Acta Cryst. (1994). D**50**, 298-301

Low-Resolution X-ray Diffraction Data Obtained from Hexagonal Crystals of Methylamine-Treated α_2-Macroglobulin

BY GREGERS R. ANDERSEN, SØREN THIRUP AND JENS NYBORG*

Department of Chemistry, University of Aarhus, DK-8000 Aarhus C, Denmark

AND KLAVS DOLMER, LINDA JACOBSEN† AND LARS SOTTRUP-JENSEN

Department of Molecular Biology, University of Aarhus, DK-8000 Aarhus C, Denmark

(*Received* 3 *September* 1993; *accepted* 23 *November* 1993)

Abstract

Two hexagonal crystal forms of tetrameric human methylamine-treated α_2-macroglobulin have been grown by vapour diffusion. One of the crystal forms diffracts X-rays beyond 9 Å resolution. The space group of this form is *P*6(2)22 or *P*6(4)22 with $a = b = 327$, $c = 219$ Å and with one dimer of α_2-macroglobulin in the asymmetric unit. Several data sets have been collected by the use of synchrotron radiation at cryogenic temperature. A native data set extending to 10 Å resolution has been obtained. The merging *R* factor of these data is 10.3%.

Introduction

α_2-Macroglobulin (α_2M) is a plasma proteinase inhibitor consisting of four identical 180 kDa subunits that are disulfide bridged in pairs. Each subunit contains one β-Cys-γ-Glu thiol ester and a 'bait region' which can be cleaved by many different proteinases. Complex formation between proteinases and α_2M is initiated by specific limited proteolysis in the 'bait region'. This induces activation of the thiol ester and a conformational change, which physically traps the proteinase inside the inhibitor. A large fraction of the proteinase molecules are covalently bound, primarily through ε-lysyl(proteinase)-γ-glutamyl(α_2M) [for reviews of the structural and functional properties of α_2M, see Sottrup-Jensen (1987, 1989)]. Upon treatment of α_2M with methylamine, the thiol ester is cleaved, resulting in inactive α_2M (Sottrup-Jensen, Petersen & Magnusson, 1980). In electron micrographs this form, and also the α_2M-proteinase complex, has a compact H-like shape (Boisset, Penczek, Pochon, Frank & Lamy, 1993; Delain, Pochon, Barray & Van Leuven, 1992; Schramm & Schramm, 1982). Proteinases appear to bind in an elongated central cavity of α_2M (Arakawa, Nishigai & Ikai, 1989; Boisset *et al.*, 1989).

The crystallization of α_2M-MA (MA = methylamine) and α_2M-proteinase complexes has been reported earlier (Andersen, Jakobsen, Thirup, Nyborg & Sottrup-Jensen, 1991; Delain *et al.*, 1992; Löbermann, 1980; Wenger, von Gruber & Schramm, 1991). Here we describe a hexagonal crystal form of α_2M-MA, which for the first time allows the processing of data from crystals of α_2M. Although the resolution of these data are much lower than that which is normally aimed for in protein crystallographic studies, it should be compared with the resolution of 30 Å in the electron-microscopy studies of this protein. A successful structure determination from these data can therefore produce a more detailed model than the one currently available thereby describing in greater detail the important structural properties such as the proteinase-containing cavity, the connection between subunits and possibly the location of the thiol ester. This could be of considerable benefit for functional studies of the protein and help in interpreting data from electron micrographs. Furthermore, it would provide a good starting model for structure determination when crystals of higher quality are available.

Materials and methods

α_2M was prepared from pooled outdated human plasma (Sottrup-Jensen *et al.*, 1980). α_2M-MA was obtained after incubating α_2M with 0.2 *M* methylamine at pH 8.0 in the presence of 10 m*M* iodoacetamide for 4 h.

Sialic acids were removed from α_2M-MA in order to improve the homogeneity of the preparation prior to crystallization. Reaction conditions for desializing were 3.5 mg ml^{-1} α_2M-MA, 50 mU ml^{-1} neuramidase type V (Sigma), 0.1 *M* sodium acetate pH 6.0, 0.025% NaN_3, 0.45 m*M* phenylmethanesulfonylfluoride (PMSF) at room temperature. Desalting on a Sephadex G25 column equilibrated with reaction buffer was performed after 6 h of reaction and the same amount of enzyme was added. Following 12 h of digestion, α_2M-MA was separated from the enzyme on a Sephacryl S200 column equilibrated with 20 m*M* Tris-HCl pH 7.7 and concentrated in Centricon-100 (Amicon Corporation) cells

* Author for correspondence: J. Nyborg, Department of Chemistry, Langelandsgade 140, 8000 Aarhus C, Denmark.

