On the Structural Changes of Native Human α₂-Macroglobulin upon Proteinase Entrapment

THREE-DIMENSIONAL STRUCTURE OF THE HALF-TRANSFORMED MOLECULE*

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The remarkable structural rearrangement of α₂M has been the subject of numerous electron microscopy studies (6). Three-dimensional reconstructions of native (7, 8), and fully transformed α₂M (5, 9) reveal molecules of very different shapes. A low resolution x-ray map of α₂M-MA (10) is in general agreement with the electron microscopy structures. These results, as well as other physicochemical studies (2), have shown that the native and transformed molecules have significantly different structures. The native structure is more globular, with dense end regions that are connected by twisted strands (7, 8). The transformed structure has a more compact central region of protein with four arms extending from its sides, similar to the letter “H” (5, 9, 10). It also migrates more rapidly on nondenaturing gels than native α₂M (1, 2). The trapped proteinases are located in an internal cavity, symmetrically above and below the minor axis of the transformed molecule (5).

With structural details of the native and transformed molecules emerging from electron microscopy reconstructions (5, 7–9) as well as a low resolution x-ray map (10), conflicting proposals have been advanced for the transformation of α₂M. It was proposed that a lateral compression and unfolding mechanism links α₂M and α₂M-MA (8). On the other hand, the observation that the native and transformed structures are constituted of two twisted, side-by-side strands of opposite handedness has led to the proposal that an unwinding and rewinding of these constituent strands leads to proteinase entrapment (7).

α-Macroglobulins (αMs) are nonspecific, irreversible inhibitors of endoproteinases found in the circulation of all vertebrates and some invertebrates (for a review, see Ref. 1). Human α₂-Macroglobulin (α₂M),1 the largest known proteinase inhibitor (Mₐ = 720,000), is a homotetramer formed by two proteomic units, each of which contains two 180-kDa subunits linked by two disulfide bonds. It has a vital role in the clearance of proteinases from the circulation and in regulating their activity in fibrinolysis, coagulation, and complement activation (2, 3). A single α₂M molecule can entrap two proteinase molecules such as chymotrypsin and trypsin and can therefore be considered to contain two functional domains (1). Each subunit of α₂M has a bait region with cleavage sites for nearly all known endoproteinases and an internal thiol ester bond. A proteinase cleaves the two bait regions within both functional units, leading to an activation and cleavage of the thiol ester bonds. Consequently, α₂M undergoes a major structural change resulting in entrapment of the proteinase and its covariant linkage to the molecule (1, 4). The bound proteinase, although inaccessible to proteins, may react with small substrates and inhibitors (2), which is in contrast to the mode of inhibition of all other natural proteinase inhibitors that bind at the proteinase’s active site. Treating α₂M with a small nucleophile such as methylamine also causes cleavage of the thiol ester bonds, leading to a structural transformation of the molecule (1). Electron microscopy reconstructions have shown that the methylamine-transformed α₂M (α₂M-MA) has a similar structure to the α₂M-proteinase complex (5).

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‡ The abbreviations used are: α₂M, native α₂-macroglobulin; α₂M-HT, half-transformed α₂-macroglobulin; α₂M-MA, α₂-macroglobulin-methylamine; ice, frozen-hydrated; stain, methylamine tungstate.
α-M-half transformed (α-M-HT) provides a plausible structural link between the native and transformed structures. This intermediate structure was obtained by reacting the native molecule with chymotrypsin covalently bound to Sepharose (11). Two bait regions and two thiol ester linkages are cleaved within this homotetrameric structure, and the proteinase is not bound. α-M-HT reacts with and traps a proteinase by cleavage of the remaining bait regions at a slower rate than the native structure. Its rate of migration on nondenaturing gels is intermediate between α-M and α-M-MA (11).

In this study, the three-dimensional structures of α-M-HT, obtained from negative stain and frozen-hydrated specimens, are compared and show excellent concordance indicating that the molecule is well preserved by both methods and that the reconstructions are reliable. Comparisons of the structure of α-M-HT with the native and transformed structures were investigated to further understand the mechanism of its structural rearrangement and identify the functional division in native α-M.

