Khan, S. A., & Erickson, B. W. (1982) *J. Biol. Chem. 257*, 11864–11867.
Kurecki, T., Kress, L. F., & Laskowski, M., Sr. (1979) *Anal. Biochem. 99*, 415–420.
Moore, W. J. (1972) *Physical Chemistry*, 5th ed., pp 449–457, Longman, London.
Pochon, F., Favaudon, V., & Bieth, J. (1983) *Biochem. Biophys. Res. Commun. 111*, 964–969.
Price, N. C., & Dwek, R. A. (1974) *Principles and Problems in Physical Chemistry for Biochemists*, pp 98–124, Clarendon Press, Oxford.
Salvesen, G. S., Sayers, C. A., & Barrett, A. J. (1981) *Biochem. J. 195*, 453–461.
Sottrup-Jensen, L., & Hansen, H. F. (1982) *Biochem. Biophys. Res. Commun. 107*, 93–100.
Sottrup-Jensen, L., Stepanik, T. M., Jones, C. M., Peterson, T. E., & Magnusson, S. (1979) in *The Physiological Inhibitors of Coagulation and Fibrinolysis* (Collen, D., Wiman, B., & Verstraete, M., Eds.) pp 255–271, Elsevier/North-Holland, Amsterdam.
Sottrup-Jensen, L., Petersen, T. E., & Magnusson, S. (1980) *FEBS Lett. 121*, 275–279.
Sottrup-Jensen, L., Hansen, H. F., Mortensen, S. B., Petersen, T. E., & Magnusson, S. (1981a) *FEBS Lett. 123*, 145–148.
Sottrup-Jensen, L., Lønblad, P. B., Stepanik, T. M., Petersen, T. E., Magnusson, S., & Jörnvall, H. (1981b) *FEBS Lett. 127*, 167–173.
Sottrup-Jensen, L., Petersen, T. E., & Magnusson, S. (1981c) *FEBS Lett. 128*, 123–126.
Steinbuch, M., Pejaudier, L., Quentin, M., & Martin, V. (1968) *Biochim. Biophys. Acta 154*, 228–231.
Straight, D. L., & McKee, P. A. (1982) *Biochemistry 21*, 4550–4556.
Swenson, R. P., & Howard, J. B. (1979a) *J. Biol. Chem. 254*, 4452–4456.
Swenson, R. P., & Howard, J. B. (1979b) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4313–4316.
Swenson, R. P., & Howard, J. B. (1980) *J. Biol. Chem. 255*, 8087–8091.
Van Leuven, F., Cassiman, J.-J., & Van den Berghe, H. (1981a) *J. Biol. Chem. 256*, 9016–9022.
Van Leuven, F., Cassiman, J.-J., & Van den Berghe, H. (1981b) *J. Biol. Chem. 256*, 9023–9027.
Van Leuven, F., Cassiman, J.-J., & Van den Berghe, H. (1982) *Biochem. J. 201*, 119–128.
Wu, K., Wang, D., & Feinman, R. D. (1981) *J. Biol. Chem. 256*, 10409–10414.

Covalent Thrombin–α_2-Macroglobulin Complexes. Evidence for Bivalent Cross-Linking of Inhibitor Chains by a Single Enzyme Molecule†

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ABSTRACT: Complexes formed between thrombin and α_2-macroglobulin (α_2M) were studied by polyacrylamide gel electrophoresis. The results provide evidence for the existence of a recently proposed novel enzyme–inhibitor species in which a single thrombin molecule forms two or more covalent bonds to two or more different α_2M chains. At least one of several slowly migrating bands (greater than 375K on nonreduced gels) that have previously been observed in the literature but not well characterized can be assigned to the new species. The involvement of the lysyl amino groups of thrombin is shown by the observation that methylation of these groups reduces the higher molecular weight bands. In addition, increasing the thrombin:α_2M ratio causes a relative decrease in the higher molecular weight species, suggesting that these complexes arise by intramolecular reactions that are susceptible to competition by solution thrombin. The data provide support for our previous proposal [Wang, D., Yuan, A., & Feinman, R. D. (1983) *Ann. N.Y. Acad. Sci. 421*, 90–97] that the 260K band seen in reduced gels is composed of two proteolyzed inhibitor subunits linked to one thrombin molecule. This intersubunit link maintains the integrity of the α_2M in sodium dodecyl sulfate, accounting for the high molecular weight bands under nonreducing conditions. Comparison with a synthetically cross-linked α_2M molecule allows a tentative but not unambiguous assignment of one of the bands to this novel structure.

