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Crystallization of human complement component C5

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Abstract

Human complement component C5 has been crystallized using a low-salt batch technique. The crystals are large hexagonal bipyramids often larger than 1.5 mm. Although these crystals were grown in low salt (0.1 *M* NaCl), they are remarkably stable for at least 2 months at 281 K and they are not dissolved in aqueous buffers containing up to 2 *M* sodium chloride. The space group is $P3_121$ or $P3_221$, and the cell parameters were determined to be $a = 144.9$, $b = 144.9$, $c = 243.1$ Å; $\alpha = 90^\circ$, $\beta = 90$, $\gamma = 120^\circ$. At room temperature and cryo-temperatures the crystals diffract at best to 6 Å using rotating-anode X-ray sources. Using synchrotron radiation with cryoprotection using 40%(*v*/*v*) PEG 400 the resolution limit can be extended to 3.3 Å. In both cases the crystals show significant anisotropy, with relatively weaker reflections at higher resolution in the a^*b^* plane.

1. Introduction

The fifth component of human complement, C5, consists of two polypeptide chains, chain α (M_r = 116 000) and chain β (M_r = 75 000) held together by a disulfide bridge (Tack *et al.*, 1979). Both human and murine C5 cDNA's have been cloned and sequenced. The derived amino-acid sequences indicate that C5 consists of 1640–1654 amino acids and that the polypeptide chains contain 2–3 potential sites for asparaginyl-linked glycosylation (Wetsel *et al.*, 1987; Haviland *et al.*, 1991).

C5 is a multi-domain protein which along with its derivatives can interact with the C5a receptor on granulocytes and with complement components C3b, C4b, C6 and C7 (Gerard & Gerard, 1991; Vogt *et al.*, 1978; Ebanks *et al.*, 1992; Arroyave & Muller-Eberhard, 1973; DiScipio *et al.*, 1983; DiScipio, 1992). Activation of C5 by C5 convertases of either the classical or alternative pathways results in the cleavage of a single peptide bond between residues 74 and 75 of the α-chain releasing a small domain, C5a (M_r = 11 000), and producing a large fragment, C5b (M_r = 185 000) (DiScipio *et al.*, 1983). C5b can be digested further with plasmin to yield fragments C5c (M_r = 140 000) and C5d (M_r = 45 000). Plasmin only cleaves the α'-chain of C5b. C5c consists of fragments derived from the amino- and carboxyl-terminal segments of the α'-chain and an intact β-chain. The amino-terminal α'-chain fragment is disulfide bridged to the β-chain and to carboxyl-terminal derived segments of the α'-chain. C5d arises internally within the α'-chain (DiScipio, 1992).

C5a which is a basic glycosylated domain of 74 amino acids is an activator and chemotactic mediator of granulocytes (Fernandez *et al.*, 1978; Fernandez & Hugli, 1978). The receptor for C5a (CD88) is found on several cell types including the granulocytes, neutrophils and eosinophils. This receptor has been cloned, and it is a member of the family of G-protein coupled receptors (Gerard & Gerard, 1991).

C5b contains a metastable binding site for C6. Immediately after activation, newly formed C5b must complex with C6 or this interaction capability of C5b decays spontaneously within a few minutes (DiScipio *et al.*, 1983). Once the C5b–6 complex forms, it initiates the assembly of the membrane attack complex (MAC) (C5b–9) on the surface of target cells. The fully assembled MAC consists of a transmembrane tabule, which has an outer diameter of 210 Å and a central channel with a diameter of 100 Å. The MAC tabule is associated with a leaflet projection. In contrast to C6, C7, C8 and C9, C5b is not integrated into the wall of the MAC cylinder, but it has been mapped to the apex of the leaflet using a gold labeling technique with electron microscopy (Tschopp, 1984).

The solution structure of a C5a has been derived from NMR spectroscopy data. This polypeptide can be considered an oblate ellipsoid whose long and short axes are 33 and 22 Å, respectively. A knot consisting of three disulfide bonds is at the center. Approximately 50% of the residues are found in four α-helices. However, the carboxyl-terminal tail consisting of the last 11 amino acids is of unordered structure (Zuiderweg *et al.*, 1989).

