The Three-dimensional Structure of the Human $\alpha_2$-Macroglobulin Dimer Reveals Its Structural Organization in the Tetrameric Native and Chymotrypsin $\alpha_2$-Macroglobulin Complexes*

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Three-dimensional electron microscopy reconstructions of the human $\alpha_2$-macroglobulin ($\alpha_2$M) dimer and chymotrypsin-transformed $\alpha_2$M reveal the structural arrangement of the two dimers that comprise native and proteinase-transformed molecules. They consist of two side-by-side extended strands that have a clockwise and counterclockwise twist about their major axes in the native and transformed structures, respectively. This and other studies show that there are major contacts between the two strands at both ends of the molecule that evidently sequester the receptor binding domains. Upon proteinase cleavage of the bait domains and subsequent thiol ester cleavages, which occur near the central region of the molecule, the two strands separate by 40 Å at both ends of the structure to expose the receptor binding domains and form the arm-like extensions of the transformed $\alpha_2$M. During the transformation of the structure, the strands untwist to expose the $\alpha_2$M central cavity to the proteinase. This extraordinary change in the architecture of $\alpha_2$M functions to completely engulf two molecules of chymotrypsin within its central cavity and to irreversibly encapsulate them.

Human $\alpha_2$-macroglobulin ($\alpha_2$M) is of broad interest to the biological and medical communities as a result of its unique function as a nonspecific proteinase inhibitor. It serves the biological role of a scavenger of most proteinases in the plasma and a receptor binding domain, and a thiol ester linkage between the numerous structural and biochemical studies of $\alpha_2$M have not yielded a convincing determination of the organization of the two disulfide-linked pairs of identical subunits that comprise the complexes. Consequently, our understanding of the mechanism of proteinase entrapment is hampered, and the structural organization of the complex remains obscure. We have proposed that the two side-by-side dimers span the length of the $\alpha_2$M and the transformed $\alpha_2$M on its major axis so that the structural and functional division lies on its major and minor axes, respectively (13). Consequently, the half-molecule (dimer) would not be capable of trapping a proteinase. In this regard, biochemical studies of the $\alpha_2$M dimeric mutants that do not associate to form a tetrameric $\alpha_2$M complex show that they are incapable of trapping proteinases, thus favoring this structural and functional organization of $\alpha_2$M (14). Contrary to this arrangement, other investigators have proposed that structural and functional division resides on its minor axis based on a comparison of the architectures of the native and transformed structures (7) and the shape of electron microscopy images of $\alpha_2$M (16). Moreover, the findings that dimers resulting from urea-dissociated $\alpha_2$M are capable of trapping trypsin non-covalently appear to support this arrangement (17). Because these structural studies lacked the resolution to trace the polypeptide chain in the molecule, and the biochemical studies were conflicting and equivocal, the arrangement of the dimer in the complex remains open to speculation. The present determi-
nation of a three-dimensional structure of the $\alpha_2$M dimer gives a plausible answer to this long standing question concerning the organization of the protomeric units in the complex, because we have been able to assemble a molecule that is very similar to the native structure from two of its constituent dimers. This knowledge combined with the comparative three-dimensional structures of $\alpha_2$M, C949S mutant $\alpha_2$M (11) and $\alpha_2$M-chymotrypsin ($\alpha_2$M-C), the latter determined herein, solidify our understanding of the structural basis of proteinase entrapment.

EXPERIMENTAL PROCEDURES

Protein Preparations—$\alpha_2$M was isolated as described previously, and based on the number of sulfhydryl groups released during the reaction with excess chymotrypsin, the preparation was judged to be 88% active (18). The $\alpha_2$M-C complex was prepared by reacting 1 mol of $\alpha_2$M with 2 mol of Worthington $\times 3$ crystallized bovine pancreas $\alpha$-chymotrypsin.