We recently presented evidence for the existence of a unique protein species in which two subunits of one protein (α_2-macroglobulin) (Figure 1) were covalently cross-linked via two bonds to a single molecule of a second protein (thrombin) (Wang et al., 1983). We proposed that this complex was one of the constituents of the covalent species that arise as part of the tight binding of proteolytic enzymes to the plasma inhibitor α_2-macroglobulin (α_2M)[1] (Cranelli-Piperno & Reich, 1978; Salvesen & Barrett, 1980; Wu et al., 1981). The details of the covalent binding are not understood although it is assumed that the bonds are γ-glutamyl–ϵ-lysyl amide bonds on the basis of an analogy with the binding of amines to an active Glu residue of the inhibitor and the demonstrated reduction in covalent binding when the lysyl amine groups of the enzymes are blocked (Wu et al., 1981; Feinman et al., 1983, references cited therein). The evidence for the unusual bivalently linked complexes came from an examination of two-dimensional electrophoresis of complexes. In particular, we called attention to a number of high molecular weight bands seen under re-

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[1] Abbreviations: α_2M, α_2-macroglobulin; DFP, diisopropyl phosphorofluoridate; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; DTNB, bis(4,4′-dithiodinitrobenzene); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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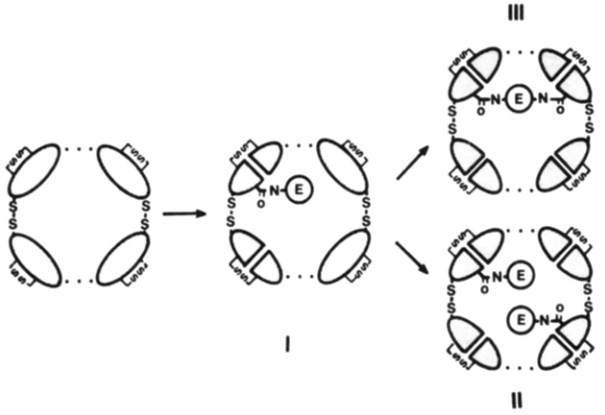

FIGURE 1: Schematic of the structure of α_2-macroglobulin and the proposed sequence of formation of thrombin–α_2M complexes. The molecule (725K) is a tetramer of four identical subunits. Ellipses indicate the 185K subunits. Broken ellipses indicate the specific proteolysis to (85/100)K fragments. Dotted lines indicate noncovalent (SDS-labile) bonds (structures I, II, and the native enzyme appear as 375K dimers in nonreduced PAGE). The proposed sequence of reaction for covalent bond formation is (1) the native molecule binds enzyme to form noncovalent complexes (not shown), (2) the bound enzyme forms a covalent attachment (presumed to be to the active glutamate residue) to form structure I, and (3) this structure can react with a second molecule of enzyme to form structure II or (4) a second covalent bond can form between the bound enzyme and another subunit chain to form structure III. The stoichiometry of subunits hydrolyzed to enzymes covalently bound is not known, and the arrangement shown is arbitrary and schematic.

