Structural Details of Proteinase Entrapment by Human \( \alpha_2 \)-Macroglobulin Emerge from Three-dimensional Reconstructions of Fab labeled Native, Half-transformed, and Transformed Molecules*

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Three-dimensional electron microscopy reconstructions of native, half-transformed, and transformed \( \alpha_2 \)-macroglobulins (\( \alpha_2 \)-Ms) labeled with a monoclonal Fab Fab offer new insight into the mechanism of its proteinase entrapment. Each \( \alpha_2 \)-M binds four Fab s, two at either end of its dimeric protomers approximately 145 Å apart. In the native structure, the epitopes are near the base of its two chisel-like features, laterally separated by 120 Å, whereas in the methylamine-transformed \( \alpha_2 \)-M, the epitopes are at the base of its four arms, laterally separated by 160 Å. Upon thiol ester cleavage, the chisels on the native \( \alpha_2 \)-M appear to split with a separation and rotation to give the four arm-like extensions on transformed \( \alpha_2 \)-M. Thus, the receptor binding domains previously enclosed within the chisels are exposed. The labeled structures further indicate that the two proteomic strands that constitute the native and transformed molecules are related and reside on each side of the major axes of these structures. The half-transformed structure shows that the two Fab s at one end of the molecule have an arrangement similar to those on the native \( \alpha_2 \)-M, whereas on its transformed end, they have rotated. The rotation is associated with a partial untwisting of the strands and an enlargement of the openings to the cavity. We propose that the enlarged openings permit the entrance of the proteinase. Then cleavage of the remaining bait domains by a second proteinase occurs with its entrance into the cavity. This is followed by a retwisting of the strands to encapsulate the proteinases and expose the receptor binding domains associated with the transformed \( \alpha_2 \)-M.

Human \( \alpha_2 \)-macroglobulin (\( \alpha_2 \)-M; \( M_r = 720,000 \)) is an essential protein present at a high concentration in the serum (\( \sim 2 \) mg/ml) that has the unusual physiological role of a nonspecific proteinase scavenger (1–3). In a presently poorly understood mechanism, native \( \alpha_2 \)-M irreversibly traps almost all known endoproteinases by undergoing a structural change that involves a large alteration in its shape. Evolutionarily related proteinases performing a similar physiological function, termed \( \alpha \)-macroglobulins, are present in all vertebrates and several invertebrates (1). Recently, an impairment in the \( \alpha_2 \)-M gene has been implicated in the etiology of Alzheimer’s disease (4).

\( \alpha_2 \)-M is a glycoprotein assembled from four identical 180-kDa subunits that are disulfide-linked in pairs to form two protomers, which, in turn, are noncovalently associated (1). Each subunit contains an approximately 40-residue-long sequence termed the “bait” region, which displays target sequences for a variety of proteinases (5). Bait region cleavage by a proteinase in turn causes the activation of a functionally important internal thiol ester bond between Cys\(^{949} \) and Glx\(^{952} \) of the subunit, which rapidly undergoes a nucleophilic attack (1). Cleavage of the thiol ester moiety triggers a major shape change, aptly termed the “mousetrap mechanism,” that causes \( \alpha_2 \)-M to internally sequester the proteinase, which typically retains its catalytic activity but is inaccessible to its target proteins (6).

An attacking proteinase cleaves two of the four bait regions of \( \alpha_2 \)-M in rapid succession (1). \( \alpha_2 \)-M can therefore entrap up to two proteinases, the size of chymotrypsin (\( M_r = 25,000 \)). Significantly, a direct nucleophilic attack by methylamine on the thiol esters of native \( \alpha_2 \)-M results in a structural change similar to cleavage by a proteinase (1). Thus, thiol ester cleavage has a pivotal role in the shape change that accompanies the entrapment of the proteinase. Transformed \( \alpha_2 \)-M obtained by either mechanism exposes receptor binding domains (RBDs) that allow its rapid endocytosis by cell-membrane receptors principally displayed by hepatocytes but also by a variety of other cells (1, 7).

