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Effect of Protease Binding by α_2-Macroglobulin on Intrinsic Fluorescence†

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ABSTRACT: We have evaluated intrinsic protein fluorescence as a method for investigating the reactions of α_2-macroglobulin (α_2M) with proteases and amines. Changes in fluorescence intensity of α_2M in the presence of proteases and amines were shown to correlate with structural and functional changes in the α_2M molecule. By intrinsic fluorescence we found that 2 mol of trypsin bound to 1 mol of α_2M whereas thrombin and plasmin each bound in a stoichiometry closer to 1:1. Studies showed that changes in fluorescence caused by ammonium ion paralleled the loss of the ability of α_2M to protect trypsin from soybean trypsin inhibitor. The exposure of sulfhydryl groups on α_2M by a small organic amine (methylamine) also correlated with fluorescence change that could be quantitatively eliminated by prior reaction of α_2M with trypsin. Cleavage of α_2M by four serine proteases (plasmin, thrombin, trypsin, and elastase) as determined by sodium dodecyl sulfate gel electrophoretic analyses and the binding of plasmin and thrombin as measured by macromolecular inhibitor assays corresponded to the increase in fluorescence intensity. In addition, the rate of thrombin inhibition for clotting fibrinogen was the same as the rate of fluorescence change observed when thrombin was incubated with α_2M. Our results indicate that intrinsic protein fluorescence is an easy and rapid technique for assessing both qualitative and quantitative aspects of protease–α_2M interactions.

While the physiological function of α_2M[1] is unknown, its ability to bind nearly all proteases (Barrett & Starkey, 1973) and its affinity for macrophages (Debanne et al., 1975; Kaplan & Nielsen, 1979a) and fibroblasts (Maxfield et al., 1978) make this large [M_r ~725 000 (Jones et al., 1972)], tetrameric glycoprotein the object of considerable current interest. In addition to the functional importance suggested by the binding, internalization, and subsequent degradation of α_2M–protease complexes by cells (Debanne et al., 1976; Kaplan & Nielsen, 1979b; Van Leuven et al., 1979; Mosher & Vaheri, 1980), α_2M has a number of other interesting and unusual characteristics. When α_2M binds an enzyme, the degree to which the activity of that enzyme is inhibited depends on the molecular size of the substrate (Rinderknecht et al., 1975; Mehl et al., 1964). Another property that distinguishes α_2M is its ability to bind and inhibit two protease molecules at a time (Ganrot, 1966; Barrett et al., 1979; Swensen & Howard, 1979a). Recent data suggest that α_2M contains a reactive site on each of its four subunits. Each of these sites, like the one present in the complement proteins C3 and C4, is sensitive to

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‡ An Investigator of the Howard Hughes Medical Institute.

[1] Abbreviations: α_2M, α_2-macroglobulin; $NaDodSO_4$, sodium dodecyl sulfate; TLCK, 1-chloro-3-(tosylamido)-7-amino-L-2-heptanone; SBTI, soybean trypsin inhibitor; *p*-NPGB, *p*-nitrophenyl *p*-guanidinobenzoate; PEG, poly(ethylene glycol).

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small amines, proteases, and denaturants (Tack et al., 1980; Sottrup-Jensen et al., 1980; Swensen & Howard, 1979b). This site has been tentatively identified as a thiol ester cross-link between cysteine and glutamic acid residues that are separated by two residues in the polypeptide chain (Sottrup-Jensen et al., 1981a; Howard, 1981). Cleavage of the thiol ester bonds results in inactivation of α_2M, exposure of four thiol groups per molecule, and a significant conformational change in the α_2M molecule (Barrett et al., 1979; Marelis et al., 1969; Jacquot-Armand & Krebs, 1973). This latter observation prompted us to determine whether a change in protein intrinsic fluorescence might occur and correlate with the reactions that change the conformation of α_2M. Initial results that we and others have recently obtained for the interaction of α_2M with trypsin support this notion but have only been partially reported (Straight & McKee, 1980; Richman & Verpoorte, 1981). We now present in full our studies of the reaction of small amines and the serine proteases plasmin, thrombin, and elastase with α_2M. We have correlated fluorescence change with results of structural and functional studies, utilizing methods of studying α_2M such as NaDodSO$_4$ gel electrophoresis, macromolecular inhibitor assays, sulfhydryl group exposure, and inhibition of the ability of thrombin to clot fibrinogen. Our results show that the reactions of α_2M with amines and proteases are easily monitored by intrinsic fluorescence and correlate both qualitatively and quantitatively with structure–function relationships of the α_2M molecule.