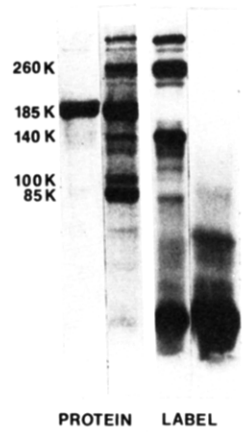

FIGURE 2: Reduced SDS–PAGE of α_2M–^{125}I-labeled thrombin complexes. Reaction mixtures contained 1 μM α_2M and 2 μM ^{125}I-labeled thrombin. Reaction time was 60 min; pH 8.3; Tris-HCl; 25 °C. Details of the gel system are given under Materials and Methods. Protein was stained with Coomassie Brilliant Blue. Protein: lane 1 (left to right), α_2M; lane 2, α_2M–thrombin. Label: lane 3, autoradiogram of the sample in lane 2; lane 4, autoradiogram of ^{125}I-labeled thrombin.

duced and nonreduced conditions, some of which had been seen in the literature but not discussed. In this paper, we have further examined the reaction of thrombin with α_2M and discuss the behavior of products on one-dimensional polyacrylamide gel electrophoresis (PAGE). We can present further evidence for covalent complexes in which a single enzyme molecule forms more than one covalent bond to different α_2-macroglobulin chains. By comparison with synthetically cross-linked α_2M we can make reasonable, if not certain, assignments to particular bands in the nonreduced SDS–PAGE. Our conclusion is also supported by studies on the effect of methylating lysyl amino groups of the enzyme, which reduces the concentration of high molecular weight species, as well as the effect of the concentration of reactants on the distribution of complexes, which is consistent with an intramolecular reaction of α_2M-bound enzyme.

Materials and Methods

Enzymes and Derivatives. Human α-thrombin was the generous gift of Dr. John Fenton, New York State Department of Health. Three methylated derivatives of thrombin containing an average of three, six, and seven free amino groups were also used in these studies. Methylation was performed by reductive methods using cyanoborohydride. The methylated derivatives have high activity toward small ester and amide substrates, although there are differences in the actual value of Michaelis constants when compared to that of native thrombin. These and other details of the preparation and properties of this material will be presented elsewhere (D. Wang et al., unpublished results). Iodination was performed by the lactoperoxidase method (Martin et al., 1976). In agreement with literature reports, iodination did not affect enzyme activity. Experiments were also run to compare the behavior of unlabeled enzyme and iodinated enzymes in gel electrophoresis under the conditions used for the experiments in this paper. In all cases, iodinated enzymes showed protein patterns identical with those of unmodified samples. One of the paradoxes in the literature is that whereas covalent binding, per se, is well documented, many reported gel electrophoresis experiments show no bands that could be attributed to enzyme complexes. Although we cannot resolve this paradox, we have observed many bands in trypsin experiments that might be attributed to proteolysis fragments. It is for this reason that we focused on the reaction with thrombin, which is simpler. To try to find the source of the conflict between reports in the literature, however, we compared the effects of our methods of treatment of samples and electrophoresis conditions with those reported by Swenson & Howard (1979). In all cases essentially identical patterns were observed with the two experimental conditions. The possibility that iodination of the enzymes introduces an artifact is excluded by the experiments described above.

Slab Gradient Polyacrylamide Gel Electrophoresis. A gel gradient of 3.5–10% acrylamide was used, unless otherwise indicated. Separation was performed at ambient temperature, at 27 mA. Gels were stained with Coomassie Brilliant Blue, destained, and vacuum dried. Autoradiography was carried out in a conventional manner by exposing X-ray film to the dried gel. The procedures for treatment of samples were described previously (Wu et al., 1981).

Intramolecular Cross-Linking of α_2-Macroglobulin. Samples of α_2M (10 mg/mL) in 0.025 Tris-HCl, pH 8, were inactivated with methylamine (1000-fold molar excess) at 25 °C for 20 h in the presence of 4,4′-dithiodipyridine. The inactivated material was dialyzed against 0.1 M triethylamine, pH 10.5, with two changes of dialyzing solution. Cross-linking was effected by addition of a 1000-fold molar excess of dimethyl adipimidate at 22 °C. The pH was maintained by addition of NaOH and allowed to proceed for 30 min, after which time the reaction mixture was dialyzed against 0.025 M Tris-HCl, pH 8.

