1/nucleosome complexes using nucleosome core particles with different nucleosomal DNA sequences (symmetric human γ -satellite DNA used in previous nucleosome crystals, mouse mammary tumor virus (MMTV) long terminal repeat (LTR) NucB nucleosome positioning sequence [17,18]) and different DNA lengths (MMTV NucB 152 and 157 bp), or with truncations in individual histone tails. Each of these variant RCC1/nucleosome complexes, with the exception of the complex containing human γ -satellite DNA which did not crystallize, produced crystals with similar morphology to the original complex containing human RCC1^{Δ1} and nucleosome core particles reconstituted from *Xenopus* core histones and the 147 bp Widom 601 DNA positioning sequence [19]. These results suggested to us that in contrast to nucleosome core particle crystals, neither the DNA ends nor the histone tails mediated critical crystal contacts in these human RCC1/nucleosome complex crystals. We also surmised that RCC1 surfaces were likely involved in crystal contacts in such RCC1/nucleosome crystals.

Crystallization of RCC1/nucleosome complexes using RCC1 from yeast and other species

We next purified hexahistidine tagged full length yeast RCC1 (gene name Srm1, but referred to here as yRCC1) which shares 28% sequence identity with human RCC1, and reconstituted the yeast protein with nucleosome core particles containing the Widom 601 147 bp sequence. Crystallization trials of the HISyRCC1/nucleosome complex produced crystals under a variety of crystallization conditions at different pH (5.5 to 8.0) and

containing different monovalent salts. Unlike the hRCC1/nucleosome crystals with their persistent hollow tube-like morphology, HISyRCC1/nucleosome crystals formed block parallelepiped crystals (Fig. 2c). Optimization of crystallization conditions containing 25 mM sodium acetate pH 5.5, 50 mM sodium citrate, 4–6 % PEG2000MME produced block crystals with dimension of up to $150 \times 150 \times 1000 \mu\text{m}$. When mounted in a capillary and exposed to home source X-ray at room temperature, these crystals produced diffraction spots to 7 Å. Similar crystals cryoprotected using 24% glycerol or a mixture of 10% MPD and 20% glycerol produced anisotropic diffraction to 4.5 Å along the best axis and about 8 Å along the other two axes at 100 K using home source X-rays. On a synchrotron source (CHESS F1 beamline), the cryoprotected crystals displayed marginal improvements in the diffraction limit with smeary diffraction to 3.5 Å in the best axis, and little change along the other axes (Fig. 3a).

Attempts to improve these HISyRCC1/nucleosome complex crystals by optimizing or altering crystallizing conditions were unsuccessful. For example, inclusion of additives such as divalent metal ions, detergents and organic solvents, as provided through the Hampton additive screen, produced similar block crystals with no improvement in diffraction quality.

Given the significant improvement in RCC1/nucleosome crystal morphology and diffraction quality switching from human to yeast RCC1, we engineered variants of the yeast RCC1 protein for cocrystallizing with nucleosome core particles. Removing the N-terminal hexahistidine tag on yeast RCC1 did not affect yRCC1/nucleosome cocrystallization nor did this improve diffraction characteristics of the crystal. We also engineered yeast RCC1 variants with N- or C-terminal truncations or with internal truncations to remove potentially flexible loop regions. The variant proteins were analyzed for solubility and nucleosome binding activity using pull-down assays. However, crystal trials using these truncation variants of the yeast RCC1 protein in complex with nucleosome core particles containing the Widom 601 DNA sequence produced crystals with similar morphology and diffraction characteristics to those RCC1/nucleosome complexes containing full length yeast RCC1. Crystal trials employing nucleosome core particles reconstituted with different DNA nucleosomal positioning sequences or removing histone tails also failed to produce better diffracting crystals.

In addition to RCC1 from *S. cerevisiae*, we cloned and expressed RCC1 from the ancient yeast *Ashbya gossypii* (75% identity with yRCC1) and from the vertebrate zebrafish (74% identity with hRCC1). Both proteins bound nucleosome core particles, but these RCC1/nucleosome complexes produced needle crystals that were not pursued further.