Experimental Procedures

Materials. Human thrombin was provided by Dr. David Aronson, Bureau of Biologics, Federal Drug Administration, Bethesda, MD. Human antithrombin III was a gift from Dr. Michael Griffith (University of North Carolina, Chapel Hill, NC). Bovine pancreatic trypsin and soybean trypsin inhibitor were purchased from Worthington, and porcine pancreatic elastase was obtained from Sigma. Synthetic substrate S-2160 (*N*-benzoyl-L-phenylalanyl-L-valyl-L-arginine-*p*-nitroanilide) was from Ortho Diagnostics and S-2251 (D-valylleucyl-lysine-*p*-nitroanilide) was from Kabi Diagnostics. The enzyme active site titrant *p*-nitrophenyl *p*-guanidinobenzoate was from Nutritional Biochemicals, and the elastase titrant diethyl *p*-nitrophenyl phosphate was from Sigma as was the 2,2′-dipyridyl disulfide. Human fresh-frozen plasma (single donor) was purchased from the American Red Cross. All chromatography resins were from Pharmacia. All chemicals were of reagent grade or better. The solutions used were buffered with 0.05 M Tris, pH 8.0; all α_2M–protease interactions were studied at ambient temperature unless otherwise stated.

α_2M Purification. α_2M was purified from fresh-frozen human plasma essentially as described (Swenson & Howard, 1979a) with the following modifications: DEAE-Sephacel chromatography (2.5 × 30 cm column) was performed in 0.025 M Tris, pH 8.0, by using a linear gradient of 0–0.15 M NaCl (500 mL of each). Before application to the DEAE-Sephacel column, the preparation was purified by Blue Sepharose CL-6B chromatography as previously described (Virca et al., 1978). Purity was determined on NaDodSO$_4$ and non-NaDodSO$_4$ gels and was >95%. Each α_2M molecule bound two molecules of trypsin according to the SBTI assay (Ganrot, 1966b) in which the synthetic substrate S-2160 was used. The concentration of the protein was determined by absorbance at 280 nm when an $E^{1\%}_{1cm}$ of 8.9 (Hall & Roberts, 1978) for α_2M was assumed [M_r 725 000 (Jones et al., 1972)].

Plasminogen Purification and Activation to Plasmin. Human plasminogen was purified from fresh-frozen plasma by lysine–Sepharose chromatography as described by Deutsch & Mertz (1970). Plasminogen was activated to plasmin at 37 °C by 300 units/mL urokinase in 0.05 M Tris–0.1 M lysine, pH 8.0, for 20 min.

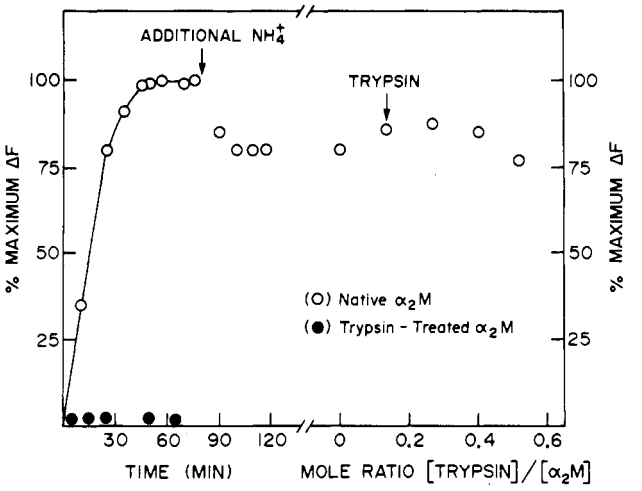

FIGURE 1: Effect of ammonium ion on the intrinsic fluorescence of α_2M (0.1 μM). Ammonium ion (0.2 M) caused an increase in protein intrinsic fluorescence of native α_2M (O). Prior reaction of α_2M with trypsin (●) prevented this effect. Additional ammonium ion (first arrow) caused no further increase in fluorescence. Treatment of α_2M with ammonium ion also eliminated any fluorescence change inducible by trypsin (second arrow). The reactants were at room temperature in 0.05 M Tris, pH 8.0.

Enzyme Active Site Concentrations. Active site concentrations for plasmin, thrombin, or trypsin were determined with *p*-NPGB in veronal buffer by the method of Chase & Shaw (1970). Elastase active sites were determined with diethyl *p*-nitrophenyl phosphate as described by Bender et al. (1966). The buffer used was 0.05 M Tris–0.15 M NaCl, pH 7.4.

Gel Electrophoresis. NaDodSO$_4$–4% polyacrylamide gel electrophoresis was performed in a Tris–borate buffer essentially as described previously (Sykes & Bailey, 1971). The final running buffer concentration was 0.1 M Tris–0.1% NaDodSO$_4$ brought to pH 8.7 with boric acid. The samples were denatured and reduced in 40 mM Tris–borate–2% NaDodSO$_4$–5% 2-mercaptoethanol, pH 8.7, at 37 °C for 45 min. Following electrophoresis of protein samples, the gels were stained with Coomassie Blue R250, destained, and then scanned on a Quick Scan (Helena Laboratories) gel scanner.

Fluorescence. Intrinsic protein fluorescence was measured with a Perkin-Elmer 650-10S fluorescence spectrophotometer. When necessary, the cell temperature was controlled by using a Lauda K-2/RD circulating water bath. In all cases fluorescence emission was measured at 340 nm after excitation at 285 nm.