Electron Microscopy—The native $\alpha_2$M (10 g/ml) was added to 0.25% methyamine tungstate stain and 10 g/ml bacitracin. After 60 s the sample was applied by the spray method to the butvar side of the carbon-coated film in order to obtain multiple orientations of the molecules (19, 20). Because the specimen was applied by the spray method, it was possible to reliably estimate the molecular composition of the preparation. Within 5000 particle images there were 1000 (20%) that corresponded to $\alpha_2$M dimers. The incubation of $\alpha_2$M in the stain at low protein concentration promoted the dissociation of the complex to dimers. The particles corresponding to dimeric and tetrameric $\alpha_2$M were selected from the same fields for their three-dimensional reconstructions (Fig. 1A). The images were recorded by conventional irradiation procedures with a JEOL JEM 1200 electron microscope operated at 100 kV at a nominal magnification of $\times 50,000$ with an underfocus of $-0.7$–$1.4$ m$\mu$. The $\alpha_2$M-C molecules (0.05 mg/ml) were added to carbon film by the drop method and stained with 0.5% uranyl acetate. The film was then folded to create a double carbon layer "sandwich" (21). Images were recorded with a Phillips EM 420 electron microscope at 100 kV with an underfocus of $-0.5$–$0.8$ m$\mu$ and a total electron dose of 20 e/Å$^2$ at a nominal magnification of $\times 50,000$. All images were recorded on Kodak SO 163 film and developed as described previously (20).

Digitization and Particle Extraction—Micrographs were digitized using a Zeiss SCAI scanner with a 16-bit dynamic range and pixel size of 5.6 Å on the specimen scale. Power spectra from the micrographs were analyzed, and those exhibiting significant astigmatism or drift were rejected. Particle images were selected in 64 × 64 pixel boxes using BoxFilament from the EMAN software package (22). The particle data sets consisted of 1742 $\alpha_2$M dimers and 1142 native and 1333 $\alpha_2$M-C images.

Two-dimensional Alignment, Classification, and Reconstruction—The data were processed using SGI Indigo$^2$ and Octane workstations. The initial model for the classification of the $\alpha_2$M dimers was generated by selective excision on the major axis of the front half of the native structure (Fig. 2A). Additionally, a model was created by excising the top half of the native structure on its minor axis in order to show that the initial model does not bias the refined structure of $\alpha_2$M-dimer reconstruction (see below) (Fig. 2A). Averaged images of the characteristic views were obtained by subjecting selected angular bins to a reference-free alignment using the SPIDER software (23). The approach developed by Kolodziej et al. (20) using three-dimensional projection alignment and iterative reconstruction in SPIDER was employed to compute the dimeric structure from images of the half-molecules. A set of quasi-uniformly distributed reference projections employing the half-model of the native molecule, and the x-ray structure of $\alpha_2$M-methylamine (10) filtered to 25 Å was generated within $\theta = 0$–$90^\circ$ and $\phi = 0$–$360^\circ$ with angular steps of 5$^\circ$ followed by refinements of 3$, 2^\circ$, and 2$^\circ$. After the initial angular alignment, a reference-based translational alignment of the boxed images was performed by cross-correlating the particle images with the respective reference projection of the preliminary structure (24). The resulting structures were used as the models to generate the reference projections for subsequent refinements until a stable resolution was achieved. The resolution values of the reconstructions (~25 Å) were measured using the Fourier Shell Correlation with a Fourier ring criterion of 0.5 (25). The structures were Fermi-filtered with a temperature parameter of 0.01 (26) to the resolution value of the reconstruction. Average images and projections were displayed using the SUPRIM software package (27). The solid-shaded structures were thresholded to a volume that corresponds to their approximate molecular weight, and the images were rendered using Explorer software and some custom modules (NAG Inc., Downer's Grove, IL).

