Structure of α_2-Macroglobulin–Protease Complexes. Methylamine Competition Shows That Proteases Bridge Two Disulfide-Bonded Half-Molecules†

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ABSTRACT: α_2-Macroglobulin (α_2M) forms several different covalent complexes with proteases. These include unusual forms in which more than one of the four identical subunits of α_2M are cross-linked by amide bonds to more than one lysyl amino group of the bound protease. The structure of these complexes and the question of how the identical subunits are arranged to form two protease binding sites are matters of current controversy. The 185-kDa subunits are arranged into two disulfide-bonded half-molecules which are, in turn, noncovalently associated. We have provided evidence that, in the major multivalent cross-linked form, proteases can span the two half-molecules, forming a covalently bonded tetramer [Wang, D., Yuan, A. I., & Feinman, R. D. (1984) *Biochemistry 23*, 2807–2811]. An alternative theory has recently been proposed in which the major high molecular weight form has two bonds to protease that are within half-molecules—a multivalent cross-linked dimer [Sottrup-Jensen, L., Hansen, H. F., Pedersen, H. S., & Kristensen, L. (1990) *J. Biol. Chem. 265*, 17727–17737]. To resolve this conflict, experiments were carried out to determine the structure of one of the high molecular weight bands (band 3) seen on SDS–PAGE. Band 3 has anomalous migration, corresponding to markers of apparent molecular mass of 550 kDa (between the tetramer and dimer). In the experiments described here, reactions of thrombin with α_2M were run in the presence of methylamine, which competes for one of the two thrombin–α_2M covalent bonds. The logic of the experiment was that the location of labeled methylamine would indicate the original covalent bridging site. The results showed that methylamine was only found in half-molecules that did not contain a bound thrombin molecule. The results can only be explained by a mechanism whereby (in the absence of amines) proteases must bridge two half-molecules. The results also support, but do not prove, the theory that two subunits from different half-molecules constitute the minimum protease binding site.

α_2-Macroglobulin (α_2M)[1] is one of the major protease inhibitors of blood. It is capable of binding (and clearing from the circulation) most proteases, and this broad specificity suggests that it represents a general defense against proteolytic activity due, for example, to microbial infection. In addition, two recent lines of research suggest that it is involved, in some way, in cell signaling and growth factor regulation. First, the α_2M receptor has been isolated and cloned, and it is, surprisingly, identical to a LDL receptor-related protein (LRP) (Ashcom et al., 1990; Herz et al., 1988; Jensen et al., 1989; Kristensen et al., 1990; Moestrup & Gliemann, 1991; Strickland et al., 1990). Several groups have also shown that α_2M can bind growth factors and cytokines [Borth & Luger, 1989; LaMarre et al., 1990; O'Connor-McCourt & Wakefield, 1987; see review by James (1990)]. In most cases, the interaction of α_2M with its receptor and the binding of growth factors are regulated by the reaction with proteases. Thus, the generation of multiprotein complexes under the control of a proteolytic event is at the heart of an understanding of α_2M function.

Although there is a good deal of knowledge about the structure of α_2M and its reaction with proteases, a complete mechanism has so far not been described [see reviews by Roberts (1986) and Sottrup-Jensen, (1989)]. Part of the problem is that several different events take place, and several different kinds of α_2M–protease complexes are formed. The native α_2M molecule is composed of four identical 185-kDa subunits. Pairs of subunits are disulfide-bonded (referred to here as the "half-molecule"), and the half-molecules are noncovalently associated (Figure 1). Thus, SDS–PAGE under nonreducing conditions shows a single band at 375 kDa (Figure 1). Binding of protease is accompanied by a specific proteolysis of the subunits. A maximum of two proteases can be bound, and generally two subunits are cleaved per protease molecule bound. The proteolysis of α_2M is accompanied by a large conformational change, and it is generally believed that this has the effect of "trapping" the protease in a clathrate-like structure (Barrett & Starkey, 1973): the hallmark of α_2M reactions is that the bound protease has a free active site and can react with small substrates but is sterically hindered from reaction with large substrates or inhibitors. In addition to noncovalent trapping, most of the bound protease (80–95% for trypsin and thrombin) is covalently bound via amide bonds formed by the attack of lysyl amino groups of the protease on an internal Cys-Glu thioester in α_2M (Cranelli-Piperno & Reich, 1978; Salvesen & Barrett, 1980; Sottrup-Jensen et al., 1990: Van Leuven et al., 1981; Wu et al., 1981). The potential for covalent bond formation is great, and one of the unusual features of the α_2M–protease reaction is that more than one bond may form between lysines of a single protease and the thioesters of α_2M (Feinman et al., 1985; Krebs et al., 1978; Wang et al., 1983, 1984). The protease may thus "bridge" two or more subunits with multiple Lys-Glu amide bonds. The nature of such complexes is currently a matter of conjecture, and this report is an attempt to characterize one such form.

