Articles

Kinetics of the Conformational Alterations Associated with Nucleophilic Modification of α_2-Macroglobulin†

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Appendix: Solution of Differential Rate Equations‡

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ABSTRACT: The effect of nucleophilic modification of α_2-macroglobulin (α_2M) with methylamine on the kinetics of sulfhydryl exposure was investigated. The generated sulfhydryl groups were detected with 4,4′-dithiodipyridine. The bimolecular rate constant for sulfhydryl exposure was determined to be 11.6 ± 0.8 M^{-1} s^{-1} at 30 °C and pH 8.0. Treatment of α_2-macroglobulin with methylamine or proteases, such as plasmin and trypsin, results in a substantial increase in the fluorescence of 6-(*p*-toluidino)-2-naphthalenesulfonic acid. This probe was used to monitor the kinetics of the conformational change occurring in α_2-macroglobulin upon treatment with methylamine. It was found that the conformational change did not occur simultaneously with the cleavage of the thiol ester bonds by the nucleophile but, rather, the conformational alterations occurred following a lag phase. The data are consistent with a mechanism requiring the random cleavage of two thiol ester bonds of a dimeric unit in the molecule prior to the unimolecular process representing the conformational change. According to this model, the two dimeric units present in α_2M act as independent entities. On the basis of the best fit with the model, the bimolecular rate constant, for hydrolysis of the thiol ester bonds, was determined to be 11.9 ± 0.7 M^{-1} s^{-1}, while the rate constant for the conformational change was (9.7 ± 2.0) × 10^{-3} s^{-1}. The measured rate of conformational change is rate limited by thiol ester cleavage at lower methylamine concentrations. The conformational change, measured with this fluorescence probe, approximately parallels the loss of trypsin binding activity of α_2-macroglobulin, measured by resistance of the bound trypsin to soybean trypsin inhibitor. A much slower loss of plasmin binding activity was observed than was found for trypsin, suggesting that the structural requirements on α_2M for the interaction with plasmin are disrupted much more slowly than the structural requirements for trypsin binding upon methylamine treatment of the molecule.

α_2-Macroglobulin (α_2M)[1] is a 718 000 molecular weight plasma glycoprotein that contains four identical subunits that are linked in pairs via disulfide bonds (Swenson & Howard, 1979a,b; Hall & Roberts, 1978). It has been proposed that α_2M (Sottrup-Jensen et al., 1980; Howard, 1981) is similar to complement components C3 and C4 (Thomas et al., 1982; Cambell et al., 1981) in that it contains internal thiol ester bonds formed from side chains of cysteinyl and glutamyl residues that are four residues apart on the polypeptide chains.

α_2M functions as a protease inhibitor and is capable of inhibiting all four classes of proteases (Barrett & Starkey, 1973). Association of a protease with α_2M is characterized by a reduced activity of the enzyme toward large molecular weight substrates with little change in activity toward lower molecular weight substrates. The inhibition of the protease by α_2M has been proposed to occur via a "trap" mechanism (Barrett & Starkey, 1973) in which limited proteolysis at the "bait" region of α_2M is followed by a conformational change in α_2M, during which the thiol ester bonds are hydrolyzed. This conformational change is postulated to result in "entrapment" of the protease and has been measured by alterations in several of the physical properties of α_2M (Gonias et al., 1982; Bjork & Fish, 1982; Straight & McKee, 1983; Barrett et al., 1979). These conformational alterations in α_2M result in the rapid clearance of the complex from the circulation via receptors in the reticuloendothelial system (Van Leuven et al., 1979; Kaplan & Nielsen, 1979; Imber & Pizzo, 1981).

It has been observed that reaction of α_2M with small amines, such as methylamine, results in incorporation of the amine into α_2M (Swenson & Howard, 1979) with liberation of free sulfhydryl groups (Salvesen et al., 1981) presumably as a result of hydrolysis of the thiol ester bonds. This interaction with nucleophiles induces a conformational change in α_2M that results in alterations in some of the physical properties of the molecule similar to those observed upon protease treatment (Gonias et al., 1982; Bjork & Fish, 1982; Straight & McKee, 1983; Barrett et al., 1979). The methylamine-treated molecule, like the α_2M–protease complex, is rapidly cleared from the circulation (Imber & Pizzo, 1981; Kaplan et al., 1981).