Results and Discussion

SDS–PAGE of Thrombin–α_2M under Reducing Conditions. The results of gel electrophoresis of α_2M and the α_2M–thrombin complex under denaturing and reducing conditions are shown in Figure 2. The most conspicuous feature of the protein pattern under these conditions is the conversion of the 185K subunit of α_2M to the (85 + 100)K fragments. This specific proteolysis is characteristic of the reaction of α_2M with

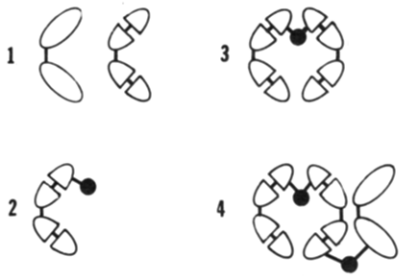

FIGURE 3: Proposed structure of α_2M–thrombin complexes seen under SDS–PAGE, nonreducing conditions. Numbers refer to the discussion in the text and bands in nonreduced gels in Figures 4 and 7.

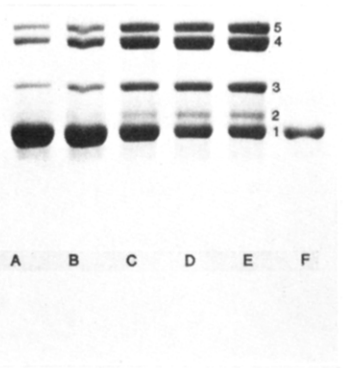

FIGURE 4: SDS–PAGE of α_2M–thrombin complexes under nonreducing conditions. Protein was stained with Coomassie Brilliant Blue. Gel system: 3.5% polyacrylamide. Reaction mixtures contained 1 μM α_2M and varying concentrations of thrombin to give the following thrombin:α_2M molar ratios: (A) 0.25:1; (B) 0.5:1; (C) 0.75:1; (D) 1:1: (E) 2:1; (F) α_2M alone.

active protease (Harpel, 1973). The (85 + 100)K bands frequently run together and are referred to in the literature simply as the 85K fragment. Comparison of the protein-stained material with the autoradiogram indicates that enzyme is not bound to the (85/100)K fragment but appears rather in a band at approximately 140K and in several bands that migrate much slower than the intact subunit. The band at 140K is presumed to be the proteolyzed fragment of the inhibitor covalently bound to the thrombin molecule, as proposed in the literature for several other enzymes (Salvesen & Barrett, 1980; Harpel & Hayes, 1979; Brower et al., 1979); this species would arise from reduction of structure I in Figure 1 or structure **2** in Figure 3. In contrast, the higher molecular weight bands have rarely been discussed in the literature. Brower et al. called attention to the smallest of these species that migrates at about 260K. They attributed this to an intact subunit covalently linked to the enzyme. We propose an alternative interpretation, namely, that they indicate two proteolyzed subunits bridged by two covalent bonds to a single molecule (derived from structure III in Figure 1 and **3** in Figure 3). We would further propose that the higher molecular weight bands represent the cross-linking of three or possibly four subunits and/or proteolytic fragments by enzyme molecules via covalent bonds to lysyl amino groups. To examine this question further, we next consider the behavior of enzyme–α_2M complexes in a denaturing, but nonreducing, gel system.

SDS–PAGE of Thrombin–α_2M under Nonreducing Conditions. The behavior of α_2M–protease complexes on SDS electrophoresis under nonreducing conditions is less widely studied than with methods involving reduction. Because the proteolyzed fragments are held together by disulfide bonds (Sottrup-Jensen et al., 1983), the major protein pattern does not change after reaction with protease—both native and protease-reacted inhibitor migrate at approximately 375K (Figure 1). The SDS gel shown in Figure 4 indicates that this is, in fact, the major band. Several other bands with slower mobility are clearly detected, however. These bands are seen in the literature but have rarely been discussed. We believe they contain the novel bivalently cross-linked species. Densitometry studies reveal that they comprise approximately 50% of the protein mass. The percent of covalent bond formation with thrombin is observed to depend on the initial conditions (D. Wang et al., unpublished results), but 50% is typical for the conditions studied here. As noted below, the higher molecular weight bands (3, 4, and 5 in Figure 4) can be the major enzyme containing species.