Crystallization of RCC1/nucleosome complexes using *Drosophila* RCC1

Both human RCC1 and *Drosophila* RCC1 (Bj1, referred to as dRCC1 here) functionally complement the tsBN2 hamster cell line which exhibits premature chromosomal condensation at non-permissive temperatures [20]. We expressed full length dRCC1 in *E. coli* and demonstrated the protein binds nucleosome core particles. However, the protein and the complex with the nucleosome tended to aggregate or precipitate, and we were unable to grow crystals of the complex. Full length dRCC1 = dRCC1(1–517) contains a C-terminal 130 amino acid extension not found in most other organisms and comprised of three Bj1 repeats. We therefore expressed and purified a truncation variant, dRCC1^{Δ1} = dRCC1(2–422) which removes the C-terminal Bj1 extension and produces a protein equivalent to hRCC1(2–422). The dRCC1^{Δ1} protein was well behaved and could be assembled into stable RCC1/nucleosome complexes. The dRCC1^{Δ1}/nucleosome complex produced single crystals which grew to dimensions of approximately $300 \times 100 \times 400 \mu\text{m}$ within 4 to 7 days at 294K in 25 mM sodium acetate pH 5.5, 25 mM sodium citrate and 6–8% PEG2000-MME (Fig. 2d). These crystals grew using a variety of crystallization setups including microbatch under

oil, sitting or hanging drop vapor diffusion. Reproducibility of crystallization could be improved by microseeding to promote nucleation. Analysis of washed crystals by SDS-PAGE confirmed the presence of RCC1 and histone protein components of the complex (data not shown).

When radiated from a home X-ray source, crystals of dRCC1 1/nucleosome core particle containing the 147 bp Widom 601 sequence mounted in a capillary produced diffraction to 6.5 Å at room temperature. Notably, the diffraction was isotropic in contrast to that collected from yRCC1/nucleosome crystals. We collected diffraction data to 5.0 Å at 100 K at the Advanced Photon Source synchrotron facility (Fig. 3b) and determined a low resolution structure of the complex by molecular replacement using human RCC1 and the nucleosome core particle crystal structures as search models. Analysis of the crystal packing showed large voids in the lattice consistent with the calculated 70% solvent content. We explored filling such voids by reconstituting a RCC1/nucleosome complex containing dRCC1 1 fused to maltose binding protein (MBP), but this complex did not produce convincing crystal hits. As we did with human and yeast RCC1, we produced crystals of dRCC1 1/ nucleosomes with different nucleosome DNA sequences or lacking histone tails but these crystals had inferior morphologies (mostly needles).

Post-crystallization soaks to improve diffraction of RCC1/nucleosome crystals

The internal order of macromolecules in a crystal can, in some instances, be improved through post-crystallization soaks in dehydrating solutions which promote more closely packed molecules in the crystal [21–23]. In particular, the diffraction of nucleosome core particle only crystals was improved by post-crystallization soaks in solutions containing methylpentanediol (MPD) [1,16]. We therefore explored different cryoprotectants and dehydrating agents including MPD, glycerol, ethylene glycol, PEG400, trehalose and combinations thereof with our best yeast RCC1/nucleosome and *Drosophila* RCC1/ nucleosome crystals. Most of these agents did not improve diffraction of the yeast RCC1/ nucleosome crystals. Modest improvement from anisotropic $4.5 \times 8.0 \times 8.0$ Å diffraction to $4.0 \times 6.0 \times 6.0$ Å was obtained by soaking these crystals grown in 25 mM sodium acetate pH 5.5, 50 mM sodium citrate and 4–6% PEG2000-MME for 5–10 minutes at room temperature in this solution but containing 15% PEG2000-MME (the increased concentration presumably dehydrates the crystal) and 20% glycerol (which acts as a cryoprotectant to prevent ice formation in the crystal during flash cooling into liquid nitrogen used for cryocrystallography data collection) [24]). Analysis of the diffraction data indicates a C2 monoclinic unit cell with cell parameters $a = 131.81$, $b = 104.83$ $c = 148.91$ Å, $\beta = 105.39^\circ$. The determined Matthews number of $3.19 \text{ Å}^3/\text{Da}$ suggests a pseudo-symmetric half of the RCC1/nucleosome complex in the asymmetric unit of the C2 space group. Further post-crystallization soaking experiments failed to improve diffraction limits or anisotropy of the yeast RCC1/nucleosome crystals.

