FOR THE RECORD

The proteasome 11S regulator subunit REG α (PA28 α) is a heptamer

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Abstract: Activity of the 20S proteasome, which performs much of the cytosolic and nuclear proteolysis in eukaryotic cells, is controlled by regulatory complexes that bind to one or both ends of the cylindrical proteasome. One of these complexes, the 11S regulator (REG), is a complex of 28 kDa subunits that is thought to activate proteasomes toward the production of antigenic peptides. REG, purified from red blood cells, is a complex of REG α and REG β subunits. We have crystallized recombinant REG α (rREG α) and collected diffraction data to 3.0 Å resolution. The self-rotation function indicates that rREG α forms a heptameric ring in the crystal. Equilibrium sedimentation demonstrates that rREG α is a heptamer in solution also.

Keywords: PA28 α ; proteasome 11S regulator; REG α ; sevenfold symmetry; X-ray crystallography

The 20S proteasome is believed to be a major contributor to non-lysosomal cytosolic and nuclear proteolysis in eukaryotic cells, where it comprises 0.1–1% of total cellular protein (Rechsteiner et al., 1993). Also known as the multicatalytic protease, MCP, and multicatalytic endopeptidase, the 20S proteasome is a ~700 kDa complex that possesses at least five endopeptidase activities that cleave peptide bonds at the carboxyl side of basic, hydrophobic, acidic, small neutral, and branched chain amino acid residues (Cardozo et al., 1994). The crystal structures of 20S proteasome from the archaeobacterium *Thermoplasma acidophilum* (Löwe et al., 1995) and from yeast (Groll et al., 1997) reveal an assembly of four stacked rings ($\alpha\beta\beta\alpha$) each of which contains seven subunits. Access to the protease active sites, which are located on the two β rings in the center of the cylindrical structure, is through the central channel that passes along the sevenfold axis of the proteasome (Wenzel & Baumeister, 1995). The observation that this channel is restricted by a 13 Å diameter annulus in the archaeobacterium 20S proteasome and is completely closed in the structure of yeast 20S

proteasome, provides an explanation for how indiscriminate degradation of folded proteins is avoided.

Activity of the 20S proteasome is important for numerous cellular processes, including degradation of abnormal or mis-folded proteins; stress responses; the regulated degradation of signaling pathway components such as Myc, Fos, Jun, homeotic proteins, cdc proteins, p53, and NF- κ B; adaptation to changing metabolic conditions; cell-cycle progression; apoptosis; and the degradation of cytoplasmic proteins to provide antigenic peptides to MHC class I molecules (reviewed in Ciechanover & Schwartz, 1994; Coux et al., 1996; Hoffman & Rechsteiner, 1996). The substrate specificity of 20S proteasomes is controlled by interacting proteins and protein complexes, the best known of which is the 19S regulatory complex (also known as “the ball,” μ -particle, 19S cap, or PA700), which binds to the ends of the proteasome in the presence of ATP to form the 26S protease (Peters et al., 1993; Rechsteiner et al., 1993; DeMartino et al., 1994; Ma et al., 1994).

A second regulator (REG) that sediments at 11S, has been identified that differentially activates peptide hydrolysis by the 20S proteasome, with hydrolysis of some peptides increased 200-fold and others only threefold (Yukawa, 1991; Di Cola, 1992; Ma et al., 1992; Dubiel et al., 1992). Also known as PA28, REG binds the proteasome reversibly ($K_d \sim 1$ nM) with no requirement for nucleotides. Despite the activity of REG in elevating peptidase activities, it does not appear to confer activity toward large folded proteins, although it is possible that REG-activated proteasomes hydrolyse proteins in vivo through interactions with other factors such as chaperones. REG purified from human red cells is composed of two subunits, REG α and REG β , of molecular weight 28.7 kDa and 27.3 kDa respectively, which share 47% sequence identity with each other. We have expressed recombinant REG α in *E. coli* and shown that the activity of rREG α is similar to that of the heteromeric REG α /REG β purified from red cells (Realini et al., 1994a; Realini et al., 1997).