RESULTS

Electron Microscopy Images of $\alpha_2$M Dimers and $\alpha_2$M-Chymotrypsin—We utilized methyamine tungstate stain to image the $\alpha_2$M dimer because the stain consistently preserves the shape of the native structure as determined by comparisons of average images of stained and frozen-hydrated molecules (18) and their corresponding three-dimensional reconstructions (13). The electron micrograph fields of stained $\alpha_2$M have the essential advantage over those obtained in ice for three-dimensional reconstruction because they afford numerous higher contrast images of dimeric $\alpha_2$M that greatly facilitates their selection (Fig. 1A). Also, as indicated previously, the lower protein concentration in stain (10 µg/ml) compared with frozen-hydrated molecules (0.1 mg/ml) favors the significant dissociation of tetrameric $\alpha_2$M to its constituent dimers.

A reference-free alignment of the particle images in selected angular bins gave average images that were very similar to the corresponding projection of the structural model of the dimer (23) (data not shown). This good match indicates that the particle images were appropriately classified and that images corresponding to the $\alpha_2$M dimers were appropriately selected.

Numerous studies comparing the stability of native and transformed $\alpha_2$M have shown that dimeric protomers in the former complex dissociate more readily than in the latter. For example, the chaotropic agent sodium thiocyanate extensively dissociated native $\alpha_2$M, whereas $\alpha_2$M-trypsin remained tet-
FIG. 2. Comparisons of the structures of dimeric and tetrameric (native \( \alpha_2 \)M) (A) and a diagram of the two subunits that comprise dimeric \( \alpha_2 \)M (B). A, the relationship between the different orientations of the dimer and native \( \alpha_2 \)M are indicated. The semi-transparent composite structure shows that there are extensive contacts and overlap between the two dimers at the top and bottom of the molecule that form its chisel-like features. The red and blue rods displayed with native \( \alpha_2 \)M correspond to its major and minor axes, respectively and also denote its structural and functional divisions. The arrows denote the small openings on the front side of the structure that lead into its cavity, and the chisel-like feature is labeled in the end view of the structure. The models initially used to align the EM images of the \( \alpha_2 \)M dimers were obtained by excising native \( \alpha_2 \)M on its major or minor axes in the plane or normal to the plane of the page, respectively (see “Three-dimensional Alignment, Classification, and Reconstruction” under “Experimental Procedures”). Because the central cavity is formed by a combination of two dimers, dimeric \( \alpha_2 \)M is not capable of trapping the proteinase as determined by biochemical studies (14). The scale bar in this and subsequent figures corresponds to 100 Å. B, the polypeptide chains are cross-linked by the two disulfide bonds in anti-parallel manner (1), presumably near the center of the dimeric structure since the dimer has C2 symmetry. The bait domains and thiol ester moieties are located near the central cavity of the structure, and the receptor binding domain is located in the C-terminal region of the subunits (see “Discussion”). The numerous intramolecular disulfide bonds associated with the subunit form loops in the polypeptide chains, which probably contribute to its domain organization and the stability of the subunit architecture.
rameric (28). Our EM studies are consistent with these results, because they show that dimeric α₂M is frequently seen in EM fields of native α₂M (Fig. 1A), whereas transformed preparations of α₂M (α₂M-C (Fig. 1B)) C949S α₂M mutant (11), and α₂M-methylamine (8) exhibit no images that correspond to the half-molecule.

A field of α₂M-C displays H- and ()-shaped views of the molecules that are characteristic of all the transformed α₂M (11) (Fig. 1B). An EM tilt experiment originally demonstrated that these shapes are side and end views of the molecules, respectively (29). The variable shapes in the field show that the α₂M-C molecules are in multiple orientations that facilitate a three-dimensional reconstruction with uniform resolution.

Three-dimensional Reconstructions of Dimeric α₂M and α₂M-Chymotrypsin—The model utilized to align the EM images was obtained by excising one-half of the tetrameric α₂M structure in the “lip” orientation (Fig. 2A) on its major axis in the plane of the page. Even though the resulting model removes some of the twist associated with the side-by-side strands (Fig. 3), reconstruction of the dimer has restored this feature as seen in the comparison of the corresponding slices of the composite and the native α₂M structures (Fig. 3).