In our initial post-crystallization soaks of the dRCC1 1/nucleosome crystals, we were able to collect synchrotron diffraction data to 5.0 Å by soaking the crystals in a solution containing 10% MPD and 24% glycerol prior to flash cooling in liquid nitrogen. We included MPD because it had been successfully used to improve diffraction from crystals of the nucleosome core particle while glycerol was included as a cryoprotectant. While exploring combinations of different cryoprotectants and dehydrating agents, we discovered that dRCC1 1/nucleosome crystals soaked into a solution containing 24% MPD at room temperature for 5–10 minutes and subsequently transferred to 4°C for 3 hours before flash cooling in liquid nitrogen produced isotropic diffraction to 3.5 Å using a home X-ray source (Fig. 3c). This encouraging result was tempered somewhat by relatively high mosaicity, increased diffuse scatter at higher resolution and in particular, the difficulty reproducing the post-crystallization soaking procedure. Nevertheless, this procedure formed the foundation

for establishing a robust and effective post-crystallization protocol for improving the crystal diffraction limits. Systematic variation of the post-crystallization soaking parameters was key to developing this protocol.

We found that the critical post-crystallization parameters were the soaking temperature and time, as well as the identity and concentration of cryoprotectants and dehydrating agents. Crystals from these post-crystallization soaking studies were analyzed using our home X-ray source. We suspected that the post-crystallization soaking temperature was an important parameter when we observed time-dependent deterioration of diffraction quality for dRCC1 1/nucleosome crystals soaked in a final concentration of 24% MPD (by successive transfers into increasing concentrations of MPD) at room temperature, but improved diffraction when soaked at 4°C. Crystals soaked in 24% MPD at room temperature for more than 30 minutes failed to diffract X-rays and higher ambient temperatures exacerbated the diffraction deterioration. The temperature effect was not reversible since crystals with reduced or no diffraction after soaking in 24% MPD at room temperature could not be rescued by soaks at 4°C. Furthermore, diffraction of crystals grown at 4°C or harvested from crystallization plates equilibrated at 4°C after growth at room temperature could not be improved by post-crystallization soaks. We also found that the age of the crystals affects post-crystallization improvements. The crystals grow to optimal size in 4–7 days, and are typically harvested for post-crystallization soaks within 10–15 days. Crystals harvested after this period were less likely to benefit from the post-crystallization soaks.

To improve the diffraction quality and reproducibility of the post-crystallization soaking protocol, we explored different cryoprotectants and dehydrating agents including glycerol, ethylene glycol, 1,6-hexanediol, PEG400 and PEG1000 at 4°C. The first three did not improve diffraction from dRCC1 1/nucleosome crystals, but replacing MPD with PEG400 produced improvements in diffraction spot quality and mosaicity. Further improvements were obtained by using smaller increments of 2% PEG400 to the final concentration of 24% over a period of 2–3 hours at 4°C. Since 24% PEG400 was sufficient to prevent ice formation during flash cooling into liquid nitrogen, we omitted glycerol from the post-crystallization soaking solution. The final optimized protocol involved using cryoloops to transfer crystals grown in 25 mM sodium acetate pH 5.5, 25 mM sodium citrate, 1 mM DTT and 6–8% PEG2000-MME into 50 µl of a base soaking solution of 25 mM sodium acetate pH 5.5, 25 mM sodium citrate and 10–12% PEG2000-MME at room temperature, and then immediately moving this to a 4°C cold room for subsequent soaks. After 2–5 minutes in this base solution, the crystals were serially transferred to 50 µl of base soaking solution containing 2 to 24% PEG400 in 2% increments for 10–15 minutes each. After soaking for 10–15 minutes in the final solution, the treated crystals were then flash cooled in liquid nitrogen and used for diffraction experiments or stored in liquid nitrogen. We found that flash cooling in liquid nitrogen was marginally better than cryocooling directly in a 100K nitrogen stream. dRCC1 1/nucleosome crystals processed through this post-crystallization soaking protocol reproducibly showed isotropic diffraction to 3.5 Å on our home X-ray source.

To collect diffraction data for structure determination, 65 crystals were processed through the post-crystallization soaks and taken to the NE-CAT 24-ID-E beamline at the Advanced Photon source. We observed diffraction to 3.1 Å for the majority of the crystals, but two crystals produced diffraction to 2.8 Å allowing full data set collection to 2.9 Å (Fig. 3d). The mosaicity and spot quality from these crystals were significantly improved over diffraction observed using the home source presumably due to a combination of factors including individual crystal quality and the tight focus and the brilliance of the 24-ID-E beamline.

