Acta Cryst. (1994). D**50**, 786–789

Crystallization of human methylamine-treated complement C3 and C3b.

By ANDERS H. SØRENSEN, KLAVS DOLMER,* SØREN THIRUP, GREGERS R. ANDERSEN, LARS SOTTRUP-JENSEN* and JENS NYBORG, *Department of Chemistry, Aarhus University, DK* 8000 *Aarhus C, Denmark*

(*Received* 14 *October* 1993; *accepted* 6 *April* 1994)

Abstract

Human methylamine-treated complement C3 (C3-MA) and C3b (C3b-MA) have been crystallized using ammonium sulfate as precipitant. The crystals of the two compounds are morphologically indistinguishable though they belong to different space groups. We show that only minor alterations in packing are responsible for the change in space group. Crystals of C3-MA are tetragonal [$P4_{1(3)}22$, $a = b = 135$, $c = 610$ Å] with two molecules per asymmetric unit. Crystals of C3b-MA are also tetragonal [$P4_{1(3)}2_12$, $a = b = 191$, $c = 610$ Å] with four molecules per asymmetric unit. The maximum diffraction observed is 7.7 Å at cryogenic temperature using synchrotron radiation.

Introduction

The third component of the complement system, C3, consists of a 75 kDa β-chain and a 115 kDa α-chain connected by a disulfide bond (Tack, Janatova, Thomas, Harrison & Hammer, 1981). C3 has an effector role in the immune response, where it is operative at a point at which the classical and the alternative pathways of complement activation converge. Activation by the C3 convertases C4b:C2a or C3b:Bb results in the formation of the fragments C3a and C3b (Müller-Eberhard, 1988). The anaphylatoxin C3a consists of the N-terminal 77 residues of the α-chain (Tack, Janatova, Thomas, Harrison & Hammer, 1981; Müller-Eberhard, 1988). The large C3b fragment binds covalently to biological targets, *e.g.* membrane constituents and antibody aggregates, through a β-Cys-γ-Glu thiol ester located in the α-chain (Law & Levine, 1977; Tack, Harrison, Janatova, Thomas & Prahl, 1980; Gadd & Reid, 1981). Different parts of the C3 structure can be recognized by receptors, which are important for the regulation of complement activity and the immune response (Ross, 1989).

The amino-acid sequence of human C3 is known (De Bruijn & Fey, 1985), as is the disulfide-bridge pattern (Huber, Scholze, Paques & Deisenhofer, 1980; Dolmer & Sottrup-Jensen, 1993) and the carbohydrate structure (Hase, Kikuchi, Ikenaka & Inoue, 1985; Hirani, Lambris & Müller-Eberhard, 1986; Welinder & Svendsen, 1986). The X-ray structure of C3a has previously been determined (Huber, Scholze, Paques & Deisenhofer, 1980).

C3 is a member of the α_2-macroglobulin (α_2M) superfamily (Sottrup-Jensen *et al.*, 1985). In contrast to α_2M, which is a tetramer in its active form, C3 and C3b function as monomers. Structural information for members of the α_2M superfamily has so far been limited to results obtained from electron microscopy (Schroeter *et al.*, 1992; Boisset, Penczek, Pochon, Frank & Lamy, 1993), although low-resolution (10 Å) X-ray diffraction data have been collected on crystals of α_2M (Andersen *et al.*, 1994). The diffraction by the crystals of C3 and C3b, reported here, only extends to 7.7 Å resolution. This resolution will allow information regarding the domain arrangement to be obtained and the position of C3a to be located with respect to the rest of the structure. A domain model of C3 may also serve as a more detailed subunit model for other members of the α_2M superfamily.

* Department of Molecular Biology.