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[1] Abbreviations: α_2M, α_2-macroglobulin; *p*NPGB, *p*-nitrophenyl *p'*-guanidinobenzoate hydrochloride; PZP, pregnancy zone protein; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; STI, soybean trypsin inhibitor.

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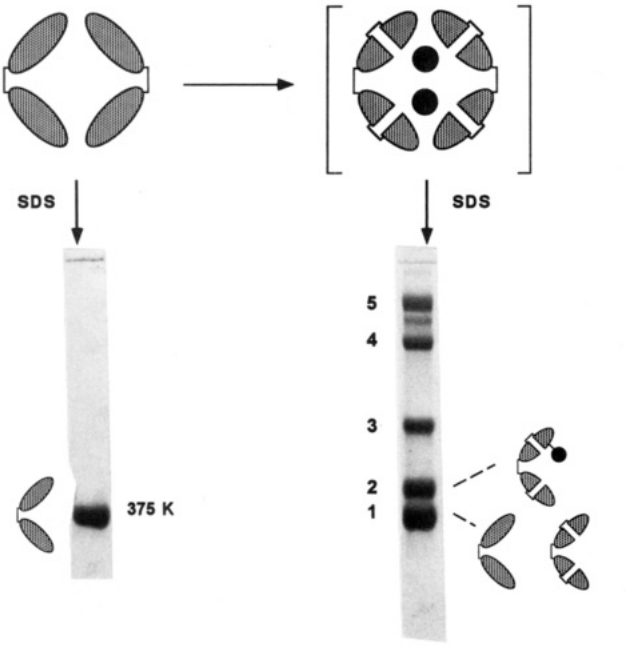

FIGURE 1: Summary scheme for the reaction of α_2M with protease and for the electrophoretic behavior on SDS–PAGE. The native α_2M molecule is composed of four identical 185-kDa subunits (ellipses). Pairs of subunits are disulfide bonded (solid lines connecting ellipses) to form "half-molecules". The half-molecules are noncovalently bonded and dissociate in SDS; the mobility on SDS–PAGE corresponds to markers or 375 kDa (Wang et al., 1983). Reaction with protease (filled circles) is accompanied by specific proteolysis of one to four subunits (broken ellipses) which are connected by disulfide bonds (solid lines connecting half-ellipses). The protease is bound through noncovalent and covalent γ-carboxy-ϵ-amino amide bonds (solid lines connecting circles and half-ellipses). The number and arrangement of covalent bonds are the subject of this investigation, but it is known that there is only one bond possible per subunit. It is generally believed, but not absolutely established, that only proteolyzed subunits form covalent bonds. Brackets are meant to indicate that the precise composition of any particular α_2M-protease complex depends on the reaction conditions. Complexes show five major bands on SDS–PAGE. There is general agreement that band 1 arises from species containing half-molecules that are not bound to protease and band 2, to species containing a single protease. Band 3 has been shown to migrate at a rate corresponding to markers of molecular mass of 550 kDa, and band 4, to have a mobility corresponding to greater than 1 MDa (Wang et al., 1983, 1984).

The cross-bridged structures are unusual in that different subunits of one protein are cross-linked by multiple covalent bonds to another. The main evidence for the existence of such species comes from the behavior of α_2M–protease complexes on nonreducing SDS–PAGE (Feinman et al., 1985; Wang et al., 1983, 1984). Such gels reveal five bands (Figure 1). Band 1 is a half-molecule formed by dissociation of unreacted α_2M (or noncovalent complexes) in SDS. Band 2 is attributed to the dimer with one protease molecule covalently linked. The attribution of bands 3, 4, and 5 has been the subject of some controversy. There are two major theories for these high molecular weight species. Our laboratory has proposed that band 3 represents a tetramer formed from two (disulfide-bonded) half-molecules bridged by bonds to a single protease (Figure 2). Because of the low mobility and the performance on two-dimensional gels (Wang et al., 1983), we attributed bands 4 and 5 to structures larger than the tetramer. Recently, Sottrup-Jensen's group (Sottrup-Jensen et al., 1990) has proposed an alternative theory, based on gel chromatography data, that band 3 is actually a half-molecule, cross-linked internally (Figure 2, mechanism II), and that bands 4 and 5 are tetramers with multiple internal cross-links.