**EXPERIMENTAL PROCEDURES**

**Protein Preparations**—Half-transformed α-M was prepared by reaction of human plasma α-M with chymotrypsin-Sepharose. Chymotrypsin-Sepharose was prepared by extensive washing with 50 mM Hepes, 100 mM NaCl, and 2 mM EDTA (pH 7.4). Approximately 5 g (wet weight) of the chymotrypsin-Sepharose was incubated with 40 mg of plasma α-M in a total volume of 12 ml. The suspension was gently rocked at room temperature, and the progress of the reaction was monitored by assaying for remaining intact thiol esters. After 5 h, the chymotrypsin-Sepharose was removed by centrifugation and the supernatant filtered through a 0.45-μm filter. Iodoacetamide (10 mM) was added to react with the free SH groups generated, and the protein was then dialyzed against 50 mM Hepes, 100 mM NaCl, and 2 mM EDTA (pH 7.4). After gel filtration on Sephadex G150, the α-M-HT was shown to contain two cleaved and two intact bait regions as well as two intact and two cleaved thiol esters as described previously (11). However, no chymotrypsin activity is associated with the α-M, and the molecule is active, since it traps one molecule of chymotrypsin (11).

**Electron Microscopy**—α-M-HT (5 μg/ml) in 0.05 M sodium phosphate with 10 μg/ml bacitracin and 0.25% m/m dimethylamine tungstate (pH 7.2) was applied by the spray method to carbon films (12). Stain images were acquired with a JEOL JEM 1200 electron microscope operating at 7.2 kV. Stain images by the spray method to carbon films (12). Stain images were kept below carbon-coated holey grid, and the excess removed by blotting with filter paper. The unstained images are presented in reverse contrast to facilitate comparison with the stain images. The pseudo-lip shape is represented for the stain and ice images by a–c and k–m, respectively. Other images represent off-axis orientations of the molecule. The scale bar in this and subsequent figures corresponds to 100 Å, and the gray scale bar indicates relative protein density high (white) and low (dark).

**RESULTS**

**Images of α-M-HT in Stain and Ice**—A gallery of stain and ice images of α-M-HT shows pseudo-lip views (Fig. 1, a–c and k–m), respectively, which exhibit similarity to the “lip” views of native α-M. These oval-shaped images have small triangular and rounded shapes of higher protein density at opposing ends connected by two curved strands. These are in contrast to a similar arrangement of two small triangular shapes of higher protein density in the lip view of the native molecule (7, 8). Pseudo-lips with various in-plane rotations in the tilted micrographs interconverted into a variety of other shapes upon tilting the microscope stage 50° (data not shown). By observing these conversions, other orientations of α-M-HT were identified in the tilt orientation and ice images and subsequently used for the refinement of the initial random conical tilt reconstruction using the three-dimensional projection alignment (20, 21). Some of these orientations, representing side and end views, are shown in Fig. 1 (d–j and n–t). It is interesting to note that none of these images resemble the single particle images of the binary chymotrypsin α-M complex (26).
result from the secondary reaction of the trapped proteinase with the remaining uncleaved $\alpha_2$M subunits in the binary complex preparation (11, 27). In the present study, the $\alpha_2$M-HT preparation was found to contain two free thiols and thiol ester moieties per molecule when the electron microscopy images were recorded. Images of $\alpha_2$M-HT molecules in methylamine tungstate stain are very similar to their corresponding views in vitreous ice, indicating that the molecular architecture is faithfully reproduced by the stain (Fig. 1). The Euler angle distribution plot of the untilted stain specimens exhibits an isotropic distribution of views showing that the molecules do not assume a preferred orientation in stain on the carbon film (Fig. 2A). In contrast, the ice images show a tendency to cluster about the $\theta$ axis and therefore under-represent the end views of the molecule (Fig. 2B).