Materials and methods

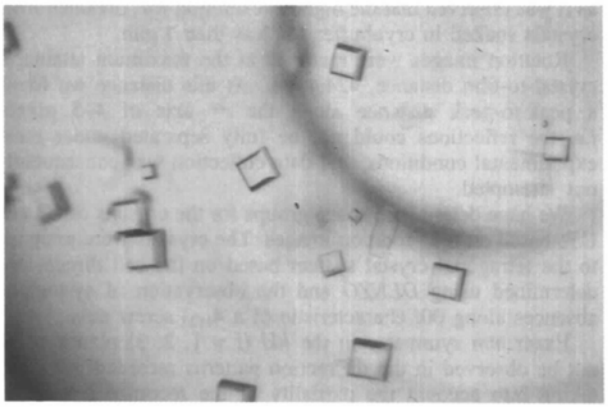
C3 was prepared from the 3–10% polyethylene glycol 6000 precipitate obtained from fresh pooled human plasma essentially according to the method of Tack, Janatova, Thomas, Harrison & Hammer (1981). C3 was treated with methylamine to cleave the internal thiol ester (Tack, Harrison, Janatova, Thomas & Prahl, 1980) and the free thiol was blocked by reaction with iodoacetamide. Further purification was achieved by Blue Sepharose chromatography (column size 5 × 24 cm, 50 m*M* Tris–HCl pH 7.4, 50–1000 m*M* linear gradient of NaCl) (Travis, Bowen, Tewksbury, Johnson & Pannell, 1976) and Mono-Q anion-exchange chromatography (column size 0.5 × 5 cm, 50 m*M* Tris–HCl pH 8.0, 50–1000 m*M* gradient of NaCl). C3b-MA was prepared from C3-MA by limited proteolysis with 1/100 (mol/mol) trypsin for 10 min at 310 K followed by Mono-Q anion-exchange chromatography as above. Tosylphenylalanine chloromethyl ketone-treated bovine trypsin was from Cooper Biomedicals. C3-MA was stored in 20 m*M* sodium phosphate buffer pH 7.4, 170 m*M* NaCl, and C3b-MA in the same buffer with 230 m*M* NaCl. Mono-Q and Sepharose CL-4B were from Pharmacia, Cibacron Blue 3G-A was from Ciba-Geigy and standard chemicals were from Merck and Sigma. Blue Sepharose was prepared from Sepharose CL-4B and Cibacron Blue (Bîhme, Kopperschlager, Schulz & Hofman, 1972).

Crystallization experiments were performed using the vapor-diffusion method with 4–6 μl sitting drops in Cryschem trays (Charles Supper Co., MA, USA). Conditions were screened and optimized by incomplete factorial experiments (Carter & Carter, 1979) using a crystallization robot (Oldfield, Ceska & Brady, 1991). Crystal densities were measured in a Ficoll gradient (Westbrook, 1985).

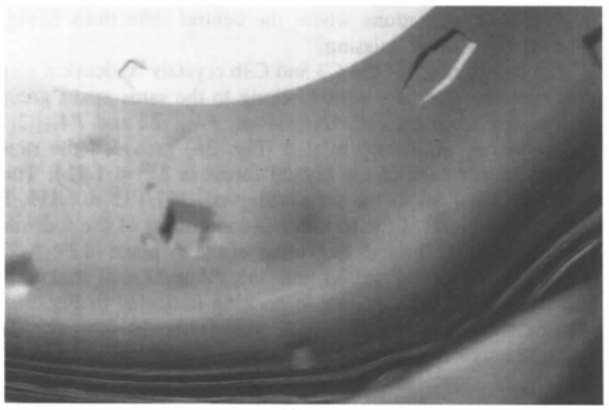
Oscillation images were recorded at beamline 7-1 SSRL, Stanford, USA, equipped with a MAR image-plate system (MAR Research, Germany), with a pixel size of 150 μm. Crystals were mounted either in glass capillaries for exposure at 291 K or in a free-standing film for exposures at 95 K (Teng, 1990). The wavelength used was 1.08 Å. Cell parameters were determined and refined using the *DENZO* program system (Otwinowski, 1991).