Molecular packing in the *Drosophila* RCC1/nucleosome complex crystals

To better understand the molecular basis for improvements in diffraction of the dRCC1/NCP crystals after post-crystallization soaks in PEG400, we compared the crystal lattice and molecular packing in the partially refined low resolution structure at 5.0 Å (crystal soaked in 10% MPD and 24% glycerol) with the high resolution structure at 2.9 Å (crystal soaked in 24% PEG400, PDB id 3MVD). In both crystals, each tricycle front wheel-like dRCC1/NCP complex stacks on each other by the pedals (RCC1) of the tricycle wheel contacting the neighboring tricycle wheel (nucleosome). Both crystals belong to $P2_1$ space group and the unit cell parameters were $104.64 \times 185.92 \times 109.55$ Å, $\beta = 97.61$ and $102.681 \times 183.036 \times 107.030$ Å, $\beta = 101.52$ for the low and high-resolution crystal lattices respectively. The unit cell volume of the high resolution crystals decreased 6.7% as compared to the low resolution crystal unit cell. The Matthews number of the low and high resolution crystals were $3.58 \text{ Å}^3/\text{Da}$ and $3.34 \text{ Å}^3/\text{Da}$, corresponding to solvent contents of 69.3% and 67.1% respectively.

The 5.0 Å diffraction of the low resolution crystals and the resulting incomplete refinement of the molecular replacement solution model limit us to only estimates for the molecular packing interactions in the low resolution crystals. However, it is clear that the PEG400 post-crystallization soaks result in increased crystal packing interactions in the high resolution crystals. The decreased solvent content and smaller cell parameters occur concomitant with subtle rearrangements of the molecules in the lattice (Fig. 4). The number of symmetry related crystal contacts increased dramatically from about 80 (low resolution crystal) to 302 (high resolution crystal) calculated using CCP4 NCONT software and a distance cutoff of 4.0 Å. Most of the increased crystal contacts in the high resolution crystal occurred at locations of existing crystal contacts in the low resolution crystal (Fig. 4). A major increase of ~22 to 102 crystal contacts occurs along the unit cell a-axis where the dRCC1/NCP molecules stack on each other related by translational symmetry ($x \pm 1, y, z$). In this direction, charged and mostly basic residues 63–66 (Glu-Arg-Lys-Arg) and 394 (Lys) of the RCC1 interacts with the symmetry-related nucleosome DNA at +2 DNA helical turns from the nucleosome dyad (DNA residues 53–56) (Fig. 4b, c, f, g). A perpendicular set of interactions occurs between only one of the two RCC1 molecules and one end of the nucleosomal DNA, increasing the number of interactions from ~18 in the low resolution crystal to 38 in the high resolution crystal.

In addition to increasing the number of existing contacts, the PEG400 post-crystallization soaks also resulted in two new sets of crystal contacts. In the first set, Thr344 of the RCC1 L-chain interacts with the DNA backbone of a symmetry related complex at –3 DNA helical turns from the nucleosome dyad. In the second interaction, displacements of 5–7 Å after the post-crystallization soaks enable RCC1 molecules from symmetry related complexes to interact with each other. The increased existing and the new crystal contacts occur along the three unit cell axes and result in a significance increase in accessible surface area excluded due to crystal contacts from 1 843 to 3 125 Å² (calculated using CCP4 AREAIMOL using a probe solvent radius of 1.4 Å).

Discussion

We recently determined the first atomic structure of a chromatin protein bound to the nucleosome [6]. Our ability to collect X-ray diffraction data to 2.9 Å can be traced to successes in two stages of the project. In the first stage, which occurred over a period of about 20 months, we managed to grow single crystals of the RCC1/nucleosome complex. In the second stage, which took about 9 months, careful optimization of dehydration and cryosoaking conditions improved the diffraction limits of the crystals from about 6 Å to 2.9 Å. Analysis of the steps taken during these two stages of the project suggest lessons that

might prove to be useful for others tackling crystallization of large macromolecular complexes such as chromatin complexes.

To grow single crystals of the RCC1/nucleosome complex, we prepared ~60 different complexes containing 22 RCC1 species or truncation variants and 18 nucleosome core particles with different histone truncations, DNA sequences and lengths. Our first crystals of the RCC1/nucleosome complex contained human RCC1 but were morphologically imperfect. We failed to improve those crystals by optimizing crystallization conditions, and then embarked on a systematic study varying individual components of the complex. This proved to be an effective approach because even negative results (i.e. no improvements in the crystals) provided us with insights about which complex components were important for the complex to pack into a crystal. For example, our finding that changing the nucleosomal DNA length did not appear to affect crystallization of the human RCC1/nucleosome complex suggested the nucleosomal DNA ends did not mediate crystal packing. Conversely, observing that switching from human to yeast RCC1 dramatically improved crystal morphology suggested that the RCC1 protein mediated major crystal packing interactions of the complex.

