Thrombin-Induced Conformational Changes of Human α_2-Macroglobulin: Evidence for Two Functional Domains†

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ABSTRACT: The interaction of thrombin with α_2-macroglobulin (α_2M) was characterized by monitoring conformational changes and measuring the increase of free sulfhydryl groups during the reaction. Under experimental conditions where [thrombin] > [α_2M], the conformational change, measured by increases in the fluorescence of 6-(*p*-toluidino)-2-naphthalenesulfonate, and thiol group appearance displayed biphasic kinetics. The initial rapid phase results in the formation of a stable complex, the appearance of two sulfhydryl groups, the cleavage of approximately half of the M_r 180 000 subunits, and a conformational change that is not as extensive as that which occurs with trypsin. The slower phase is associated with the appearance of two additional sulfhydryl groups, increased cleavage of the M_r 180 000 subunit, and additional conformational changes. The available evidence suggests that the slow phase results from hydrolysis of the M_r 180 000 subunit(s) due to proteolysis of the α_2M–thrombin complex by free thrombin. Experiments with ^{125}I-thrombin document the binding of 1 mol of thrombin/mol of α_2M that is not dissociated upon sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the complex. At higher ratios of thrombin to α_2M, a second mole of thrombin will reversibly associate with the 1:1 α_2M–thrombin complex. Under conditions where [thrombin] < [α_2M], biphasic kinetics were not observed, and the conformational change, sulfhydryl appearance, and hydrolysis of the M_r 180 000 subunit were found to follow second-order kinetics. The second-order rate constants obtained for these events were $(3.2 \pm 0.5) \times 10^3$ M^{-1} s^{-1}, $(2.5 \pm 0.4) \times 10^3$ M^{-1} s^{-1}, and $(4.9 \pm 0.7) \times 10^3$ M^{-1} s^{-1}, respectively. The similarity of these rate constants is consistent with a concerted mechanism or with a sequential mechanism in which either thrombin association or cleavage of the α_2M subunit(s) is rate limiting and the subsequent events (i.e., thiol ester hydrolysis and conformational change) occur at more rapid rates.

α_2-Macroglobulin (α_2M)[1] is a plasma glycoprotein (M_r 718 000) that contains four identical polypeptide chains, linked in pairs via a single disulfide bond (Swenson & Howard, 1979a,b; Hall & Roberts, 1978; Sottrup-Jensen et al., 1984), and functions as a protease inhibitor. The amino acid sequences of the subunits have been reported, and each contains 1451 amino acid residues with 8 asparagine-linked oligosaccharide groups (Sottrup-Jensen et al., 1984). In addition, each subunit contains a labile bond that can be cleaved upon denaturation (Harpel et al., 1979; Howard et al., 1980). The covalent incorporation of methylamine occurs at this site (Howard et al., 1980) and results in the appearance of free sulfhydryl groups (Sottrup-Jensen et al., 1980), with a decrease in the ability of the inhibitor to bind several proteinases (Steinbuch et al., 1966). On the basis of these and other observations, it has been proposed that α_2M (Sottrup-Jensen et al., 1980; Howard, 1981), like complement components C3 and C4 (Thomas et al., 1982; Cambell, 1981), contains internal thiol ester bonds formed from the β-sulfhydryl group of Cys-949 and the γ-carbonyl group of Glx-952 (Sottrup-Jensen et al., 1984).

As a protease inhibitor, α_2M is capable of inhibiting all four classes of proteases (Barrett & Starkey, 1973). The inhibition mechanism appears unique among protease inhibitors and has been proposed to occur via a "trap" mechanism (Barrett & Starkey, 1973). According to this proposal, limited proteolysis at a specific region on the α_2M subunit is followed by a conformational change, during which the thiol ester bonds are hydrolyzed. This conformational change results in "entrapment" of the protease and is characterized by a reduced activity of the enzyme toward large molecular weight substrates, with very little change in activity toward lower molecular weight substrates.

While the interactions of several different enzymes with this inhibitor have been characterized (Gonias et al., 1982; Björk & Fish, 1982; Barrett et al., 1979; Harpel, 1973; Straight & McKee, 1982; Bieth & Meyer, 1984; Virca & Travis, 1984), several questions remain regarding the reaction mechanism, including the detailed sequence of events that occurs upon proteolysis of α_2M which leads to inhibition of the protease. In the present study, the reaction of thrombin with α_2M has been characterized by monitoring conformational changes and by quantitating the appearance of free thiol groups in α_2M. The results of this study suggest that α_2M contains two functional domains and are supportive of a mechanism in which thrombin associates with a binding site on one of these domains, resulting in proteolysis and a conformational change occurring within this region. The α_2M–thrombin complex contains a second site that is not readily cleaved by thrombin but is accessible to trypsin. Further, the data are consistent with a concerted mechanism or with a sequential mechanism in which either thrombin association or cleavage of the α_2M

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[1] Abbreviations: α_2M, α_2-macroglobulin; NPGB, *p*-nitrophenyl *p*-guanidinobenzoate hydrochloride; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TNS, 6-(*p*-toluidino)-2-naphthalenesulfonic acid; DAPA, dansylarginine *N*-(3-ethyl-1,5-pentanediyl)amide; PPACK, D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; AT-III, antithrombin III; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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subunit(s) is rate limiting and the subsequent events (i.e., thiol ester hydrolysis and conformational change) occur at more rapid rates.

Materials and Methods

Materials

5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB), *p*-nitrophenyl *p*-guanidinobenzoate hydrochloride (NPGB), methylamine hydrochloride, and HEPES were obtained from Sigma Chemical Co. 6-(*p*-Toluidino)-2-naphthalenesulfonic acid (TNS) was obtained from Eastman. Crude lyophilized *Echis carinatus* venom was obtained from the Miami Serpentarium. Antithrombin III was a gift from Dr. Milan Wickerhauser of the American Red Cross, Bethesda, MD. Human cryosupernatant was obtained from the Washington Regional Blood Services, American Red Cross. Cohn fraction III paste was obtained from the Michigan Department of Public Health (East Lansing, MI). Dansylarginine *N*-(3-ethyl-1,5-pentanediyl)amide (DAPA) was synthesized as described by Nesheim et al. (1979).

Methods

Proteins. Trypsin, obtained from Calbiochem, was dissolved in 1 mM HCl. Active-site titration with NPGB (Chase & Shaw, 1970) gave an active-site concentration of 0.70 mol/mol of protein. The venom coagulant enzyme from *Echis carinatus* was purified from crude lyophilized venom (Rhee et al., 1982). Thrombin was prepared from Cohn fraction III paste on the basis of the procedure outlined by Fenton et al. (1977) except that the protein from *Echis carinatus* venom was used to activate prothrombin rather than thromboplastin and DEAE-Sephacel was used to remove the *Echis carinatus* just prior to the second CG-50 column. The thrombin preparation was homogeneous as judged by SDS disc gel electrophoresis (Weber & Osborn, 1969). Thrombin prepared in this manner typically had activities ranging from 2500 to 3000 NIH clotting units/mg and contained 0.70–0.80 mol of active site/mol of enzyme based on active-site titration with NPGB (Chase & Shaw, 1970).