Results

Crystals of C3-MA used for diffraction experiments were grown by mixing 3 μl of protein (9.2 mg ml^{-1}) in storage buffer with 3 μl of reservoir consisting of 33% ammonium sulfate, 5.5 m*M* Tris, 10 m*M* CH_3COOH pH 4.3. Crystals of C3b-MA were grown in the same way except for the protein concentration being 9.8 mg ml^{-1} and the reservoir



(*a*)



(*b*)

Fig. 1. (*a*) Crystals of C3-MA and (*b*) C3b-MA. The size of the crystals is ~0.15 × 0.15 × 0.4 mm.

containing 36% ammonium sulfate. All experiments were performed at 293 K. Crystals appeared after 1–3 weeks, and growth continued for several weeks. The crystals of C3-MA and C3b-MA were both tetragonal bipyramids and were indistinguishable from each other (Fig. 1).

The maximum size obtained was 0.2 × 0.2 × 0.4 mm for C3-MA and 0.15 × 0.15 × 0.4 mm for C3b-MA. When exposed to synchrotron radiation at 293 K, the crystals decayed after a few minutes. The decay was avoided by flash freezing the crystals in a free-standing film. Prior to freezing, the crystals were transferred to a buffer containing 30% glycerol as cryoprotectant and 30% ammonium sulfate. The best results, in terms of the observed diffraction, were obtained with a soaking time of approximately 30 s. Flash freezing the crystals at 95 K in a free-standing film, gave the crystals a lifetime of more than 1 h in the beam.

Crystals of C3-MA diffracted to 8.0 Å resolution, and crystals of C3b-MA diffracted to 7.7 Å resolution. Typical exposure times were 10–20 min for a 0.2° rotation photograph. An example of the diffraction from a crystal of C3b-MA is given in Fig. 2.

For C3-MA the cell parameters were determined as: $a = b = 135$, $c = 610$ Å; and the most likely space group was $P4_{1(3)}22$. For C3b-MA the cell parameters were determined as: $a = b = 19$, $c = 610$ Å; and the most likely space group was $P4_{1(3)}2_12$.

Crystals of C3-MA and C3b-MA had a density of 1.12 (1) g cm^{-3} using the Ficoll method. Assuming that the density of the mother liquor in the crystal becomes 1 g cm^{-3} when suspended in the Ficoll gradient (Westbrook, 1985), C3-MA and C3b-MA were found to contain two and four molecules, respectively, per asymmetric unit.

Discussion

The lifetime of the crystals was very short at room temperature but could be increased significantly by using cryotechniques.

