The EMBO Journal vol.4 no.1 pp.85 – 89, 1985

Electron microscopy of the conformational changes of alpha 2-macroglobulin from human plasma

Jacqueline Tapon-Bretaudière, Andrée Bros, Evelyne Couture-Tosi[1] and Etienne Delain[1]

Laboratoire d'Hématologie, CHU Necker-Enfants Malades, 156, rue de Vaugirard, 75015 Paris, and [1]Laboratoire de Microscopie cellulaire et moléculaire, (L.A. 147 du CNRS), Institut Gustave-Roussy, Rue Camille Desmoulins, 94805 Villejuif Cédex, France

Communicated by E.Kellenberger

High resolution electron microscopy reveals that fully active alpha 2-macroglobulin (α2M) from fresh human plasma presents a very characteristic tetrameric structure. This native conformation of the α2M molecule is described here for the first time, along with its various orientations in negatively stained preparations. Although the native form is sensitive to inactivation, glutaraldehyde fixation is not necessary for its observation except when ammonium salts are used. The tetrameric structure of α2M undergoes a drastic conformational change when the protein is treated either with trypsin, thrombin or methylamine, as evidenced by the appearance of the typical)+(structure already described in the literature. The various aspects of this second conformation correspond to different orientations of the molecules in the stain film, and depend upon the nature of the support.

Key words: α2-macroglobulin/conventional and scanning transmission electron microscopy/native form/negative staining/protease inactivated form

Introduction

Human alpha 2-macroglobulin (α2M) is a plasmatic tetrameric glycoprotein of mol. wt. 725 000. It binds and inhibits nearly all endoproteases. It can also be inactivated by primary amines (see James, 1980; Barrett, 1981; Van Leuven, 1982; Travis and Salvesen, 1983; Feinman, 1983). Its structural modifications were described as being initiated by either a proteolytic cleavage or the hydrolysis of the thioesters which are required for the α2M inhibition function; both reactions lead to similar changes in the conformation of the α2M molecules (Björk and Fish, 1982; Van Leuven *et al.*, 1982). Two distinct forms of α2M were identified by non-denaturing polyacrylamide gel electrophoresis: the 'slow' S-form which corresponds to the native form, and the 'fast' F-form which corresponds to the α2M complexed with a protease or inactivated by amines or ammonium ions (Barrett *et al.*, 1979).

Data obtained from small angle X-ray scattering (Branegård *et al.*, 1982) and various physical measurements (Pochon *et al.*, 1978; Gonias *et al.*, 1982; Straight and McKee, 1982) provided information on the shape of the two forms of α2M. Additional evidence for these conformational changes was obtained from electron microscopic observations indicating a more compact shape for α2M-protease complexes as compared with native α2M (Höglund and Levin, 1965; Bloth *et al.*, 1968; Barrett *et al.*, 1974; Schramm and Schramm, 1982).

Here we show that negatively stained native α2M from fresh human plasma consists of a population of tetrameric molecules which have a conformation completely different from any previous description. However these molecules are capable of undergoing a drastic conformational change upon binding of proteases, or by methylamine inactivation. This inactivated form is similar to the one previously described for α2M molecules.

Results

Native α2M

Figure 1a shows native α2M negatively stained with uranyl acetate. The molecules typically present two aspects. The clearest one consists of a tetrameric structure 220 – 250 Å wide composed of four spherules each of which is of 85 ± 5 Å in diameter. The tetramers appear to be held together and are more or less densely packed, as shown in Figure 1b. Other structures, looking like doughnuts of average diameter 155 ± 10 Å, are made up of less distinguishable subunits arranged around a space of 55 ± 5 Å which is filled with electron-dense stain (Figure 1c). These molecules sometimes present an asymmetry with a lateral expansion (Figure 1c). Other molecules have an intermediate aspect with four visible subunits and a dense central zone (Figure 1d). The different aspects of α2M molecules correspond to various orientations of the same tetramer on the supporting film.