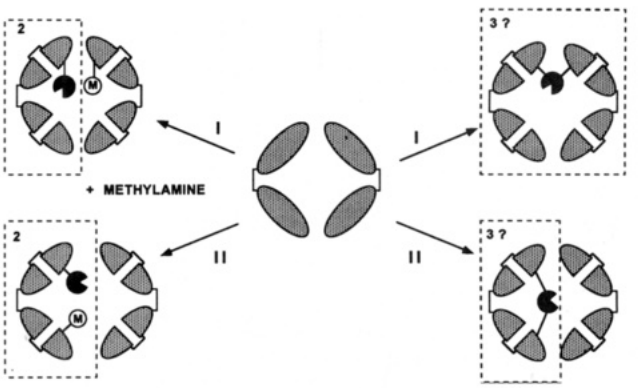

FIGURE 2: Proposed mechanisms for the formation of multivalent cross-linked α_2M–protease complexes and for protease-induced incorporation of methylamine. The schemes on the right side show the proposed mechanism in the absence of methylamine and, on the left, in its presence (graphic symbols as in Figure 1). Mechanism I proposes that protease binding leads to the proteolysis and activation of at least two subunits on different (disulfide-bonded) half-molecules; mechanism II proposes the activation of at least two subunits on the same half-molecules. Proteases form Lys-Glu amide bonds with both sites unless methylamine is present, in which case amine binds to one (and only one) of the sites. Reacted subunits are shown as completely proteolyzed in keeping with data from two-dimensional gel studies (Wang et al., 1983).

In this report, we focus on band 3 and attempt to distinguish between the two proposed models for this species. To do this, we take advantage of the reaction of α_2M with the reagent methylamine. It has long been known that methylamine reacts with the thioesters of α_2M (Swenson & Howard, 1979; Sottrup-Jensen et al., 1980). This reagent has been used as a probe for covalent reactions of α_2M with proteases. If α_2M is incubated with methylamine before addition of protease, there is a slow reaction of amine with the thioesters and concomitant inactivation of protease binding capability. If the protease and amine are added together, the amine incorporation is greatly accelerated. Surprisingly, when amine is added with protease, there is limited inhibition of enzyme binding (Chen et al., 1990; Salvesen et al., 1981; Sottrup-Jensen et al., 1981). Thus, there is an apparent paradox: there is substantial amine incorporation with little inhibition of covalent protease binding even though amine and protease compete for the same site. We recently showed that the explanation is that methylamine does not compete for the first covalent bond formed between the protease and α_2M and, hence, does not reduce total covalent bonding—the concentration of band 2 actually increases. Methylamine does compete for the second (bridging) bond, causing a reduction in the amounts of bands 3, 4, and 5. Here we take advantage of this observation to ask about the pattern of amine and enzyme incorporation. From this pattern, we can deduce the original bridging site in band 3, and we find that the bridge is across two half-molecules.

MATERIALS AND METHODS

Chemicals. [^{14}C]Methylamine, specially purified by distillation, was purchased from New England Nuclear. 4,4′-Dithiopyridine was obtained from Aldrich Chemical Co. and used without further purification. *p*-Nitrophenyl *p*′-guanidinobenzoate hydrochloride (*p*NPGB) was obtained from ICN Pharmaceuticals. All other reagents were the highest grade commercially available.

Proteins. Human α-thrombin was the generous gift of Dr. John W. Fenton II of the New York State Department of Health, Albany, NY. Trypsin was obtained from Sigma [bovine pancreatic trypsin (TRL 36P704)]. Trypsin and