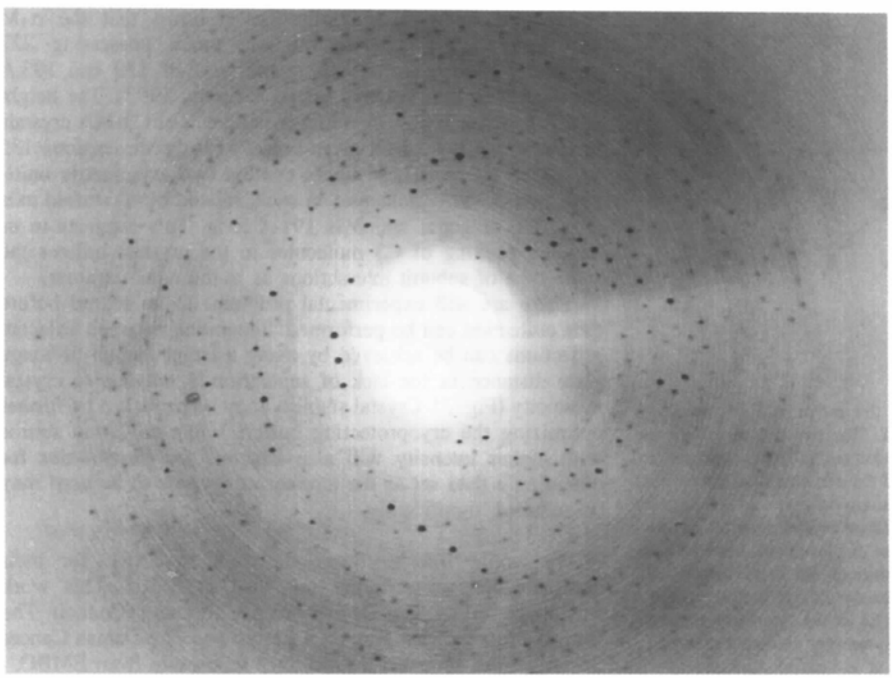


Fig. 2. 0.2° rotation image of a C3b-MA crystal. Crystal-to-film distance 424.2 mm. The wavelength used was 1.08 Å and exposure time was 1200 s. c^* is nearly parallel to the beam. a^* and b^* are both at an angle of ~45° to the rotation axis. It is seen that the lunes of layers $l = 0$, 1 and 2 are separated at low resolution indicating that reflections do not overlap due to mosaicity.

The composition of the cryobuffer had, prior to the diffraction experiments, been optimized with respect to maintaining macroscopic crystal stability. With respect to the diffraction experiments described here we have not used the optimal buffer as it was observed that the highest resolution was obtained from crystals soaked in cryobuffer for less than 1 min.

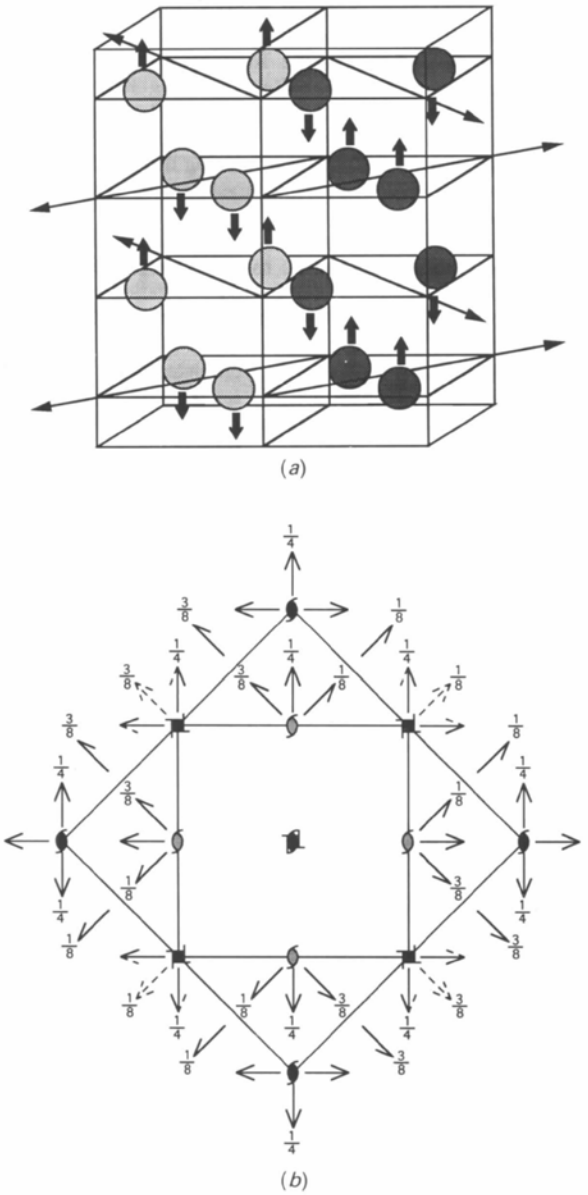


Fig. 3. (*a*) Schematic representation of the movement of molecules that will transform $P4_122$ into $P4_12_12$. The two molecules of one asymmetric unit are indicated as one shaded circle. The contents of one unit cell are shown in light shading and the contents of the second in dark shading. Movement of molecules along the *c* axis is indicated by arrows. Arrows along the diagonals indicate crystallographic twofold axes which, by means of the molecular displacement, become non-crystallographic. The 4_1 axis passing through the center of the unit cell will become a 2_1 axis whereas the 4_1 axis at the edge of the unit cell will remain a 4_1 axis. (*b*) Overlay of the symmetry elements of space groups $P4_122$ and $P4_12_12$. Symmetry elements present in $P4_122$ only are shown as dashed lines or as shaded symbols.