A likely physiological role of REG is to activate the proteasome for production of antigenic peptides to be displayed on MHC class I molecules (Yewdell & Bennink, 1992; Germain, 1994). Proteasome inhibitors prevent production of peptide ligands for MHC class I molecules from ovalbumin (Rock et al., 1994). The cytokine INF γ , which activates REG expression (Realini et al.,

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1994a; Ahn et al., 1995), is released during viral infection to signal production of numerous components of the antigen processing and presentation machinery, including TAP1/TAP2 transporters, MHC class I/II molecules, β_2 -microglobulin (Townsend & Bodmer, 1989), and the specialized proteasome subunits LMP2 and LMP7 (Driscoll et al., 1993; Gaczynska et al., 1993). The preference of MHC class I molecules for peptides with large hydrophobic C-terminal residues (Falk & Rotzschke, 1993) matches the sequence preference of 20S proteasomes activated by authentic REG and by rREG α (Realini et al., 1994a; Ustrell et al., 1995). Furthermore, increased expression levels of REG α result in more efficient recognition of virus-infected cells by cytotoxic T lymphocytes (Groettrup et al., 1996). Finally, REG alters the cleavage pattern of proteasomes to enhance a double-cleavage mechanism that favors the production of peptides that are a suitable length for presentation on MHC class I molecules (Dick et al., 1996).

To further understand the mechanism and function of REG we are determining the crystal structure of rREG α . Crystals have been grown and data collected to 3.0 Å resolution. The self-rotation function shows that rREG α forms a heptameric ring structure in the crystal. Equilibrium sedimentation further demonstrates that the heptameric oligomerization state is maintained in solution.

Materials and methods: Recombinant human erythrocyte REG α was expressed in *E. coli* and purified by anion exchange and size exclusion chromatography as described (Realini et al., 1994a). Protein purity was assessed by SDS PAGE and activity was monitored by a fluorometric peptidase assay as described (Realini et al., 1994a). For crystallization, rREG α was dialyzed into 10 mM MOPS pH 7.2, 0.2 mM EDTA, 1 mM DTT, and concentrated to 10 mg mL⁻¹ (Amicon, Beverly, MA). Crystallization was performed by sitting drop vapor diffusion at 4°C. The reservoir solution was 12% PEG 6 K, 100 mM Na MOPS pH 7.2, 2 M NaCl, and 1 mM ZnCl₂. The initial drop comprised 3 μ L each of the protein and reservoir solutions, from which plate-like crystals (0.3 $\times$ 0.3 $\times$ 0.05 mm) grew in four days. The crystals were shown to contain full-length rREG α by SDS-PAGE analysis of washed crystals.

For data collection at 100 K, crystals were cryoprotected in a solution of 12% PEG 6 K, 100 mM Na MOPS pH 7.2, 2 M NaCl, and 20% glycerol. Crystals were first washed in fresh well solution, which was sequentially replaced with solutions containing increasing concentrations of glycerol (2%, 4%, 6%, ..., 20%). At each increment of glycerol concentration the crystals were allowed to equilibrate for several minutes after gentle mixing. After the final equilibration step the crystals were suspended in rayon loops and cryo-cooled by plunging into liquid nitrogen. The crystals were initially characterized from data collected on an RAXIS IIC imaging plate detector mounted on a Rigaku RU200 rotating anode generator. A full data set was collected on a MAR imaging plate detector at beamline 7-1 of the Stanford Synchrotron Radiation Laboratory. All data processing was performed with DENZO and SCALEPACK (Otwinowski, 1993). Most crystallographic computations used programs from the CCP4 suite (CCP4, 1994).

Equilibrium sedimentation experiments were performed using a Beckman XL-A Optima analytical centrifuge. Samples, at protein concentrations of 0.25, 0.125, 0.0725, and 0.035 mg mL⁻¹, were analyzed in the sizing column buffer (10 mM Tris HCl pH 7.5, 200 mM KCl, 10 mM NaCl, 1.1 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT). Samples were spun at 13,000 rpm at 21°C, and scanned at 280 nm after 42 and 48 h. Absorbance profiles of these two scans were overlaid to verify that the samples had reached equilibrium.