The events that occur in α_2M as a result of treatment with nucleophiles have been examined by several laboratories, and

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[1] Abbreviations: ANS, 8-anilino-1-naphthalenesulfonate; bis(ANS), 5,5′-bis(8-anilino-1-naphthalenesulfonate); TNS, 6-(*p*-toluidino)-2-naphthalenesulfonic acid; NPGB, *p*-nitrophenyl *p*-guanidinobenzoate; α_2M, α_2-macroglobulin; SBTI, soybean trypsin inhibitor; SDS, sodium dodecyl sulfate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; PEG, poly(ethylene glycol); Tris, tris(hydroxymethyl)aminomethane; DFP, diisopropyl fluorophosphate.

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conflicting results have been reported. A report by Straight & McKee (1982) has concluded that hydrolysis of the thiol ester bonds, measured by reaction of generated sulfhydryl groups with 2,2′-dithiodipyridine, occurs simultaneously with intrinsic fluorescent changes in α_2M. Van Leuven et al. (1982) have reported that the incorporation of [^{14}C]methylamine into the thiol ester site and the resulting conformational change in α_2M, as measured by alterations in electrophoretic mobility, are sequential events in time. In order to clarify the sequence of events occurring in this molecule upon hydrolysis of the thiol ester bonds, and to gain further insight into the function of these bonds in the molecule, a study was initiated examining the kinetics of thiol ester hydrolysis and conformational change in α_2M upon reaction with methylamine.

Materials and Methods

Materials

4,4′-Dithiodipyridine, *p*-nitrophenyl *p*-guanidinobenzoate hydrochloride, methylamine hydrochloride, and HEPES were obtained from Sigma Chemical Co. 8-Anilino-1-naphthalenesulfonate (ANS) and bis(ANS) were obtained from Molecular Probes. 6-(*p*-Toluidino)-2-naphthalenesulfonic acid (TNS) was obtained from Eastman. Human cryosupernatant was obtained from the Washington Regional Blood Services, American Red Cross.

Methods

Proteins. Trypsin, obtained from Calbiochem, was dissolved in 1 mM HCl/7.5 mM $CaCl_2$. Active-site titration with NPGB (Chase & Shaw, 1970) gave an active-site concentration of 0.87 mol/mol of protein. Plasmin, prepared as described previously (Strickland et al., 1982), was stored at −70 °C in 50% glycerol, 100 mM lysine, and 0.05 M sodium phosphate, pH 8.0. Immediately before use, an aliquot was desalted on a Sephadex G-25 column previously equilibrated with 50 mM HEPES, 0.15 M NaCl, and 10% glycerol, pH 8.0. The active-site concentration, determined by titration with NPGB, was 0.86 mol/mol of protein. Affinity chromatography variant I was used in all cases. α_2M was prepared from fresh frozen plasma by precipitation with poly(ethylene glycol) (PEG) (Harpel, 1978), zinc chelate chromatography (Kurechi et al., 1979; Sottrup-Jensen et al., 1980), and gel chromatography on Bio-Gel A-5m. The following procedure was used. Sixty milligrams of SBTI was added to 1 L of cryosupernatant plasma. Fibrinogen was removed by precipitation with PEG 4000 (4% final concentration). α_2M was then precipitated with 12% PEG, resuspended in 200 mL of 0.02 M sodium phosphate and 0.15 M NaCl, pH 6.0, dialyzed against the buffer overnight at 4 °C, and applied to a zinc chelate column (2.5 cm × 30 cm) equilibrated in the same buffer. The column was washed with 2 L of buffer and the α_2M eluted with 0.1 M EDTA, pH 6.0. The protein fraction was concentrated and applied to a Bio-Gel A-5m column (5.0 × 100 cm) equilibrated with 0.05 M NaH_2PO_4/0.15 M NaCl, pH 7.2. The α_2M peak was pooled, concentrated, and stored frozen at −70 °C. The purified protein gave a single band in SDS gel–polyacrylamide gel electrophoresis (Weber & Osborn, 1969) and in immunoelectrophoresis against anti-human serum. Traces of immunoglobulin M (IgM) could be detected by radial immunodiffusion at levels of 0.5%. The preparation protected 2.0 mol of trypsin/mol of α_2M from inhibition by SBTI. No amidolytic activity was found to be associated with the preparation.