Rotation images were recorded at the maximum attainable crystal-to-film distance, 424.4 mm. At this distance we found a peak-to-peak distance along the c^* axis of 4–5 pixels, *i.e.* the reflections could not be fully separated under these experimental conditions, and data collection was consequently not attempted.

We have determined space groups for the crystals of C3 and C3b based on 0.2° rotation images. The crystals were assigned to the tetragonal crystal system based on the cell dimensions determined using *DENZO* and the observation of systematic absences along 00*l* characteristic of a $4_{1(3)}$ screw axis.

Exact *mm* symmetry in the *hkl* (l = 1, 2, 3) planes could not be observed in the diffraction patterns recorded but when taking into account the partiality of the recorded reflections violation of *mm* symmetry could not be found either. The twofold screw axis of C3b was indicated by two sets of three neighbouring reflections where the central reflections having an odd index were missing.

The morphology of the C3 and C3b crystals is identical so it was expected that they would belong to the same space group with nearly identical cell dimensions. $P4_{1(3)}22$ and $P4_{1(3)}2_12$ are, however, intimately related (Fig. 3*b*) provided the ratio between the *a* axes of the crystal forms is $2^{1/2}$ = 1.414. This is almost identical to the ratio observed, 191/135 = 1.415. It can be shown that if the translation component of the fourfold screw axis going through the center of the *xy* plane in $P4_{1(3)}22$ is not fulfilled (see Figs. 3*a* and 3*b*), $P4_{1(3)}22$ will transform into $P4_{1(3)}2_12$. If the change in packing indicated in Fig. 3(*a*) is sufficiently small the C3b-MA crystals should show pseudo $P4_{1(3)}22$ symmetry with cell dimensions identical to those of the C3-MA crystals. In the rotation photograph in Fig. 2 it is seen that reflections with $h + k = 2n + 1$ are in general weaker than the $h + k = 2n$ reflections. If the change in packing along the *c* axis is the only change the reflections in the *hk*0 plane should be truly absent for $h + k = 2n + 1$. As this is not the case the change in packing also involves translations perpendicular to the *c* axis and/or rotation of the molecules.

Electron microscopy studies have shown that the α_2M tetramer forms roughly a rhombic prism possessing 222 symmetry with diagonals along the base of 130 and 193 Å (Boisset, Penczek, Pochon, Frank & Lamy, 1993). The height of this prism is 144 Å. Dividing the unit cell of C3-MA crystals in four along the *c* axis gives boxes with the dimensions 135 × 135 × 152 Å. These boxes contain two asymmetric units, with two C3-MA molecules in each, related by a twofold axis along the diagonal which is 191 Å long. This suggests to us that the packing of C3 molecules in the crystals utilizes the same type of subunit interactions as in the α_2M tetramer.

There are still experimental problems to be solved before data collection can be performed. Separation between adjacent reflections can be achieved by using a larger crystal-to-image plate distance as the lack of separation is not due to crystal mosaicity (Fig. 2). Crystal stability may be increased by further optimizing the cryoprotecting buffer. Using an X-ray source with higher intensity will also improve the possibilities for obtaining a data set as the number of crystals to be used may be reduced significantly.

We would like to thank the staff at SSRL for their excellent assistance during our visit at SSRL. This work was supported by The Danish Science Research Council, The Danish Biomembrane Research Centre and The Danish Cancer Society. GRA received a short-term fellowship from EMBO.