Measurement of the Effect of Ammonium Ion on the Intrinsic Fluorescence of α_2M. α_2M (0.1 μM) was mixed with ammonium ion (0.2 M) and incubated in the fluorescence spectrophotometer with the excitation shutter closed; periodic readings were taken until a maximum was reached. α_2M that had been saturated with trypsin was treated similarly. α_2M that had been reacted with ammonium ion and allowed to reach maximum fluorescence was then treated with additional ammonium ion and subsequently with trypsin at the mole ratios shown in Figure 1. The fluorescence was measured as above.

Rate of Ammonium Ion Reaction with α_2M. The rate of reaction of ammonium ion with α_2M was measured by two methods: (1) by the rate of change in intrinsic protein fluorescence induced by ammonium ion and (2) by the rate of loss of α_2M's ability to protect trypsin from SBTI. Since

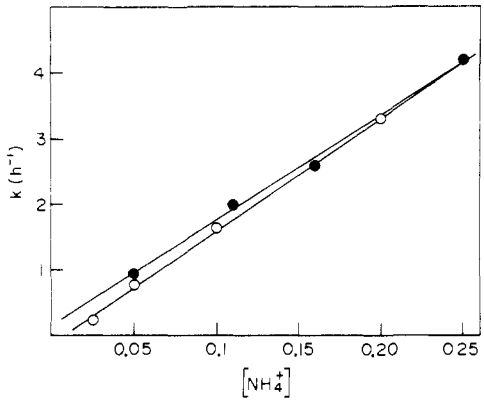

FIGURE 2: Plot of pseudo-first-order rate constants (k) vs. ammonium ion concentration for the reaction of α_2M with ammonium ion. Constants were determined from semilog plots of rate of fluorescence change (○) and rate of loss of the ability of α_2M to protect trypsin from SBTI (●). The slopes of these lines are equal to the second-order rate constants of the reactions and are essentially identical. Reactions were run at room temperature in 0.05 M Tris, pH 8.0.

the ammonium ion concentrations for both experiments (Figure 2) were in extreme excess to α_2M (0.1 μM), the conditions were pseudo first order; thus, the pseudo-first-order rate constants could be determined from semilogarithmic plots of percent activity remaining vs. time. The constants (k) were then plotted against ammonium ion concentrations to obtain the second-order rate constants (equal to the slopes). Alternatively, after the addition of ammonium ion to α_2M, portions of the mixture were removed and reacted with trypsin for 15 min (>2 mol of trypsin/mol of α_2M). The reaction of trypsin with α_2M is assumed to be much faster than the amine reaction and thus quenches the latter reaction. The trypsin not bound to α_2M was then inactivated by reaction with SBTI for 15 min, and residual trypsin activity (i.e., that bound to α_2M since it possesses full activity toward small substrates) was measured with S-2160.

Measurement of the Rate of Fluorescence Change and Sulfhydryl Exposure by Methylamine. The rate of fluorescence change in the presence of methylamine was determined as above for ammonium ion except that the sulfhydryl reagent 2,2′-dipyridyl disulfide was present. In an identical experiment the rate of sulfhydryl appearance was determined by measuring the absorbance change at 343 nm. Both experiments were performed at room temperature with 1 μM α_2M in 0.02 M sodium phosphate–0.1 M NaCl–0.1 M methylamine, pH 7.4. The sulfhydryl reagent was dissolved in H_2O to give ~1.3 mM solution. The molar extinction coefficient at 343 nm was 7060 M^{-1} cm^{-1} (Grassetti & Murray, 1967).

Measurement of Residual Methylamine-Induced Fluorescence Change after Treatment of α_2M with Various Concentrations of Trypsin. To determine the quantitative relationship between methylamine-induced fluorescence change and that caused by proteases, α_2M (50 nM) was reacted with various mole ratios of trypsin at 4 °C overnight. Each sample was then treated with 0.2 M methylamine at 25 °C and the change in fluorescence measured until a maximum was reached. The change in a sample not treated with trypsin was taken as 100%.

Measurement of Cleavage and Fluorescence Change due to Proteases. The change in fluorescence caused by the four serine proteases was compared to the cleavage of α_2M subunits as observed on polyacrylamide gels of samples treated with $NaDodSO_4$ and 2-mercaptoethanol. The gel experiments were performed as described previously (Swensen & Howard, 1979a) and consisted of reacting 20 μg of samples of α_2M with increasing mole ratios (0 to >3) of protease for 1 h followed by denaturation and reduction of the samples at 37 °C for 45 min. The samples were then analyzed by $NaDodSO_4$ electrophoresis as described above. Trypsin, which is the only enzyme not immediately inactivated by $NaDodSO_4$, was inactivated by TLCK prior to denaturation.

Fluorescence experiments were performed by reacting a series of samples of α_2M at a constant concentration with increasing mole ratios of protease for 1 h. Elastase and plasmin were reacted with 80 nM α_2M while thrombin and trypsin were each incubated with 60 nM α_2M. The fluorescence due to α_2M or protease alone was determined for samples that contained only one or the other and subtracted from the values obtained for the α_2M–protease mixtures. This gave the amount of fluorescence change due to the interaction of α_2M with the protease.