† Present address: Department of Molecular Genetics, University and Biocenter Vienna, A-1030 Wien, Austria.

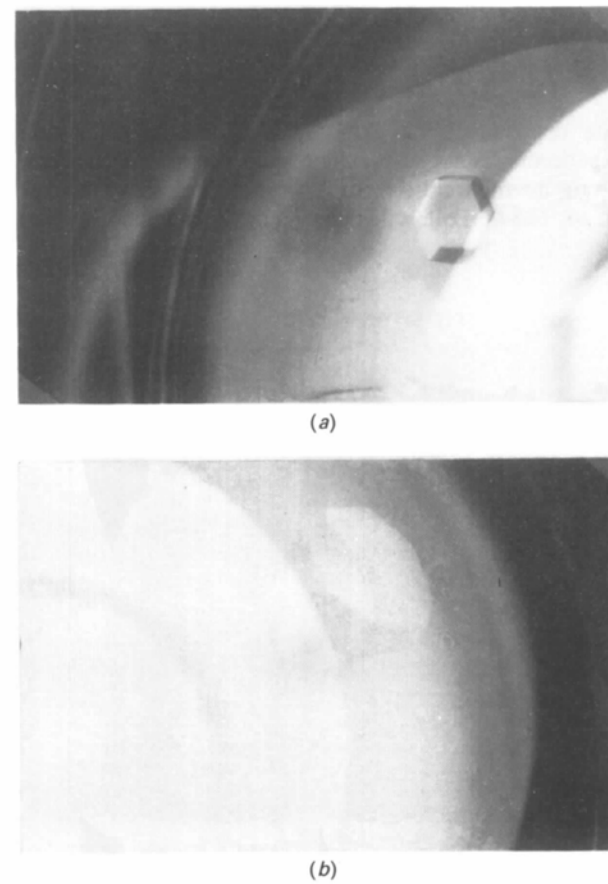
to 7.5 mg ml^{-1}. The desialization was more than 95% complete as measured by the 2-thiobarbituric acid assay (Warren, 1959). Screening for crystallization conditions was performed by vapour diffusion in Cryschem trays (Charles Supper Co., Massachusetts, USA) by the use of a modified Gilson crystallization robot (Oldfield, Ceska & Brady, 1991). Crystallization at 277 K was performed in a cooling incubator. 2-Methyl-2,4-pentanediol (MPD) used for crystallization was of MicroSelect quality from Fluka, Switzerland. All other chemicals were of analytical quality. Prior to mounting by the loop method (Teng, 1990) and flash freezing at 95 K in a stream of nitrogen, crystals were gradually transferred to a cryoprotective solution to avoid formation of ice during flash freezing (see below in *Results*). Data were collected with an MAR imaging plate using synchrotron radiation, λ = 1.08 Å, at beamline 7-1, SSRL, CA, USA. The density of the crystals was determined by transferring crystals to 200 µl drops of Ficoll solution until a solution was found which made the crystal float in the middle of the drop for at least 15 min. The drops were calibrated with small drops mixed from bromobenzene and *o*-xylene. This procedure was preferred to the normal gradient procedure (Westbrook, 1985), since centrifugation of the gradient rapidly dissolved the crystals. The programs *IMSTILLS*, *ROTAVATA*, *POSTREF* and *AGROVATA* from the *CCP*4 package (SERC Daresbury Laboratory, 1979) were used for spot scanning, scaling, post refinement and merging, while *REFIX* (Kabsch, 1988) was used for initial indexing. The program *DENZO* (Otwinowski, 1991) was used for refinement of orientation and integration. Temperature factors were not refined during scaling.

Results

Hexagonal crystals of α_2M-MA grew in two different morphologies depending on the concentration of NaCl in the experiments (Fig. 1). Hexagonal rods with maximal dimensions 0.2 × 0.2 × 0.7 mm formed in 2–3 weeks by mixing α_2M-MA, 7.5 mg ml^{-1} in 20 m*M* Tris-HCl pH 7.7, with reservoir buffer containing 13% MPD, 0.5 *M* NaCl, 21 m*M* 2-(*N*-morpholino)ethanesulfonic acid (MES) and 0.5 m*M* $ZnSO_4$ in a 2:1 ratio. The growth time was 2–4 weeks at 277 K. Chunky hexagonal crystals with maximum dimensions 0.8 × 1.0 × 1.0 mm were grown by the same procedure, except that the concentration of NaCl was 1.0 *M*. For these crystals, the growth time was 3–6 d at 277 K. The apparent pH was 6.5 at 277 K.