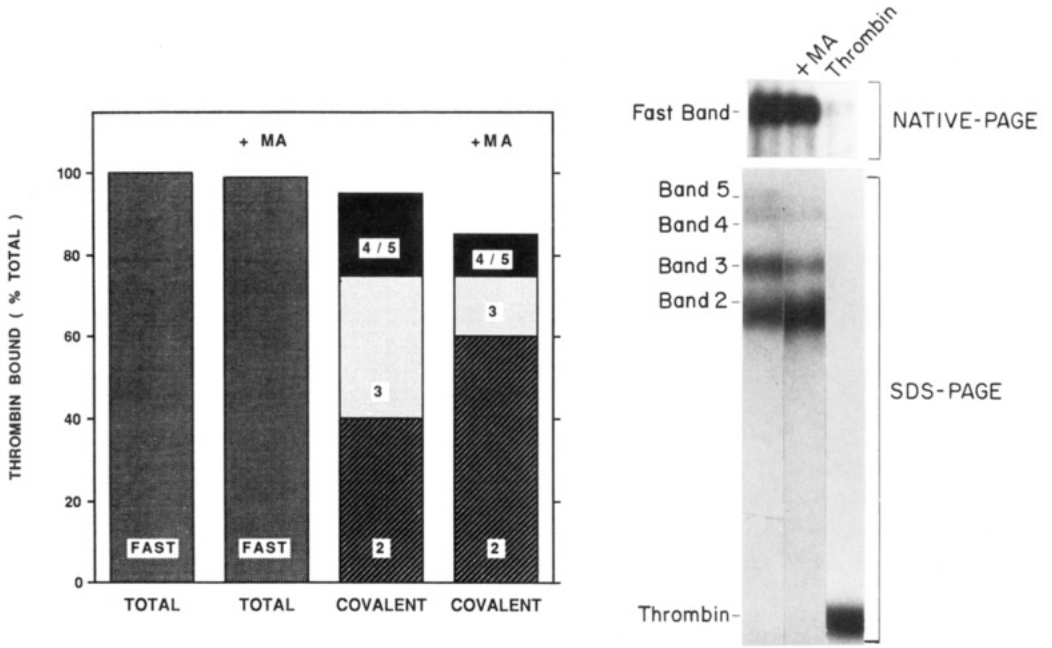

FIGURE 3: Electrophoretic behavior of α_2M–^{125}I-thrombin complexes and the effect of methylamine. Samples of α_2M (1 μM) were allowed to react with ^{125}I-thrombin in a 1:1 molar ratio, in the absence or presence (+MA) of 30 mM methylamine. Total protease incorporation was determined from label appearing in the "fast" band (Barrett et al., 1979) by densitometry of autoradiograms from native polyacrylamide gel electrophoresis (NATIVE-PAGE). Covalent binding was determined by densitometry of SDS gel electrophoretograms (SDS–PAGE). Areas of the individual components of the stacked histogram show percent of total protease binding for the indicated species. Band 1 is not seen on autoradiograms because it does not contain protease.

thrombin activities were assayed by active-site titration using *p*NPGB or by use of a chromogenic trypsin substrate, *N*-benzoyl-L-isoleucyl-L-glutamylglycyl-L-arginine-*p*-nitroanilide hydrochloride in the presence of methylamine. Iodination of α_2M and native trypsin or thrombin with ^{125}I in the presence of lactoperoxidase was performed according to the procedure of Martin et al. (1976).

α_2M was purified by a combination of zinc chelate chromatography using the method of Virca et al. (1978) and chromatography on Cibacron Blue–Sepharose according to Kurecki et al. (1979), as described previously (Feinman et al., 1985).

Electrophoresis. Polyacrylamide gel electrophoresis was carried out with slabs on a Gelbond support. The analytical gel used for quantitating total ^{125}I-enzyme bound to α_2M or the amount of [^{14}C]methylamine incorporated into α_2M was a gradient of 3.5–10% acrylamide. For quantitation of covalent complex formation, a 3.5% gel containing 0.1% SDS was used. Reactions were stopped with diisopropyl phosphorofluoridate or ice-cold 10% (w/v) trichloroacetic acid. In the latter case, samples were centrifuged, and the recovered pellet was washed extensivley, neutralized with ammonia vapor, and solubilized with 1% (w/v) SDS/25 mM Tris-HCl buffer, pH 8.0, before being applied to the gel. Normal Tris–glycine buffers for gel systems were used as electrophoresis buffer. The method of stopping the reaction is critical. Soybean trypsin inhibitor (STI) is inadequate, and its use leads to proteolysis of the α_2M–protease complexes, presumably due to the reversibility of the trypsin–STI reaction (Laskowski, 1971; Wange et al., 1981, and references cited therein). Thus, Crews et al. (1988), who used STI, reported that methylamine, when added with chymotrypsin, prevented covalent bond formation. We found, using their conditions, almost total hydrolysis of α_2M complexes (data not shown). Therefore, their report that chymotrypsin bound to α_2M has the same tumbling rate as solution chymotrypsin is probably an artifact due to measuring chymotrypsin–α_2M fragments of much lower molecular weight.

RESULTS

Rationale. In order to determine the structure of multivalent cross-linked species, we focused our attention on the major such form that gives rise to band 3 on SDS–PAGE (Figures 1, 3, and 4). Experiments were designed to test whether this band is a tetramer formed from the cross-linking of two half-molecules (mechanism I in Figure 2), or whether it is a dimer in which subunits within one half-molecule are cross-linked (mechanism II). The logic is to take advantage of the fact that methylamine prevents the formation of band 3 by competing for a second (bridging) covalent bond (Chen et al., 1990). Proteases that would normally appear in band 3, in the presence of methylamine, are in band 2 (the monovalent complex). The methylamine itself will appear in that subunit that contained the second site. The two proposed models make different predictions as to the location of this site (Figure 2). In particular, if the bridge is *across* half-molecules, then methylamine should be incorporated into a different half-molecule than the protease and hence will not appear in band 2. If the cross-links are *within* the half-molecule, then amine should be incorporated into the same half-molecule and will appear in band 2. We first describe the effect of methylamine on the α_2M–protease reaction.