Rate of Reaction of α_2M and Thrombin. The reaction of α_2M with thrombin was determined by the loss of fibrinogen clotting activity as described by Downing et al. (1978). In a parallel experiment, thrombin was added to a solution of α_2M and incubated in the fluorescence spectrophotometer with the excitation shutter closed. Readings were taken periodically until a maximum was reached. Both clotting and fluorescence experiments were performed at 0.4 μM α_2M and 0.18 μM thrombin at 37 °C in 0.05 M Tris–0.1 M NaCl–1% PEG-4000, pH 7.4. The second-order rate constants were determined as previously described (Downing et al., 1978) except the inhibitor concentration was taken to be twice that of α_2M, i.e., two potential thrombin binding sites per α_2M molecule.

Measurement of Plasmin and Thrombin Binding to α_2M by Molecular Inhibitor Assays. α_2M (1.4 μM) was reacted with various mole ratios of plasmin for 1 h at room temperature. Excess plasmin was inactivated by SBTI (30 μM, 15 min). Samples were then assayed for residual activity with the substrate S-2251. Various mole ratios of thrombin were also reacted with α_2M (1.1 μM) overnight at 4 °C. Nonbound thrombin was reacted with antithrombin III (6 μM) for 30 min at room temperature, and residual thrombin activity was measured with the substrate S-2160 by using the following substrate concentrations: S-2160, 0.1 mM; S-2251, 0.3 mM. In all assays 0.5 mL of substrate was reacted with the selected amount of the enzyme–α_2 complex. The reaction was quenched with 0.2 mL of glacial acetic acid and the absorbance at 410 nm then measured.

Results

Effect of Ammonium Ion on the Intrinsic Fluorescence of α_2M. Small amines and ammonium ion have been known to inactivate α_2M for some time (Mehl et al., 1964). As shown in Figure 1, ammonium ion treatment of α_2M increased protein fluorescence significantly. The results shown are normalized, the actual increase in fluorescence being 30%. As is evident in Figures 5 and 6, protease treatment of α_2M also caused a fluorescence increase, but results in Figure 1 show that when α_2M saturated with trypsin was treated with ammonium ion, no increase occurred. While trypsin has been shown to bind and cleave amine-inactivated α_2M (Wang et al., 1981b), there is not a significant increase in fluorescence associated with this event. Furthermore, addition of more ammonium ion to α_2M previously inactivated by ammonium ion did not cause any further increase in fluorescence.

Rate of Ammonium Ion Reaction with α_2M. The second-order rate of the irreversible inactivation of α_2M by ammonium ion was measured by two methods: fluorescence and loss of α_2M's ability to protect trypsin from SBTI. Figure 2 shows that both methods gave a second-order rate constant of ~0.3

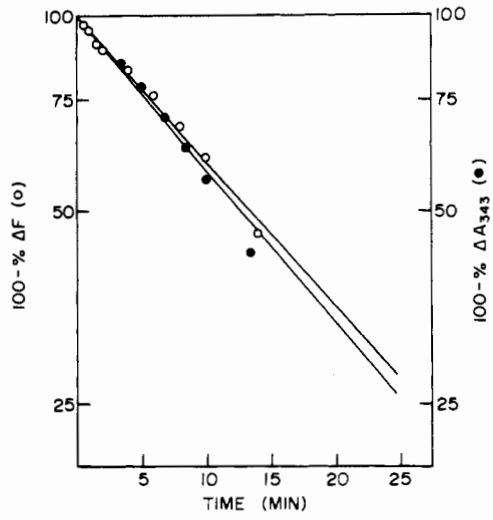

FIGURE 3: Comparison of the rate of change in intrinsic fluorescence (○) and exposure of sulfhydryl groups (●) in α_2M in the presence of methylamine. One micromolar α_2M (0.02 M sodium phosphate–0.1 M NaCl, pH 7.4) was reacted with 0.1 M methylamine, and the change in fluorescence was measured. Alternatively, the appearance of sulfhydryl groups was determined with 2,2'-dipyridyl disulfide (0.1 mM) by measuring ΔA at 343 nm. The sulfhydryl reagent was also present during the fluorescence experiment. Reactions were carried out at room temperature.

M^{-1} min^{-1}, suggesting that the structural change as measured by fluorescence does correspond to a change in function of the α_2M molecule.

Comparison of the Rate of Fluorescence Change and Sulfhydryl Group Exposure Induced by Methylamine. Recently two groups (Howard, 1981; Sottrup-Jensen et al., 1980) have shown that reaction of α_2M with methylamine or proteases causes exposure of four sulfhydryl groups on α_2M. Figure 3 gives the semilogarithmic plots showing the coincidence of the rate of fluorescence change and the rate of sulfhydryl appearance when α_2M is incubated with methylamine; in both cases the $t_{1/2}$ is ~13 min. It should be pointed out that the fluorescence experiment was done with a thiol reagent that absorbs at 343 nm and therefore quenching of protein fluorescence was observed (~8%). During the course of this reaction, we have routinely observed that 3.8 of the theoretically expected 4.0 sulfhydryl groups per α_2M molecule were exposed.

