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Structure of native α_2-macroglobulin and its transformation to the protease bound form

JEAN-PIERRE BRETAUDIERE*, JACQUELINE TAPON-BRETAUDIERE†, AND JAMES K. STOOPS‡

*University of Texas Health Science Center, Department of Pathology and Laboratory Medicine, P.O. Box 20708, Houston, TX 77225; †Faculté de Médecine Necker-Enfants Malades, Département d'Hématologie, 156, rue de Vaugirard, 75015 Paris, France; and ‡Verna and Marrs McLean Department of Biochemistry, Baylor College of Medicine, Houston, TX 77030

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ABSTRACT **Well-preserved structures of native and α-chymotrypsin-bound α_2-macroglobulin were obtained by electron microscopy. Computer processing of these images has shown that the native structure has the shape of a padlock 19 nm long. It is proposed that the native α_2-macroglobulin consists of the juxtaposition of two protomers with one protomer shaped like a distorted letter "S" and with the other its reverse image, to form a binding site between the two protomers near the bottom of the complex. On cleavage of the subunits with chymotrypsin, the native structure condenses to 16.7 nm and rearranges so that the interaction between the protomers is near the middle. Two images of the α_2-macroglobulin–chymotrypsin conjugate were obtained. We suggest that these images represent the end and side view of this complex. Based on the manner in which the native structure is assembled, we propose that the proteolyzed form of α_2-macroglobulin is functionally asymmetric in that both protease binding sites reside on the same half of the complex.**

α_2-Macroglobulin (α_2M) is one of the major antiproteases found in the plasma of vertebrates. It has the capacity to inhibit not only endoproteases normally present in the plasma but also proteases from other origins (1). α_2M, isolated from human plasma, is a large glycoprotein (M_r, 725,000) composed of four identical subunits (M_r, 180,000) (2, 3). The quaternary structure consists of two noncovalently bound protomers; each protomer is made up of two subunits covalently linked by two disulfide bonds (4). When exposed to an endoprotease, a limited proteolysis of α_2M occurs at a site called the "bait" region, located near the middle of the protein, resulting in a change in its structure (5–7). The structural transformation has been identified by increases in the sedimentation coefficient (8) and in the electrophoretic mobility (9) and by decreases in the radius of gyration (10) and in the Stokes' radius (11). The inactivated protease may form a covalently bound complex with α_2M by reacting with an intramolecular thiol ester linkage between glutamine and cysteine residues. Even though there are four "bait" sites available, it has been proposed that α_2M has two independent and identical proteinase binding sites (12–14). By using energy transfer experiments, Pochon *et al.* (12) demonstrated that the two sites are 4.4 nm apart. The binding of 2 mol of proteases per mol of α_2M has been observed only with smaller proteases like trypsin and chymotrypsin (M_r, 25,000); larger proteases like plasmin (M_r, 81,000) display a 1:1 molar binding ratio (15).

There have been conflicting reports relating the various structures of α_2M obtained by electron microscopy to the native and protease bound forms. This discrepancy seems to have resulted from the study of α_2M that had undergone proteolysis during its isolation (16). Early studies reported the presence of two forms (7, 17–19): an ovate structure shaped like two crescents facing each other with a bar in the center (the "closed" form) and a larger structure, conventionally described as a cyrillic Ж (the "opened" form). Some authors suggested that the opened form was the native structure whereas the closed form was the result of the exposure to a protease (7, 18–20). A speculative model based on these two forms has been proposed to correlate the structure of native and protease-exposed α_2M (20).

A significant contribution to the understanding of the structural forms seen by electron microscopy has been reported by Tapon-Bretaudiere *et al.* (16). These authors suggested that the so-called open and closed forms of α_2M were in fact the same structure viewed from the side and the end, respectively. They further demonstrated that these views of the complex existed only after proteolysis. The existence of a third form was assigned to the native structure, and these results were confirmed by Nishigai *et al.* (11). In these studies, electron micrographs of native α_2M consisted of only a few ordered structures in the shape of crowns with a distribution of matter resembling the petals of a flower. Even less-frequent shapes consisting of "tetrades" and "crosses" were found. Because of the variability of the structures and the presence of a large number of particles with no ordered structure, these authors did not propose a model for native α_2M.

It is apparent that the proposed models relating the structure of the native and proteolyzed forms of α_2M are questionable and that the structure of native α_2M remains obscure. In this study, by using electron microscopy and image processing, we report the native structure of α_2M and relate it to its proteolyzed forms.