α_2M was prepared according to Imber & Pizzo (1981). The amount of active α_2M was quantitated by titrating the sulfhydryl groups released during reaction with an excess of trypsin. Routinely, an aliquot of α_2M is placed in a cuvette containing 50 μM DTNB in 50 mM HEPES–150 mM NaCl, pH 8.0, buffer (final concentration of α_2M = 1–2 μM). After thermal equilibrium is reached, a 4–10 molar excess of trypsin in 20 μL or less of 1 mM HCl, pH 3.0, is added. The rapid release of sulfhydryl is measured until the absorbance change has stabilized, and the amount of intact α_2M is quantitated by using an E value of 13 600 M^{-1} cm^{-1} for the 3-carboxylato-4-nitrothiophenolate anion at pH 8.0 (Ellman, 1959). It is assumed that trypsin treatment of fully active α_2M would result in the release of 4 mol sulfhydryl/mol of protein (Sottrup-Jensen et al., 1980). Typically, the preparations contained 90% active α_2M. In all experiments, the indicated concentration of thrombin, trypsin, α_2M represents the active-site concentration.

Protein Concentrations. These were determined spectrophotometrically by using the following values for $E^{1\%}_{280nm}$ and molecular weight, respectively: α_2M, 8.93 and 718 000 (Jones et al., 1972; Hall & Roberts, 1978); trypsin, 15.4 and 23 300 (Robinson et al., 1971; Walsh & Neurath, 1964); thrombin, 18.3 and 38 500 (Fenton et al., 1977); antithrombin III, 6.5 and 58 000 (Nordenman et al., 1977).

Free Sulfhydryl Determination. Sulfhydryl appearance was measured on a Perkin-Elmer Lambda 5 spectrophotometer equipped with a programmable thermostated cell that can hold six sample cells and six reference cells. The temperature was maintained at 30 °C, and the slits were maintained at 4 nm. An aliquot of α_2M (final concentration 1 μM) was added to both sample and reference cuvettes containing 50 μM DTNB in 50 mM HEPES–0.15 M NaCl, pH 8.0. The measurement of free sulfhydryl appearance was initiated by the addition of thrombin to the sample cuvettes and the appropriate amount of buffer to the reference cuvettes after the cuvettes had reached thermal equilibrium. Usually three sample cuvettes were monitored at one time. The reaction was monitored at 410 nm, and an E of 13 600 M^{-1} cm^{-1} was used (Ellman, 1959). The concentration of DTNB in the cuvette was sufficient to ensure that the reaction of free sulfhydryl with DTNB was not rate limiting. Control experiments have shown that incubation of α_2M with DTNB does not result in the appearance of sulfhydryl groups. To minimize oxidation of the 3-carboxylato-4-nitrothiophenolate anion, all buffers were extensively deoxygenated with nitrogen prior to use.

Hydrolysis of the M_r 180 000 Subunit. α_2M (2 μM) was incubated with thrombin (2 μM) at 30 °C, and at selected time intervals, the reaction was terminated by adding an aliquot to an excess of AT-III–heparin. Following an incubation period of 3 h in SDS–sample buffer containing 5% β-mercaptoethanol, 18 μg of protein was loaded on each gel and subjected to electrophoresis using a 7.5% separating gel and a 4% stacking gel (Laemmli, 1970). The gels were stained and scanned on a Gilford 250 spectrophotometer fitted with a gel scanning stage. The decrease in areas corresponding to the M_r 180 000 subunit was used to estimate the rate constant. Each sample contained an internal standard, and the data were normalized to that standard.

Fluorescence Measurements. TNS fluorescence was monitored in a Perkin-Elmer MPF-4 fluorometer equipped with a thermostated cell maintained at 30 °C. The excitation wavelength was 320 nm, and the slit widths were 15 nm. Measurement of the rate of conformational change was carried out by adding an aliquot of α_2M (final concentration 1 μM) to a solution containing 50 μM TNS in 50 mM HEPES–0.15 M NaCl, pH 8.0. After thermal equilibrium was obtained, the reaction was initiated by the addition of thrombin, and the fluorescence change was monitored continuously. This same procedure was used with trypsin or methylamine. Fluorescence emission spectra were recorded at 30 °C with an excitation wavelength of 320 nm and slit widths of 10 nm.

Kinetics. The reaction was assumed to be an irreversible bimolecular reaction as shown in eq 1:

$$\alpha_2\text{M} + \text{thrombin} \rightarrow \alpha_2\text{M–thrombin} \quad (1)$$

Thus

$$\text{velocity} = kIE \quad (2)$$

where k is the second-order rate constant, $I = [\alpha_2\text{M}]$, and E = [thrombin]. When $I_0 > E_0$, integration of eq 2 results in the expression given by Jencks (1969):

$$\frac{1}{I_0 - E_0} \ln \frac{E_0(I_0 - x)}{I_0(E_0 - x)} = kt \quad (3)$$

where E_0 and I_0 are the initial thrombin and α_2M concentrations, respectively, t is time, and x is the concentration of the thrombin–α_2M complex at time t. In these experiments, it was assumed that $x = E = E_0(F - F_0)/(F_{max} - F_0)$ for the TNS experiments and $x = E = E_0(A - A_0)/(A_{max} - A_0)$ for the sulfhydryl experiments. F and A are the fluorescence and the absorbance at time t, respectively, while F_0 and A_0 are the fluorescence and the absorbance at time zero and F_{max} and A_{max} are the fluorescence and the absorbance at infinite time.

Therefore, by plotting $[1/(I_0 - E_0)]$ ln $\{[E_0(I_0 - x)]/[I_0(E_0 - x)]\}$ vs. t, one can obtain a straight line with a slope of k. When $E_0 = I_0$, the integrated rate expression is

$$E_0/E = E_0kt + 1 \quad (4)$$

In these experiments where product formation is measured, $E_0/E = (F_{max} - F_0)/(F_{max} - F)$ or $(A_{max} - A_0)/(A_{max} - A)$. When $F_0 = 0$ (or $A_0 = 0$), a plot of $F_{max}/(F_{max} - F)$ vs. t [or $A_{max}/(A_{max} - A)$] will give a straight line with a slope of E_0k and an intercept of 1. In experiments where reactant loss is measured, a plot of A_0/A was used. If the substitution for E_0/E is made in cases when $E_0 = I_0$ or the substitution for x is made when $E_0 < I_0$, nonlinear curve fitting can be employed, and F_{max} (or A_{max}) need not be known. Both linear curve fitting and nonlinear curve fitting were employed to analyze the data and yielded equivalent results.