Each plot represented the average of 99 replicates scanned at 0.001 cm radial spacing. Background absorbance was corrected empirically by allowing the baseline to float during the fitting calculations. Solvent density was measured to be 1.016 g mL⁻¹. The calculated partial specific volume was estimated to be 0.748 mL g⁻¹ based on amino acid composition (Laue et al., 1992). Data were analyzed with software incorporated into IGOR-PRO (Wave-metrics, Lake Oswego, OR) generously provided by Dr. Preston Hensley (Brooks et al., 1994).

Results and discussion: X-ray diffraction data were collected from a cryocooled crystal (Table 1). The space group is C2 with cell dimensions $a = 162.8$ Å, $b = 134.2$ Å, $c = 116.2$ Å, $\beta = 90.86^\circ$. The data are 89.5% complete to 3.0 Å resolution, and have an overall $R_{\text{merge}}(I)$ of 7.6%. Post-refinement indicates that the mosaicity is 1.0°. Consideration of the Matthews parameter, V_m (Matthews, 1968), suggests that there are likely to be between 5 ($V_m = 4.42$ Å³/Da; solvent content = 72%) and 12 ($V_m = 1.84$ Å³/Da; solvent content = 33%) rREG α subunits per asymmetric unit. As discussed below, the self-rotation function indicates that the true number is 7, for which $V_m = 3.16$ Å³/Da and the estimated solvent content is 61%.

The self-rotation function (Fig. 1) shows that rREG α forms a heptameric ring in the crystal. A peak at 95% of the origin height is centered on kappa sections that correspond to sevenfold rotation, i.e., $\kappa = 51.4^\circ, 102.9^\circ$, and 154.3° (Fig. 1A). The $\kappa = 180^\circ$ section shows seven twofold rotation axes that are 90° away from the sevenfold rotation axis (Fig. 1B). One of these twofold axes is coincident with the crystallographic b axis, with the others at approximately 95% of the origin height. These features in the self-rotation function indicate that rREG α packs in the crystal as two seven-member rings stacked face to face (Fig. 1C, 1D).

The self-rotation function shows that two heptameric rings are related to each other by a twofold axis that is parallel to the crystallographic diad, although this does not distinguish between the situation where these axes are coincident (one heptamer per asymmetric unit), and where the axes are parallel but not coincident.

Table 1. Data collection statistics^a

Resolution (Å)	$I/\sigma(I)$	$R_{\text{merge}}(I)^b$	% Complete
30.0–7.05	19.5	4.7	96.9
7.05–5.60	15.5	6.8	97.8
5.60–4.89	14.8	7.4	97.8
4.89–4.44	14.3	7.2	97.4
4.44–4.13	12.7	7.7	96.5
4.13–3.88	10.6	8.8	93.6
3.88–3.69	9.6	9.1	89.0
3.69–3.53	8.5	9.4	87.2
3.53–3.39	7.2	9.8	83.7
3.39–3.27	5.4	12.1	83.6
3.27–3.17	4.4	14.4	79.7
3.17–3.08	3.5	19.3	80.1
3.08–3.00	3.1	22.1	79.5

Cumulative $I/\sigma(I) = 10.4$, $R_{\text{merge}} = 7.6\%$, Completeness = 89.5%.

^aData were collected at SSRL 7-1 and processed with DENZO and SCALEPACK (Otwinowski, 1993)

^b $R_{\text{merge}}(I) = 100 * \sum_{hkl} \sum_i |I_i - \langle I \rangle| / \sum_i \langle I \rangle$.

Fig. 1. rREG α is a heptameric ring in the crystal. **A:** Self-rotation function calculated with GRLF (Tong & Rossman, 1990) (resolution 20–7 Å, Patterson integration radius 25 Å, $\kappa = 51.43^\circ$ ($= 360^\circ/7$)). Contours are in units of standard deviation starting from two standard deviations. **B:** Same as A but for $\kappa = 180^\circ$. **C:** Sketch of the single seven-member ring in the asymmetric unit with a second crystallographically related ring. These simple sketches are not intended to imply that the two rings necessarily stack with individual subunits exactly eclipsing each other. **D:** Sketch along the sevenfold axis showing the six non-crystallographic twofolds that are generated by combination of the sevenfold axis with the crystallographic twofold, to give the seven twofold axes seen on the $\kappa = 180^\circ$ section.

dent (two heptamers per asymmetric unit). The absence of a pseudo-origin peak in the native Patterson function indicates that the crystallographic twofold axis and the twofold axis relating two adjacent rREG α rings are in fact coincident. This confirms the presence of only one heptamer in the crystallographic asymmetric unit, consistent with the usual solvent content for protein crystals.

