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Proliferating cell nuclear antigen (PCNA) is the component of the chromosomal DNA replication machinery in eukaryotic cells that confers high processivity upon DNA polymerase δ and ε. It has been proposed that PCNA functions by forming a trimeric complex with a ring-like structure through which DNA is threaded. PCNA from the yeast Saccharomyces cerevisiae has been crystallized in a cubic space group (P₂₁₃, a = 121.1 Å). Unexpectedly, a mercury derivative of PCNA yields crystals that diffract significantly better than crystals of the unmodified protein (2.4 Å and 3.0 Å resolution, respectively). Mass spectrometry reveals that the derivative results from the addition of two mercury atoms to the protein. Although crystals of the mercurated protein show evidence of non-isomorphism, the anomalous diffraction signal is strong and phases may be determined by multi-wavelength anomalous diffraction (MAD phasing).

Keywords: DNA replication; DNA polymerase; processivity; crystallography

DNA polymerases that are involved in chromosomal replication achieve high processivity by the attachment of their catalytic subunits to a "sliding DNA clamp", which prevents dissociation of the polymerase from the template during replication (Kornberg & Baker, 1991; Kuriyan & O'Donnell, 1993). The best understood of these polymerases are DNA polymerase III holoenzyme (Pol III) of Escherichia coli (McHenry, 1991), the DNA polymerase of T4 bacteriophage (Chu & Alberts, 1988) and the eukaryotic DNA polymerases δ and ε (So & Downey, 1992). Each consists of three components: an enzymatic core that carries out polymerization and proof-reading, a DNA clamp (β subunit in E. coli, gene 45 protein in T4 phage and PCNA§ in eukaryotic cells), and a set of ATP-dependent proteins that are required to load the clamp onto DNA (γ complex in E. coli, proteins encoded by genes 44 and 62 in T4 phage and the RF-C complex in eukaryotic cells).

The DNA clamps are not nucleic acid binding proteins in the conventional sense and have no intrinsic affinity for nucleic acids. They are bound to DNA by virtue of their topology and have to be assembled on DNA by other proteins, in an ATP dependent process (Stukenberg et al., 1991; Burgers & Yoder, 1993). The crystal structure of the DNA clamp (β subunit) of Pol III has been determined previously (Kong et al., 1992). The structure is that of a closed ring formed by a dimer of β subunit, with approximate diameter 80 Å, with a hole of diameter ~35 Å in the middle. The structure is highly symmetric, and each monomer consists of three domains of identical topology. In addition, the topology of each domain is 2-fold symmetric, leading to the formation of a simple two-layer structure that has 12 α-helices lining the central hole, flanked by six β sheets on the outside (Kong et al., 1992). The clamping action of β subunit is readily
explained by assuming that DNA passes through the ring-like molecule during replication, and that the \( \gamma \) complex is required to initially load the clamp onto DNA since the closed circle would not normally bind DNA.

Given the functional similarity between PCNA and \( \beta \) subunit, a natural question is whether they have any structural similarity. They have no significant sequence identity, and PCNA is approximately two-thirds the size of \( \beta \) subunit (Saccharomyces cerevisiae PCNA: 28.8 kDa, 258 residues; \( \beta \) subunit: 40.6 kDa, 366 residues). Despite this, a sequence alignment between them was generated by matching hydrophobic residues, and it was suggested that a trimeric, rather than dimeric, assembly of PCNA molecules could have a ring-shaped structure similar to that of \( \beta \) subunit (Kong et al., 1992). A similar model was proposed for T4 phage gene 45 protein. The sequence alignment between PCNA and \( \beta \) subunit is extremely weak (~5% sequence identity), and the recently developed profile method of sequence comparison (Bowie et al., 1991) fails to detect significant similarity between these proteins. A reliable model for PCNA function thus requires an independently determined crystal structure, and we report here the crystallization of PCNA as a first step towards this end.

PCNA from S. cerevisiae was overexpressed in E. coli and purified as described (Bauer & Burgers, 1988; Yoder & Burgers, 1991). The purified protein was dialyzed against 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT and 0.1 M NaCl (the salt is required to prevent aggregation of the protein). The protein was concentrated to 20 \( \mu \)g/ml for crystallization using ultrafiltration (Centricon-10, Amicon). A mercury derivative of the protein was prepared by dialyzing the protein for 24 hours against 0.1 M NaCl, 25 mM Hepes (pH 8.0), 0.4 mM HgCl\(_2\). The unreacted mercury was removed by dialysis, prior to crystallization.

X-ray data collection by the oscillation method utilized a Rigaku R-AXIS IIC imaging phosphor detector mounted on a Rigaku RU-200 rotating anode X-ray generator. To minimize radiation damage, crystals were flash frozen using 25% (v/v) glycerol as the cryoprotectant. The space group and unit cell were determined by indexing single images using software provided by Rigaku, by testing the symmetry properties of oscillation data sets, and by examining peaks in difference Patterson maps. The program DENZO (Z. Otwinowski, personal communication) was used for processing the oscillation data frames.