The entire molecule of C5 has been visualized by negative contrast staining using electron microscopy as a heart-shaped structure whose maximal dimensions are 152 × 150 × 93 Å (DiScipio *et al.*, 1983; DiScipio, 1992). It was hypothesized further that the two 'lobes' of the heart-shaped molecule were the α- and β-chains of C5 (DiScipio, 1992). However, a very different model for the tertiary structure of C5 was proposed based on data from neutron and X-ray scattering. From these data a model of C5 was conceived as consisting of two distinct domains which may be described as a 'raft' associated with a 'sail'. One domain, the 'raft', was estimated to have a length of ∼180 Å and a maximum width of ∼80 Å. At right angles to the 'raft' was positioned the second domain, a 'sail', of ∼80 Å height. Both 'raft' and 'sail' were predicted to be rather thin, ∼20 Å (Perkins *et al.*, 1990). Only the determination of the three-dimensional structure of C5 by X-ray diffraction could provide conclusive evidence on which of these models is correct.

We report here the crystallization of human complement component C5. By applying synchrotron radiation to crystals of C5 a diffraction limit of 3.3 Å was obtained. At this resolution the overall folding and the domain arrangement of C5 can be determined.

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2. Materials and methods

Human complement component C5 was purified from frozen recovered plasma by 4.5–12% polyethylene glycol precipitation, DEAE–Sephadex column chromatography, gel filtration on Sephacryl S300, dextran sulfate Sepharose, and affinity absorption of contaminants (DiScipio & Pinz Sweeney, 1994). C5 at a concentration between 0.5 and 1.5 mg ml^{-1} was stored in 20 m*M* imidazole pH 6.5, 0.3 *M* NaCl, 5 m*M* EDTA at 277 K for at most 2 months prior to crystallization. The protein was concentrated to 10–15 mg ml^{-1} by precipitation with 50% ammonium sulfate. Then C5 was dialyzed several times *versus* 1 m*M* HEPES pH 7.6/0.1 *M* NaCl.

After dialysis imidazole buffer pH 6.6 to a final concentration of 40 m*M* was added, and aggregates were removed by centrifugation through 0.22 µm filters (Millipore). Crystallization was performed by a batch method using 10–15 mg ml^{-1} C5 in 200 µl of 1 m*M* HEPES, 40 m*M* imidazole HCl pH 6.6, 100 m*M* NaCl in conical polypropylene tubes at 281 K. Usually crystals appeared within two weeks, and had grown to full size by the third week. The crystal density was measured using a Ficoll gradient (Westbrook, 1985). Examination of C5 by synchrotron radiation was performed at the beamline 'X-ray Diffraction' in Trieste, Italy. After cryo-preserving the crystals in 40%(*v*/*v*) PEG 400, 100 m*M* NaCl, 10 m*M* MES 6.6, the crystals were mounted by the loop method (Teng, 1990) and flash frozen at 100 K in a stream of cold nitrogen. The programs *DENZO* and *SCALEPACK* were used for indexing, integration, scaling and merging of the data (Otwinowski, 1991).

3. Results and discussion

Since C5 is a particularly large protein and is a euglobulin, we examined whether the protein could crystallize in the absence of precipitants. The strategy we employed was to examine the precipitation of concentrated solutions (5–15 mg ml^{-1}) of this protein as a function of pH ranging from 6 to 8.0. The protein was concentrated using 50% ammonium sulfate precipitation and dialyzed *versus* pH 7.6, 0.1 *M* NaCl. Then to each sample of 50 µl was added 1 µl of 2 *M* imidazole buffer ranging from pH 6 to 8. The samples were placed at 281 K and examined periodically for crystallization or precipitation.

Crystals of C5 were observed to form spontaneously in batch using these conditions within a narrow pH region of 6.6 to 6.8. Below pH 6.5 only amorphous precipitates are observed, and above pH 6.9 C5 neither precipitates nor do crystals form. The optimal pH for large crystals was pH 6.6. Furthermore, C5 crystallization required that C5 be concentrated to at least 5 mg ml^{-1}. The largest crystals of C5 were obtained when these were grown in conical polypropylene tubes.