Some preparations were negatively stained with uranyl acetate in low salt conditions (~1 mM NaCl) (Figure 1e). Under these conditions, most of the molecules had the tetrameric structure already shown in Figure 1b. Glutaraldehyde fixation (3% v/v) was found not to be necessary for protection of the molecules stained with uranyl acetate, as no ultrastructural difference was observed between fixed and unfixed specimens. This is not true for ammonium molybdate (pH 8) staining of the native α2M which needs aldehyde fixation. This difference could be related to the particular sensitivity of α2M to ammonium ions and to the high pH. Native α2M samples diluted in Michaëlis buffer at a pH between 5 and 8 in the presence of glutaraldehyde, then negatively stained with uranyl acetate, exhibit the same usual tetrameric morphology.

α2M-trypsin complexes

When trypsin is added to α2M solutions at a molar ratio of 2/1, the conformation of all α2M molecules is changed from the native state to a new, completely different one, as seen with the electron microscope. The transformation appears immediately and does not need any incubation time. The preparations appear to be stable even for several days at room temperature. Samples prepared with excess trypsin are degraded slowly. The shape of the trypsin-complexed α2M is similar to that already described in the literature, i.e., it looks like the cyrillic character)+(. In thin uranyl acetate stainings, all the molecules had the same dimensions: 155 ± 5 x 230 ± 16 Å (Figure 2a). The molecules had two main aspects: the first showed a very clear)+(structure with two well individualized