Differential Scanning Calorimetry. Calorimetric measurements were made with a heat capacity calorimeter manufactured by Microcal, Inc., Amherst, MA. The heat capacity of the sample (5.2 μM α_2M and 0–15.7 μM thrombin in 50 mM HEPES, pH 8.0) was scanned from 25 to 90 °C at a rate of 1 °C/min. The enthalpy associated with the 82 °C transition was plotted vs. the ratio of thrombin to α_2M. The method of data analysis has been reported previously (Ploplis et al., 1981; Sturtevant, 1974).

Binding of ^{125}I-Thrombin to α_2M. α_2M (2.1 μM) was incubated with a 0–4-fold excess of ^{125}I-thrombin in 50 mM HEPES–150 mM NaCl, pH 8.0, at room temperature. Following incubation for 1 or 24 h, thrombin was inhibited by the addition of DAPA (20 μM). After 10 min, 50-μL aliquots were mixed with 50 μL of the appropriate buffer and analyzed by SDS–PAGE or Tris–borate–PAGE. Following electrophoresis, the gels were stained, destained, sliced, and counted.

Bound Thrombin Activity. This measurement was based on the observation that thrombin bound to α_2M is not inhibited by AT-III (Straight & McKee, 1982). α_2M (160 nM) was incubated with 0–800 nM thrombin in 50 mM HEPES–150 mM NaCl, pH 8.0 at room temperature. After 1 and 5 h, 100 μL of each sample was added to 700 μL of AT-III–heparin to give final concentrations of 2000 nM AT-III, 20 nM α_2M, and 0.2 mg of heparin. After incubation at room temperature, hydrolysis of S-2238 was measured at 405 nm by adding 100 μL of sample to a cuvette that contained 50 μM S-2238 in 1000 μL of 50 mM HEPES–150 mM NaCl, pH 8.0, buffer.

Electrophoresis. SDS–PAGE was performed as described by Laemmli (1970), using 5% separating gels and 3% stacking gels or 4% separating gels. Tris–borate gel electrophoresis was performed as described (Nelles et al., 1980).

RESULTS

Monitoring the Conformational Changes in α_2M with TNS. Previous studies have shown that TNS is a useful probe for monitoring conformational changes occurring in both human and bovine α_2M (Strickland & Bhattacharya, 1984; Strickland et al., 1984). The uncorrected emission spectra of this dye with α_2M and α_2M–thrombin complexes at selected time intervals following the addition of an excess of the enzyme are shown in Figure 1. The interaction of thrombin with α_2M is associated with an increase in intensity of fluorescence, as well as a blue shift in the wavelength of maximal emission (curves 5–8, Figure 1). Even after incubation for a period of 84 min, the fluorescence intensity did not reach the level obtained when trypsin was added to α_2M (curve 9, Figure 1). However, addition of trypsin to the thrombin complex after a 40-min incubation (curve 7, Figure 1) resulted in a rapid

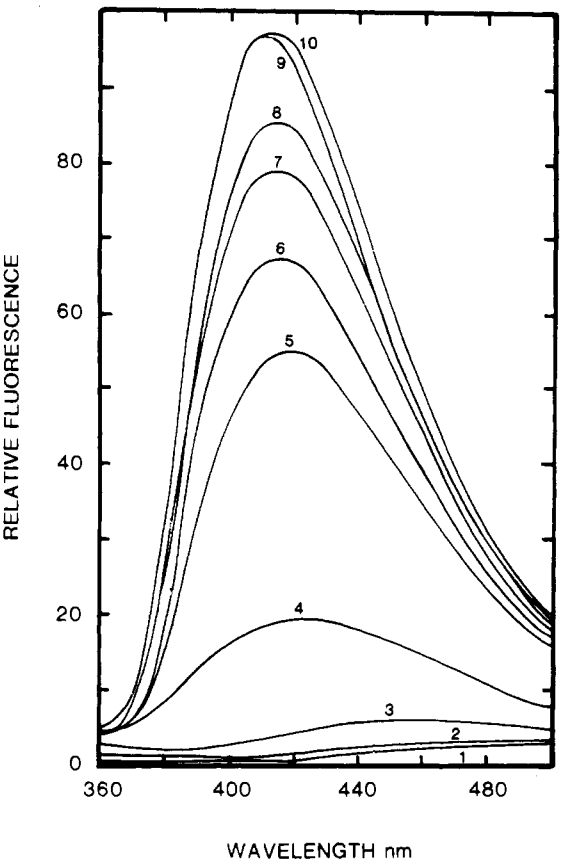

FIGURE 1: Uncorrected emission spectra of TNS in the presence of (1) buffer, (2) 4 μM trypsin, (3) 4 μM thrombin, (4) 1 μM α_2M, (5) α_2M–thrombin (4 μM thrombin, 1 μM α_2M), 5-min incubation, (6) α_2M–thrombin, 13-min incubation, (7) α_2M–thrombin, 40-min incubation, (8) α_2M–thrombin, 84-min incubation, (9) α_2M–trypsin (4 μM trypsin, 1 μM α_2M), 1-min incubation, and (10) trypsin added to α_2M–thrombin after 40-min incubation.

increase (<1 min) to the maximal level (curve 10, Figure 1) observed when trypsin reacted with α_2M. In a control experiment, TNS was added to a sample containing thrombin (curve 3) and the emission spectrum recorded. While some interaction between the dye and thrombin is apparent, the majority of the signal increase and wavelength shift appears to be associated with α_2M. This is further supported by the observation that the emission spectra of several α_2M–protease complexes are quite similar and virtually identical with the emission spectra obtained for the methylamine-modified molecule (Strickland & Bhattacharya, 1984).

The time course of thrombin-induced increase in TNS fluorescence was monitored (Figure 2), and the reaction appears biphasic, with the initial more rapid phase representing approximately half the signal observed when α_2M was reacted with trypsin or methylamine. In these experiments, [thrombin] > [α_2M]. Further additions of trypsin or methylamine to the incubation mixture during the slow phase resulted in an increase in TNS fluorescence to the maximal level observed when α_2M was treated with trypsin or methylamine alone (Figure 2). Control experiments have indicated that the activity of thrombin is not inhibited by TNS. In a separate experiment, the change in TNS fluorescence with time correlated well with thrombin binding to α_2M (data not shown), as measured by protection of thrombin in the α_2M–thrombin complex from inhibition by AT-III–heparin (Ganrot, 1966; Straight & McKee, 1983). These results indicate that TNS is sensitive to conformational changes occurring in α_2M resulting from thrombin binding and proteolysis.