MATERIALS AND METHODS

Preparation of the α_2M Specimens. The purification of native α_2M from human plasma was performed as described by Tapon-Bretaudiere *et al.* (16). The purity of α_2M was examined by $NaDodSO_4$/polyacrylamide gel electrophoresis (21). The activity of the purified α_2M was tested by measuring its capacity to inhibit thrombin in a clotting assay. The absence of bound and unbound proteases in the preparation was verified by measuring the amidolytic activity with a synthetic chromogenic substrate (S2160 from Kabi, Stockholm). The preparation of the protease complex was performed by reacting 1 mol of α_2M with 2 mol of Worthington three-times crystallized bovine pancreas α-chymotrypsin as described (16). The α_2M (1.4 μM) was incubated with 2.6 μM α-chymotrypsin for 3 min at 25°C in 0.10 M sodium citrate (pH 6.0).

Electron Microscopy. The α_2M specimens were deposited on collodion-coated grids by the drop method and then

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Abbreviation: α_2M, α_2-macroglobulin.

negatively stained with a 0.25% solution of methylamine tungstate at pH 7.2 (22). The grids were visualized with a JEOL 1200 electron microscope at an instrumental magnification of ×72,000 with an accelerating voltage of 100 kV. The underfocus was estimated at ≈500 nm, consistent with our optical diffraction measurements of the phase-contrast transfer function. Micrographs of those fields where the particles are totally embedded in the stain were used for image processing.

Image Processing. All image processing was carried out on a Digital Equipment VAX 11/785 with the SUPRIM software system designed in this laboratory for electron microscopy. Micrographs were digitized as 1536 × 1536 arrays of optical densities with an Eikonix 78/99 digitizer. Each set of individual particles was extracted interactively by using a 60 × 60 window from a display of the micrograph on a raster monitor. Particles were selected visually according to their similarity in shape. Within each set, all particles were aligned with respect to a reference. The alignment is based on methodology described by Frank (23) that utilizes the auto- and cross-correlation functions. Three to 5 iterations of alignment were performed for each set. The first iteration used a well-preserved particle as reference whereas the next two used the average image of the preceding iteration. After alignment, the motif of interest in each particle was masked off (24), and correspondence analysis was applied to the resulting data set as described by Van Heel and Frank (25). A reduced set of eigenvector coordinates was selected and used as features for clustering by hierarchical ascendant classification (26). The index of dissimilarity, based on the median rule or Ward's criterion (27), is computed from the euclidean distance in the reduced eigenvector space. The