Protein Concentrations. These were determined spectrophotometrically by using the following $\epsilon^{1\%}_{280nm}$ and molecular weight values, respectively: Lys_{77}-plasmin, 17.0 M^{-1} cm^{-1} and 84000 (Barlow et al., 1969); α_2M, 8.93 M^{-1} cm^{-1} and 718000 (Jones et al., 1972; Hall & Roberts, 1978); methylamine-treated α_2M, 8.93 M^{-1} cm^{-1} and 718000 (Gonias et al., 1982); trypsin, 15.4 M^{-1} cm^{-1} and 23300 (Robinson et al., 1971; Walsh & Neurath, 1964).

Kinetics of Free Sulfhydryl Appearance. Sulfhydryl appearance was measured on a Cary 118 spectrophotometer equipped with a thermostated cell holder maintained at 30 °C. All reagents were maintained at this temperature prior to addition to the cuvettes. An aliquot of α_2M (final concentration 1 μM) was added to two cuvettes containing 2 mM 4,4′-dithiodipyridine (Castillo et al., 1979) in 50 mM HEPES/0.15 M NaCl, pH 8.0. Measurement of the free sulfhydryl appearance was initiated by the addition of methylamine to the sample cuvettes and the appropriate amount of buffer to the reference cuvette. The reaction was carried out at 30 °C and the absorbance monitored at 324 nm. An ϵ value of 19800 M^{-1} cm^{-1} at 324 nm was used (Grassetti & Murray, 1967).

TNS Fluorescence. TNS fluorescence was monitored in a Perkin-Elmer MPF-4 fluorometer equipped with a thermostated cell. The temperature was maintained at 30 °C, the slits were 10 nm, and the excitation wavelength was 315 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. The reaction was initiated by the addition of methylamine, and the fluorescence change was continuously monitored. The data were fit to several models by iteratively fitting the data to curves described by the appropriate equations. The first model is shown in Scheme I. This model

Scheme I

$$P + CH_3NH_2 \xrightarrow{k} PS \xrightarrow[\Delta F]{k_c} P^*S$$

assumes that the four subunits of α_2M are independent of one another and the appearance of P*S, the conformationally altered subunit with the thiol ester hydrolyzed, can be determined from standard kinetic equations derived for two consecutive first-order processes:

$$[P^*S] = [P_0]\left[1 + \frac{1}{k' - k_c}(k_c e^{-k't} - k' e^{-k_c t})\right] \quad (1)$$

where $[P_0]$ = the initial concentration of the α_2M subunit, $[P^*S]$ = the concentration of the conformationally altered subunit, k' = the pseudo-first-order rate constant for hydrolysis of the thiol ester bond by the nucleophile, and k_c = the first-order rate constant for the conformational change occurring in α_2M giving rise to increased TNS fluorescence.

The second model is shown in Scheme II. This model

Scheme II

$$P + CH_3NH_2 \underset{k}{\overset{k}{\rightleftharpoons}} PS_1 + CH_3NH_2 \underset{k}{\overset{k}{\rightleftharpoons}} PS_1S_2 \xrightarrow[\Delta F]{k_c} P^*S_1S_2$$
$$\qquad\qquad\qquad\qquad\quad PS_2 + CH_3NH_2$$

assumes that the random hydrolysis of two thiol ester bonds of a dimeric unit of α_2M precedes the conformational change. The integrated rate equations have been derived by Dr. Steven Olson (Henry Ford Hospital) (see the Appendix), and the appearance of $P^*S_1S_2$ is determined from

$$[P^*S_1S_2] = \frac{-2k_c[P_0]}{k_c - k'}e^{-k't} + \frac{k_c[P_0]}{k_c - 2k'}e^{-2k't} - \frac{2k'^2[P_0]}{(k_c - k')(k_c - 2k')}e^{-k_c t} + [P_0] \quad (2)$$

where $[P_0]$ = the initial concentration of the dimeric unit,

$[P^*S_1S_2]$ = the concentration of the molecule with an altered conformation, k' = the pseudo-first-order rate constant for hydrolysis of thiol esters, and k_c = the first-order rate constant for conformational change.