The temperature was found to be essential for crystal growth. Only very small crystals of the chunky form of α_2M-MA could be grown at 293 K. If drops containing crystals were moved from 277 to 293 K, the crystals dissolved rapidly. Also a strict control of the concentration of $ZnSO_4$ was necessary. At the optimized conditions containing 0.5 m*M* $ZnSO_4$ in the reservoir, millimetre-sized crystals were obtained in virtually every experiment. The density of the chunky crystals was found to be 1.10 (1) g cm^{-3}.

Both forms diffracted X-rays when exposed to synchrotron radiation. For the chunky hexagonal crystal form of α_2M-MA, weak reflections were observed beyond 9 Å resolution on small angle oscillation images (Fig. 2). 1° oscillation images with useful reflections extending to 10 Å could be collected within 3–5 min of exposure with synchrotron radiation. The rod-shaped hexagonal crystals did not diffract beyond 15 Å. No data were collected for this crystal form. For both crystal forms, the flash freezing and exposure at cryogenic temperature was found to be essential. If not frozen, the crystals ceased to diffract rapidly. For the chunky crystal form, diffraction extending to at least 15 Å was still observed after 10 h of exposure following soaking of crystals in a cryoprotective solution containing 1 *M* NaCl, 30%(*v*/*v*) glycerol, 45 m*M* $CdCl_2$ pH 6.5 at 277 K and subsequently flash freezing. Concentrations ranging from 0.0 to 0.5 m*M* $ZnSO_4$ in the soak solutions were tested, but all failed to give a diffraction pattern of the quality obtained when $CdCl_2$ instead of $ZnSO_4$



(*a*)

(*b*)

Fig. 1. (*a*) Hexagonal rod-shaped crystals of α_2M-MA. (*b*) Chunky hexagonal crystals of α_2M-MA.

was added to the soak solution prior to flash freezing. The chunky hexagonal crystals belong to space group *P*6(2)22, alternatively *P*6(4)22, as evidenced by systematic extinction along the c^* axis observed on oscillation images, and also confirmed by inspection of the integrated (00l) intensities.

Several data sets obtained from the chunky crystal form were examined. The mosaicity of the best crystals following flash freezing was approximately 0.5°. The refinement during data processing with *DENZO* generally resulted in χ^2, defined as (observed error)^2/(predicted error)^2, for the positions refining to below 1.5, while χ^2 for the partiality refined to between 2 and 3 for the best crystals. It was possible to process data and scale these together from two crystals with reasonable results, although some variations in the cell parameters were observed. The results of data processing are summarized in Table 1. A merging *R* factor of 10.3% was obtained with data extending to 10 Å resolution.

A large amount of anisotropic background was observed for this crystal form as was also observed for the tetragonal crystals (Andersen *et al.*, 1991). In particular, a very strong halo surrounding the (0,0,12) reflection (not shown), which is also the most intense reflection in the data set, was observed.

Table 1. *Breakdown of the observed intensities as a function of resolution as output from the AGROVATA program*

A total of 17 778 reflections were processed and merged to 3800 independent reflections.

d_{min} (Å)	R_{merge}	R_{cum}	$\langle I/\sigma(I)\rangle$	% complete
32.1	0.074	0.074	6.6	68.0
26.3	0.058	0.065	7.9	95.8
22.8	0.051	0.059	13.1	99.1
20.4	0.057	0.059	11.1	97.4
18.7	0.073	0.061	9.1	97.5
17.3	0.074	0.063	8.9	102.3
16.2	0.091	0.066	6.7	98.0
15.3	0.087	0.068	7.1	101.9
14.5	0.095	0.070	7.0	98.4
13.8	0.112	0.072	6.1	96.8
13.2	0.156	0.076	4.3	98.4
12.7	0.132	0.078	5.1	98.6
12.2	0.149	0.081	4.6	97.5
11.8	0.143	0.083	5.2	96.0
11.5	0.171	0.086	4.2	97.6
11.1	0.170	0.089	4.1	96.3
10.8	0.233	0.092	3.1	97.8
10.5	0.201	0.095	3.5	91.9
10.3	0.264	0.098	2.8	95.4
10.0	0.405	0.103	1.8	98.6

Discussion

The dependence on Zn^{2+} ions in the crystallization experiments is not surprising considering the well known strong interaction between α_2M and Zn^{2+} ions (Pratt & Pizzo, 1984). Following mixing of 7.5 mg ml^{-1} protein solution and reservoir solution containing 0.5 m*M* Zn in a 2:1 ratio, the number of Zn^{2+} ions per tetramer of α_2M-MA is 24. Considering the large number of binding sites for Zn^{2+} in α_2M-MA (Pratt & Pizzo, 1984), the concentration of free Zn^{2+} ions in the crystallization droplet could be very low. This could explain the difficulties in soaking crystals in cryoprotective solutions containing Zn^{2+}, where the concentration of free Zn^{2+} would be high compared to the concentrations in the crystallization experiments.