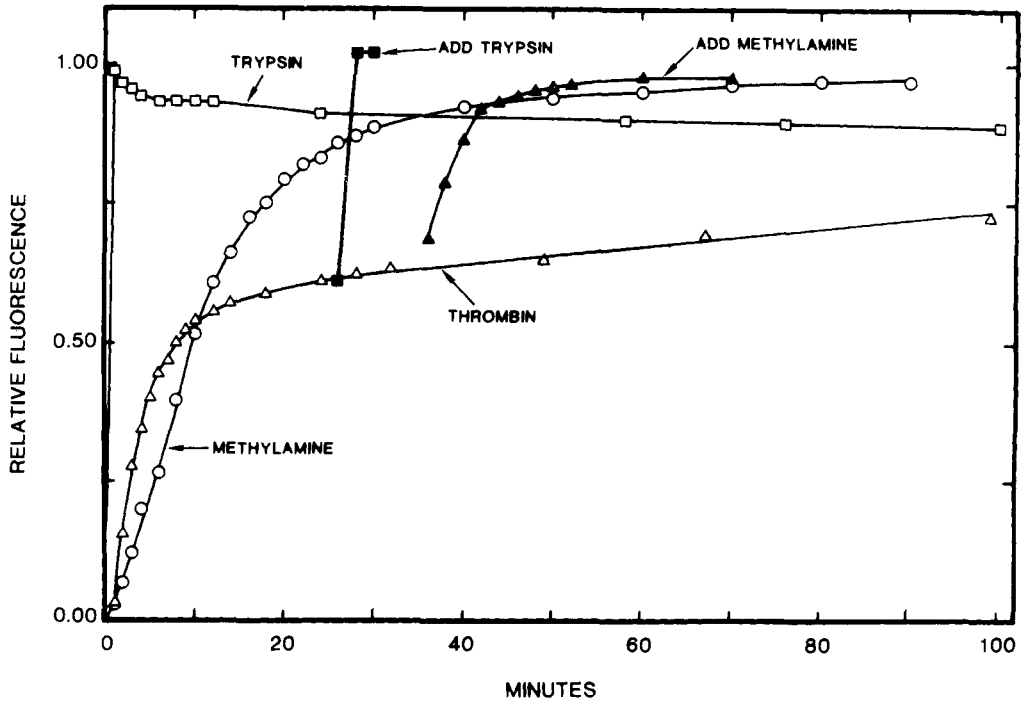

FIGURE 2: Time course of the TNS fluorescence change induced by treatment of α_2M (0.5 μM) with trypsin (2 μM), thrombin (2 μM), or methylamine (200 mM). In a duplicate experiment, trypsin (2 μM) or methylamine (200 mM) was added at the indicated times to the α_2M–thrombin complex.

The changes occurring in α_2M resulting from interaction with thrombin were examined by disc gel electrophoresis in a Tris–borate system. At a protease to inhibitor ratio of 1 (Figure 3A), the slower migrating band, attributed to native α_2M, is converted to two faster migrating bands. These bands did not comigrate with the "fast" form resulting from formation of a trypsin–α_2M complex, even after prolonged incubation (6 h). Increasing the protease to inhibitor ratio to a value of 2 (Figure 3B) results in a more rapid disappearance of native α_2M, with the formation of the two faster migrating bands, one of which disappears upon longer incubation with the concurrent appearance of a new band. This new band most likely results from a slow proteolysis of α_2M–thrombin by free thrombin, since very little, if any, of the faster migrating species is seen at lower α_2M:thrombin ratios, where very little free thrombin is present (Figure 3A). At this point, it is not known if these bands resulting from the association of thrombin with α_2M represent different conformational states of the complex or result from charge differences due to limited proteolysis. Of interest in this regard is the report by Nelles et al. (1980), who observed up to five bands upon electrophoresis of purified α_2M that were attributed to different conformational states of the inhibitor.

Analysis of the extent of cleavage of α_2M subunits was examined by SDS–PAGE under reducing conditions (Figure 4). Untreated α_2M displays the characteristic M_r 125 000 band associated with fragmentation due to denaturation (Harpel et al., 1979). Some cleavage of the M_r 185 000 subunit is evident and represents <10% of the native molecule (see Methods). The reaction of thrombin with α_2M is associated with an increased appearance of the M_r 85 000–100 000 bands, with concomitant disappearance of the M_r 185 000 subunit. Even in the presence of a large excess of thrombin and for extended time periods, the M_r 185 000 band is not completely hydrolyzed. The addition of AT-III following a 60-min incubation with thrombin (Figure 4) inhibits further hydrolysis of the M_r 185 000 band, suggesting that cleavage during the slow phase is dependent on the presence of free thrombin. In addition, M_r 140 000 and 360 000 bands are also apparent. These bands appear to form during the fast phase of the reaction, while a M_r >360 000 band appears during the slow phase. The origin of these bands has been postulated to involve covalent complexes of thrombin with α_2M subunits (Wang et al., 1984).

Monitoring the Appearance of Free Sulfhydryl from α_2M with DTNB. Trypsin, thrombin, and methylamine are reported to release 4 mol of sulfhydryl/mol of α_2M (Björk et al., 1984; Christensen & Sottrup-Jensen, 1983; Sottrup-Jensen et al., 1980; Straight & McKee, 1983; Salvensen et al., 1981). The appearance of free sulfhydryl groups upon treating α_2M with methylamine, trypsin, and thrombin is shown in Figure 5. Incubating α_2M with trypsin or methylamine resulted in the rapid appearance of 4 mol of sulfhydryl available for titration with DTNB. In contrast, incubating α_2M with thrombin, under conditions where [thrombin] > [α_2M], resulted in the biphasic appearance of sulfhydryl groups, with only 2 mol of sulfhydryl generated in the initial phase. Adding trypsin or methylamine to the incubation mixture during the slow phase resulted in the appearance of the two remaining sulfhydryls in the molecule. These results indicate that two of the thiol ester bonds remain intact and that the slow phase does not result from buried sulfhydryl groups with low reactivity toward DTNB. Quantitating the amount of trypsin bound, using the assay described by Ganrot (1966), indicated that the initial α_2M–thrombin complex can incorporate 1 mol of trypsin/mol of complex. Further addition of a 7-fold molar excess of thrombin to the incubation mixture during the slow phase results in only a slight increase in the rate of the slow phase (data not shown). The biphasic nature of sulfhydryl appearance upon association of thrombin with α_2M is similar to the results obtained in the TNS fluorescence studies (Figure 2). It should be noted that the slow phase was only measurable when thrombin concentrations exceeded α_2M concentrations. Attempts to monitor the slow phase in its entirety were hampered by the slow rate and the slow spontaneous hydrolysis of DTNB.