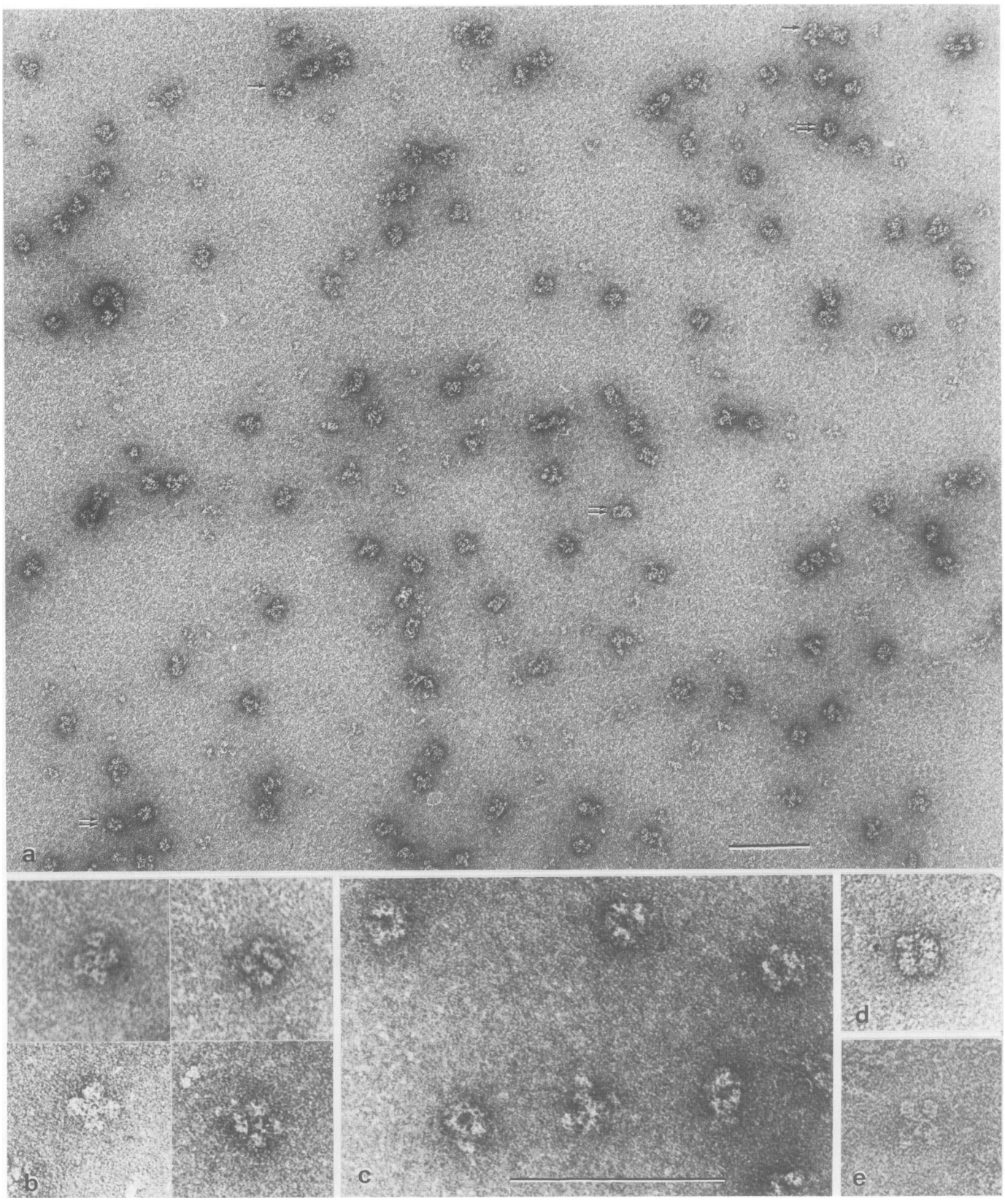

Fig. 1 (a) Representative large field of native α2M from human plasma negatively stained with 2% aqueous uranyl acetate pH 5. (Bar = 100 nm) with the different aspects enlarged thereafter. **(b)** High magnification of selected α2M molecules showing typical tetrameric structure: see → in **a**. (For **b**–**f**, the bar represents 10 nm). **(c)** Selection of molecules having a doughnut-like structure: see ⇉ in **a**. **(d)** Molecule with four visible subunits and a dense central zone. **(e)** In very low ionic strength the tetrameric organization of native α2M is clearly visible.