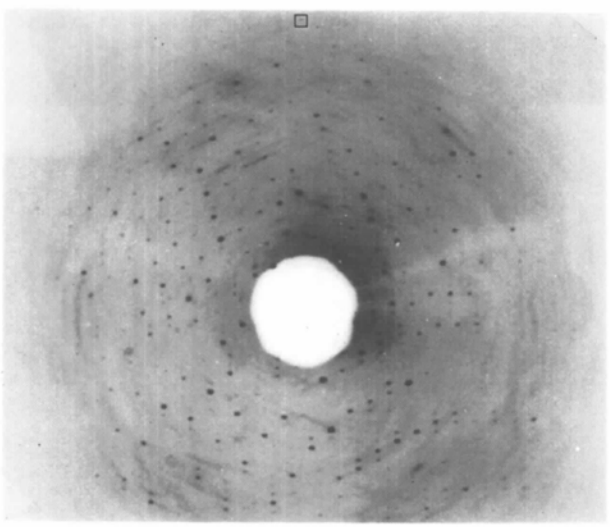


Fig. 2. 0.1° oscillation image recorded at SSRL by exposure of a chunky hexagonal crystal at 115 K. The exposure time was 600 s and the crystal-to-film distance 424 mm. The resolution of the reflection in the black box is 8.5 Å.

Using the model in Westbrook (1985), the theoretical density in the Ficoll system is 1.09 and 1.17 g cm^{-3} for one and two dimers per asymmetric unit, respectively. It is, therefore, most likely that the asymmetric unit contains the dimer and has a solvent content of 73.5%. This results in a value of V_m = 4.8 Å^3 Da^{-1}, which is above the usual range of 1.68–3.53 Å^3 Da^{-1} for protein crystals (Matthews, 1968). It is, however, significantly lower than the value of 6.0 Å^3 Da^{-1} obtained for the tetragonal crystals of α_2M-MA (Andersen *et al.*, 1991). The tighter packing in the hexagonal crystals could be the reason for the increased quality of these crystals compared with the tetragonal crystals. Tetragonal crystals of α_2M-MA could also be flash frozen, but the quality of the diffraction pattern obtained was inferior compared with that of the hexagonal crystal described here. The hexagonal crystal form therefore seems suitable for obtaining a low-resolution structure of α_2M-MA.

We would like to thank the staff at SSRL for their excellent assistance during our visit to SSRL and the staff at The Blood Bank, Aarhus University Hospital, for providing outdated plasma. 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., JAKOBSEN, L. THIRUP, S. NYBORG, J. & SOTTRUP-JENSEN, L. (1991). *FEBS Lett.* **292**, 267-270.

ARAKAWA, H., NISHIGAI, M. & IKAI, A. (1989). *J. Biol. Chem.* **264**, 2350-2356.

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

BOISSET, N., TAVEAU, J.-C., POCHON, F., TARDIEU, A., BARRAY, M., LAMY, J. N. & DELAIN, E. (1989). *J. Biol. Chem.* **264**, 12046-12052.

DELAIN, E., POCHON, F., BARRAY, M. & VAN LEUVEN, F. (1992). *Electron Microsc. Rev.* **5**, 231-281.

KABSCH, W. (1988). *J. Appl. Cryst.* **21**, 67-71.

LÖBERMANN, H. (1980). Diplomarbeit, Ludwig-Maximillian-Univ., München, Germany.

MATTHEWS, B. W. (1968). *J. Mol. Biol.* **33**, 491-497.

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.

PRATT, C. W. & PIZZO, S. V. (1984). *Biochem. Biophys. Acta*, **791**, 123-130.

SCHRAMM, H. J. & SCHRAMM, W. (1982). *Hoppe Seylers Z. Physiol. Chem.* **363**, 803-812.

SERC Daresbury Laboratory (1979). *CCP4. A Suite of Progams for Protein Crystallography.* SERC Daresbury Laboratory, Warrington WA4 4AD, England.

SOTTRUP-JENSEN, L. (1987). *The Plasma Proteins*, Vol. 5, pp. 191-291. Orlando: Academic Press.

SOTTRUP-JENSEN, L. (1989). *J. Biol. Chem.* **264**, 11539-11542.

SOTTRUP-JENSEN, L., PETERSEN, T. E. & MAGNUSSON, S. (1980). *FEBS Lett.* **121**, 275-279.

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

WARREN, L. (1959). *J. Biol. Chem.* **234**, 1971-1975.

WENGER, T., VON GRUBER, M. & SCHRAMM, H. J. (1991). *Biol. Chem. Hoppe Seyler*, **372**, 147-148.

WESTBROOK, E. M. (1985). *Methods Enzymol.* **114**, 187-196.