Native and transformed \( \alpha_2 \)-Ms display significantly different physico-chemical properties, including migration speeds on nondenaturing gels (8) and Stokes radii (9). Three-dimensional electron microscopy reconstructions have shown that the two forms also exhibit markedly different shapes (10–12). The ambiguity in relating structural features between the native and transformed molecules has therefore led to conflicting models for the structural rearrangement involved in the transformation of \( \alpha_2 \)-M (10, 12). The recently published structure of half-transformed \( \alpha_2 \)-M (\( \alpha_2 \)-M-HT), which has only two cleaved bait regions and two hydrolyzed thiol esters in its bottom half, has

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provided an important link in understanding the process leading from native to transformed $\alpha_2$M (13). In the present study, we have employed cryoelectron microscopy to obtain structures of native $\alpha_2$M ($\alpha_2$M-N), $\alpha_2$M-HT, and $\alpha_2$M-methylamine ($\alpha_2$M-MA) labeled with four monoclonal Fab fragments that bind to a common epitope on all three structural forms of the molecule and permit an assignment of related features.

**EXPERIMENTAL PROCEDURES**

**Protein Preparations—**Purification protocols for native, half-transformed, and methylamine-transformed $\alpha_2$Ms have been previously described in Kolodziej et al. (12), Qazi et al. (13), and Schroeter et al. (11), respectively. Monoclonal antibody 6E8 binds to a 55-kDa fragment and subsequent figures correspond to native $\alpha_2$M (12), 2M (12), and fully transformed $\alpha_2$M-MA (13). In the present study, the Fab fragments from 6E8 were previously shown to recognize both native and fully transformed $\alpha_2$M with a stoichiometry of approximately 4 mol of Fab bound/mol of $\alpha_2$M (12). This electron microscopy study has further confirmed the binding of four Fabs to the native, half-transformed, and fully transformed $\alpha_2$Ms.

**Electron Microscopy—**A 6–8 M excess of 6E8 monoclonal Fab was added to the $\alpha_2$M, $\alpha_2$M-HT, or $\alpha_2$M-MA (transformed $\alpha_2$M) preparations so that the resulting $\alpha_2$M concentration was 0.1 mg/ml. A 3-pj sample of each Fab-labeled $\alpha_2$M was added to a glow-discharged carbon-coated holey grid for cryoelectron microscopy. After removing the excess sample by blotting with filter paper, the grid was rapidly cooled by immersion in liquid ethane. A Gatan cold-holder was used to maintain the specimens below ~170 °C. Images were then acquired with a JEOL JEM 1200 electron microscope operating at 100 kV with an underfocus of ~1.7 μm and an exposure of ~9 e/Å² on Kodak SO 163 film (15).

**Digitization and Particle Extraction—**Micrographs were digitized using an Eikonix 1412 scanner with a 12-bit dynamic range and a pixel size of 5.7 Å on the specimen scale. Power spectra from the unaligned micrographs were analyzed for astigmatism and drift. Micrographs showing frost, significant astigmatism, or drift were rejected. Representative particles were selected in 64×64-pixel boxes using the SUPRIM software package (16). The $\alpha_2$M-N, $\alpha_2$M-HT, and transformed $\alpha_2$M data sets contained 2567, 3498, and 2900 particle images, respectively.

**Three-dimensional Alignment and Classification—**Previously obtained unlabeled reconstructions (15) from single-particle images using the methylamine tungstate stain and carbon support film (17) were used as initial models for the alignment and refinement, i.e. three-dimensional projection alignment and iterative reconstruction, using the SPIDER software (18, 19).

In the case of $\alpha_2$M-N and $\alpha_2$M-HT, the particles were initially aligned to isotropic projections from the unlabeled stain models spaced 2° apart (19). Correspondence analysis, followed by hierarchical ascendent classification (20) using the SUPRIM software, was then used to identify and remove misaligned particles in each projection direction. The edited particle data sets (1564 particles, $\alpha_2$M-N; 3026 particles, $\alpha_2$M-HT) were then aligned to projections of these reconstructions at 2° intervals and used to obtain reconstructions with resolutions of 41 Å ($\alpha_2$M-N) and 38 Å ($\alpha_2$M-HT) using a Fourier shell correlation criterion of 0.67 (21).