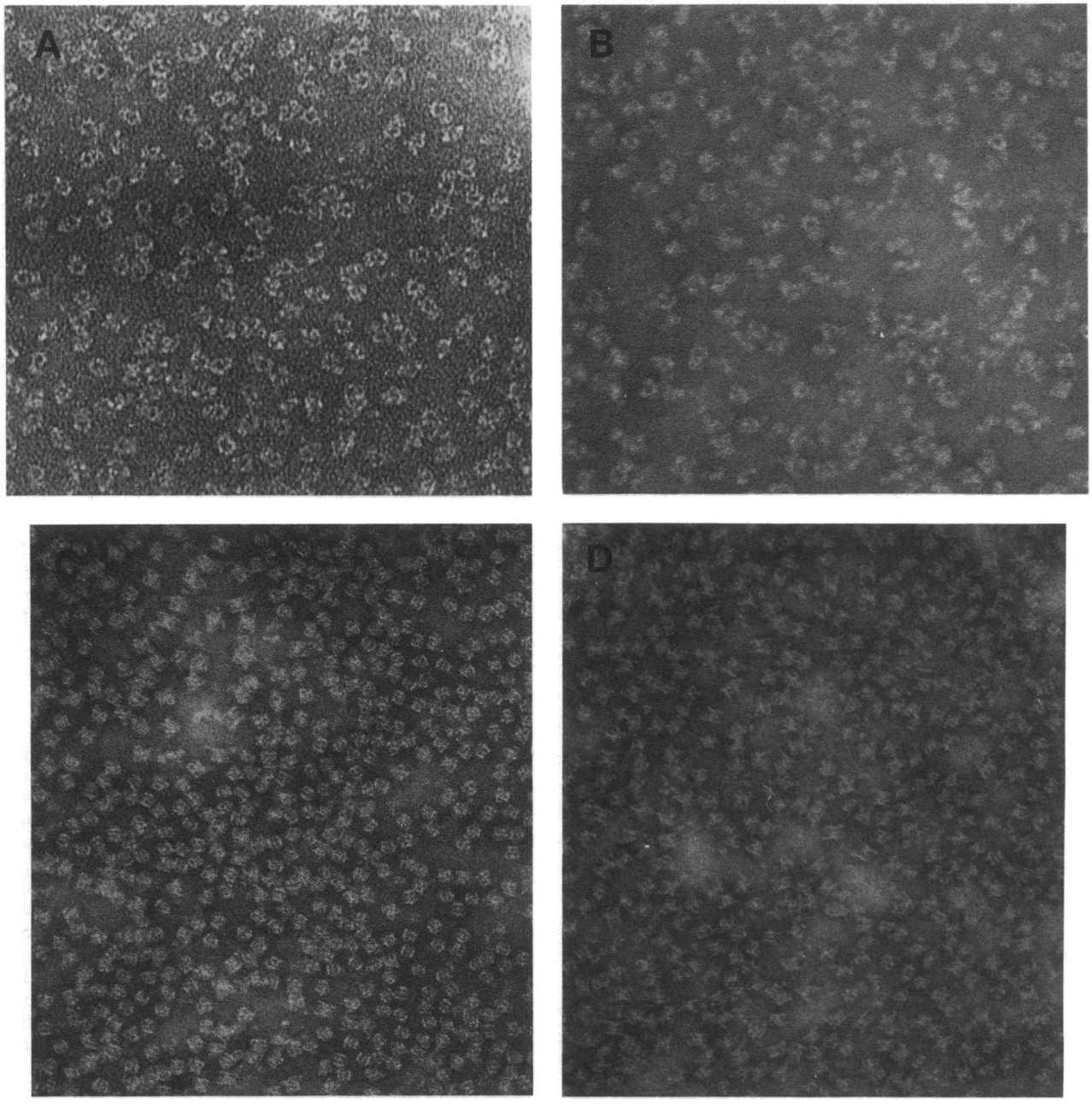

FIG. 1. Electron micrographs of native and chymotrypsin-bound α_2M. (×194,400.) (*A*) Native α_2M under focus to highlight the structures. (*B*) Representative field of native α_2M used for computer processing. (*C*) Chymotrypsin-bound α_2M predominantly end views of the structure. (*D*) Chymotrypsin-bound α_2M predominantly side views of the structure.

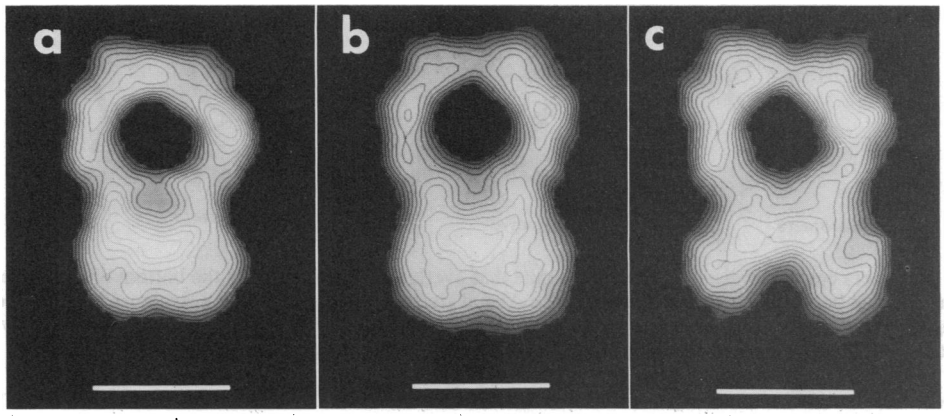

FIG. 2. Contoured cluster average images of native α_2M. Clusters in *a*, *b*, and *c* are composed of 263, 124, and 85 individual images, respectively. The distances between contour levels indicate an increment of 0.0075 absorbance unit. (Bar = 10 nm.)

resulting hierarchical tree was cut at an appropriate level after examination of the dissimilarity indices of the nodes.

RESULTS

Fig. 1 *A* and *B* shows micrographs of native α_2M. The field consists of numerous well-preserved structures shaped like a padlock. The structures have a 2-fold axis of symmetry on the major axis and are otherwise asymmetric. Fig. 1 *C* and *D* shows representative electron microscope fields of the two forms of α_2M after treatment with 2 mol of chymotrypsin per mol of the antiprotease. There is a predominance of the ovate structure in Fig. 1*C*, whereas Fig. 1*D* shows a majority of structures resembling the symbol ⋈. Although both shapes are seen in each micrograph, the reason for the predominance of one shape over the other in selected areas on the grid is not known. It may possibly result from preferential interactions with the nonuniform grid film. The dimensions of these two shapes (given below) and their presence in the same preparation lead us to believe that these two forms are likely to be two different views of the α_2M–protease complex as suggested (16). Consequently, we identify end views as the particles with an ovate shape and side views as the particles shaped like ⋈.