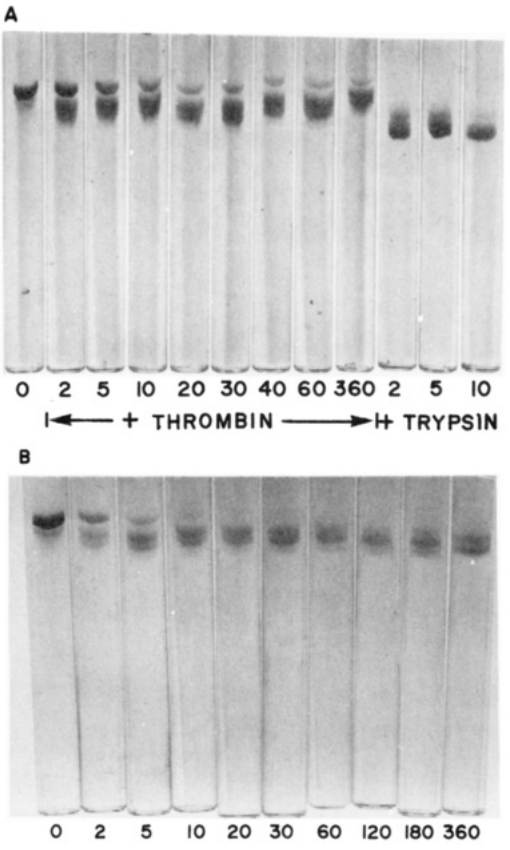

FIGURE 3: (A) Nondenaturing gel electrophoresis of α_2M (1 μM) incubated with thrombin (1 μM) or trypsin (2 μM). At the indicated time intervals (minutes), an aliquot was removed from the reaction mixture, and the reaction with thrombin was stopped by the addition of DAPA (60 μM) while the reaction with trypsin was stopped by the addition of benzamidine (75 mM) prior to electrophoresis. (B) Nondenaturing gel electrophoresis of α_2M (1 μM) reacted with thrombin (2 μM). At the indicated time intervals, an aliquot was removed from the reaction mixture, and the reaction was stopped by the addition of DAPA (60 μM) prior to electrophoresis.

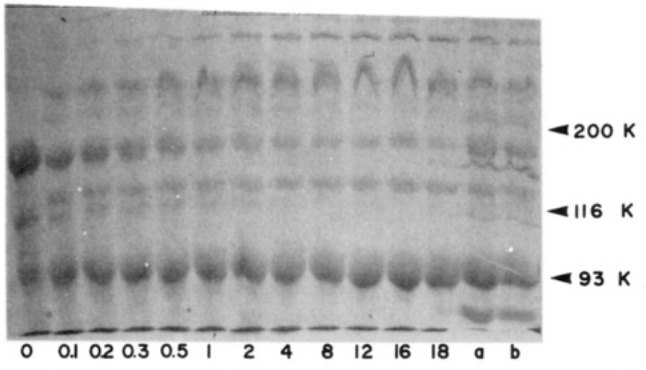

FIGURE 4: SDS–polyacrylamide gel electrophoresis under reducing conditions of α_2M incubated with thrombin for various time periods. α_2M (3 μM) was incubated with thrombin (12 μM) at 25 °C. At indicated intervals (hours), an aliquot (50 μL) was removed and added to SDS–sample buffer (100 μL) containing β-mercaptoethanol and PPACK (20 μM). Ten micrograms of sample was applied to each gel. In two cases, a sample was removed after 0.5-h incubation and added to a 5-fold (a) or a 10-fold (b) molar excess of AT-III–heparin. The migration positions of standards are indicated by arrows.

A titration of sulfhydryl groups with DTNB at increasing mole ratios of α_2M:thrombin is shown in Figure 6. In these experiments, the α_2M concentration was fixed, and increasing amounts of thrombin were added to vary the ratio. The sulfhydryl groups generated during the rapid phase were quantitated by extrapolation of the slow phase from the time course data to time zero (as shown by the dashed line in Figure 5). It is evident that even at a 6-fold molar thrombin excess, only two out of four sulfhydryls are available for rapid DTNB titration. However, in a separate experiment, after an 18-h incubation, all four sulfhydryl groups were available for titration with DTNB, and, surprisingly, the thrombin binding stoichiometry did not appear to change significantly. While the mechanism of increased sulfhydryl generation with time remains unknown, this slow process could result from proteolysis of α_2M subunit(s) by bound and/or free thrombin, thereby allowing hydrolysis of the intact thiol ester bonds. Alternately, the formation of a thrombin–α_2M complex could result in a conformational change in the molecule that would render the intact thiol ester bonds more susceptible toward nucleophilic attack.

Stoichiometry of the Thrombin–α_2M Complex. α_2M was titrated with varying amounts of thrombin, and the effect was monitored by measuring the conformational change occurring in α_2M or by measuring thrombin binding to α_2M (Figure 7; see Methods for descriptions). In all cases, the stoichiometry of the thrombin–α_2M interaction appears to be 1, in agreement with several reports in the literature (Pochon et al., 1983; Straight & McKee, 1984). The binding stoichiometry is only slightly altered by increasing the incubation time periods. Further, in several cases, a slight positive slope is observed at higher thrombin concentrations. This is due in part to a kinetic effect since in several cases the slow phase was incomplete when the measurements were made.

To examine the binding stoichiometry of thrombin to α_2M more directly, ^{125}I-thrombin was used. Various concentrations of ^{125}I-thrombin were incubated with fixed levels of α_2M for 18 h, and the extent of "covalent" and noncovalent binding was quantitated by subjecting the incubation mixture to electrophoresis under denaturing and nondenaturing conditions. Here "covalent" binding is defined as binding that cannot be dissociated upon SDS–PAGE of the complex. Analysis of the incubation mixture by SDS–PAGE under nonreducing conditions indicated that ^{125}I-thrombin was distributed between two major species with M_r 360 000 and $M_r \geq 760\,000$ (Figure 8, curves 1 and 2, respectively). The sum of these two curves (Figure 8, curve 3), representing the total covalent binding, indicates that the initial slope is equivalent to binding measured by nondenaturing gel electrophoresis. The near-identity of these slopes suggests that all of the thrombin bound, at thrombin:α_2M mole ratios of <1, it not dissociated upon SDS–PAGE of the complex. Further, the data suggest that when the thrombin:α_2M ratio is <1, the majority of thrombin is associated with α_2M. Precise estimation of the amount of free thrombin is hampered by the errors associated with measurements using this system. A sharp break in the extent of covalent binding occurs as more thrombin is added. In contrast, a continuous increase in bound thrombin was obtained when the incubation mixture was analyzed under nondenaturing conditions (Figure 8, curve 4). Taken together, these results suggest that 1 mol of thrombin is bound to α_2M in a manner that is not dissociated by SDS–PAGE, while a second molecule associates reversibly with α_2M. These experiments were repeated at shorter incubation times, and similar results were obtained (data not shown).