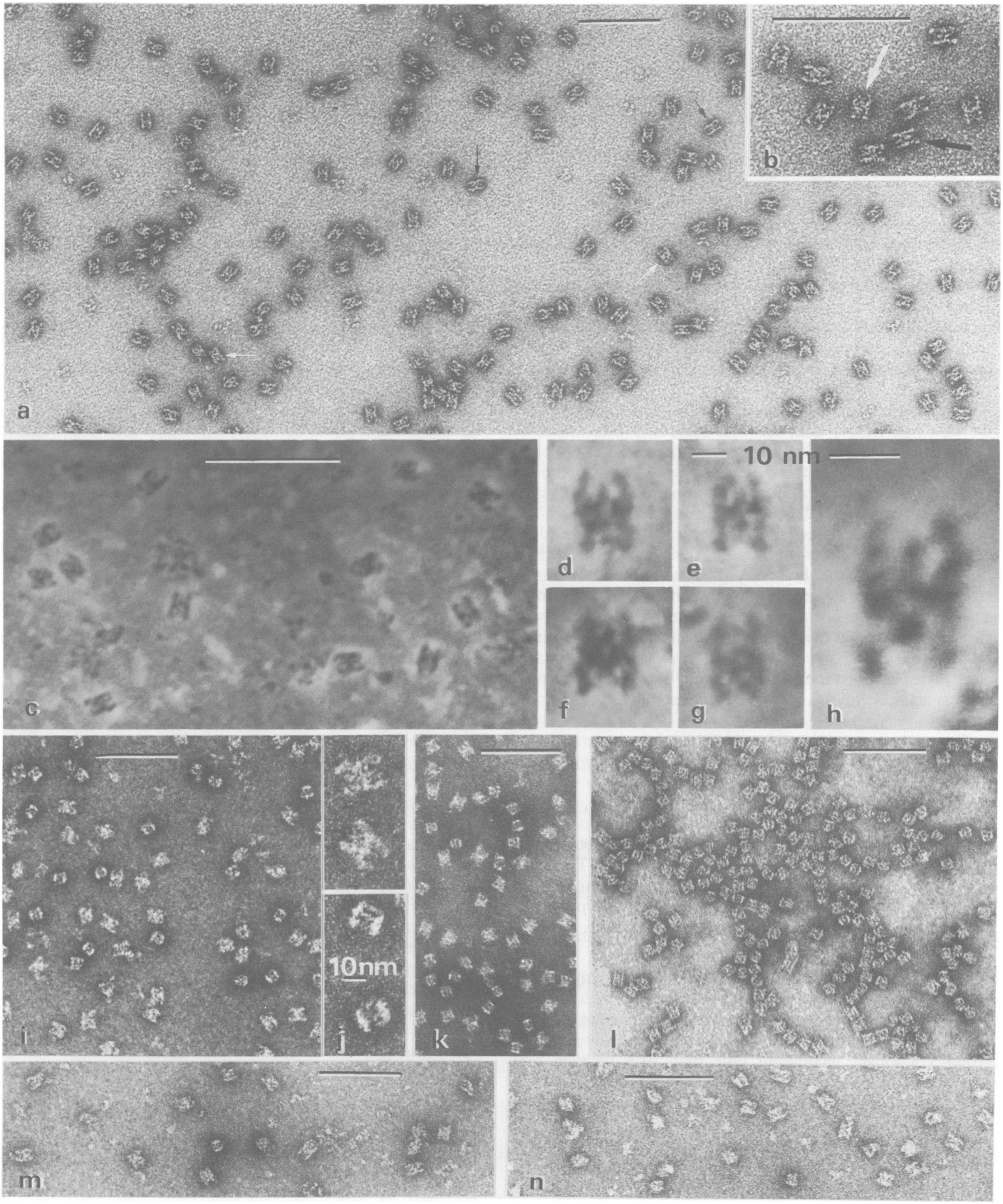

Fig. 2 (a) Representative large field of α2M-trypsin complexes negatively stained as for Figure 1. (Unless specified, the bars represent 100 nm). **(b)** Different aspects of the molecules. **(c – h)** Z-contrast pictures obtained by STEM of α2M-trypsin molecules prepared as for **a (d – h**: bars = 10 nm). **(i)** Thick uranyl acetate staining showing horizontal and vertical views of α2M-trypsin molecules. **(j)** Detail of the two orientations (bar = 10 nm). **(k)** Thick sodium phosphotungstate staining showing the same two orientations. **(l)** Most of the molecules absorbed to mica have a vertical orientation. **(m)** α2M complexed with thrombin. **(n)** α2M inactivated with methylamine.

walls and an evident internal cross-like structure (Figure 2a,b: black arrows); the second presents the same regular profile but showed a complex structure between the two walls (Figure 2a,b: white arrows). These two structural aspects suggest that the molecules, when lying on their larger side, have only two possible orientations. No vertical top views were detectable in thin preparations.

Scanning transmission electron microscopy (STEM) of thin negatively stained samples produced particularly good pictures of the molecules when the in-line processing of the image signals was used. The atomic number (Z)-contrast procedure especially enhances the contrast of biological molecules. With STEM the pictures of α2M-trypsin molecules show their characteristic structure very well (Figure 2c–h). There is some variability in the shape of the walls of the molecules, which appear straight, distorted or bent at one end. The internal organization is complex and variable, and probably depends on the orientation of the molecules deposited on the carbon film.

The morphology of the molecules varies somewhat with the thickness of the stain film. As seen on Figure 2a, molecules embedded in a thin film of uranyl acetate have a very regular appearance. In thick films of uranyl stain (Figure 2i, j), or in neutral sodium phosphotungstate (Figure 2k), molecules have more varied aspects. Some molecules present the usual)+(aspect when they lie down, others are surrounded by a dark halo of stain, which indicates that they are taller than the non-haloed form. It is very likely that this latter form represents a vertical view of the α2M molecules. Such molecules have a square or rectangular section (155 Å wide) with two thick somewhat curved walls; the other dimension varies between 100 and 150 Å (Figure 2j). The presence of dark and clear zones between the two dense walls shows that the molecule is actually hollow, as already suggested by its horizontal view.