At a high contour level the native molecule separates into two V-shaped components whose open ends face each other across the minor axis of the structure (13), suggesting that these less robust contacts between the top and bottom halves of the structure are representative of the contacts between the adjacent dimers. Thus, the structural division lies on the minor axis of the molecule (Fig. 2A). Accordingly, a half-molecule generated in this manner was also used as a model to align the data set, and the resulting reconstruction (not shown) after refinement was very similar to the structure of the dimer shown in Fig 2A. Consequently, we concluded that the structure of the dimer correctly represents its disposition in tetrameric α₂M.

The 2-fold related structures of dimeric α₂M (red and blue) when joined produce a molecule (Fig. 2A, Composite) that nicely reproduces the architecture of tetrameric native α₂M. The length (190 Å) and width (150 Å) of the dimer are very similar to the corresponding dimensions of native α₂M in the lip orientation (Fig. 2A). The composite structure was obtained by joining the outside and inside orientations of the dimers so that their 2-fold axes of symmetry coincide at the center of the molecule (Fig. 2A). The only dimension of the composite that is uncertain is its depth, and this dimension was set to match the corresponding value of the native complex (150 Å). This composite gives a reasonable estimation of the degree of overlap between the two halves of the molecule as shown in their semi-transparent presentation (Fig. 2A).

The good correspondence between the composite structure and native α₂M shows that upon dissociation the dimers maintain their architecture, a result that is consistent with studies of sodium thiocyanate (28) and urea-treated (17) tetrameric α₂M. Three studies show that the potentially labile thiol ester moieties are maintained upon dissociation and that the dimer is active because it reacts with trypsin (28).

The protein density distribution of serial sections of the composite and native α₂M confirms the close correspondence between the two structures and shows that the side-by-side dimers have a clockwise twist about the major axes of the structures (Fig. 3). Both structures have a centrally located ellipsoid cavity and display major and minor contacts between the two dimers at both ends and near the central portion of the structures, respectively (Fig. 3A).

The α₂M-C structure (Fig. 4A) is similar to the methylamine-transformed and C949S mutant α₂M (11). This structure, with
higher and more uniform resolution than our previous random conical tilt structure (8), identifies more precisely the location of chymotrypsin inside the complex and its surrounding cavity (see below). Serial slices of this reconstruction also identify the location of the two chymotrypsin molecules and show that the two side-by-side dimers have the opposite handedness of the native $\alpha_2M$. The approximate location of the receptor binding domains (red) are indicated by the arrows. The location of the Cys-949 moieties on the wall of the cavity are indicated by the solid and hatched oval in front of and behind the chymotrypsin molecules, respectively. The bait domains are within 17 Å of the Cys-949 residues inside the cavity (31). The difference map analysis of $\alpha_2M$-C and C949S mutant $\alpha_2M$ indicated the primary position of the chymotrypsin molecule in the cavity. The application of a variable threshold to the difference map indicated that chymotrypsin may less frequently occupy other positions within the cavity, probably because of its restricted freedom to move within the cavity (41), and the variation in the amino acid residues of chymotrypsin that are cross-linked to Glu-952 of the thiol ester moieties of $\alpha_2M$ (43). Consequently, the orientation of the chymotrypsin molecule is arbitrary, and the ribbon presentation of the structure is not presented.

DISCUSSION

Structure-Function Relationships—The $\alpha_2M$ dimer is an extended strand-like structure 190 Å in length and ~50 Å thick with C2 symmetry (Figs. 2A and 3). Sequence analysis shows that the two polypeptide chains are cross-linked by two disulfide bonds in an anti-parallel manner (Fig. 2B), and immuno-electron microscopy has determined that the subunits are laterally associated and arranged head-to-tail (9). Thus, it is apparent from this side-by-side arrangement of the dimers in tetrameric $\alpha_2M$ that two of them are required to form the proteinase-trapping cavity (Fig. 2A). This structural organization suggests that the N termini of the subunits and their associated cross-links are located near the minor axis of the structure and may form the wall of its central cavity (Figs. 2A and B). The bait domains are located near the middle of the polypeptide chain, and the thiol ester moieties are located ~250 residues further toward the C terminus (Fig. 2B) (1). The low resolution x-ray structure (10) and fluorescence spectroscopy (30) studies show that the Cys-949 thiol groups of $\alpha_2M$ are flanked by thiol ester cleavage by methylamine are located ~44 Å apart inside the central cavity (Fig. 4B). The fluorescence studies indicated that the bait domains were within 11–17 Å from Cys-949 and were also proposed to reside inside the cavity of the trans-
formed structure (31) (see below).