Effect of Methylamine on α_2M–Protease Complexes. We previously showed that if thrombin and α_2M are allowed to react in the presence of a large excess of methylamine, there is a substantial reduction in the concentration of bands 3, 4, and 5 compared to the amount formed in the reaction without amine. Band 2 increases in the presence of amine. This is consistent with the notion that band 3 arises from band 2 and that amine blocks the conversion by interfering with formation of the second covalent bond. We repeated the previous experiments using a thrombin:α_2M ratio of 1:1 in order to simplify the analysis and to avoid cooperative effects (Christensen & Sottrup-Jensen, 1984; Gonias & Pizzo, 1983b; Steiner et al., 1985). The results of native and SDS gel elec-

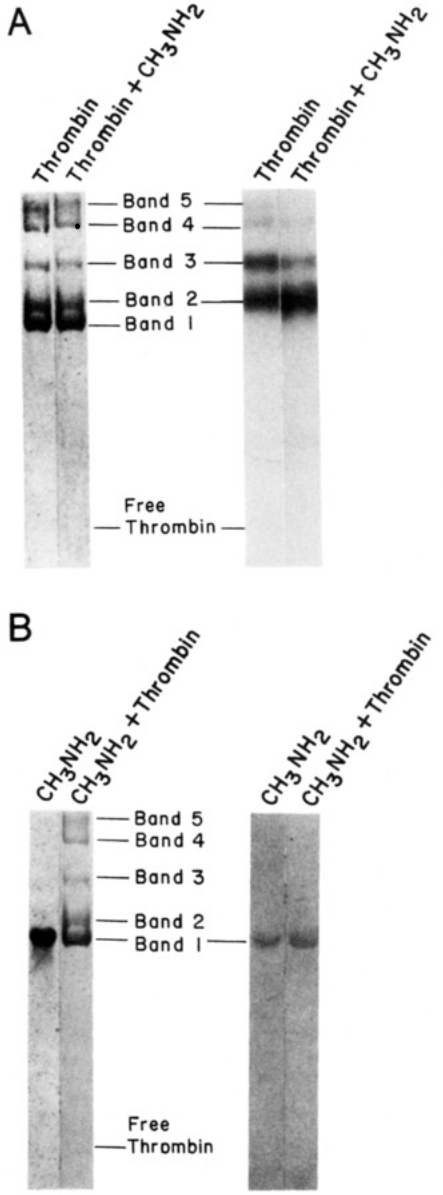

FIGURE 4: Identification of species containing thrombin and methylamine. (A) Samples of α_2M (1 μM) were allowed to react with ^{125}I-thrombin (1 μM) in the absence or presence of a 30 000-fold molar excess of methylamine. Protein was stained with Coomassie Brilliant Blue (lanes 1 and 2), or autoradiograms were prepared (lanes 3 and 4). (Lanes 1–4 from left to right.) (Lanes 1 and 3) α_2M + ^{125}I-thrombin; (lanes 2 and 4) α_2M + ^{125}I-thrombin + methylamine. Densitometry of lane 4 revealed that the region of band 1 contained 3% of the total radioactivity and band 2 contained 63%. (B) Reactions were run under the same conditions as in (A) except that unlabeled thrombin and [^{14}C]methylamine were used. Results are also shown for the uncatalyzed incorporation of [^{14}C]methylamine into α_2M, in the absence of thrombin. Autoradiograms were prepared (lanes 3 and 4). For comparison, protein gels for the reaction are also shown (lanes 1 and 2). (Lanes 1 and 3) α_2M + [^{14}C]-methylamine; (lanes 2 and 4) α_2M + thrombin + [^{14}C]methylamine. Densitometry of lane 4 revealed that band 1 contained 70% of the total radioactivity and the region of band 2 contained 7%.

trophoresis are shown in Figure 3. The results are similar to earlier observations, but more dramatic: under conditions of equimolar protease and α_2M, methylamine had virtually no effect on the total amount of protease binding as shown by the amount of incorporation into the "fast" form on native gels [the conformational change due to protease binding is seen as an increase in mobility (Barrett et al., 1979)]. Covalent binding was, similarly, not substantially inhibited: covalent binding, normally 95% of total, was only reduced to 85%,