The procedure using ammonium molybdate-protein mixture absorbed to mica gives a majority of vertically oriented α2M-trypsin molecules (Figure 2l). These vertically oriented molecules are also visible, but in a lower proportion, in α2M-trypsin preparations diluted in glutaraldehyde-containing Michaëlis buffer (pH 7–8). Their two-order symmetry is not as obvious as it is in thickly stained preparations. The molecules have a square section 130 Å wide, identical to that of the few horizontal molecules present in the same preparation. These square sections also show a hollow structure.

Thrombin and methylamine-treated α2M

Thrombin- (Figure 2m) or methylamine- (Figure 2n) treated α2M molecules are quite similar to those treated with trypsin. However, the pictures of the former do not have the fineness of the trypsin-inactivated α2M pictures and their appearance requires a longer incubation time than with trypsin. The kinetics of α2M inactivation by trypsin, thrombin and methylamine are different as already mentioned (Van Leuven *et al.*, 1982). In the progressive inactivation of α2M by thrombin and methylamine, intermediate structures are observed.

Discussion

The ultrastructure of human, rabbit or rat α-macroglobulins was first described by Höglund and Levin (1965), and then by Bloth *et al.* (1968), Gauthier *et al.* (1974) and Lebreton de Vonne and Mouray (1974). None of these reports described the existence of two distinct forms of α-macroglobulin. Morelis *et al.* (1969) found that electron microsope images of trypsin-complexed rabbit α1M [which is morphologically similar to rabbit α2M and human α2M (Bloth *et al.*, 1968)] were more contrasted than those of untreated molecules and had the shape of a butterfly. Their pictures are similar to the vertical α2M-trypsin molecules that we observed in thick negative stainings (Figure 2i,j,k). A protease inhibitor isolated from reptilian egg white was shown recently to be homologous but distinct from mammalian α2M (Ikai *et al.*, 1983). The ultrastructural appearance of both molecules shows striking analogies in both their native or trypsin-inactivated forms.

The description by Barrett *et al.* (1974) and by Starkey and Barrett (1977) of control α2M and trypsin-complexed α2M corresponds with the two horizontal and vertical orientations of our inactivated molecules. Schramm and Schramm (1982) working with a commercial preparation of human α2M, only 0.5% active, obtained negative stainings with uranyl salts totally comparable with our thick ones of inactivated α2M. Several reasons could account for the discrepancies between the previously published results and ours; all are related to the origin of the α2M (fresh or frozen plasma, or serum) and its method of purification. Previous reports on the ultrastructure of α2M did not fully specify the origin and purification procedure of the protein. α2M is a polyvalent plasmatic inhibitor of several serine proteases which are generated during blood coagulation from inactive zymogens. To obtain native α2M it is necessary to avoid the formation of α2M-protease complexes. This is achieved by starting from fresh plasma, and not serum, by using plastic ware, and adding, at each purification step, inhibitors to either avoid the generation of these serine proteases or at least to neutralize the small amounts formed. Plasma fractionation by ammonium sulfate should be avoided, as mentioned by Barrett (1981), because of the high sensitivity of α2M to ammonium ions. Finally it is necessary to check both the integrity of the purified α2M, by measuring its inhibitory capacity as reported by Pochon *et al.* (1978), and the absence of α2M-protease complexes, by the procedure described in Materials and methods.

We consider that the various aspects of α2M described in the literature correlate into a unique structure which looks like a)+(. All our native preparations contain molecules as shown in Figure 1. A few)+(forms appear progressively in old samples, or in solutions frozen and thawed several times. Treatments of native α2M with low concentrations of proteases or amylamine, not sufficient to allow a full conformation change, lead to complex intermediate forms difficult to compare with the well distinguishable native forms that we describe here. We think that fully active native α2M from fresh human plasma does not contain any)+(forms.