The crystals grow as hexagonal bi-pyramids with approximate dimensions of 1.5 × 0.8 × 0.8 mm (Fig. 1). The crystals are stable and do not dissolve in 10 m*M* HEPES pH 7.4 in absence or presence of 0–2 *M* NaCl. However, crystals can be slowly dissolved in 10 m*M* HEPES pH 7.5, 0.2*M* sodium bromide (a weak chaotroph) after 2 d at 277 K. C5 derived from dissolved crystals using this method retained full hemolytic activity, and the protein could be re-crystallized using the original procedure. Using Cu $K\alpha$ radiation from a rotating anode at 296 K, the crystals diffracted to at best 6 Å resolution, and suffer from radiation damage decaying within a few hours.

We have analyzed the effect of various additives. For example, addition of either 3 m*M* Ca^{2+} or 3 m*M* Mg^{2+} to the buffer increases the nucleation and growth rates but does not result in crystals that diffracted to a better resolution. Neither re-crystallizing nor gradual soaking of the crystals in 20% polyethylene glycol, 1 *M* Li_2SO_4, or 30% glycerol results in better diffracting crystals.

Several trials were needed to determine optimal conditions for cryo-protection of crystals analyzed with synchrotron radiation. Simply placing or gradually transferring C5 crystals into concentrated solutions of glycerol or PEG resulted in the crystals cracking and distorting. However, by placing crystals in dialysis buttons obtained from Hampton Research and subsequent dialysis against first 20%(*v*/*v*) PEG 400, 100 m*M* NaCl, 10 m*M* MES 6.6, and later 40% PEG the crystals could be transferred to the cryo-protective solution without damage. The dialysis was carried out at 281 K using membranes with a molecular weight cutoff of 12–14 kDa.

Oscillation images in ranges of 1° were collected at the 'X-ray Diffraction' beamline in Trieste with 5–10 min exposure. The space group of the crystals was determined to be $P3_121$ or $P3_221$ based on the symmetry of the diffraction patterns and the systematic absences along 00*l* characteristic of a threefold screw axis with cell parameters $a = b = 144.9$, $c = 243.1$. A complete native data set to 4.0 Å resolution with an R_{sym} of 5.1% was obtained from a single crystal (Table 1). An example of the diffraction pattern from a crystal of C5 is shown in Fig. 2. Although the crystals diffract to 3.3 Å, the anisotropy observed in the a^*b^* plane makes the collected data usable to only 4.0 Å.

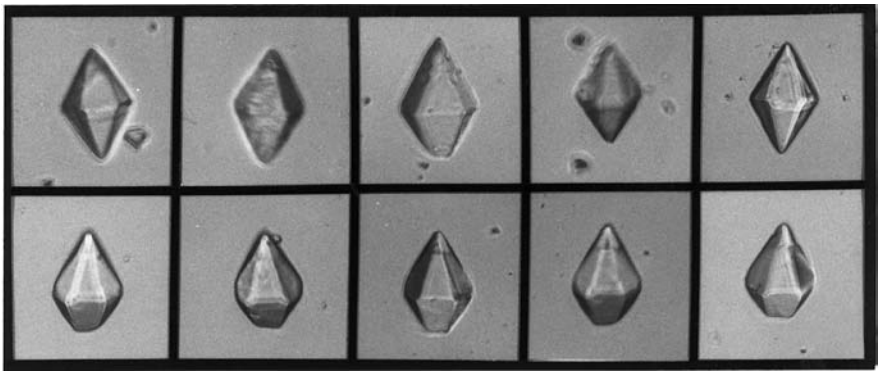

Fig. 1. Crystals of human complement component C5. Crystals are hexagonal bi-pyramids, and are more than 1.5 mm long.