In addition to the unproteolyzed subunit (band 1 in Figure 4), it is possible to distinguish a second band in this region with a slightly lower mobility. This band, referred to as 2, has been shown by autoradiography to contain labeled enzyme (Wang et al., 1983; see Figure 7) and is sensibly attributed to a single enzyme molecule bound to the half-molecule of the inhibitor (expected molecular weight = 375K + 36K = 411K). This is shown schematically in Figure 3. In our previous paper (Wang et al., 1983), we attributed band 3 to a structure with molecular weight of 750K composed of two half-molecules bridged by two covalent bonds to a thrombin molecule (Figure 3). Our primary evidence was that when band 3 is electrophoresed in a second dimension under reducing conditions, the 260K complex described above is observed but little intact subunit is found (Wang et al., 1983). The proposed structures for the 260K band and structure **3** are, thus, consistent with each other, although neither is proved. The comparison with high molecular weight standards, discussed below, is somewhat ambiguous, and it is not excluded that band 4 is the smallest structure with the molecular weight of the intact molecule. If our assignment of band 3 is correct, however, the other high molecular weight bands in Figure 3 must be of significantly greater molecular weight. Any assignment of these bands at this time would be highly speculative, but because of its high mass we feel a structure of the type shown in Figure 3 (structure **4**) is possible. Such a species involves the cross-linking of more than one α_2M molecule. It should also be mentioned, however, that other modes of covalent bonding might be possible for α_2M. During the reaction with enzyme, α_2M passes through a highly reactive intermediate that is manifested in increased rate of reaction with amines (Van Leuven et al., 1981b; Salvesen & Barrett, 1981). It is conceivable that such an intermediate reacts with amino groups on other α_2M molecules. The dependence on the concentration of enzyme and the requirement for intact lysyl amino groups, discussed below, make such an idea unlikely, if not absolutely excluded. Also, to eliminate the possibility that inhibitor chains were being cross-linked by disulfide bonds due to release of new thiol groups (Howard, 1981; Tack et al., 1980; Sottrup-Jensen et al., 1980), reaction were carried out in the presence and absence of DTNB with no observed difference. It is, therefore, most likely that covalent bond formation between α_2M chains requires the involvement of enzyme.

Determination of Molecular Weight of Bands on SDS Electrophoresis. Although bands 3–5 clearly are very different in mobility from the native half-molecule, or the presumed monovalent enzyme complex, the attribution of molecular weights to the bands in nonreduced gels is complicated by the lack of reliable high molecular weight standards and by what we believe is the anomalous behavior of α_2M–enzyme complexes. Figure 5 indicates that band 3 has approximately the same mobility as human platelet glycoprotein G, molecular weight 540K. Because this is substantially less than that

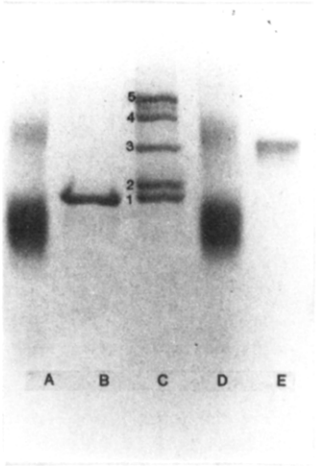

FIGURE 5: SDS–PAGE of α_2M–thrombin complexes and synthetically cross-linked α_2M under nonreducing conditions. Protein was stained with Coomassie Brilliant Blue. The cross-linked material was prepared as described under Materials and Methods. (A and D) α_2M cross-linked with dimethyl adipimidate; (B) α_2M alone; (C) α_2M–thrombin complex; (E) purified human platelet glycoprotein G.