Electron microscopy studies have shown that bovine REG forms ring structures that can bind one or both ends of the proteasome (Gray et al., 1994). The diameter of the REG ring is ~ 100 – 110 Å, with a depth of ~ 70 – 80 Å. An object with dimensions of the REG seen by electron microscopy can be accommodated in the rREG α

asymmetric unit and packed against a crystallographically related rREG α heptamer to give the arrangement indicated by the self-rotation function (Fig. 1).

rREG α is also a heptamer in solution. Dynamic light scattering (DLS) and sizing chromatography are consistent with either a hexamer or a heptamer (data not shown). Because these techniques are unable to distinguish between hexamer and heptamer, we have used the method of equilibrium sedimentation, which provides a shape-independent estimate of mass. Data from individual equilibrium sedimentation scans were initially fit assuming a single species, and provided an estimated molecular weight of 192,500

Da with chi-squared of 1.180×10^{-3} (data not shown). The slightly non-random distribution of residuals suggested that the sample could be more accurately modeled as a self-associating system. The known monomer molecular weight (28.7 kDa) was then fixed and the N-mer term (number of monomers that associate to form a multimer) was allowed to vary. The result was N-mer of 6.908, with chi-squared of 1.175×10^{-3} , suggesting that monomers associate to a heptamer. A multiple-species analysis was then performed with the N-mer term fixed at 7.0. The residual plot shows a random distribution for a self-associating model of monomers to heptamers (Fig. 2B). A species concentration plot (Fig. 2D) shows that equal amounts of monomer and heptamer are present in a solution of approximately $1 \mu\text{M}$ concentration. This value corresponds to a monomer:heptamer equilibrium constant of $7.8 \times 10^{-41} \text{ M}^6$. A monomer:hexamer model was then tested by fixing

the N-mer term at 6.0. The resulting residual plot shows a highly non-random distribution (Fig. 2A), indicating that rREG α does not associate as a hexamer in solution.

It has previously been estimated from gel filtration and sedimentation through glycerol gradients, that REG purified from red cells, which is a heteromeric complex of REG α and REG β subunits, is *hexameric* (Ma et al., 1992). Immunoprecipitation data have also been interpreted to show that red cell REG is a hexameric ring of alternating α and β subunits (Ahn et al., 1996; Song et al., 1996). Our results, however, clearly demonstrate that recombinant REG α has a *heptameric* ring structure. It is possible that, unlike rREG α , the heteromeric REG purified from tissues is hexameric, although this apparent discrepancy is surprising.

Structure determination of rREG α , which is currently in progress by the method of isomorphous replacement, should reveal interactions that stabilize the seven-member ring assembly and surfaces that bind to the 20S proteasome. The structure may also suggest functional roles for the KEKE sequences of REG α that have been hypothesized to mediate protein-protein interactions (Realini et al., 1994b).

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Fig. 2. Equilibrium sedimentation analysis of rREG α . A and B show the residuals when data were fit to a self-associating monomer:hexamer model (A) and a self-associating monomer:heptamer model (B). The highly non-random distribution in A when compared to the random distribution of residuals in B indicates that rREG α associates to form heptamers in solution. (C) The overall fit of the monomer:heptamer model to the data (dark line through data points) as well as the calculated absorbance due to heptamer alone (thin dotted line), and the monomer alone (solid line with extremely shallow curve above the ordinate). The species concentration plot (D) shows the relative amounts of monomer and heptamer at a given concentration. The thickened region of each species curve shows the concentration range that was sampled in the experiment. The species curves cross at about $1 \mu\text{M}$, indicating that a solution of this concentration contains 50% of each species.

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