For $\alpha_2$M-HT, a further pass of refinement was carried out with 2-fold symmetry imposed on its major axis. This was done to give equal prominence to all Fabs. The final reconstruction, which was not further symmetrized, had a resolution of 38 Å.

The $\alpha_2$M-MA data set was initially examined using a reference-free, K-means clustering algorithm in SPIDER with 50 clusters and 100 iterations (22). Of these, 41 average cluster images were retained and aligned to a recently obtained, refined $\alpha_2$M-MA stain model. A reconstruction from these averages, which prominently displayed the Fabs, was used to obtain a final refined reconstruction (39 Å resolution) from the entire 2900 particle data set.

**Display—**The reconstructions were corrected for the contrast transfer function of the electron microscope as described previously (23–25). Bandpass Fermi filtering (26) was applied to retain information between Fourier space radii of 12 pixels (1/30.4 Å⁻¹; close to the contrast-transfer cutoff and providing the best match with the unlabeled structures) and 1 pixel (1/364.8 Å⁻¹), with a temperature parameter of 0.01. $\alpha_2$M-N and $\alpha_2$M-MA reconstructions revealed 2-fold symmetry along their major axes, and consequently they were 222-symmetrized for display. The distal ends of the Fabs appeared wedge-shaped in the average images (see Fig. 2) and mushroom-shaped in the surface-rendered structures (data not shown), presumably because their site of attachment is not rigid. In the solid-shaped structures, their external ends were trimmed to give them a rod-like appearance so that their contact with the surface can be more readily discerned.

**RESULTS**

**Electron Microscopy—**In the galleries of frozen-hydrated images (Fig. 1), four Fab fragments from the monoclonal antibody 6E8 clearly bind to each of the three structural variants of $\alpha_2$M. On most particles, the Fabs can be discerned as rod-shaped protrusions with knob-like extremities (Fig. 1). Typical shapes such as “lip” views of $\alpha_2$M-N (particles a, c, and g) (12), “pseudo-lip” views of $\alpha_2$M-HT (particles j and m) (13), as well as “H” views of $\alpha_2$M-MA (particles f and u) (11) can be identified and are similar to their unlabeled counterparts (11–13). Thus, the Fabs do not appear to perturb the $\alpha_2$M molecules (11–13).

Cryoelectron microscopy was utilized as the imaging technique in the present study because it permits the use of higher protein concentrations in the sample preparation than stain electron microscopy. This assured that the four Fab binding sites are occupied (Fig. 1) (dissociation constants of the $\alpha_2$M Fab complexes are in the nM range (12)). As often observed in the imaging of nonviral proteins in vitreous ice, the $\alpha_2$Ms tended to assume a preferred orientation in relation to the air-water interface (27–29). However, we obtained a sufficient number of off-axis, “rocking” views in all data sets, which permitted the computation of three-dimensional structures with significant spatial information in all directions (27).

**Three-Dimensional Alignment and Reconstruction—**Our previous reconstructions of the unlabeled $\alpha_2$Ms (11–13) (with resolutions near 30 Å) were obtained from single particles imaged in an amorphous layer of methylamine tungstate stain containing 10 μg/ml bacitracin over carbon support film (17). Specimen application by the spray method (17) and the inclusion of bacitracin minimized the interaction of the particles with the support film and provides multiple orientations of the molecules (28). Our technique provides high contrast images of well preserved molecules, and the multiple orientations of the par-
A comparison of the projections of the Fab-labeled three-dimensional structures and the corresponding two-dimensional average images (Fig. 2) clearly indicate that the processes of alignment and reconstruction have correctly preserved the location of the Fab labels. The Fabs are somewhat less prominent than the 90° rotation exhibited by the strands (Figs. 3 and 4). 