A set of images from 510 native α_2M particles was extracted interactively from eight digitized micrographs. After alignment, the set was submitted to correspondence analysis and classification. The classification resulted in three major clusters for which average images are shown in Fig. 2. The resolution of the average images was found to be ≈3.2 nm as determined by phase residual (28). The three forms of native α_2M show basically the same structural motif. They differ only in their degree of compactness with respect to both the "arms" of the loop and the bottom of the structure. The average image is 19 nm high and 13 nm wide. The upper loop has an inside diameter of 7.5 nm and narrows down to 9 nm wide near the middle of the structure. The fact that no one cluster has a predominant membership indicates that the molecule can take any of these three forms or possibly intermediates thereof.

Sets of images from 150 and 52 particles were used for the image analysis of the end and side views, respectively, of the proteolyzed form. Correspondence analysis of the aligned images followed by classification revealed in each case one major cluster with large membership and a constellation of small clusters that represented disformed or damaged views. The corresponding average images are shown in Fig. 3. The end and side views can be inscribed in boxes whose dimensions are 11.6 nm × 8 nm and 16.7 nm × 11.8 nm, respectively. The end view consists of two crescent-shaped protein masses with concave surfaces facing so that a central protein mass is sandwiched between them. The central protein (width of 4.5 nm) is topped along the major axis by two smaller structures on each end. The side view is 9.6 nm wide near the center of the particle. Average images of both the native and chymotrypsin exposed forms show a number of regions of variable mass density that may correspond to the domain structure of the complexes. The structures should contain multiple domains, since each subunit has 11 intramolecular disulfide bonds (3).

DISCUSSION

A plausible model for the structure of native α_2M consists of the juxtaposition of two protomers with one protomer shaped like a distorted letter S and the other its reverse image. Each protomer is composed of two covalently linked subunits. The noncovalent interactions between the two protomers would take place at the apex of the structure, where the ends meet, and at the bottom, where the two strands either crossover or overlap. This model accounts for the presence of the loop at the top and for the increased protein density in the center of the bottom part. It also accounts for the variability of the shape whereby the structure could be in a compact state (Fig. 2*a*) or in a more opened state (Fig. 2*c*) with cluster b (Fig. 2*b*) representing an intermediate form between the two states. The flexibility can be easily explained by the noncovalent association of the two protomers since the strongest interaction occurs at the crossover or overlap. A parallel orientation between the two subunits—i.e., head-to-head or tail-to-tail—can be readily

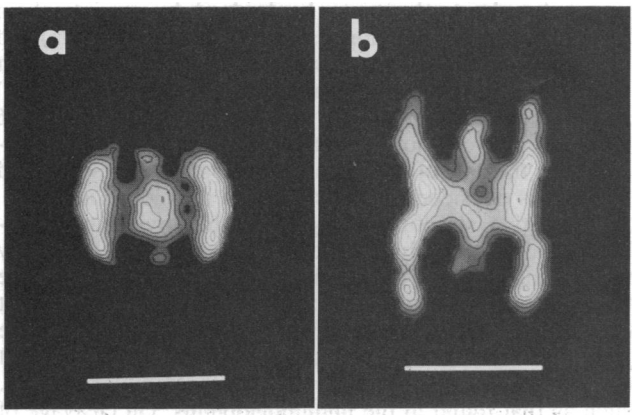

FIG. 3. Average images of chymotrypsin-bound α_2M. (*a*) End view (n = 130). (*b*) Side view (n = 37). The distances between contour levels indicate an increment of 0.012 absorbance unit. (Bar = 10 nm.)

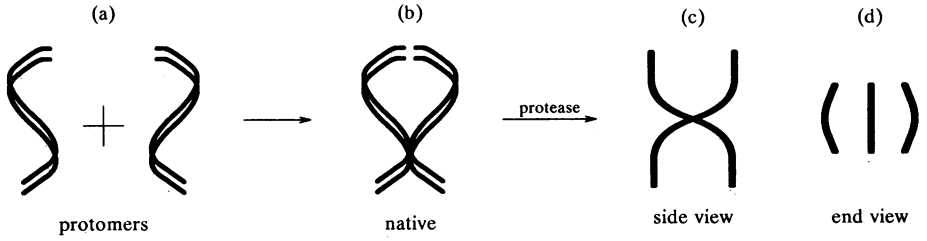

FIG. 4. (*a*) Two protomers consist of disulfide-linked subunits that overlap or crossover near the bottom of the complex to form the major noncovalent site of interaction between the protomers of native α_2M and give rise to the padlock-like structure in *b*. On proteolysis of the native structure, the binding site between the protomers rearranges toward the center of the complex giving rise to the side view (*c*) and end view (*d*). The shape of the protomers depicted here only implies their shape in the tetrameric complex.

related to the asymmetric structure of α_2M in which the major binding interaction between the two protomers is located at the bottom of the model (Fig. 4). A report of the anti-parallel orientation of the two disulfide bonds between the two subunits does not comment on the relative orientation of the two subunits within the protomer (4).