The C-terminal region of the subunits comprise the receptor binding domains, which are buried between the ends of the two dimers that form the chisel-like feature of tetrameric α2M (32–33) (Fig. 2A). Changes in the structure that are related to the exposure of the receptor binding domain and proteinase entrapment are described below.

**Mechanism of Proteinase Entrapment**—The length of the major axis and the width and depth of the native and transformed α2M are very similar (Figs. 2A and 4A), indicating that the subunits maintain these dimensions after bait domain and thiol ester cleavages. In contrast, because the disposition and shape of the dimers in native α2M were unknown, representative dimensions of the two structures were proposed to undergo a major change (the “accordion model” of transformation, Ref. 7).

We proposed that the cleavage of the thiol ester moieties (one per subunit), which are presumably located in the central cavity of α2M, results in a remarkable change in the disposition of the C-terminal region of the subunits. A comparison of the structures of Fab-labeled native and methylamine-transformed α2M showed how the two disparate ends of these molecules are related. Recall that the monoclonal Fab that binds near the C termini of the subunits showed that the chisel-like ends of the native molecule (Fig. 2) split so that the epitopes increase their separation from 120 to 160 Å upon thiol ester cleavage (methylamine-treated α2M). This increase in separation of the epitopes also involves their 90° rotation about the major axis of the structure at both ends (13). A comparison of the protein density distribution of serial slices of α2M-methylamine, C949S mutant (11), and α2M-C (Fig. 3) shows that the two dimers, which have separated at opposite ends as described above, have the twisted strand-like appearance of the native molecule but with opposite handedness. Consequently, we conclude that the transformed α2M also has the dimers arranged with their structural division on the major axis of the molecule.

The comparisons of these Fab-labeled structures and the structure of the dimer offer a plausible explanation for the location of the sequestered receptor binding domains associated with the native molecule. These domains are located within the join between the C-terminal ends of the subunits that form the chisel-like component of native α2M (Fig. 2). As described above, upon cleavage of the thiol esters the ends separate to expose the receptor binding domains at the ends of the arms of the structure (Fig. 4B). Within the context of the present study, the red and blue strands separate at the top and bottom of the composite structure (Fig. 2).

However, the manner in which the proteinases enter the cavity is not apparent because the central cavities of both structures are inaccessible to most proteinases. For example, the chymotrypsin molecule is approximately two times larger than the openings to the α2M cavity (see Figs. 2A and 4B). Structural studies of α2M in which two bait domains and two thiol ester moieties were cleaved by chymotrypsin (34) (half-transformed α2M) gave insight into the phenomenon of proteinase entrapment. Using the Fab-labeled half-transformed α2M in the studies described above, the structure showed the epitopes rotated 45° at one end of the molecule, resulting in the untwisting of the two strands and a doubling of the size of the openings to the cavity to ~50 Å so that the proteinase may enter (Fig. 5). The half-transformed α2M may be representative of the intermediate structure of α2M that forms in the initial cleavage event of α2M with a proteinase. The upper half of the cavity also becomes accessible to a proteinase after the second cleavage event, after which the strands retwist to close the openings and entrap the proteinase (13).

The entry of the proteinase into the cavity appears to be assisted, because more than 95% of the chymotrypsin-transformed α2M molecules are occupied by 1–2 chymotrypsin molecules (1). We propose that the bait domains, externally disposed on the native structure, are readily accessible to proteinases irrespective of their size. Upon bait domain and thiol ester cleavages, the bait domains and their bound proteinases are internalized through the large openings afforded by the half-transformed structure (Fig. 5). Once inside, the geometric constraints of the cavity prevent the bait domain from binding to the proteinase active site (see below). This process may be analogous to the bait region cleavage of serpins by a proteinase, which involves the formation of an acyl-enzyme intermediate. The intermediate serves to anchor the proteinase for its transport to another location in the serpin structure (35).