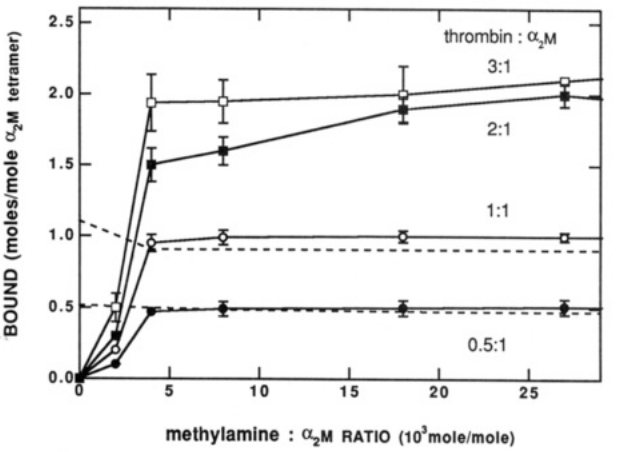

FIGURE 5: Dependence of [^{14}C]methylamine incorporation on concentration. Samples of α_2M (1 μM) were allowed to react with thrombin at the indicated molar ratios in the presence of increasing thousand-fold molar excesses of [^{14}C]methylamine. Reactions were stopped with a 10^4 molar excess of unlabeled methylamine and 10% cold trichloroacetic acid, and incorporated label was counted. The dashed line shows the incorporation of thrombin in parallel experiments with cold amine and ^{125}I-thrombin (dashed line) at 1:1 and 0.5:1 molar ratios (thrombin to α_2M). All data points are averages of at least triplicate experiments.

even though, as shown below, there is substantial amine incorporation. [An earlier report (Crews et al., 1988) that methylamine prevented covalent bond formation appears to be an artifact of the inhibitor used to stop the reaction (see Materials and Methods).] The effect of methylamine was only to change the distribution of complexes between monovalent complexes (band 2 increased from 39% of the total bound protease to 60%) and the proposed multivalent complexes (band 3 was reduced from 35% to 15%). Although there are precedents in the literature (Salvesen et al., 1981; Sottrup-Jensen et al., 1981), this primary result is surprising: at less than saturating protease, methylamine (which *is* incorporated into α_2M) does not compete for total or covalent protease binding.

Incorporation of Methylamine. Under the conditions in Figure 3 (1:1 thrombin:α_2M ratio, 35 000-fold molar excess of amine), 1 mol of methylamine is incorporated into α_2M. Figure 5 extends our previous results to show that the protease-catalyzed incorporation of amine reaches a maximum for a given amount of enzyme. The stoichiometry of incorporation is, at most, 1 amine:1 enzyme. In addition, SDS–PAGE under reducing conditions shows that methylamine is incorporated into proteolyzed subunits (100 kDa) rather than native subunits (data not shown). The results are consistent with a mechanism whereby protease binding is accompanied by proteolysis of at least two subunits and the activation of at least two thioesters. One of the activated thioesters reacts with the Lys groups of the bound protease, another, with amine. In the absence of methylamine, this second site is the one that reacts to form a second covalent bond with the protease.

Location of the Bridging Site in α_2M. With the above results as background, we now asked what is the nature of the bridged structure in band 3. The assumption is that, in the presence of methylamine, the protease that would have been in band 3 is now present in structures that produce band 2. If our theory is correct (mechanism I in Figure 2), then this half-molecule should not contain any methylamine; the incorporated methylamine is predicted to be in band 1. In other words, mechanism I predicts that half-molecules contain protease or methylamine but never both. On the other hand, if mechanism II is correct, half-molecules will contain *both*

enzyme and amine or will contain *neither*. A third possibility is that the subunits are arranged in such a way that bound enzyme has access to all subunits (both mechanism I and mechanism II can occur). In this case, methylamine should appear in *both* enzyme-containing (band 2) and unreacted half-molecules (band 1). We incubated ^{125}I-thrombin at a 1:1 ratio to α_2M with a 30 000-fold molar excess of cold methylamine. In a parallel experiment, we used cold thrombin and [^{14}C]methylamine. The results of SDS electrophoresis of the reaction mixtures are shown in Figure 4: thrombin radioactivity (Figure 4A) appeared almost entirely in band 2 as expected. The thrombin label in band 1 (native half-molecule) was essentially at background. The fate of the labeled amine is shown in Figure 4B where it can be seen that it was almost entirely in band 1 with virtually no incorporation into higher bands. Thus, protease is incorporated into one half-molecule and methylamine into a different half-molecule as predicted by model I. Since the label in band 2 is essentially at background, it was necessary to estimate the limits of detectability of the labeled methylamine. Electrophoresis was run with serially diluted samples of the reaction mixture, and the amount of label in band 1 (the amine containing band) was determined. We found that radioactivity was no longer detectable in band 1 for samples at a 4% dilution. Thus, the concentration of [^{14}C]methylamine in band 2 is less than 4% of that in band 1. There were, thus, virtually no species containing both enzyme and methylamine on SDS gels. We conclude from this that, in the absence of methylamine, the second bridging site would be on a half-molecule that had no protease bound and hence band 3 is a tetramer spanned by protease.