Quantitative Effect of Prior Treatment of α_2M with Trypsin of Amine-Induced Fluorescence Change. As shown in Figure 4, α_2M reacted with various mole ratios of trypsin and then treated with methylamine showed a stoichiometric loss of amine-inducible fluorescence. These results indicate that no further change in fluorescence can be caused by methylamine after α_2M has been incubated with trypsin in a mole ratio of one α_2M to two trypsins.

Rate of α_2M Reaction with Thrombin. Binding of thrombin to α_2M inhibits the ability of thrombin to catalyze fibrinogen clotting. Downing et al. (1978) have reported a second-order rate constant for the inhibition of thrombin by α_2M using the fibrinogen clotting assay. In Figure 5, we have compared this rate of loss of thrombin activity with the rate of fluorescence change when thrombin reacts with α_2M. The second-order rate constants as determined from the slopes of the lines in Figure 5 were 1.4×10^5 M^{-1} min^{-1} and 1.5×10^5 M^{-1} min^{-1} for the clotting assay and fluorescence change, respectively. Again this shows close correspondence between a change in fluorescence and the inhibitory function of α_2M.

Proteolytic Cleavage and Fluorescence Changes in α_2M. Proteolytic cleavage of α_2M, which is apparently necessary

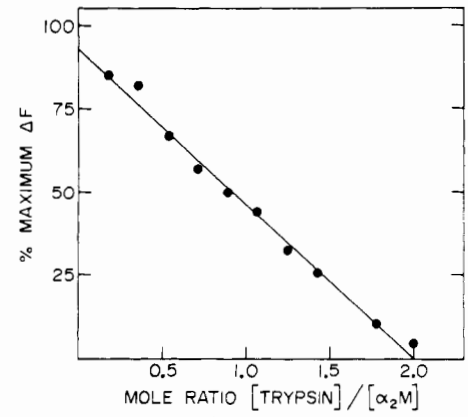

FIGURE 4: Residual methylamine-induced fluorescence change after treatment of α_2M with various concentrations of trypsin. α_2M (50 nM) was reacted with increasing mole ratios of trypsin (overnight, 4 °C). Samples were then placed in the fluorescence spectrophotometer, and the change in fluorescence in the presence of 0.2 M methylamine was measured (25 °C). Data are presented as the percent of amine-induced fluorescence change measured in an α_2M sample not treated with trypsin. All reactions were in 0.05 M Tris, pH 8.0.

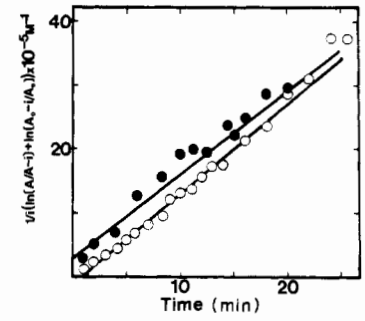

FIGURE 5: Determination of the second-order rate constants for the α_2M–thrombin reaction determined by loss of fibrinogen clotting activity (●) and change in fluorescence (○). α_2M (0.4 μM) was reacted with 0.18 μM thrombin at 37 °C. The second-order rate constants may be determined from the slopes of the lines and appear to be similar. Reactants were in 0.05 M Tris–0.1 M NaCl, pH 7.4, and 14.0% PEG. The symbols as previously described (Downing et al., 1978) are defined as follows: A_0 = initial inhibitor concentration; A = inhibitor concentration at a given time; $i = A_0$ – initial thrombin concentration.

for functional binding of the protease to α_2M, may be observed on $NaDodSO_4$–polyacrylamide gels of samples reduced with 2-mercaptoethanol. The M_r 185 000 subunit of α_2M is cleaved to yield two chains of approximately M_r 85 000. The extent of cleavage was quantitated by scans of electrophoretic gels and then examined for correlation with fluorescence change in response to increasing mole ratios of protease to α_2M. As shown in Figure 6, plasmin, thrombin, trypsin, and elastase each gave cleavage patterns and increases in fluorescence that reached maximum values at approximately 2 mol of protease/mol of α_2M. Although about 100% cleavage of α_2M was noted when trypsin or elastase were reacted with α_2M, the maximum cleavage observed with plasmin or thrombin was 75–80% of the α_2M subunits. Incomplete cleavage of α_2M has also been noted by Sottrup-Jensen et al. (1981b) and for chymotrypsin by Howell & Hunter (1981). Possible explanations for incomplete cleavage of α_2M by certain proteases will be discussed later.