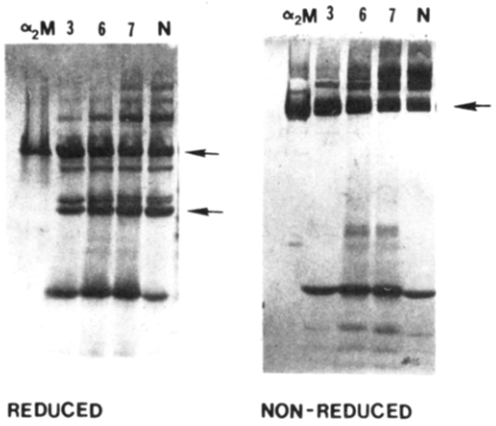

FIGURE 6: Effect of methylation of thrombin lysyl amino groups on reaction with α_2-macroglobulin. The derivatized thrombin samples contained an average of three, six, or seven free amino groups, as indicated. Native thrombin is in the lane marked N. In all cases, the thrombin:α_2M ratio was 4:1. Arrows: (reduced gel) 185K subunit and (lower) 85K proteolysis fragment; (nonreduced gel) 375K half-molecule. Gel system as in Figure 2.

deduced from two-dimensional electrophoresis, and because this band runs so much slower than band 2, which is almost surely equal to 411K, we prepared a sample of intramolecularly cross-linked α_2M for comparison. This material contained two major diffuse bands that are presumed to be of molecular weights 375K and 750K. Figure 5 indicates that the lower of these bands migrates much faster than the native half-molecule. The presumed 750K band migrates close to band 3. The likely explanation is that the synthetically cross-linked α_2M molecules do not unfold in SDS and hence have faster mobility than expected. We would offer the same explanation for what would be anomalously high mobility of band 3 if we are correct in assigning it to a structure of molecular weight 750K. The possibility that band 3 is a species containing two enzyme molecules is remote but not excluded. In this case, band 4 would be of approximately 750K and would have the structure of **3** in Figure 3. This band also shows the 260K band after reduction and electrophoresis in a second dimension (Wang et al., 1983). Other analytical methods, however, will be required to resolve this ambiguity.

Effect of Lysyl Amino Group Modification on Formation of Enzyme Complexes. It is now well established that covalent bond formation between proteases and α_2M is inhibited by blocking the lysyl amino groups of the enzyme. Samples of ^{125}I-labeled proteases in which the lysyl amino groups have been blocked by methylation or acylation show no incorporation of radioactive label into α_2M protein after treatment with SDS. The protein pattern, however, indicates that the 185K subunit has been cleaved by these enzymes to the (85/100)K fragments, although, of course, the protein bands corresponding to the labeled regions of the autoradiogram are no longer present (Wu et al., 1981; Wang et al., 1981; Van Leuven et al., 1981b). A complete loss in covalent binding, similar to that observed with lysyl-blocked trypsin, is seen, in fact, when human α-thrombin is methylated exhaustively (to a point where 22 of 24 amino groups are modified). We inquired, however, about the effect of partial methylation. In the trypsin system, the total covalent binding shows a nonlinear dependence on the number of free amino groups; the fraction of covalent complexes decreases rapidly when only a few amino groups are left (Wu et al., 1981). In the work reported here we used samples of thrombin that had been methylated to a level where an average of three, six, or seven free amino groups was left. The behavior of these preparations on reduced and nonreduced PAGE is shown in Figure 6. It should be emphasized that these derivatives are enzymatically active toward small substrates although, as discussed below, they show differences in response to protein or cell substrates compared to that of native thrombin. The behavior of the methylthrombin preparation with only seven free amino groups is essentially indistinguishable from that of the native enzyme. When the number of free amino groups is reduced to an average of six, however, there is a substantial decrease in the high molecular weight bands, particularly the two slowest migrating. With three amino groups unmodified, the slow bands are almost completely lost, although the 140K band in the reduced gel persists. This behavior is consistent with a role for lysyl amino groups in the formation of high molecular weight covalent complexes. The dependence of covalent complex formation on the degree of methylation is strikingly similar to that of methylthrombins in other systems (D. Wang et al., unpublished results); fibrinogen clotting activity, platelet activation, and reaction with plasma antithrombin all show a dramatic decrease in going from samples with seven free amino groups to those with six.