Three-dimensional Reconstructions—Accordingly, the easily recognizable pseudo lip images were used in the initial stain random conical tilt reconstruction (41-Å resolution), and this structure served as a model to align the nontilted stain images in multiple orientations using the three-dimensional projection alignment method as described previously (20, 21). The wide distribution of projection angles (Fig. 2A) ensures a reconstruction with more uniform resolution than the random conical tilt structure. Using the previous structure as a model, iterations of refinement were carried out until a stable resolution value was achieved (32 Å, Fig. 3). The frozen-hydrated images were aligned using the random conical tilt stain structure as a model, and further refinement was achieved using the resulting ice structure as the model (34-Å resolution). An ice reconstruction using the refined stain model was similar, and the resolution was not improved. The random conical tilt structure is significantly narrower than the refined stain and ice structures (height-width ratios of 1.5 and 1.6, respectively), and the vertical strand across the front of the random conical tilt structure is incomplete (Fig. 3). These differences may be related to the “missing cone” of information associated with the random conical tilt structure (17).
The stain and ice structures, their projections and the corresponding average images show good concordance (Fig. 3). Comparisons of the protein density distribution between 5.8-Å-thick slices of the stain and ice structures also show good agreement (Fig. 4). It is interesting that the less robust features associated with the central slices (8–16) in the stain and ice structures have similar densities. This contrasts with a comparison of the stain and ice structures of the *Saccharomyces cerevisiae* fatty acid synthase where the stain was found to enhance the less robust features (21). The agreement obtained in the present comparisons indicates that the architecture of a\textsubscript{2}M-HT has been faithfully reproduced by both methods of imaging the molecule and that the reconstructions are reliable.

Stereo views of the stain structure show an oval-shaped structure (195 height × 130 width × 137 Å depth) with four large openings; 45 Å in diameter leading into an internal cavity (Fig. 5). The top of the structure is chisel-shaped, whereas its bottom is broad and bulbous. The two ends of the structure are joined by four strands (Fig. 5) whose protein densities are considerably diminished from those associated with its two ends (Fig. 4). The variability between the size and density of the strands and different shapes of the two ends results in a structure that lacks symmetry. The absence of 2-fold symmetry on the major axis of the structure was unexpected, since it traps one molecule of proteinase (11). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that chymotrypsin-Sepharose cleavage is specific for the bait region (11). The oblique side view average images in Fig. 3 also exhibit this asymmetry and the projections of the structure in 15° angular bins agree well with the average images from the candidates assigned to these bins by the three-dimensional projection alignment method (data not shown). Finally, a refinement using a random conical tilt model with 2-fold symmetry imposed on its major axis gave a structure similar to that shown in Fig. 5 (data not shown). These results indicate that the data set is self-consistent and further support our proposal that the reconstructions are reliable.

A threshold level that doubled the volume of the structure resulted in a global swelling but revealed no additional features.

**Structural Comparisons of a\textsubscript{2}M, a\textsubscript{2}M-HT, and a\textsubscript{2}M-MA—**

The protein density distributions in serial slices 5.8 Å thick cut normal to the major axes of the structures show the variation in the central portion (body) of the molecules (Fig. 6). In this figure, the native and half-transformed structures are aligned so that the tops of the chisel-shaped bodies are in the same orientation and the strands associated with the native and a\textsubscript{2}M-MA structures are oriented in corresponding positions in slice 12. In the native structure, the strands at the top and bottom (slices 1–5 and 20–25, respectively) are joined; they separate on either side of its middle (slices 6–9 and 15–18) to form two openings to the cavity approximately 25 Å in diameter. As the strands course through the body of the structure, they exhibit a 90° clockwise twist. The strands associated with

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**Fig. 4.** Relative protein density distribution in slices from the stain and ice structures. Slices (5.8 Å thick) were cut normal to the major axes of the structures and contour lines were included as an aid in depicting the relative protein density distribution. The good agreement between the corresponding slices supports the proposal that the reconstructions are reliable.