**Three-dimensional Structures and Location of Fab Labels**—All three Fab-labeled reconstructions of α₂M closely resemble their unlabeled stain analogs that were used as the initial models for three-dimensional alignment, further supporting the reliability of the reconstructions (Fig. 2). The 222-symmetric α₂M-N structure presents an overall twisted appearance. In its characteristic lip view, the structure appears as two Z-shaped protein strands that merge at the top and bottom to form regions of high protein density. The strands exhibit a 90° clockwise rotation about their major axis and form the walls of the cavity (Fig. 4a). The two dense ends of the molecule clearly display a chisel-like appearance as observed in the unlabeled stain structure (Figs. 2 and 3). Furthermore, the elbow-shaped bends of the two Z-shaped strands are superficially connected by low density, bridge-like features. Four small (~20 Å diameter) openings to the interior of the structure are located above and below each of the surface bridges on the front and back of the molecule.

The end view of α₂M-N shows that the Fabs bind on the elbow-like bends of the Z-shaped protein strands 120 Å apart and protrude diagonally outward from either side of the base of the chisel (Fig. 3). A closer examination of the “figure-8” end views shows that the Fabs are arranged in a staggered configuration, i.e. located slightly away from the symmetry planes bisecting the structure along its major axis. On each Z-shaped protomer, the two Fabs are located near the ends of antiparallel-linked subunits and consequently form an angle greater than the 90° rotation exhibited by the strands (Figs. 3 and 4).

α₂M-HT is a functional intermediate between the native and transformed molecules in which two of the four bait domains and thiol ester moieties have been cleaved by chymotrypsin bound to Sepharose (30). Its top has a chisel-like shape analogous to α₂M-N and is similarly flanked by two Fab labels 120 Å apart on either side, analogous to the native structure. However, the center of the molecule shows an approximately 2-fold widening of the openings to its internal cavity, whereas the superficial, bridge-like features of α₂M-N are absent (Fig. 3).

The bottom of α₂M-HT presents a bulbous, rounded appearance. The two strands that comprise the native molecule appear to have split into four that exhibit little twist (Fig. 4a). The two Fabs that bind at the bottom appear to be rotated ~50° with respect to the Fabs at the top of the structure and are located laterally 120 Å from each other as in α₂M-N (Fig. 3).

α₂M-MA forms a more compact, cage-like structure, with
four arm-like features that extend, two from each end of the molecule (Fig. 3). As seen in the H and X views, the molecule is formed by two relatively straight strands of protein density that form major connections near the two ends of the structure. The H view exhibits a groove, which appears as a pronounced gap at an increased threshold and separates the body of the structure into two strands of protein density that twist 45° counterclockwise (Fig. 4, a and b). The side X view shows that each strand is broad at the center and tapers at the two ends to form the arm-like extensions (Fig. 3). This structure is similar to the α2M-MA reconstruction reported previously without the Fab labels (11). Two Fabs are located at each end near the tapered base of its arm-like extremities 160 Å apart, in a noticeably staggered fashion. The Fabs show the same 45° rotation exhibited by the two strands (Figs. 3 and 4a). In the three structures, the epitopes are located near the upper and lower ends of their internal cavities with a vertical separation of ~145 Å.

DISCUSSION

Structural Organization of the α2M Variants—A variety of hypotheses have been proposed for the structural transformation that links the quite dissimilar structures of the native and transformed α2Ms (10, 12). The bases for these proposals have ranged from a correlation of surface features (10) to a comparison of the protein density distributions in the two structures (12, 13). As discussed below, previous immunoelectron microscopy studies of individual stained particles have provided significant structural information for the location of domains in transformed α2M (31, 32). However, a structural correspondence was not established with antibody-labeled α2M-N, because the images of stained specimens exhibited variable shapes, making their interpretation questionable. The acidic uranyl salts (pH < 4) used in these studies seemingly perturbed the more labile native molecule. We have shown that a reconstruction obtained from a specimen stained with the neutral pH methylamine tungstate (27) gives excellent correspondence with that obtained from frozen-hydrated specimens (Fig. 2).