DISCUSSION

Organization of α_2M Subunits. The functional arrangement of the four identical subunits of α_2M that allow it to bind up to two protease molecules remains unknown. It is generally believed that at least two subunits are required to form a protease binding site. Dissociation by mild reduction (presumably breaking disulfide bonds, producing dimers of subunits that are held together by noncovalent forces) has been reported to produce an active molecule (Gonias & Pizzo, 1983a). Dissociation by urea, acid, or Cd(II) is also reported to produce active (disulfide-bonded) half-molecules (Pochon et al., 1987; Thomas et al., 1988; Liu et al., 1989). There have been several criticisms of both of these methods: dissociation by reducing agents is difficult and produces low yields, and evidence has been presented that activity in urea is due to only a fraction of existing tetramers or to re-formation of tetramers during complex formation (Roche et al., 1988; Sjöberg et al., 1991), and it is thus not absolutely excluded that the intact tetramer is the minimum structure required for protease binding. On the other hand, dimeric macroglobulins such as PZP are disulfide-bonded and may be analogs of the half-molecule. If, in fact, both methods of dissociation produce active species, the α_2M molecule may be functionally symmetrical, any two subunits producing a protease binding site (Liu et al., 1987). The proposed central location of proteases within the α_2M molecule (Gettins et al., 1990; Pochon et al., 1981), and the close proximity of proteases when two are bound, is consistent with this idea. Likewise, spectroscopic measurements indicate that the distance between any of the released thiol groups is probably substantially less than 8 nm (Gettins et al., 1988). Thus, there are no known constraints on which subunits can be cross-linked. The results presented here show that the major bivalent cross-linking to protease occurs across two (disulfide-bonded) half-molecules, rather than within half-molecules. This suggests that two subunits in noncovalent association (i.e., from different half-molecules) form a protease binding site as suggested by Feldman et al. (1985). The results do not exclude, however, that the bridging bond might form after protease binding to a site localized to the (disulfide-bonded) half-molecule.

Proposed High Molecular Weight Structures. The results of the competition by methylamine for the second cross-linking site seem unambiguous in indicating a tetrameric structure for band 3. There are, however, a number of questions. The results are in conflict with conclusions of Sottrup-Jensen et al. (1990) based on denaturing gel chromatography where only two peaks were eluted from α_2M–protease complexes. One of these had a mobility similar to unreacted or to methylamine-treated α_2M, and this one contained band 3 when run on SDS–PAGE. Although this is prima facia evidence against our model, if band 3 is a cross-linked tetramer, it would be expected to be more compact than the native form and run faster on denaturing gel chromatography and therefore might elute with the dimers rather than in the normal place for tetramers. Band 3, in fact, has anomalous mobility on SDS–PAGE, running in the range of 550-kDa standards. If our model is correct, it would be running fast (actual M_r = 750K), whereas a dimer (375 kDa) would be running slowly. Since a compact structure for the protease complex is more consistent with physical studies in the literature, a tetramer with higher mobility seems like the more likely explanation of the anomalous behavior on gels and might possibly explain the chromatographic data as well. Also, if bands 4 and 5 are tetramers as proposed by Sottrup-Jensen, they are running uncharacteristically slowly (markers > 1 MDa).