The single most important parameter for RCC1/nucleosome crystallization was, in fact, the RCC1 species used to reconstitute the complex. We were fortunate that it was relatively straightforward to express and purify recombinant RCC1 from different orthologs, allowing us to screen RCC1 from five (excluding *Xenopus*) different species in our RCC1/nucleosome crystallization trials. Each of the monodisperse RCC1 proteins could be reconstituted with our recombinant nucleosome core particles, but not all RCC1/nucleosome complexes could be crystallized. Only RCC1/nucleosome complexes containing yeast or *Drosophila* RCC1 produced single crystals with good morphologies.

The use of orthologs to grow crystals is a well-known practice in macromolecular crystallography and its mechanistic basis is also understood: different orthologs will likely share similar three-dimensional structures since they serve similar biological functions. However, the surface residues not involved in the biological function will be under less selective pressure during evolution and therefore more likely to differ among different species. Since crystal contacts are typically mediated by surface residues, different orthologs offers an opportunity to explore different crystal contacts using natural occurring proteins. The RCC1/nucleosome crystal structure determination allows us to now explain why the precise RCC1 ortholog was so critical for growing RCC1/nucleosome crystals: RCC1 mediates all the crystal contacts in the RCC1/nucleosome crystal. However, it should be noted that our understanding is also incomplete. We cannot explain, for example, why *Drosophila* RCC1 produced the best RCC1/nucleosome crystals or why zebrafish RCC1 failed to produce RCC1/nucleosome crystals.

Optimum dehydration promotes subtle rearrangement of molecules that improves diffraction quality

Growing single RCC1/nucleosome crystals was the first stage of the process to collecting sufficient quality diffraction data. The second stage was improving the internal order of the crystals. The native resolution limit of freshly grown dRCC1/nucleosome crystals was about 6 Å, as shown by diffraction studies using capillary mounted crystals at room temperature without post-crystallization treatments. Dehydration/cryosoaking experiments produced improvements to 3.5 Å using 24% MPD, but such results were not reproduced consistently. Substituting PEG400 for MPD improved the reproducibility of the dehydration/cryocooling experiments

We standardized the protocol for post-crystallization treatments that reproducibly improve the diffraction quality and limit of the *Drosophila* RN complex crystals. The diffraction limit of the crystals was improved from 5 Å to 2.9 Å. This improvement is attributed to optimum dehydration of the crystals by gradual soaking into 24% PEG400 at reduced temperature. It is not clear why the reduced temperature was so critical for the dehydration step, or why PEG400 proved to be a more reliable dehydrating agent than MPD. It is possible that the lower temperature allowed for slower dehydration and correspondingly slower or more controlled rearrangement of molecules in the crystal. A characteristic of the dehydrating agent that could be relevant here is viscosity. The rate of diffusion for the dehydrating agent to enter the crystal depends on the viscosity of the dehydrating agent. Slower diffusion of MPD at 4°C due to increased viscosity at the lower temperature could partly account for the increased order in the crystal and resulting diffraction when the MPD soaks were performed at 4°C instead of room temperature. Furthermore, since MPD is significantly less viscous than PEG400 (34 cP at 20°C for MPD vs ~110 cP at 20°C for PEG400), the improved diffraction observed using PEG400 as the dehydrating agent instead of MPD could reflect slower diffusion of PEG400 into the crystal. These considerations are admittedly speculative, and it is likely that the success we enjoyed using PEG400 depends on many more aspects than simply the diffusion of the reagent into the crystal [25]. Furthermore, we cannot anticipate that PEG400 will be the appropriate dehydrating agent for other chromatin factor/nucleosome complex crystals, yet alone other macromolecular crystals. An empirical search of different dehydrating agents employed at different temperatures may be necessary to determine an optimal dehydration protocol for a particular macromolecular crystal.

Although we developed a relatively robust dehydration protocol for our RCC1/nucleosome crystals, we observed differences in the diffraction limits and mosaicity in different crystals. While some of these differences might reflect unintended variations in the dehydration soaks or cryocooling process, it is also likely that some of the differences can be attributed to differences in inherent order within individual crystals. Thus, we found it beneficial to screen multiple crystals during diffraction data collection, allowing us to collect data to 2.9 Å versus 3.1 Å for the vast majority of the crystals. We also found that the tightly focused beam available at the APS 24-ID-E beamline allowed us to aim the beam at particular sections of each crystal, which helped to reduce the mosaic spread.