An alternative proposal for the bait domain location and proteinase trapping is also considered because studies of bait domain variants of native α2M led to the proposal that they reside inside the native and transformed structure (36). The finding that cross-links formed between and within dimers indicated that the bait domains reside in or near the interface between strands. However, the location of the bait domain at the interface does not distinguish between an internal or external location for this component.

If the bait domains reside inside of native α2M, the two strands must separate to an extent that permits the entry of a proteinase the size of chymotrypsin (~50 Å, Fig. 4B) to cleave the bait domains. Following their cleavage, the connectivity between the two strands increases significantly, and the proteinase is entrapped (Fig. 3). This mechanism appears to be supported by the propensity of tetrameric α2M to dissociate to dimers at low protein concentration (Fig. 1A) suggesting that the connectivity between the strands is minimal. However, this proposal requires that there is a large conformational variabil-
ity associated with the native molecules, which is not significantly manifested in its resolution estimation (the native and transformed structures have similar resolution values, see “Experimental Procedures”). Moreover, comparison of the native and composite structures do not reveal any significant difference that can be contributed to the protein flexibility of the native αM (Fig. 2A). In any event, the disposition of the functionally important bait domain and the mechanism of proteinase entrapment require further investigation to resolve these proposals.

The strads of half-transformed αM do not appear to interact with each other in the bottom half of the molecule to the extent of those in the native and transformed αM (see Figs. 3 and 5B). Consequently, an extended plasmin molecule with a length greater than 200 Å may be able to protrude from the bottom of this structure to ultimately produce a structure in which half of the plasmin molecule extends from one end of the αM-plasmin complex (37).

We proposed that the functional division resides on the minor axis of the αM structure because the top half of the half-transformed structure is very similar to the corresponding portion of native αM (Figures 2A and 5A), and the orientation of the Fab labels are the same in the two structures (13). This proposal is further supported by the αM-C structure that shows the chymotrypsin molecules are entrapped, one on each side of the αM minor axis (Fig. 4B). Moreover, because the half-transformed molecule lacked the arm-like extensions of transformed αM, the cleavage of the remaining two thiol esters appears to be required to permit complete separation of the C-terminal ends of the subunits and exposure of the receptor binding domains (13).

In 1973 Barrett and Starkey (38) proposed the trap hypothesis for proteinase inhibition by αM because a number of studies showed that the proteinases are physically sequestered by αM without major interaction with the proteinase active site (39–40). This mode of inhibition is unique because most proteinase inhibitors are targeted to a specific class of proteinase (e.g. pancreatic trypsin inhibitor) and bind reversibly at the enzyme active site with high affinity. Their proposal is further supported by electron spin resonance studies of chymotrypsin inside the αM cavity, which showed that it is nearly as free to spin as non-sequestered chymotrypsin (41). In these studies the chymotrypsin was not covalently attached to αM (see below). The mobility of chymotrypsin in the cavity requires that there are no significant physical interactions of αM with chymotrypsin and that the cavity is significantly larger than the chymotrypsin molecules (41). Our difference map analysis of αM-chymotrypsin and αM C949S mutant indicates that the chymotrypsin molecule has a preferred position within the cavity that is significantly larger than the prolate ellipsoid-shaped chymotrypsin molecule (Fig. 4B). Even though the above study shows that the inhibition of chymotrypsin by αM does not require its covalent cross-linking to the cavity, the preferred position in the αM-chymotrypsin structure (Fig. 4B) probably results from the reaction of the thiol ester moieties with the side chains of amino acid residues (1) to stabilize the chymotrypsin molecule as a consequence of its covalent attachment to αM.

Our three-dimensional EM studies of native (9), methyla-