Our interpretation of the results in Figure 6 is that, after the formation of a covalent bond between a protease molecule and α_2-M, there are several possibilities: the complex may react with a second molecule of protease or the bound protease may form a second covalent bond with the reactive Glu residue on a different chain or both of these effects may occur. This is shown schematically in Figure 1. The noncovalent bonds that are broken when samples are treated with SDS are shown as dotted lines in the figure. Subunits with bound enzyme are shown as proteolyzed. This is based on the assumption that proteolysis is a necessary condition for covalent bond formation, an idea that is consistent with the fact that the inactive anhydrotrypsin does not cause proteolytic cleavage of α_2-M and does not bind covalently (Tsuru et al., 1978; Sayers & Barrett, 1980; Wu et al., 1981). It should be emphasized, however, that this representation is schematic; the stoichiometry of proteolysis and binding is not known. Indeed, examination of the reduced gels in Figures 2 and 6 shows that complete proteolysis never occurs with thrombin.

Effect of Concentration on Distribution of High Molecular Weight Bands. We asked next whether it was possible to change the ratio of the high molecular weight species by

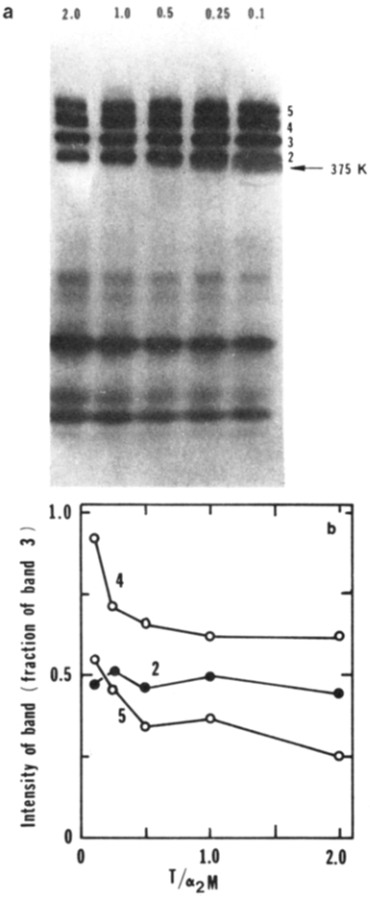

FIGURE 7: Effect of thrombin concentration on high molecular weight bands in electrophoresis of thrombin–α_2M. (a) Radioautograph of SDS–PAGE of thrombin–α_2M complexes. Reaction mixtures were incubated for 1 h at 25 °C, pH 8.3. The gel system is as described in the legend to Figure 2. The concentration of ^{125}I-labeled thrombin was maintained at 1.1 μM. The α_2M concentration was varied so as to give molar ratios of thrombin:α_2M as shown. (b) Integrated densitometry peaks from experiments shown in Figure 5A.

manipulating the reactant concentrations. The rationale of the experiment is that higher thrombin concentrations should favor the conversion of I to II rather than I to III (Figure 1). Under these conditions, higher molecular weight species should be decreased and species of type II favored. That is, it is to be expected that multiple cross-links will be less likely if a reactive Glu residue reacts with a second enzyme molecule rather than one that is already present on the inhibitor. The experiment was performed by incubating a fixed concentration of ^{125}I-labeled thrombin with varying amounts of inhibitor so that the enzyme:α_2 ratio varied from 0.1 to 2.0. The autoradiogram of a nonreduced gel is shown in Figure 7. The integrated values from densitometry in Figure 7a are plotted in Figure 7b. The intensity of bands 2, 4, and 5 is plotted as a fraction of band 3. It is clear that the relative amounts of bands 2 and 3 (the lower molecular weights) do not change significantly as the enzyme:α_2 ratio changes. There is, however, a significant decrease in the higher molecular weight bands 4 and 5 compared to band 3, as the molar ratio of thrombin to α_2M increases. An examination of Figure 7 indicates that the majority of the radioactivity is found in the high molecular weight bands even under conditions of highest thrombin concentrations.