Kinetics of the Rapid Phase. The kinetics of the thrombin reaction with α_2M were measured by monitoring the changes occurring in TNS fluorescence, the release of sulfhydryl groups with DTNB, and hydrolysis of the M_r 180 000 polypeptide chain. These data were obtained under second-order conditions, where [α_2M] $\geq$ [thrombin], in order to eliminate any potential problems due to free thrombin. Under these con-

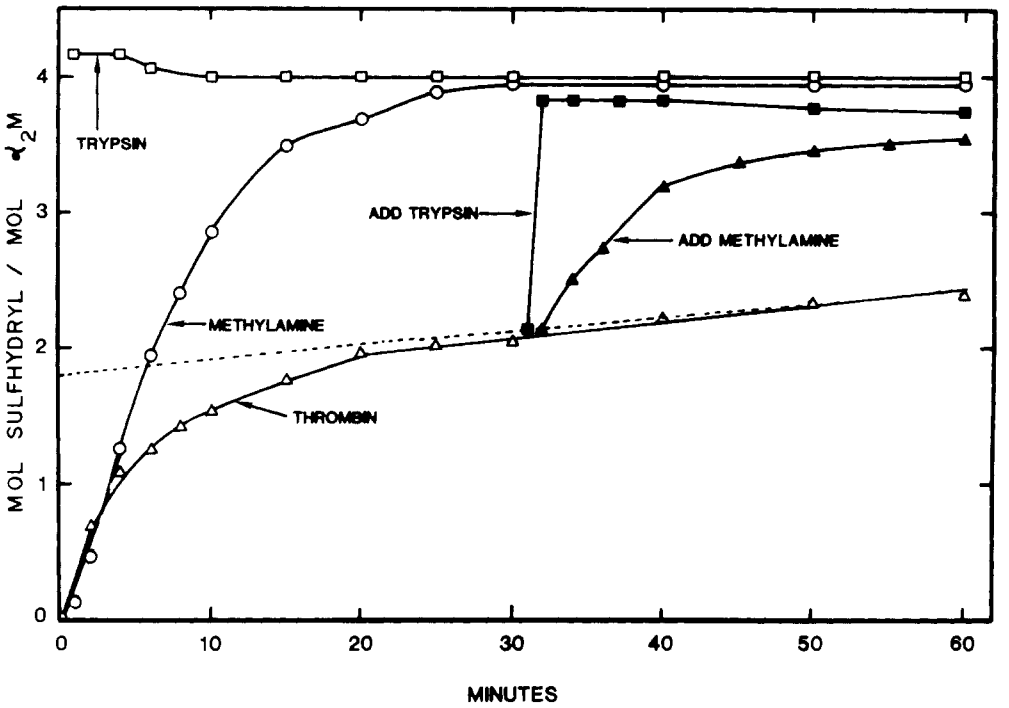

FIGURE 5: Time course of sulfhydryl generation when α_2M (1.6 μM) was incubated with trypsin (3.2 μM), thrombin (8.2 μM), or methylamine (200 mM). The generation of thiols was continuously monitored in the presence of 40 μM DTNB in 50 mM phosphate buffer (150 mM NaCl and 2 mM EDTA, pH 8.0) at 30 °C. In a duplicate experiment, trypsin (3.2 μM) and methylamine (200 mM) were added to the thrombin–α_2M complex at the indicated times.

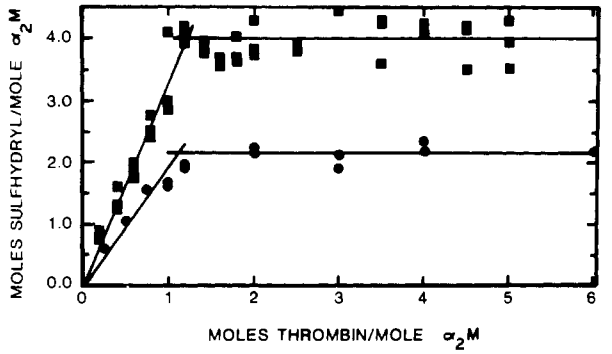

FIGURE 6: Titration of sulfhydryl groups with DTNB at increasing mole ratios of thrombin to α_2M. In these experiments, the concentration of α_2M was constant (1 μM), and the ratio was altered by increasing the thrombin concentration. The sulfhydryl groups initially exposed were quantitated by continuously monitoring the time course of thiol generation and extrapolation of the plateau region to time zero (●). In a separate experiment, α_2M was incubated with a given amount of thrombin for 17 h prior to the addition of DTNB (■).

ditions, no slow phase was observed. An example of the data obtained is shown in Figure 9 and demonstrates that an excellent fit to eq 4 was obtained. The inset is a representation of the data in the form of first- and second-order plots, where it is apparent that the reaction of thrombin with α_2M can be described by second-order kinetics. In the experiments measuring the kinetics of the conformational change and sulfhydryl appearance, the concentration of α_2M was varied and little effect on the rate constants noted. The second-order rate constants obtained for the conformational change, sulfhydryl appearance, and cleavage of the M_r 180000 polypeptide chain were (3.2 ± 0.5) × 10^3 M^{-1} s^{-1}, (2.5 ± 0.4) × 10^3 M^{-1} s^{-1}, and (4.9 ± 0.7) × 10^3 M^{-1} s^{-1}, respectively (Table I). It is apparent that these rate constants are virtually identical. It should be pointed out that the error in estimating the rate constant for hydrolysis of the M_r 180000 polypeptide is probably larger than the standard deviation reported for this

Table I: Kinetic Constants for the Rate of TNS Change, Sulfhydryl Appearance, and Cleavage of the M_r 180000 Subunit for Thrombin with α_2M at pH 8.0 and 30 °C

method	k (M^{-1} s^{-1})	no. of determinations
TNS	(3.2 ± 0.5) × 10^3 [a]	16
DTNB	(2.5 × 0.4) × 10^3 [a]	11
SDS–PAGE	(4.9 ± 0.7) × 10^3 [b]	1

[a] Rate constant ± standard deviation based on the number of indicated determinations. The concentration of α_2M was varied between 1 and 6 μM, while the concentration of thrombin was maintained at 0.5 or 1.0 μM. [b] Rate constant ± standard deviation derived from the fitting procedure based on one determination.

single determination due to the method used. The kinetics of thrombin reacting with α_2M have been reported by three different groups. Downing et al. (1978) reported a second-order rate constant of 0.49 × 10^3 M^{-1} s^{-1} using clotting assays to quantitate free thrombin. Dangott et al. (1983) reported a pseudo-first-order rate from which an apparent second-order rate constant of 1.2 × 10^3 M^{-1} s^{-1} can be derived. Their data were based on small UV differences occurring upon complex formation. Straight & McKee (1982) reported a second-order rate constant of 2.5 × 10^3 M^{-1} s^{-1} based on changes in intrinsic fluorescence in α_2M. These latter data are in close agreement with the present study.