**Fig. 5.** Stereo views of the stain structure. The structure is asymmetric and has four large openings to the cavity which may facilitate the entrapment of the proteinase. The major shape difference between the top and bottom of the structure and the top's similarity to the native structure indicate that the structural change has primarily occurred in the bottom half of the molecule.
the chisel-shaped body of the $\alpha_2$M-HT structure are significantly larger than they are in the native molecule (slices 1–6), and they separate into four lower density, thin strands in the body of the structure (slices 7–18) and coalesce into a broad oval-shaped body at the base of the molecule (slices 19–24). Significantly, the strands exhibit little rotation through the structure's body, and the four openings are approximately two times larger than those associated with the native molecule. The $\alpha_2$M-MA structure (fully transformed) consists of four higher density filaments which are joined by protein of lower density to form a pair of strands. The two strands that traverse the fully transformed structure twist 90° with a handedness opposite to that of the native structure and form external grooves. The major top-bottom asymmetry associated with the

**Fig. 6. Comparisons of the relative protein density in slices obtained from native, half-transformed, and fully transformed $\alpha_2$M ($\alpha_2$M-MA).** The top panel shows the solid shaded structures viewed from the front and top. The slices were produced by cutting the structures normal to their major axes. The strands rotate clockwise and counter clockwise 90° in the native and $\alpha_2$M-MA structures, respectively, and separate into four strands without a twist in $\alpha_2$M-HT. It is apparent that the cavity is more open and therefore accessible to the proteinase in $\alpha_2$M-HT. The $\alpha_2$M-MA is from Andersen et al. (10) and was filtered to 30 Å resolution.
α_M-HT structure and the similarity between the chisel-shaped body at its top with that of the native structure suggest that the lower portion of the molecule has reacted with the proteinase (see below).

**DISCUSSION**

**Proposals for the Structural Transformation of α_M**—The unusual structural change that permits α_M to function as a universal proteinase scavenger is not readily apparent by a comparison of the rather dissimilar structures of the native and transformed molecules. Their dissimilarity has resulted in numerous and divergent proposals concerning the proteinase trapping phenomenon from electron microscopy studies (6, 7, 8, 10, 16, 28, 29). The structure of the intermediate functional state represented by α_M-HT can be analyzed for possible similarities linking it to native and transformed α_M. Hypotheses based on three-dimensional structures to explain the transformation of α_M (7, 8, 10) can then be further examined.

Boisset et al. (8) proposed that the transformation of α_M occurs with a compression along its major axis coupled with a lateral expansion. Thus, the lip view of α_M changes into a shape that corresponds to the end view of α_M-MA. However, it is difficult to reconcile the shape of α_M-HT with their proposed rearrangement. Andersen et al. (10) simulated the structure of native α_M by a vertical displacement of the subunits and some rearrangement of an internal region of protein density of their x-ray structure. The shapes of the four individual subunits in the structure were left unchanged. Their proposed transformation is inconsistent with the reversal of the handedness of the twist in the two strands between α_M and α_M-MA (7) and does not account for the partial untwisting and separation of the strands in α_M-HT.

Kolodziej et al. (7) showed that the two strands that traverse the body of the native and α_M-MA structures are a common feature and are arranged with the opposite handedness (Fig. 6). They proposed that the rearrangement involves a separation of the strands at the opposite ends of the native molecule, their untwisting to open the proteinase binding cavity and a retwisting around the proteinase with the opposite handedness to entrap it (Fig. 7). The untwisting-retwisting hypothesis of proteinase entrapment is supported by the present studies (see below). This structural change is based on the consideration that the two oppositely twisted strands are the protomeric units that are noncovalently bound by interactions occurring primarily at the ends and near the middle of the native and transformed molecules, respectively (Fig. 7, a and c). Each protomeric unit contains two disulfide-linked 180-kDa subunits in an anti-parallel arrangement (7, 30), and the four thiol ester bonds maintain the strands in the twisted state. In this model, the lateral arm-like extensions on α_M-MA (Fig. 7c) result from a separation of the strands that are joined to form the chisel-like ends of α_M. This hypothesis is supported by immunoelectron microscopy studies of monoclonal Fab-labeled α_M and α_M-MA. The Fabs are located near the chisel-shaped bodies of the native structure and near the arms of α_M-MA (7).