Binding of Plasmin and Thrombin to α_2M As Measured by Macromolecular Inhibitors. Protease–α_2M complexes retain hydrolytic activity toward small substrates even in the presence of other specific macromolecular inhibitors, presumably due to the protection of the protease by α_2M. These features form the basis of a method which shows that 2 mol

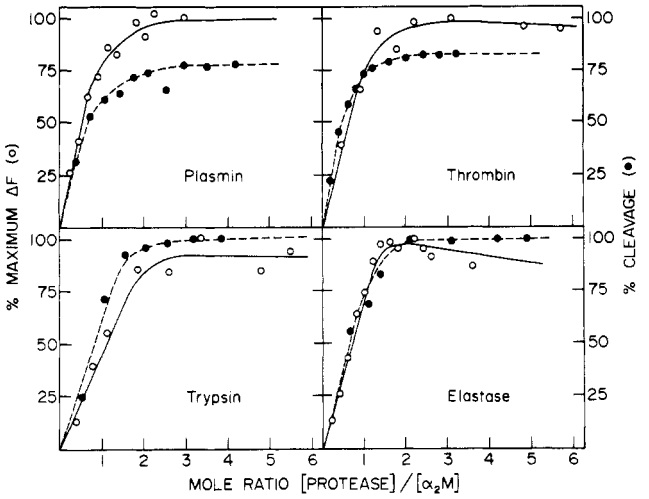

FIGURE 6: Comparison of the interaction of α_2M with plasmin, thrombin, trypsin, or elastase as measured by intrinsic fluorescence (O) with the extent of cleavage observed on $NaDodSO_4$ gels under reducing conditions (●). Various mole ratios of protease were reacted with α_2M. Reactions were performed in 0.05 M Tris, pH 8.0, at room temperature. Plasmin samples contained a final concentration of 0.02 M lysine. In the fluorescence experiments the concentration of α_2M was 80 nM for reaction with elastase and plasmin and 60 nM for reaction with thrombin and trypsin. Fluorescence was measured relative to blanks containing only protease and only α_2M. $NaDodSO_4$ gel experiments consisted of reacting 20 μg of α_2M with the required amount of protease. Samples were denatured and reduced prior to electrophoresis. The gels were then stained, destained, and scanned as described under Experimental Procedures.

of trypsin bind to 1 mol of α_2M (Ganrot, 1966a; Swensen & Howard, 1979a). Just as reported by those investigators, we also found that trypsin bound to α_2M in a mole ratio of 2:1. Although Bieth et al. (1981) and Wang et al. (1981a) have shown recently that SBTI will inhibit trypsin when bound to α_2M, this effect is slow and not likely to be a significant factor in the assay method we used in our studies. Were SBTI to be inhibiting the trypsin complexed with α_2M, we should have observed a ratio less than the consistently sharp 2:1 that we measured. On the basis of these observations we used this assay as another test of the correlation of plasmin or thrombin binding to α_2M and change in intrinsic fluorescence. In brief, α_2M was reacted with plasmin after which excess plasmin was inhibited by SBTI; the α_2M-bound plasmin was then measured by its activity toward S-2251. Since we could not demonstrate that α_2M-bound plasmin become inhibited by SBTI under these conditions, the residual proteolytic activity was used to indicate the amount of plasmin bound to α_2M. In a similar experiment α_2M was reacted with thrombin and the subsequently treated with antithrombin III to inhibit nonbound thrombin. Since antithrombin III did not inhibit α_2M-bound thrombin, the hydrolysis of S-2160 could be used as a measure of the amount of thrombin bound to α_2M. In Figure 7, the binding of plasmin and thrombin to α_2M as measured by SBTI and antithrombin III, respectively, is compared with the fluorescence results from Figure 6. Again, there is a good correlation between the two methods, suggesting that intrinsic fluorescence is sufficiently sensitive to reflect the decreased binding ratios for thrombin or plasmin with α_2M. In each case our analyses indicated mole ratios of about 1.4:1 or less; neither plasmin nor thrombin gave the sharply defined 2:1 mole ratio that we found when trypsin reacted with α_2M.

Discussion

Our studies show that intrinsic fluorescence correlates qualitatively and quantitatively with changes in the structure and function of α_2M during its interaction with a protease.

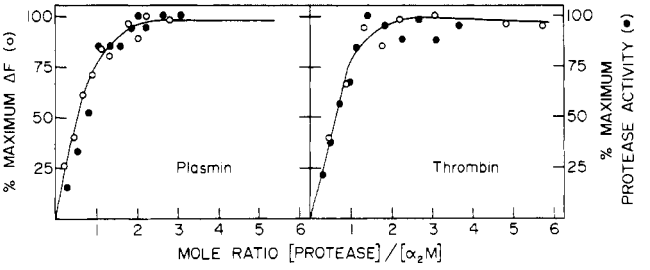

FIGURE 7: Comparison of fluorescence and macromolecular inhibitor results for the interaction of α_2M with plasmin and thrombin. The fluorescence results from Figure 6 (O) are compared to results from experiments assessing plasmin and thrombin binding to α_2M by macromolecular inhibitor assays. α_2M (1.1 or 1.4 M, respectively) was reacted with thrombin or plasmin. Nonbound plasmin was inhibited with SBTI and free thrombin by antithrombin III. Residual protease activity was measured with the synthetic substrate S-2251 (plasmin) or S-2160 (thrombin).