Summary and Conclusions

The major conclusion from this work is that the existence of a unique structure, two polypeptide chains of one protein bridged by covalent bonds to a second protein, is supported by SDS–PAGE patterns of thrombin–α_2M complexes. The new evidence presented here is summarized as follows. (1) Covalent modification of lysyl amino groups of thrombin prevents covalent bond formation; the relevant bands disappear from gels of complexes formed with methylthrombin derivatives that have had lysyl amino groups blocked, and the higher molecular weight species show the largest decrease when lysyl groups are modified. (2) Increasing the thrombin:α_2M ratio causes a relative decrease in the larger species observed on SDS–PAGE. This is explained by the idea that at higher concentrations of enzyme an enzyme–α_2M complex will be more likely to form a covalent bond with a new enzyme molecule than with the enzyme already bound to another inhibitor subunit.

Recently, Sottrup-Jensen et al. (1983) have isolated fragments from α_2M complexes whose sequence is consistent with γ-glutamyl–ϵ-lysyl amide bonds between enzyme and inhibitor. This is the first direct evidence for the type of bond originally proposed on the basis of indirect experiments with modified enzymes (Wu et al., 1981). It is interesting that the two major sites for covalent bond formation with α_2M found by Sottrup-Jensen et al. were on opposite sides of the trypsin molecule. This may be a reflection of the ability of the enzyme to engage in bivalent cross-links to different subunits as proposed here.

References

Brower, M. S., Harpel, P. C., Hayes, M. B., & Fenton, J. W. (1979) *Blood 54* (*Suppl. 1*), 272A.

Cranelli-Piperno, A., & Reich, E. (1978) *J. Exp. Med. 148*, 223–234.

Feinman, R. D., Wang, D., Windwer, S., & Wu, K. (1983) *Ann. N.Y. Acad. Sci. 421*, 178–187.

Harpel, P. C. (1973) *J. Exp. Med. 138*, 508–521.

Harpel, P. C., & Hayes, M. B. (1979) in *Physiological Inhibitors of Blood Coagulation and Fibrinolysis* (Collen, D., Wiman, B., & Verstraete, M., Eds.) pp 231–238, Elsevier/North-Holland, Amsterdam.

Howard, J. B. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2235–2239.

Martin, B. M., Wasiewski, W. W., Fenton, J. W., II, & Detwiler, T. C. (1976) *Biochemistry 15*, 4886–4893.

Salvesen, G. S., & Barrett, A. J. (1980) *Biochem. J. 187*, 695–701.

Salvesen, G. S., Sayers, C. A., & Barrett, A. J. (1981) *Biochem. J. 195*, 453–461.

Sayers, C. S., & Barrett, A. J. (1980) *Biochem. J. 189*, 255–262.

Sottrup-Jensen, L., Petersen, T. E., & Magnusson, S. (1980) *FEBS Lett. 121*, 275–279.

Sottrup-Jensen, L., Hansen, H. F., & Christensen, U. (1983) *Ann. N.Y. Acad. Sci. 421*, 188–208.

Tack, B. F., Harrison, R. A., Janatova, J., Thomas, M. L., & Prahl, J. W. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5764–5768.

Tsuru, D., Kado, K., Fujiwara, K., Tomimatsu, M., & Ogita, K. (1978) *J. Biochem.* (*Tokyo*) *83*, 1345–1353.

Van Leuven, F., Cassiman, J.-J., & Van den Berghe, H. (1981a) *J. Biol. Chem. 256*, 9016–9022.

Van Leuven, F., Cassiman, J.-J., & Van den Berghe, H. (1981b) *J. Biol. Chem. 256*, 9023–9027.

Wang, D., Wu, K., & Feinman, R. D. (1981) *Arch. Biochem. Biophys. 211*, 500–506.

Wang, D., Yuan, A., & Feinman, R. D. (1983) *Ann. N.Y. Acad. Sci. 421*, 90–97.

Wu, K., Wang, D., & Feinman, R. D. (1981) *J. Biol. Chem. 256*, 10409–10414.