In conclusion, our studies with the RCC1/nucleosome have provided guiding principles for crystallizing and improving the diffraction of nucleosome complexes. These principles include the importance of the identity of the macromolecule to be crystallized and post-crystallization soaking parameters such as the dehydration agent and temperature. However, these principles are best paired with systematic analyses to efficiently guide empirical searches of crystallization and post-crystallization parameters.

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Superdex 200HR size exclusion (2.2:1 RCC1:NCP)

Figure 1.

Purification of RCC1/nucleosome core particle complex. (a) Superdex 200HR size exclusion chromatography of human RCC1 1/nucleosome core particle (NCP) complex reconstituted in 2.2:1 molar ratio (red), nucleosome core particles (blue) and hRCC1 1 (green), (b) Coomassie stained SDS-PAGE gel of size exclusion chromatography fractions shown in (a). Purified hRCC1 1, nucleosome core particles and the input for size exclusion chromatography fractionation are shown in lanes 1 to 3 respectively.

Figure 2.

RCC1/nucleosome crystals. (a) hRCC1 Δ 1/nucleosome crystals grow as hollow tubes. (b) SDS-PAGE of washed crystals similar to those shown in (a) confirm contents of these crystals (lane 3). Purified nucleosome core particles are shown in lane 1, while the same preparation of hRCC1 Δ 1/nucleosome used to set up the crystallization trials is shown in lane 2. Molecular weight markers are shown in lane 4. (c) HIS-tagged yRCC1/nucleosome crystals. (d) dRCC1 Δ 1/nucleosome crystals.

Figure 3.

Diffraction studies of RCC1/nucleosome crystals. (a) Anisotropic diffraction of HIS-tagged yRCC1/nucleosome crystals, final soak in 20% MPD, synchrotron source: CHES F1 beamline. (b) Diffraction of dRCC1 Δ 1/nucleosome crystals, final soak in 24% glycerol, synchrotron source: APS NECAT ID-24-E beamline. (c) Diffraction of dRCC1/nucleosome crystals, final soak in 24% MPD, home X-ray source: MicroMax 007HF. (d) Diffraction of dRCC1/nucleosome crystals, final soak in 24% PEG400, synchrotron source: APS NECAT ID-24-E beamline.

Figure 4.

RCC1/nucleosome crystal packing interactions in refined 2.9 Å structure (panels a-d) and in unrefined molecular replacement model at 5.0 Å (panels e-h). (a) and (e) Overview of crystal packing interactions shown in space filling representation. (b) and (f) Closeup of boxed region from panels (a) and (e) with RCC1 atoms from yellow complex within 4.0 Å of the nucleosome molecule from the green complex shown in orange-red. More extensive crystal contact interactions are observed in the 2.9 Å crystal than in the 5.0 Å crystal. The DNA bases, phosphate backbone and histone components of the nucleosome are shown in different shades of green. (c) and (g) Crystal packing interactions with DNA shown in stick representation and RCC1 shown in cartoon representation. Same orientation and coloring as for panels (b) and (f). (d) and (h) Crystal packing interactions in space filling representation, rotated 60° from orientation shown in (b) and (f).

Table 1

Crystallography data collection and model refinement statistics for low resolution crystal of RCC1/nucleosome core complex. Data statistics for the previously published 2.9 Å crystals provided for comparison.

	Low-resolution data	High-resolution data
X-ray source	APS synchrotron, 24-ID-E	APS synchrotron, 24-ID-E
Detector	ADCS Q315	ADCS Q315
Temperature (°K)	100	100
Wavelength (Å)	0.9792	0.9792
Space group	P2 ₁	P2 ₁
Unit cell (Å, °)	a= 104.64, b= 185.92 c=109.55, =97.61	a= 102.68, b= 183.04 c=107.03, =101.52
Unit cell volume (Å ³)	2 112 400	1 971 000
Complex in asymmetric unit	one NCP and two RCC1	one NCP and two RCC1
V _m (Å ³ /Da)	3.58	3.34
Solvent content (%)	69.3	67.1
% Decrease volume of unit cell		6.7%
Mosaicity (°)	0.49–0.88	0.15–0.44
No of measured reflections	120 281	329 804
No of unique reflections	16 264	85 040
Resolution range	40–5.20 (5.29–5.20)	50–2.90 (2.95–2.90)
Multiplicity	7.4	3.9
Average I/I	36.62 (3.33)	22.25 (1.67)
Completeness (%)	99.8 (100)	98.5 (98.0)
R _{merge}	5.5 (44.8)	5.8 (59.4)