The concordance between the sizes of the minor dimension of the side view (11.8 nm) and the major dimension of the end view (11.6 nm) and their simultaneous presence in the same preparation and on the same grid suggest that the two forms are likely to be related. It is easy to reconcile the two shapes as two projections differing by a 90° rotation of the prototype molecule. These images were obtained for the exposed forms of α_2M (11, 16), and our interpretation of the relatedness of the two shapes is in agreement with the proposal by Tapon-Bretaudiere *et al.* (16).

Our computer images of the native α_2M (Fig. 2) contradict the proposed hollow cylinder model for this structure (10, 20, 29). The hollow cylinder model deduced from low-angle x-ray-scattering studies (10) is of low resolution and these data, therefore, are not inconsistent with our model. Furthermore, the similarity of the views of the chymotrypsin-treated α_2M obtained in this investigation (Fig. 3) and views reported for the native and proteolyzed preparations (10, 20) suggests that the preparation used in the previous studies had undergone proteolysis during their isolation. This has apparently given rise to the mistaken relationship between the so-called opened (native) and closed (protease-bound) forms of the structure.

Chymotrypsin treatment of the native α_2M results in a decrease in the size of the major dimension from 19 nm to 16.7 nm and in a small decrease in the size of the minor dimension from 12.8 to 11.8 (Figs. 2 and 3). This reduction in size results apparently from a condensation of the structure and not from a loss of matter after proteolysis since the bait region has been shown to be bridged by an intrachain disulfide bond (3). Our dimensions of the native (19 nm) and chymotrypsin-treated (16.7 nm) α_2M are in good agreement with the length of the molecule calculated from the Stokes' radius determined by others: native [18.8 nm (30) and 17.6 nm (11)] and chymotrypsin-treated [15.8 nm (11)]. A decrease in the size of α_2M after proteolysis is also supported by various physicochemical studies as mentioned above.

The native structure of α_2M may be envisioned to rearrange after proteolysis to form the symbol ⋊ by simply undergoing a shift of the binding domain from the end of the two protomers toward their middle with a concomitant opening of the four arms (Fig. 4). We propose that the junction of the arms and the central mass form a hinge that could correspond to the bait region of the complex (Fig. 4), such as that found in the immunoglobulins. On cleavage of the complex with an endoprotease it would be expected that the asymmetry is maintained thus giving rise to a pseudo 2-fold axis of symmetry through the minor axis. For the complex to be truly symmetrical the head-to-head orientation of the two subunits would have to become head-to-tail resulting from a 180° reorientation of one of the subunits with respect to its neighbor. The multiple interactions between the two subunits would be expected to prohibit such a rearrangement.

An asymmetric model for the proteolyzed form of α_2M with the head-to-head relationship between the two subunits requires that both protease binding sites are on the same side of the minor axis of the complex. This arrangement is consistent with fluorescence energy transfer studies that indicate that the bait regions are in close proximity and that the bound chymotrypsin molecules are within 0.4 nm of each other (12). Furthermore, such an arrangement explains the proposed steric hindrance that prevents the second molecule of plasmin from binding to α_2M (15). The location of the two chymotrypsin molecules in the complex is not apparent from the computer image of the side view. Since chymotrypsin is a compact molecule of ellipsoidal shape with major and minor axes of 5 nm and 4 nm, respectively (31), its position in the complex might have been expected to be discernible. However, a chymotrypsin molecule only comprises ≈3% of the mass of the complex, and its location could be obscured by α_2M, especially, if it is located near the center of the complex.

This study of the structure of native and proteolyzed α_2M has yielded a detailed model of the native complex and has suggested a plausible relationship between the structures of native and chymotrypsin-treated α_2M. Immunoelectron microscopy with Fab fragments derived from IgG antibodies raised against the amino- and carboxyl-terminal peptides as well as the bait and thioester region may yield considerable insight into arrangement and function of these regions in the complex. Furthermore, the detailed models of these structures can be verified by small-angle neutron scattering of the native and proteolyzed form of α_2M (32).

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