Discussion

The present investigation has examined the interaction of thrombin with α_2M by monitoring both conformational changes and the appearance of thiol groups in α_2M. Under conditions where [thrombin] > [α_2M], both processes exhibit biphasic kinetics. The initial phase of thrombin interaction with α_2M results in the formation of a stable complex that has the following characteristics: (1) it does not dissociate during SDS–PAGE; (2) two of the four thiol ester bonds remain intact; (3) the complex can incorporate 1 mol of trypsin/mol of complex; (4) approximately 50% of the 180000-dalton

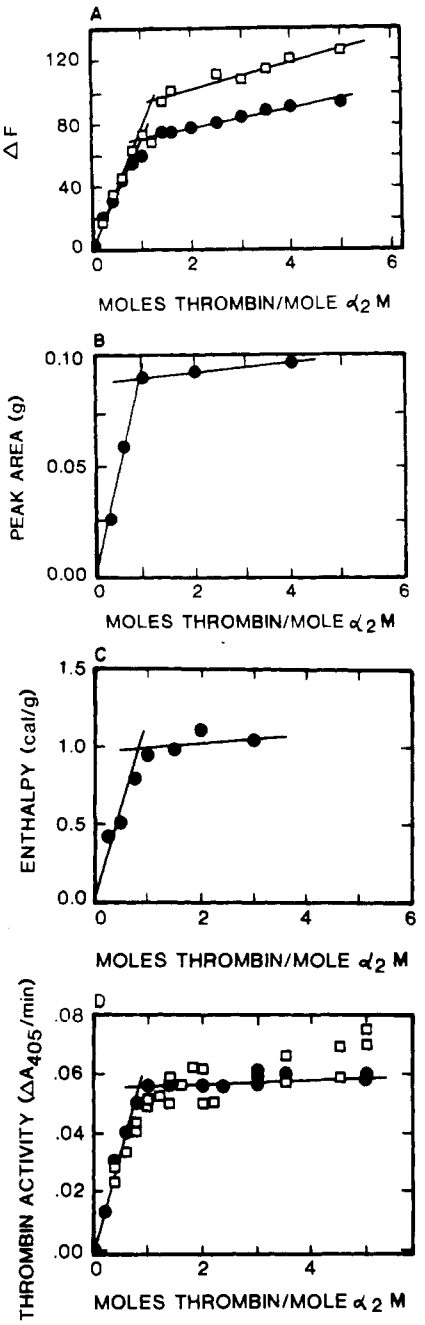

FIGURE 7: Titration of α_2M with increasing levels of thrombin as monitored by (A) changes in TNS fluorescence, (B) proteolysis of α_2M, (C) increases in the 82 °C transition measured by differential scanning calorimetry, and (D) bound thrombin activity. In the experiments measuring TNS fluorescence changes or bound thrombin activity (A and D), measurements were made after incubation periods of 1 (●) or 5 h (□). Incubation periods in the titrations measuring enthalpy and proteolysis (B and C) were carried out for 60 min.

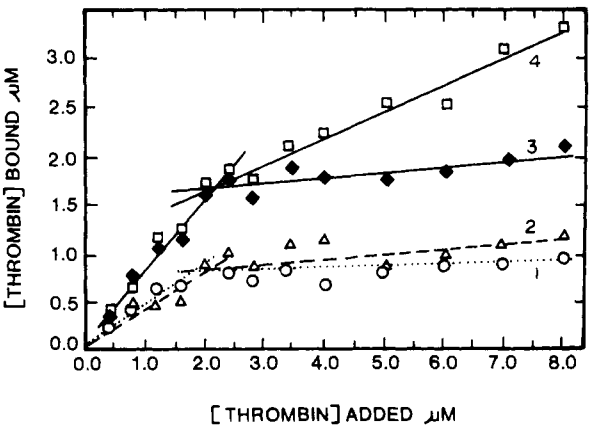

FIGURE 8: Quantitation of the amount of ^{125}I-thrombin bound to α_2M. α_2M was incubated with increasing amounts of ^{125}I-thrombin for 24 h and the incorporation of ^{125}I-thrombin assessed by PAGE under denaturing (curves 1, 2, and 3) and nondenaturing conditions (curve 4). (○) ^{125}I-Thrombin associated with the M_r 360 000 species; (△) ^{125}I-thrombin associated with the M_r ≥720 000 species; (♦) sum of the ^{125}I-thrombin amounts associated with M_r 360 000 and ≥720 000 species; (□) ^{125}I-thrombin associated with the native molecule, analyzed under nondenaturing conditions.

subunit is cleaved; (5) the conformational change, measured by increases in TNS fluorescence and by migration in a Tris–borate gel system, is not as extensive as that which occurs with trypsin.

The biphasic nature of the conformational change and the appearance of sulfhydryl groups resulting from the interaction of thrombin with α_2M are supportive of a mechanism in which thrombin first associates with a binding site located on one of two functional domains. The reaction sequence may be initiated by cleavage of one or both polypeptide chains within this "half-molecule", followed by hydrolysis of two thiol ester bonds and a conformational change occurring within this region. An additional binding site, located on the other functional unit, appears intact and is readily accessible to proteolysis by trypsin, which results in the hydrolysis of the remaining two thiol ester bonds and a further conformational change. Once thrombin is bound to the first site, the second site does not appear readily accessible to thrombin, and rates for the conformational change and thiol ester hydrolysis, associated with thrombin cleavage of polypeptide chain(s) in the second half-molecule, are extremely slow and only observed when large excesses of thrombin are present. The biphasic nature of the conformational change, observed when [thrombin] > [α_2M], might be a general phenomenon of the reaction of proteases with α_2M since a recent study has observed that the reaction of trypsin with α_2M, monitored by stopped-flow fluorescence, appears biphasic (Dangott et al., 1983).

The concept of two functional units within the α_2M molecule is consistent with several reports in the literature. In a study measuring the kinetics of nucleophilic modification of α_2M, it was concluded that the two half-molecules behaved as independent entities within this inhibitor and there were apparent interactions between the individual subunits within the half-molecule (Strickland & Bhattacharya, 1984). Additionally, Gonias & Pizzo (1983) have reported the isolation of α_2M half-molecules by limited reduction and alkylation, each consisting of two subunits, that appear to contain functional properties of the intact molecule. Although it is likely that these half-molecules obtained by limited reduction are identical with the two functional regions postulated to exist in the intact molecule, this remains to be established. Finally, Björk et al. (1984) have examined the stoichiometry of polypeptide cleavage, thiol ester hydrolysis, and conformational change upon trypsin treatment of α_2M. They found a stoichiometry of 2 for all reactions, suggesting that α_2M contains two independent functional units within the molecule.