Preliminary results by small angle X-ray diffusion of native α2M and α2M-protease or methylamine-treated specimens show that large differences are detectable between the gyration radii of the two conformations (73.8 ± 1.5 Å for native and 66.0 ± 1 Å for the other forms); these are much larger than those already described by Branegård *et al.* (1982). Although we have described a new form of the native α2M, it remains unclear how the tetrameric native α2M can undergo a transformation to a complex hollow structure in which the original tetrameric arrangement is no longer detectable on micrographs. Our description of the tetrameric organization of human native α2M, and the existence of various aspects of

the protease- or methylamine-inactivated form provide a new basis for understanding the conformational change of α2M upon protease trapping or primary amine inactivation.

Materials and methods

Chemicals

Methylamine was purchased from Sigma Chemical Co. (USA). Sepharose 6B was obtained from Pharmacia Fine Chemicals (Sweden). All other chemicals were reagent grade.

Proteins

α2M was purified from fresh human plasma according to Steinbuch and Blatrix (1970) with the following inhibitors added: soybean trypsin inhibitor (Sigma), Polybrène® (Aldrich Chem. Comp.), and aprotinine (Choay, France). In some cases, the protein was further purified by gel filtration on a Sepharose 6B column in order to eliminate IgA which usually co-purifies with α2M. The purity of α2M was checked by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) and by electron microscopy. The activity of the purified α2M was tested by measuring its capacity to inhibit thrombin in a clotting assay. By using a chromogenic substrate (S2160 from Kabi, Sweden) able to reveal an amidolytic activity, we checked the absence of bound protease in native α2M preparations. Protein assays were carried out according to Lowry *et al.* (1951) or by radial immunodiffusion (Mancini *et al.*, 1965).

Human α-thrombin (activity 99%) was kindly supplied by J.W.Fenton II (N.Y. State Department of Health, Albany, NY). Bovine trypsin purchased from Sigma was 70% active as determined by active site titration (Chase and Shaw, 1967).

Preparation of protease-α2M complexes and methylamine-treated α2M

Mixtures of proteases (trypsin or thrombin) or methylamine and α2M at various ratios were incubated 15 min at 37°C in Hepes HCl buffer 50 mM, NaCl 100 mM, pH 8.2, as recommended by Björk and Fish (1982). The formation of the complex α2M-protease was checked by measuring the residual proteases as described in the previous paragraph.

Electron microscopy

Samples of α2M-protease or α2M-methylamine mixtures were diluted to a final concentration ~20 μg/ml, and negatively stained by 2% aqueous uranyl acetate. 10 μl of solution were deposited on a carbon or collodion-carbon film which was made hydrophilic by a glow discharge. Some samples were prepared by the procedures described by Horne and Pasquali-Ronchetti (1974); a mixture of concentrated protein (a few mg/ml) was mixed with an equal volume of 2% ammonium molybdate pH 8, deposited on freshly cleaved mica sheet, dried in air and covered with a carbon film deposited in vacuum. The carbon film with the adsorbed proteins was then floated off onto a 2% aqueous uranyl acetate solution. The film was picked up with bare grids, blotted and air dried.

The negative stains used were: 2% aqueous uranyl acetate pH 4.5, 2% potassium phosphotungstate pH 7.2, 2% ammonium molybdate pH 8. Some preparations were diluted in 3% (v/v) glutaraldehyde. The effect of pH was tested by diluting α2M and α2M-trypsin samples in glutaraldehyde-containing Michaëlis 0.1 M veronal-acetate buffer (pH 5 – 8) and subsequent observation in 2% uranyl acetate.

Conventional microscopy was performed with a Philips EM300 equipped with an anti-contamination device. STEM observations were made with a VG-HB 501 electron microscope under conditions already described by Curgy *et al.* (1984). The principles of the atomic number (Z)-contrast procedure and its applications to biological specimens were described by Colliex *et al.* (1984).

Acknowledgements

We would like to thank F.Pochon and A.Tardieu for helpful discussions, Elissa Sena for correcting the English text, and E.Chiric for micrograph printing. The help of C.Mory, C.Colliex and P.Trebbia was necessary for Z-contrast picturing in the STEM. This work was supported in part by CNRS and Gustave-Roussy Institute (Contrat de Recherche Clinique 81 D 12).