References

ANDERSEN, G. R., THIRUP, S., NYBORG, J., DOLMER, K., JACOBSEN, L. & SOTTRUP-JENSEN, L. (1994). *Acta Cryst.* D**50**, 298–301.

BÎHME, H. J., KOPPERSCHLAGER, G., SCHULZ, J. & HOFMANN, E. (1972). *J.Chromatogr.* **69**, 209–214.

BOISSET, N., PENCZEK, P., POCHON, F., FRANK, J. & LAMY, J. (1993). *J. Mol. Biol.* **232**, 522–529.

CARTER, C. W. JR & CARTER, C. W. (1979). *J. Biol. Chem.* **254**, 12219–12223.

DE BRUIJN, M. H. L. & FEY, G. H. (1985). *Proc. Natl Acad. Sci. USA*, **82**, 708–712.

DOLMER, K. & SOTTRUP-JENSEN, L. (1993). *FEBS Lett.* **315**, 85–90.

GADD, K. J. & REID, K. B. M. (1981). *Biochem. J.* **195**, 471–480.

HASE, S., KIKUCHI, N., IKENAKA, T. & INOUE, K. (1985). *J. Biochem. (Tokyo)*, **98**, 863–874.

HIRANI, S., LAMBRIS, J. D. & MÜLLER-EBERHARD, H. J. (1986). *Biochem. J.* **233**, 613–616.

HUBER, R., SCHOLZE, H., PAQUES, E. P. & DEISENHOFER, J. (1980). *Hoppe-Seyler's Z. Physiol. Chem.* **361**, 1389–1399.

LAW, S. K. & LEVINE, R. P. (1977). *Proc. Natl Acad. Sci. USA*, **74**, 2701–2705.

MÜLLER-EBERHARD, H. (1988). *Annu. Rev. Biochem.* **57**, 321–347.

OLDFIELD, T. J., CESKA, T. A. & BRADY, R. L. (1991). *J. Appl. Cryst.* **24**, 255–260.

OTWINOWSKI, Z. (1991). *DENZO. A Film Processing Program for Macromolecular Crystallography*. Yale Univ., New Haven, USA.

ROSS, G. D. (1989). *Curr. Opin. Immunol.* **2**, 50–62.

SCHROETER, J. P., KOLODZIEJ, S. J., WAGENKNECHT, T., BRETAUDIERRE, J.-P., TAPON-BRETAUDIERRE, J., STRICKLAND, D. K. & STOOPS, J. K. (1992). *J. Struct. Biol.* **109**, 235–247.

SOTTRUP-JENSEN, L., STEPANIK, T. M., KRISTENSEN, T., LÖNBLAD, P. B., JONES, C. M., WIERZBICKI, D. M., MAGNUSSON, S., DOMDEY, H., WETSEL, R. A., LUNDWALL, Å. B., TACK, B. F. & FEY, G. H. (1985). *Proc. Natl Acad. Sci. USA*, **82**, 9–13.

TACK, B. F., HARRISON, R. A., JANATOVA, J., THOMAS, M. L. & PRAHL, J. W. (1980). *Proc. Natl Acad. Sci. USA*, **77**, 5764–5768.

TACK, B. F., JANATOVA, J., THOMAS, M. L., HARRISON, R. A. & HAMMER, C. H. (1981). *Methods Enzymol.* **80**, 64–101.

TENG, T.-Y. (1990). *J. Appl. Cryst.* **23**, 387–391.

TRAVIS, J., BOWEN, J., TEWKSBURY, D., JOHNSON, D. & PANNELL, R. (1976). *Biochem. J.* **157**, 301–306.

WELINDER, K. G. & SVENDSEN, A. (1986). *FEBS Lett.* **202**, 59–62.

WESTBROOK, T. M. (1985). *Methods Enzymol.* **114**, 187–196.