As shown in Figures 1 and 6, the reaction of α_2M with either small amines or proteases causes an increase in fluorescence intensity. In either case, the change in fluorescence is due to an increase in intensity rather than a shift in wavelength maximum. Importantly, our data in Figure 1 provide evidence that a native, fully functional α_2M molecule is necessary for the amine to cause an increase in fluorescence. Moreover, as shown in Figure 2, once reacted with the small nucleophile, α_2M loses its ability to protect trypsin from SBTI in parallel with the change in fluorescence.

Recent reports indicate that the reaction of α_2M with small amines causes cleavage of thiol ester bonds and the exposure of four sulfhydryl groups (Sottrup-Jensen et al., 1980; Howard, 1981). In fact radiolabeled methylamine incorporation by α_2M paralleled the development of four titratable sulfhydryl groups in the α_2M molecule (Sottrup-Jensen et al., 1980). Our data in Figure 3 show that the rate of exposure of the sulfhydryl groups indeed correlates the rate of fluorescence change caused by methylamine. Results somewhat different from these have been interpreted as showing that the reaction of α_2M with methylamine is not pseudo first order (Van Leuven et al., 1981). At this time we cannout reconcile these latter findings with our data and those of Sottrup-Jensen et al. (1980), but the apparent discrepancy may relate to differences in methods. At this time we interpret our data in Figures 1–3 as indicating that the increase in intrinsic fluorescence of amine-treated α_2M is due to a conformational change triggered by the scission of thiol ester bonds.

Since preincubation of α_2M with trypsin prevents the effect of amines on fluorescence (Figure 1), we used this observation to explore the reaction of α_2M with trypsin in a more quantitative manner as shown in Figure 4. Here we demonstrate that reaction of α_2M with trypsin causes a stoichiometric decrease in the amine-inducible fluorescence change. This decrease is complete at a mole ratio of two molecules of trypsin per molecule of α_2M. Since α_2M has been shown to bind two molecules of trypsin (Ganrot, 1966a), this result once again indicates a quantitative relationship between the intrinsic fluorescence change and α_2M function. Our results clearly show that thiol ester cleavage (by amines) does cause the fluorescence change without proteolytic cleavage or protease binding, (Figures 1–3) and that this effect is quantitatively eliminated by protease binding and cleavage (Figure 4). Hence, the cleavage of the thiol ester bonds must be the primary trigger event for the change in fluorescence that accompanies the interaction with proteases. We do point out, however, that the amine-induced fluorescence change is somewhat lower than that with proteases (30% vs. 40%),

suggesting that the protease-α_2M interaction may also contribute to the fluorescence change. In future studies, we hope to isolate the steps in this interaction, i.e., protease binding, proteolytic cleavage, and thiol ester cleavage, in order to define the contribution of each event to the observed fluorescence change.

As shown by Downing et al. (1978) binding of thrombin to α_2M inhibits the ability of thrombin to catalyze fibrinogen clotting. Since these investigators were able to determine a second-order rate constant for the inhibition of thrombin by α_2M using the fibrinogen clotting assay, we could compare a function that is potentially important in vivo, i.e., inhibition of thrombin toward fibrinogen (Vogel et al., 1979), with our fluorescence assay. In Figure 6 are shown results for the apparent second-order rate constants (slopes) for the loss of fibrinogen clotting activity by thrombin when in the presence of α_2M and the change in intrinsic protein fluorescence that occurs when thrombin reacts with α_2M. These rates are faster than those reported by Downing et al. (1978); however, they are presented not as absolute values but instead as evidence that the observed fluorescence change is a direct measure of the functional interaction of α_2M and thrombin. The basis for the difference between the second-order rate constants that we derived and those obtained by Downing et al. (1978) is uncertain, but errors in the determination of this value should give a slower rate rather than the faster one we observed.

When a native protease reacts with α_2M, proteolytic cleavage of the α_2M molecule occurs during binding (Harpel, 1973). In results shown in Figure 5 we have compared the extent of cleavage on $NaDodSO_4$ gels under reducing conditions with changes in fluorescence for four serine proteases. As expected, both parameters were saturable and the curves for both experiments were similar. The results for trypsin and elastase are straightforward since both cleavage and fluorescence reach a maximum at ~2 mol of protease/mol of α_2M. This is in accord with reports that these proteases have a binding ratio of α_2M of 2:1 (Ganrot, 1966a; Bieth et al., 1970). Importantly, our data show that cleavage of the α_2M subunits was 100% when two molecules of either protease became bound to α_2M.