References

Barrett,A.J. (1981) *Methods Enzymol.*, **80**, 737-754.

Barrett,A.J., Brown,M.A. and Sayers,C.A. (1979) *Biochem. J.*, **181**, 401-418.

Barrett,A.J., Starkey,P.M. and Munn,E.A. (1974) in Fritz,H., Tshesche,H., Green,L.J. and Truscheit,E. (eds.), *Proteinases Inhibitors,* Springer-Verlag, Berlin, pp. 72-77.

Björk,I. and Fish,W.W. (1982) *Biochem. J.*, **207**, 347-356.

Bloth,B., Chesebro,B. and Svehag,S.-E. (1968) *J. Exp. Med.*, **127**, 749-763.

Branegård,B., Österberg,R. and Sjöberg,B. (1982) *Eur. J. Biochem.*, **122**, 663-666.

Chase,T. and Shaw,E. (1967) *Biochem. Biophys. Res. Commun.*, **29**, 508-514.

Colliex,C., Jeanguillaume,C. and Mory,C. (1984) *J. Ultrastruct. Res.*, in press.

Curgy,J.J., Iftod,F., Pérasso,R., André,J., Colliex,C. and Mory,C. (1984) *Biol. Cell,* **50**, 247-254.

Feinman,R.D., ed. (1983) *Chemistry and Biology of Alpha 2-Macroglobulin, Ann. N.Y. Acad. Sci.*, **421**, 478 pp.

Gauthier,F., Leng,M. and Mouray,H. (1974) *C.R. Hebd. Seances Acad. Sci., Paris D,* **279**, 1409-1412.

Gonias,S.L., Reynolds,J.A. and Pizzo,S.V. (1982) *Biochim. Biophys. Acta,* **705**, 306-314.

Höglund,S. and Levin,Ö. (1965) *J. Mol. Biol.*, **12**, 866.

Horne,R.W. and Pasquali-Ronquetti,I. (1974) *J. Ultrastruct. Res.*, **47**, 361-383.

Ikai,A., Kitamoto,T. and Nishigai,M. (1983) *J. Biochem.*, **93**, 121-127.

James,K. (1980) *Trends Biochem. Sci.*, **5**, 43-47.

Laemmli,U.K. (1970) *Nature,* **227**, 680-685.

Lebreton de Vonne,T. and Mouray,H. (1974) *Ann. Biol. Clin.*, **32**, 185-196.

Lowry,O.H., Rosebrough,N.J., Farr,A.L. and Randall,R.J. (1951) *J. Biol. Chem.*, **193**, 265-275.

Mancini,G., Carbonara,A.O. and Heremans,J.F. (1965) *Immunochemistry,* **2**, 235-254.

Morelis,P., Ambrosioni,J.-C., Got,R. and Fontanges,R. (1969) *C.R. Hebd. Seances Acad. Sci. Paris D,* **269**, 1453-1454.

Pochon,F., Amand,B., Lavalette,D. and Bieth,J. (1978) *J. Biol. Chem.*, **253**, 7496-7499.

Schramm,H.J. and Schramm,W. (1982) *Hoppe-Seyler's Z. Physiol. Chem.*, **363**, 803-812.

Starkey,P.M. and Barrett,A.J. (1977) in Barrett,A.J. (ed.), *Proteinases in Mammalian Cells and Tissues,* Elsevier/North-Holland Biomedical Press, pp. 663-696.

Steinbuch,M. and Blatrix,C. (1970) *Rev. Fr. Transfus. Immuno-Hematol.*, **13**, 141-151.

Straight,D.L. and McKee,P.A. (1982) *Biochemistry (Wash.),* **21**, 4550-4556.

Travis,J. and Salvesen,G.S. (1983) *Annu. Rev. Biochem.*, **52**, 655-709.

Van Leuven,F. (1982) *Trends Biochem. Sci.*, **7**, 185-187.

Van Leuven,F., Cassiman,J.-J. and Van den Berghe,H. (1982) *Biochem. J.*, **201**, 119-128.

Received on 10 August 1984; revised on 2 November 1984