Table 1. *Statistics on intensities as output from SCALEPACK*

A total of 25 552 unique reflections were recorded. The data set is complete to 96.1% with an average multiplicity of 2.2

R_{min} (Å)	R_{merge}	$I/\sigma(I)$
30.00–10.70	0.022	40.09
10.70–7.49	0.027	32.73
7.49–6.33	0.047	21.15
6.33–5.67	0.058	13.46
5.67–5.21	0.075	10.16
5.21–4.88	0.088	7.93
4.88–4.62	0.118	6.72
4.62–4.40	0.139	5.35
4.40–4.22	0.197	3.81
4.22–4.07	0.296	2.50
4.07–4.00	0.361	2.25
All reflections	0.051	15.26

In a Ficoll density gradient from 1.13 to 1.28 g cm^{-3} the crystal density was measured to be 1.19 g cm^{-3} (Westbrook, 1985). Using an M_r per monomer of 191 kDa, density values of 1.11 and 1.22 g cm^{-3} can be calculated for one and two molecules per asymmetric unit, respectively. Therefore, the crystals probably contain two molecules rather than one per asymmetric unit, and have a very low solvent content of 36%. This gives rise to a V_m of 1.92 Å^3 Da^{-1}, which is within the usual range observed for macromolecules. The low solvent content is consistent with the good resistance of the crystal to mechanical damage during routine manipulations.

The X-ray structural studies of C5 will enable the derivation of the domain arrangement of this member of the complement system, and the determined structure will also serve as a model for other members of this family. Like its homologue C3, C5 is capable of multiple specific protein–protein interactions. The C5a portion of the molecule is recognized by a specific G-coupled receptor on granulocytes (Gerard & Gerard, 1991). The binding site for C3b has been determined to within the C5d domain. Although the binding sites of C6 and C7 are believed not to be on the C5d domain their precise location in C5c has not been defined further (DiScipio, 1992). C5 also interacts with C4b through its β-chain, but the binding region on C5 has not been determined (Ebanks *et al.*, 1992).

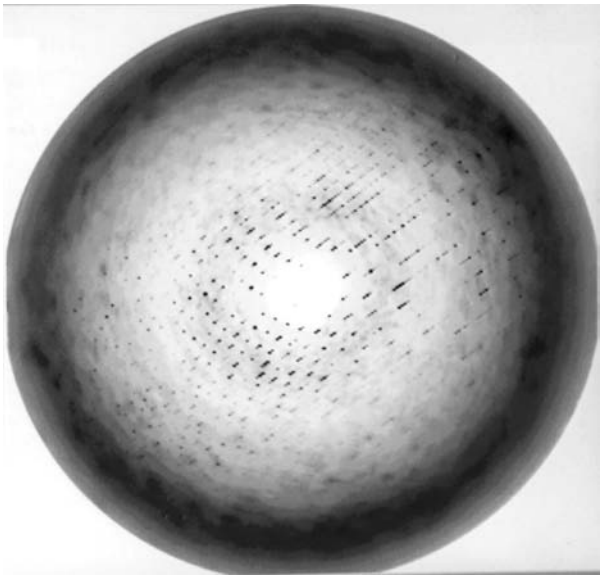

Fig. 2. A 1° oscillation image of a C5 crystal with an exposure time of 10 min. The image was collected at the 'X-ray Diffraction Beamline' in Trieste, Italy, on a MAR imaging plate. The crystal-to-detector distance was 240 mm. The wavelength was 1.07 Å.

The disulfide bridges in human complement component C3 have been determined, and those of C5 have been assigned by inference (Dolmer & Sottrup-Jensen, 1993). This information can prove useful in matching the polypeptide chain to the electron-density map, when it becomes available.

Recently several proteins of the homology family to which C5 belongs have been crystallized. These include the methylamine-treated α_2-macroglobulin, C3b, and methylamine-treated C3 (Andersen *et al.*, 1991; Sorensen *et al.*, 1994). The structure of methylamine-treated α_2-macroglobulin was solved to a resolution of 10 Å. This tetramer is nearly spherical with a diameter of 120–130 Å. It contains a central cavity made up to two irregular ellipsoidal compartments. One possible model for the monomer of α_2-macroglobulin shows a compact multilobal globular structure, which would resemble images of C3 and C5 obtained from electron microscopy (Andersen *et al.*, 1995).

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