Crystallization conditions were scanned using a sparse matrix method using hanging drops (Janecaric & Kim, 1991). A number of conditions that included polyethylene glycols yielded crystals that diffracted very poorly (~5 Å resolution). Systematic screening of various salt precipitants led to large (0.4 mm x 0.4 mm x 0.4 mm) single crystals of the unmodified protein that are obtained from 40 to 45% saturated ammonium sulfate and 100 mM sodium citrate (pH 5.6), at room temperature. These crystals diffract relatively poorly (at best to 3.5 Å resolution) and are extremely radiation sensitive. The diffraction pattern decays within a period of hours upon exposure to X-rays, with the resolution limit dropping from 3.5 Å to below 5 Å. The effect of adding various metal ions to the crystallization mixture was tested. Crystals obtained from similar conditions, but with the addition of 10 mM CuSO\(_4\), diffract significantly better (3 Å), although the radiation sensitivity is not improved. The radiation sensitivity problem has been overcome by flash freezing the crystals (held by surface tension within a wire loop) to about ~180°C, and by collecting data at that temperature (Hope, 1990). The flash freezing procedure involves transferring the crystals to cryoprotectant solutions containing 25% glycerol, 2 M ammonium sulfate and 100 mM sodium citrate, pH 5.6. The composition of the cryoprotectant was arrived at by trial and error, and the final freezing procedure results in diffraction patterns with relatively low mosaicity (~0.6 to 0.7°) and no detectable radiation sensitivity over a period of days.

Attempts to soak crystals in solutions containing various metals have not led to useful heavy-atom derivatives. PCNA contains four cysteine residues, and we decided to try reacting the protein first with mercury, and then crystallizing it. Very similar crystals are obtained, except that the optimal ammonium sulfate concentration is lower (35% saturated). A striking difference between crystals of the unmodified and mercerated protein is the significant improvement in data quality obtained upon mercuration. Diffraction spots from these crystals extend beyond 2.4 Å (Figure 1). This improvement is quite reproducible, and does not vary from batch to batch of protein.
Crystallization Notes

Figure 2. Matrix-assisted laser desorption mass spectra for unmodified PCNA (left) and mercurated PCNA (right). The peaks in the latter spectrum are marked with the corresponding number of mercury atoms attached to PCNA.

Crystals grown in the presence or absence of Cu$^{2+}$ are isomorphous. The space group is cubic, $P2_13$, with $a=123.8$ Å at room temperature or 4°C, and $a=121.8$ Å at $-160^\circ$C, for unmercurated PCNA. Crystals of mercurated PCNA are in the same space group, with $a=121.1$ Å at $-160^\circ$C. The fractional volume occupied by solvent is estimated to be 75% or 51% if we assume that there are one or two molecules in the asymmetric unit, respectively (Matthews, 1968). We measured the density of the crystals using an organic solvent density gradient with salt solution calibration (Matthews, 1985). The crystals were cross-linked with glutaraldehyde to prevent dissolution (Quiocho & Richards, 1964). This gave a density estimate of 1.077 g/cm$^3$ and indicates that there is only one molecule per asymmetric unit, with a large solvent content (Matthews, 1985).

Matrix-assisted laser desorption mass spectra (Hillenkamp et al., 1991) of unmodified and mercurated PCNA (both from solution and from dissolved crystals) were measured using a time-of-flight mass spectrometer constructed at the Rockefeller University (Beavis & Chait, 1990). One µl of protein solution (20 µM PCNA in 0.1 M NaCl, 25 mM Hepes buffer, pH 8) was mixed with 9 µl of α-cyano-4-hydroxy-cinnamic acid (10 g/l in formic acid/water/isopropanol (1:3:2, by vol.), and 0.5 µl of this solution was placed on the tip of the mass spectrometer probe. The probe tip was dried and irradiated with 10 ns pulses of light (wavelength 355 nm) from a Nd(YAG) laser. The resulting ions were analyzed by time-of-flight mass spectrometry. To improve statistics, 200 individual spectra were added together.

The mass of unmodified PCNA was measured to be $28,913 \pm 8$ daltons by matrix-assisted laser desorption mass spectrometry, consistent with the mass of 28,916 daltons calculated from the gene sequence (Bauer & Burgers, 1990). The mass spectrum for the mercurated protein shows a major component with a mass of $29,299 \pm 19$ daltons (Figure 2). The measured mass difference of $379 \pm 21$ daltons between the major component of the mercurated protein and unmodified PCNA is interpreted as resulting from the addition of two mercury atoms and the loss of two protons (399-2 daltons). Spectra obtained from dissolved crystals have broader peaks but are qualitatively similar.

The anomalous difference Patterson map for data collected from crystals of the mercurated protein is of high quality, with Harker section peak heights greater than 8 σ in a map calculated using data to 3 Å resolution. Two mercury atoms, located at (0.04, 0.03, 0.97) and (0.02, 0.75, 0.87) in fractional cell coordinates, suffice to explain all the significant peaks in the Patterson map. The relative occupancies of the two metal atoms were estimated to be 1.0 and 0.6, by comparing simulated Patterson maps with those derived from the experimental intensities. The presence of a partially occupied site is consistent with the mass spectra, which show the presence of a minor protein fraction that contains a single mercury atom (Figure 2).

In contrast to the high quality anomalous difference Patterson maps, the differences in diffraction intensity between native and mercurated crystals yield relatively poor Patterson maps (highest peak height in the Harker section is 4.2 σ). We interpret this to mean that there is significant non-isomorphism between the two crystals forms. The presence of strong peaks in the 30 Å resolution anomalous difference Patterson map indicates that the mercury atoms are well ordered and are likely to provide good phasing power in a MAD experiment (Hendrickson, 1991). We have obtained data at three wavelengths at beamline X4A, National Synchrotron Light Source, Brookhaven, using a single frozen crystal of mercurated PCNA and analysis of these data for MAD phasing is in progress. We note that the use of a single crystal in this experiment avoids complexities in the analysis that would arise from non-isomorphism due to the variable level of mercuration (Figure 2) or from the freezing process.

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References


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