Our Fab-labeled three-dimensional structures have provided the first definitive structural comparisons of antibody-labeled α2Ms, making it possible to relate the morphological changes upon transformation by methylamine or chymotrypsin. An initial consideration of the locations of the four Fab binding epitopes, near the ends of all three structural forms of α2M, clearly shows that individual 180-kDa subunits are present in an antiparallel or head-to-tail orientation within the two disulfide-linked protomers that noncovalently associate to form the tetrameric α2M. This is a structural validation of the proposal from previous sequencing studies that the two 180-kDa subunits in each protomer are linked antiparallel by two disulfide bonds near their N termini (1, 33) and a previously reported comparison of two-dimensional average images of Fab-labeled α2Ms (12).

![Fig. 4](image-url) Protein density distribution in slices of the α2M structures (a) and the proposed arrangement of the protomers (b). a, slices 5.7 Å thick were cut perpendicular to the major axis of the α2M-N and α2M-HT stain structures and the labeled α2M-MA ice structure (with the Fabs removed) as shown at the top of the figure. An extensive comparison of the slices has been previously presented (13). Two major strands that appear to split in α2M-HT are the putative dimeric protomers that constitute all three structures. The proteinase-entrapping mechanism appears to involve rotation and separation of the two strands at the top and bottom of α2M. The two Z-shaped protomers of α2M-N rotate 90° clockwise between slices 2 and 6 and merge at each end of the structure to form chisel-like features. In α2M-MA, the two protomers rotate by 45° in the anticlockwise sense between slices 1 and 7, forming significant connections with each other near the ends of the structure, giving it a cage-like appearance. In α2M-HT, the chisel-shaped top splits into two relatively untwisted strands that split further (slice 4), remerge, and undergo a 45° rotation accompanied by a broadening near the bottom of the structure without the separation seen in α2M-MA (b). The arrangement of the protomers in α2M and α2M-MA is apparent upon displaying the structures at a high threshold level. Each protomer has been shaded at a different gray level for ease of viewing. The arrows indicate the approximate locations of the Fab epitopes.
Locations of the RBDs—

The Fabs used in the present study are incapable of trapping proteinases (34). Slices of 2M-MA obtained perpendicular to their major axes (Fig. 4) reveal two twisted strands of high protein density that form arm-like features of 2M. For clarity, the rotation and translation are depicted with arrows only at the top of the molecule. The Fabs at the bottom rotate in the opposite direction.

A comparison of the three structures of α2M (Fig. 3) indicates that in each case, the top and bottom pairs of Fab epitopes are separated along the major (long) axis by a constant distance of approximately 145 Å. However, the lateral distance between the epitopes in each of these pairs is variable, ranging from 120 Å in the native and half-transformed structures to 160 Å in the transformed molecule (Fig. 5). This indicates that the transformation of α2M, which allows the physical entrapment of a proteinase after the bait domains and thiol ester moieties of α2M have been cleaved, involves a rearrangement of protein density about its major axis to this regard, the “accordion folding” model in which a lateral compression and vertical stretching of the molecular was proposed for this transformation of α2M is inconsistent with the disposition of the Fab labels on the native and transformed structures (10). Our observation agrees with the proposed localization of the two disulfide-linked dimers in the three variants of α2M reported here (12, 13), one on either side of the major axis of the structure, and is supported by studies that showed dimeric variants are incapable of trapping proteinases (34). Slices of α2M and α2M-MA obtained perpendicular to their major axes (Fig. 4e) reveal two twisted strands of high protein density that form major connections near the ends of the molecules thereby leading to the structural division proposed (Fig. 4b).

Relatedness of Structural Features in the α2M Variants and Locations of the RBDs—The Fabs used in the present study bind transformed α2M near the base of the arm-like extensions on the top and bottom of the molecule. Previous immunoelectron microscopy studies with other monoclonal antibodies showed that the RBDs are located near the tips of these arms and allow for the rapid endocytosis of α2M-proteinase complexes (32, 35, 36). It was further shown that an antibody to the RBD prevented the binding of α2M to its receptor (32, 35). However, the antibody did not bind α2M-N, indicating that the RBDs are internally sequestered (35). As the antibody 6E8 used in the present study binds native, half-transformed, and transformed α2Ms, its binding site appears to be distinct from the RBD.