Another odd feature of the high molecular weight structures is that two-dimensional electrophoresis studies (Wang et al., 1983) and assays for sulfhydryl groups after reaction show that band 3 contains no intact thioesters whereas bands 2, 4, and 5 do; a similar result showing that band 3 has no methylamine-reactive site has been found by Sottrup-Jensen's group (Sottrup-Jensen et al., 1990), and personal communication). Thus, it might be expected that the sites for methylamine incorporation are randomized on the α_2M molecule, or even that amine might be incorporated into band 3. The fact that this is not observed seems to indicate that regardless of how many thioesters are activated, only two are involved in the bridging structure and, in the case of amine incorporation, amine is only incorporated into one site in the half-moleucle opposite the one in which the α_2M–protease bond is formed. It should be pointed out that although one would assume that the competition of amine for the second bridging site is controlled by steric factors there may be a chemical component as well. Thus, Salvesen et al. (1981) showed that, by using stronger nucleophiles such as hydroxamic acids, it was possible to compete for covalent binding to protease. Thus, it is conceivable that all of the thioesters are activated but only some are capable of reacting with amine. An alternative explanation of this phenomenon is that band 3 contains two molecules of protease and would therefore have a higher degree of bait region cleavage and thioester destruction. The reactions studied here were performed at a 1:1 protease:α_2M ratio, and this would require that a second protease was bound while there were still unreacted α_2M molecules present. This is not implausible since several groups have shown evidence for highly cooperative protease binding (Christensen & Sottrup-Jensen, 1984; Feinman et al., 1985; Gonias & Pizzo, 1983b; Steiner et al., 1985; Straight & McKee, 1984), although it would require that the molecule be inherently assymetrical.

Significance of High Molecular Weight Species. The reaction of proteases with α_2M involves the generation of a highly reactive state in which multiple covalent bonds are formed. The high susceptibility to nucleophilic reaction, like the ability to trap proteases by conformational changes and the existence of multiple "bait" regions, may be a reflection of the evolution of a mechanism for binding proteases of different structure and substrate specificity. Thus, surface lysyl amino groups provide many targets on proteases, and the ability to attack two subunits of α_2M, like the trapping mechanism, provides a way for the protease to be surrounded on more than one side, making the active site inaccessible. Pregnancy zone protein (PZP) is a dimeric homolog of α_2M which forms complexes in which two PZP molecules become cross-linked by protease into a tetramer (Christensen et al., 1989; Gettins & Sottrup-Jensen, 1990). Since earlier phylogenetic forms of α_2M tend to be dimers (Quigley & Armstrong, 1985), it is conceivable that the formation of bivalent cross-links is an evolutionary stage that precedes stable tetramers.

The activation of α_2M that leads to the formation of multiple covalent bonds extends beyond the reaction with the protease itself. In addition to amide bonds to proteases, α_2M forms disulfide bonds with growth factors, presumably via the thiol group released by reaction of the thioester. In many cases, these α_2M–growth factor complexes have very low mobility on SDS–PAGE, migrating slower than markers of 550 kDa (Dennis et al., 1989; O'Connor-McCourt & Wakefield, 1987), suggestive of the multivalent species discussed here. Also, the protease-catalyzed incorporation of amines, studied here with methylamine as a model system, has long been known to include proteins (Salvesen et al., 1980; Sottrup-Jensen et al., 1981a,b), and a recent report suggests that this may have physiologic importance in the incorporation of insulin (Chu et al., 1991). The latter may also involve ester bonds as previously described for the protease reactions (Sottrup-Jensen et al., 1990). Interleukins also form complexes with α_2M that appear to be Zn-chelate compounds (Borth et al., 1990). The interaction between these various sites of covalent reaction is unknown. It is reasonable to assume that the formation of growth factor–α_2M complexes competes with protease–α_2M bond formation since the Cys residue that forms the bond to growth factor is presumed to arise from the thioester and is therefore in close proximity to bound protease (Zhao et al., 1988). This competition may be for the second bridging site in a manner analogous to the reactions studied in this paper. The growth factor interaction is, in fact, frequently inhibited when the activating protease is trypsin or thrombin (LaMarre et al., 1990; Borth, personal communication), both of which have a high degree of covalent bonding to α_2M and form multivalent cross-links. On the other hand, it may be that multivalent cross-links are necessary for the maintenance of the integrity of the α_2M molecule under conditions where other covalent and noncovalent interactions are going on. In this regard, Moestrup and Gliemann (1991) reported that high-affinity binding of α_2M is dependent on binding of two neighboring receptors to (tetrameric) α_2M and that lower affinity binding occurs if there is only one point of contact, for example, at low receptor density, or if the ligand is a chymotrypsin complex with α_1-inhibitor (a monomeric protein homologous to a single α_2M subunit). The interaction between these covalent reactions can be expected to be important in the emerging picture of growth factor regulation: growth factors have specific cellular receptors as targets, but under conditions where α_2M is activated by proteases, their availability may be regulated by the reaction with α_2M and clearance through the α_2M receptor (LaMarre et al., 1991; O'Connor-McCourt & Wakefield, 1987). The possibility that this, in turn, is controlled by the nature of the protease complex (determined by the type and concentration of protease) adds another level of regulation to the process of cell signaling.

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