**Structure-Function Relationships**—It is established that the initial proteinase reaction with α_M involves the cleavage of two bait regions and the hydrolysis of two thiol esters to give an intermediate form that reacts more slowly with the second proteinase (1). We propose that α_M-HT is representative of the intermediate structure. The α_M-HT structure supports the proposed rearrangement and gives further insight into proteinase entrapment. The Sepharose-bound chymotrypsin cleaves two bait regions located on the same side of the minor axis of the structure resulting in the untwisting of the strands (Fig. 6) and their partial separation in the bottom half of the structure to form its bulbous shape (Fig. 7b). The complete separation of the strands and their retwisting to form the arms associated with the transformed structure are evidently hindered by the intact thiol esters in the upper half of the molecule. In this proposal, one-half of each protomeric unit is cleaved in the bottom portion of the structure and therefore the functional division is on the minor axis. This assignment of the functional division is supported by the three-dimensional structure of the α_M-chymotrypsin ternary complex, which showed that two molecules are encapsulated on either side of its minor axis (5). In contrast, the top half of the structure has the chisel-shaped feature of the native structure, which is probably maintained by the two intact thiol esters. However, the changes in the bottom portion of the structure result in a significant enlargement of the chisel-shaped body and a partial separation of the strands in its upper half (Figs. 6 and 7). These changes may be related to the reduced reactivity of the remaining bait domains to subsequent proteolysis (1, 11). It is interesting, though, that the remaining thiol esters exhibit the same reactivity to methylamine as in the native structure (11). Their unchanged reactivity may be related to their similar disposition in the two molecules.

In addition to the untwisting of the two strands after the initial cleavage event, they separate to form four strands of lower protein density resulting in a central cavity that is more accessible to the proteinase (Figs. 6 and 7). The proteinase enters the open cavity of α_M-HT by passive diffusion or is pulled into the cavity as a consequence of its binding to the bait.
domain. In this regard, the bait domain is not accessible to proteolysis in α₂M-MA, suggesting that it is internalized (31).

We have ascribed the major top-bottom asymmetry of α₂M-HT to the preferential cleavage of the bait and thiol ester domains on one side of the structure’s minor axis. The minor left-right asymmetry associated with the variable size and densities of the four strands may be related to the multiplicity of cleavage sites for chymotrypsin in the bait region (32).

Disposition of Sites of Biological Interest—The cleavage of the thiol esters by methylamine or as a result of reacting α₂M with a proteinase exposes receptor binding domains. As a consequence, the transformed α₂M binds to receptors on hepatocytes and is internalized and degraded (1, 2). Immunoelectron microscopy showed that these domains are associated with the arms of the transformed α₂M (33). We propose that these domains are sequestered in the native structure where the two strands meet to form the chisel-shaped bodies at both ends of the structure. The separation and untwisting of the strands upon thiol ester cleavage exposes these domains near the tips of the arms of the transformed molecule (Fig. 7). It is not known if these domains are exposed in α₂M-HT.

The four thiol groups (Cys-949) resulting from cleavage of the functional thiol esters by methylamine have been located on the interior walls near the center of the x-ray structure (10). The internal distance between the thiols varies from 31 to 44 Å, which is in agreement with values reported from fluorescence spectroscopy (34). The location of the thiol esters in the native structure is not known.

It was proposed that the four bait domains are located inside the cavity of α₂M-MA 11–17 Å from the Cys-949 (10, 35) and that this domain is also inside the native structure (10). Since new inter and intramolecular disulfide bonds were formed in a variant that contained a single cysteine residue within the bait region, it was proposed that the four bait domains are located in bait proximity in the center of the molecule (36). However, the location of the new disulfide bonds in the amino acid sequence was not determined so that it is equally possible that they involve residues outside the bait regions and consequently their location and putative proximity remains ambiguous. Our structural studies suggest that the small ~25 Å diameter openings of the native structure (7) appear too small to allow access to an interior bait region by proteinases which vary in molecular weight from 25 (chymotrypsin) to 85 kDa (plasmin). Furthermore, the α₂M-HT structure suggests that the remaining bait domains reside near the chisel-shaped body where they may function to maintain the contact between the two strands (37). An alternative proposal is that the bait domain lies externally near the bridge structures that serve as a gateway to the internal cavity (7). (The bridge structures are two strand-like features that form the external wall of the cavity near its middle). The α₂M-HT appears to lack these bridges that may be related to the diminished reactivity of the bait regions to proteolysis.

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