The changes in TNS fluorescence have allowed the kinetics of the thrombin–α_2M reaction to be measured. Previous investigations have observed that the association of α_2M with thrombin (Downing et al., 1978), factor Xa (Ellis et al., 1981), elastase (Bieth & Meyer, 1984; Virca & Travis, 1984), and kallikrein (Virca & Travis, 1984) follows second-order kinetics. The present investigation further substantiates that the reaction of thrombin with α_2M can be characterized by second-order kinetics when [thrombin] < [α_2M]. Of particular interest is the observation that the rate constants for the appearance of

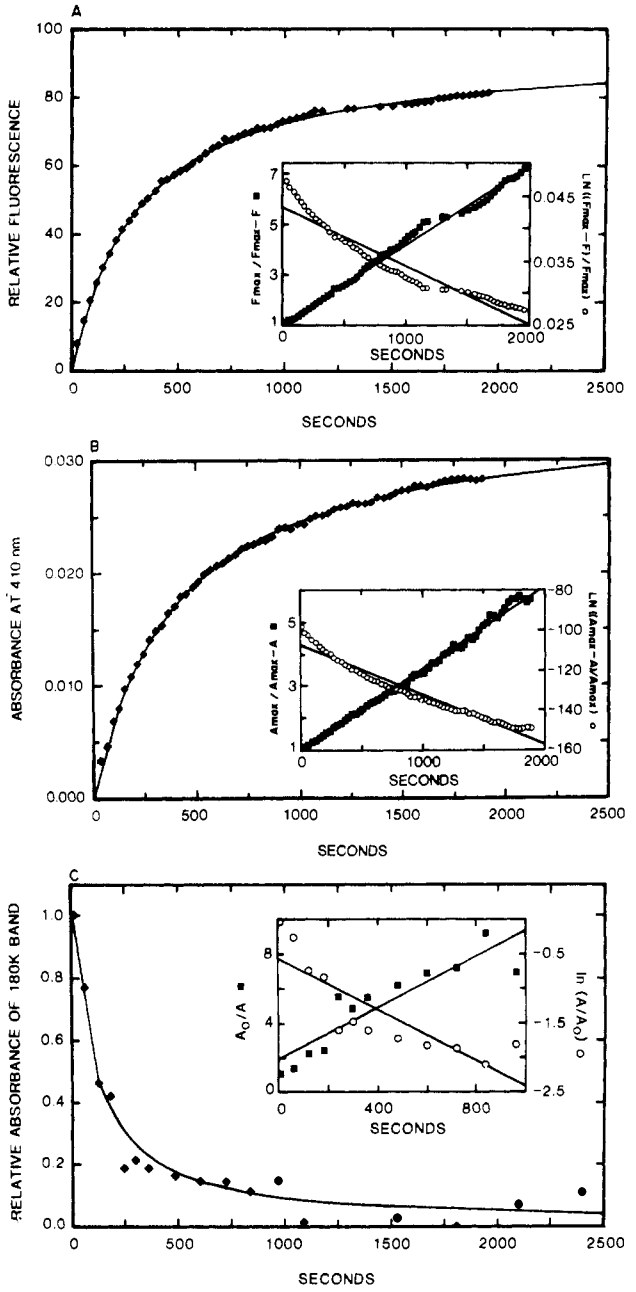

FIGURE 9: Determination of the rate of thrombin association with α_2M measured by changes in TNS fluorescence (A), appearance of sulfhydryl groups (B), or hydrolysis of the M_r 180 000 subunit (C). Thrombin (1 μM) was added to α_2M (1 μM) to initiate the reaction, and the appropriate change was monitored as described under Methods. The curve shows the best fit to eq 4. (Inset) Representation of the data in the form of first-order (O) and second-order (■) plots. The straight lines represent the best-fit line determined by linear regression.

sulfhydryl groups, the conformational change, and cleavage of the M_r 180 000 subunit are virtually identical. The data are consistent either with a sequential mechanism in which the first step is rate limiting or with a mechanism in which all of the processes occur simultaneously. Previous studies on the steps associated with the conformational change occurring in α_2M as a result of methylamine treatment have suggested that the mechanism is sequential, where the conformational change is preceded by hydrolysis of the thiol ester bonds (Strickland & Bhattacharya, 1984). Thus, the integrity of the thiol ester bonds appears to be important in maintaining the conformation of the protein. Further, the rate of conformational change resulting from proteolysis of α_2M by some proteases, such as trypsin, is very rapid, whereas with other proteases, such as thrombin, it is much slower. Since it is likely that the rate of conformational change is, for the most part, independent of the protease, this suggests that association of thrombin with α_2M or cleavage of the M_r 180 000 polypeptide chain(s) is rate limiting and that the events occurring subsequent to cleavage (i.e., thiol ester hydrolysis and conformational change) occur relatively rapidly.

In addition to the rapid phase, a slower phase is observed when [thrombin] > [α_2M]. This phase is associated with a slow cleavage of the M_r 180 000 subunits, hydrolysis of the remaining thiol ester bonds, and a conformational change measured by an increase in TNS fluorescence. While the exact mechanism by which this slow phase occurs is unknown, the available evidence suggests that this phase is dependent upon thrombin activity, and it is conceivable that either free or bound thrombin is capable of catalyzing this event. The rate of the slow phase is increased when a large excess of thrombin is added and appears to be inhibited by AT-III–heparin or DAPA. Further, the binding stoichiometry, determined from titration curves measuring the extent of conformational change, sulfhydryl appearance, and thrombin binding, is not altered when the incubation period is extended from 1 to 18 h. Direct binding experiments document that the binding of a second molecule of thrombin does occur but that this binding is reversible.

Several models have been proposed to account for the varying binding ratios observed for different α_2M–protease complexes. One model suggests that the rate of association of the protease with α_2M is important in determining the binding ratio (Straight & McKee, 1984; Howell et al., 1983). An underlying assumption of this proposal is that the binding of the first protease molecule results in a conformational change in the molecule, thereby inhibiting binding of proteases to the second site. However, at least with thrombin, this does not appear to be the case since the second site appears rapidly accessible to trypsin. These observations are consistent with other observations in the literature documenting that the α_2M–plasmin complex is capable of binding 1 mol of trypsin (Ganrot, 1967; Jacquot-Armand & Guinand, 1976). The slow rate of thrombin-catalyzed hydrolysis at this second site suggests that the α_2M–thrombin complex is no longer a good substrate for thrombin. This could result from a conformational alteration occurring in this site as a consequence of complex formation with thrombin or could simply result from steric hindrance.

Finally, a number of higher molecular weight components have been observed when the thrombin–α_2M complex was examined upon SDS–PAGE (Wang et al., 1984). These complexes were postulated to represent cross-linking of subunits by thrombin molecules via covalent bond formation involving nucleophilic groups on thrombin. The data from the present study indicate that the association of thrombin with α_2M results in a complex in which two of the four thiol ester sites remain intact. These sites would be available for covalent bond formation with nucleophilic groups and might provide some explanation for the mechanism by which these higher molecular weight complexes form. However, further studies are required to determine if this is indeed the case.

Registry No. Thrombin, 9002-04-4; trypsin, 9002-07-7.

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