The disposition of the epitopes of 6E8 on α2M-N and α2M-MA (Fig. 5) reveals a structural relationship of major significance in the biology of α2M. A comparison of the end views of the native and transformed structures (Fig. 5) shows that the two protomers that merge to form the chisels in α2M-N undergo a separation of 40 Å, along with an opposite 90°-rotation at each end. This results in the emergence of arms and exposure of the RBDs at the two ends of the arm-like features of the transformed structure. It appears that the chisels of α2M-N enclose the RBDs (Fig. 5), and this finding nicely correlates with the inability of the RBD binding antibody (7H11D6) to bind to the native molecule (35).

Structural Basis of Proteinase Entrapment—α2M-HT is a functional intermediate that was obtained by reacting an excess of α2M-N with immobilized chymotrypsin so that bait domain cleavage and thiol ester hydrolysis occurred in only two of its four subunits (30). In the top half of α2M-HT, the chisel-shaped feature and the arrangement of the two Fabs closely resemble α2M-N, whereas the bottom half is broad and bulbous, and the two Fabs have rotated −45° (Fig. 6). Therefore, we conclude that the bait domain and thiol ester on one subunit in each dimer that may be in close proximity have reacted with chymotrypsin in the bottom portion of the structure. Therefore, the minor axis of α2M-N represents its functional division. The intact subunit in each protomer maintains the shape of the chisel-like feature of the native molecule. This proposal agrees with the antiparallel arrangement of the subunits described above.

The untwisting of the strands in α2M-HT (Fig. 6) appears to lead to an approximately 2-fold widening of the openings to the cavity (Fig. 4, cf. α2M-N and HT), making the cavity accessible to proteinase entry. However, the Fabs show that there is no separation of the strands at the ends of the molecule, and the arm-like features of α2M-MA have not formed. Consequently,
the RBDs are not exposed (Fig. 3). In an apparent contradiction to this proposal, a binary chymotrypsin α₂M complex that may share the cleavage arrangement of α₂M-HT (two bait domains and thiol esters cleaved) appears to interact with an anti-RBD antibody (35, 37). However, it was noted (30, 37) that thiol ester cleavage is more extensive in the binary complex preparation, and this may have resulted in the IgG binding to some of the α₂M molecules seen in immunoelectron microscopy (35). We propose that transformed α₂M, which is targeted for endocytosis, requires cleavage of intact thiol esters in the RBDs are exposed and the complex is removed from the circulation. Thus, the presence of two intact bait domains and thiol ester moieties in the native half of the intermediate structure imposes constraints on the complete transformation of α₂M (13). In this regard, a recent study (38) suggested that the four bait regions are in contact with one another near the center of the structure. It was reported that disulfide cross-links between two dimeric protomers blocked the structural change induced by thiol ester cleavage that, however, occurred upon cleavage of bait domains. The need to cleave bait regions in both halves of the α₂M tetramer to enable complete transformation of the molecule shown in this study may, therefore, arise from the need to allow for complete reorganization of the dimer-dimer interface.

The entrapment of two proteinases by α₂M is known to occur in two steps with a fast reaction occurring between α₂M-N and the first targeted proteinase (1). The proteinase may enter the cavity through the enlarged openings where the ε-amino group of its lysine moiety is typically cross-linked to the Glx362 of α₂M (39). The thiol esters are therefore analogous to harpoons that tether the proteinase to the interior of the cavity of the α₂M and, thus, may have a role in maintaining its irreversible attachment. The intermediate or half-transformed α₂M can subsequently undergo further cleavage by a second proteinase at a reduced rate (30) in the native portion of the structure, resulting in a similar entrapment, followed by a 45° counterclockwise rotation of the strands to encapsulate the proteinases and expose the receptor binding domains (Fig. 5).

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