Our results for plasmin and thrombin also show that the cleavage of α_2M by either enzyme occurs in parallel with the change in intrinsic fluorescence. Both the maximum increases in fluorescence and cleavage occurred at a ratio of 2 mol of protease/mol of α_2M. However, in contrast to the interaction of trypsin or elastase with α_2M, only about 80% of the α_2M subunits were cleaved despite an excess of thrombin or plasmin. Interestingly, Howell & Hunter (1981) noted that only 75% of the α_2M subunits were cleaved in fully saturated complexes of α_2M–chymotrypsin. Similarly, although not given in detail, Sottrup-Jensen et al. (1981b) observed that neither plasmin nor thrombin completely cleaved the α_2M subunits. The reason for the partial cleavage of α_2M by certain proteases remain unclear; however, it probably is a function of the protease:α_2M binding ratio. In the case of thrombin the binding ratio is unknown; for plasmin, however, some have suggested that it binds to α_2M in a 1:1 ratio (Ganrot, 1967; Pochon et al., 1978). Because of the apparent difference between these results and ours, we used a third approach to investigate the binding of thrombin and plasmin to α_2M. In Figure 7 the fluorescence results from Figure 6 are compared with the results of macromolecular inhibitor assays when plasmin or thrombin is reacted with α_2M. Although inhibition of α_2M-bound trypsin by SBTI has been described (Bieth et al., 1981; Wang et al., 1981a), we emphasize that α_2M-bound plasmin was not inhibited by SBTI nor was α_2M-bound thrombin inhibited by antithrombin III under the conditions of our assays. The results shown in Figure 7 correspond closely to those in Figure 6 and therefore indicate that plasmin and thrombin each bind to α_2M in a stoichiometry closer to 1:1 than to 2:1.

There appear to be at least three possible explanations for the results we obtain with the plasmin–α_2M and thrombin–α_2M complexes. First, either protease may bind at a 2:1 mole ratio, but unlike trypsin, the second protease that binds does not give the same amount of cleavage, fluorescence change, or synthetic substrate activity. Second, certain proteases may readily bind with a 1:1 ratio and, due to their size or binding orientation, block the second site on α_2M (Ganrot, 1967; Jacquot-Armand & Guinand, 1976; Pochon et al., 1981). In this instance additional free protease could cleave some of the 1:1 α_2M–protease complexes to yield a greater than 50% cleavage of α_2M subunits. This explanation receives some support from our observation (not shown) that plasmin, but not thrombin, can readily cleave amine-activated α_2M. Third, protease binding may occur in a manner that results in some α_2M molecules binding 1 mol of protease while others bind 2 mol (Howell & Hunter, 1981) to give an average binding ratio between 1 and 2. Distinguishing between these possible mechanisms is beyond the scope of this study. Although our results indicate that thiol ester cleavage is crucial, additional studies will be required to definitively document that this event is indeed primarily responsible for the fluorescence change. On the basis of our present findings, however, we do conclude that intrinsic fluorescence is a sufficiently sensitive and rapid method for monitoring the qualitative and quantitative changes that occur during the formation of protease–α_2M complexes.

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Lipid-Induced Ordered Conformation of Some Peptide Hormones and Bioactive Oligopeptides: Predominance of Helix over β Form†

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ABSTRACT: The conformation of several naturally occurring peptide hormones and bioactive oligopeptides in phospholipid solutions was studied by circular dichroism. Phosphatidylcholine induced a partial helix in human gastrin I at neutral pH, but phosphatidylserine did not unless the five consecutive glutamic acid residues in gastrin were protonated. Reduced somatostatin with two lysines and substance P with one arginine and one lysine were partially helical in phosphatidylserine, but not phosphatidylcholine, solution. Both lipids induced a helical conformation in glucagon and its COOH-terminal fragment (19–29) probably because the helical segment is primarily located at the uncharged COOH terminus. Thus, polypeptides with a helix-forming potential can have the helical conformation only when the peptides carry no charge or charges opposite to those on the polar head of the lipid. Renin substrate, which has potentials for the β form and β turn, seemed to form a mixture of the two conformations in phosphatidylserine solution. Angiotensin I with a strong probability for the β form adopted the β form in phosphatidylserine solution and sleep peptide with no structure-forming potential remained unordered in lipid solutions. The helix usually predominated over the β form in lipid solutions if the peptide has potentials for both conformations. This could account for the preponderance of helices in bacteriorhodopsin of the purple membrane, which according to its amino acid sequence would have favored the β form.

Previously, we reported that surfactants in solution, which provide a hydrophobic environment, may stabilize an ordered conformation in polypeptides and proteins that would have otherwise been disrupted by the peptide backbone–water interactions (Wu & Yang, 1978). We have hypothesized that the induced conformation of a polypeptide is related to the structure-forming potential of the peptide segments as dictated by the amino acid sequence of the peptides. Oligopeptides and short polypeptides in surfactant solutions can be classified into four types according to their induced conformation: (1) helix forming, (2) β forming, (3) either helix forming or β forming, and (4) unordered (Wu & Yang, 1978). Charged side groups of a peptide having the same sign as the surfactant and located

† From the Cardiovascular Research Institute, University of California, San Francisco, California 94143. *Received January 28, 1982.* This work was supported by U.S. Public Health Service Grant GM-10880. Presented in part at the 73rd Annual Meeting of American Societies for Experimental Biology, New Orleans, LA, April 1982.

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0006-2960/82/0421-4556$01.25/0