The appearance of sulfhydryl groups is given by

$$[S] = 2[P_0](1 - e^{-k't}) = 4[\alpha_2M]_0(1 - e^{-k't}) \quad (3)$$

where [S] = the total concentration of sulfhydryl groups and $[\alpha_2M]_0$ = the initial concentration of α_2M.

A third model that was examined involves the random hydrolysis of all four thio esters present in α_2M prior to the conformational change. The rate equation for the appearance of P^*S_{1-4} has been derived by Dr. Steven Olson (Appendix) and is given by

$$[P^*S_{1-4}] = [P_0]\left[1 - \frac{4k_c}{k_c - k'}e^{-k't} + \frac{6k_c}{k_c - 2k'}e^{-2k't} - \frac{4k_c}{k_c - 3k'}e^{-3k't} + \frac{k_c}{k_c - 4k'}e^{-4k't} - \frac{24k'^4}{(k_c - k')(k_c - 2k')(k_c - 3k')(k_c - 4k')}e^{-k_ct}\right]$$

where P^*S_{1-4} = α_2M with an altered conformation and all thiol ester sites hydrolyzed, $[P_0]$ = the initial concentration of α_2M, k' = the pseudo-first-order rate constant for hydrolysis of thiol esters, and k_c = the first-order rate constant for the conformational change.

Experimentally, the nucleophilic modifications were carried out under pseudo-first-order conditions. The bimolecular rate constant, k, was determined from the pseudo-first-order rate constant, k', by using the relationship $k' = k$[methylamine]. The uniqueness of the fit was investigated with a parameter-dependency study by using an approach similar to those described by Endrenyi & Kwong (1973) and Ingham et al. (1975). This method consists of fixing the parameter value above and below its determined value and determining the best fit while allowing the remaining parameters to vary. By observing the change in the standard deviation, one can assess the uniqueness with which the test parameter has been determined.

Effect of Methylamine on Protease Binding to α_2M. In these experiments, α_2M was inactivated with 50 mM CH_3NH_2 at 30 °C in 0.05 M HEPES/0.15 M NaCl, pH 8.0. At selected time intervals, 50-μL aliquots were withdrawn and added to a solution containing a 5-fold molar excess of trypsin, or plasmin in 50 mM Tris, pH 7.2. After 5 min, the amount of protease bound was quantitated according to the method of Ganrot (1966) by using a 2-fold molar excess of SBTI over protease to inhibit free protease activity. Trypsin amidolytic activity was measured by using H-D-Phe-Pip-Arg-p-nitroanilide (S-2238, Kabi) while plasmin amidolytic activity was measured by using H-D-Val-Leu-Lys-*p*-nitroanilide (S-2251, Kabi).

Results

Kinetics of Free Sulfhydryl Group Appearance upon Reaction with Methylamine. Previous studies have demonstrated that upon treatment of α_2M with methylamine, four free sulfhydryl groups are generated per mole of protein (Sottrup-Jensen et al., 1980, 1981a; Howard, 1981; Salvesen et al., 1981). The kinetics of this process, at varying concentrations of methylamine, were measured by continuous monitoring of the liberated sulfhydryl groups upon reaction with 4,4′-dithiodipyridine (Figure 1). Initial experiments were performed in which the concentration of 4,4′-dithiodipyridine was varied between 0.2 and 2.5 mM. At a methylamine concentration of 200 mM, the measured rate of sulfhydryl liberation was independent of 4,4′-dithiodipyridine concentration when present above concentrations of 0.8 mM. In the experiments shown in Figure 1, the concentration of 4,4′-dithiodipyridine was kept above this concentration to ensure that cleavage of the thiol ester bonds was rate limiting. Identical data were obtained when 5,5′-dithiobis(2-nitrobenzoate) was used. The data are expressed as the fraction of absorbance change, where A_{max} represents the maximum absorbance change and corresponds to 3.7 mol of sulfhydryl/mol of protein. Control experiments have demonstrated that TNS does not alter the rate of sulfhydryl release using the sulfhydryl reagents. Additionally, the presence of the sulfhydryl reagent does not affect the rate of conformational change measured by TNS fluorescence alterations. A replot of the data in the form of a first-order plot is shown in Figure 2, where it is apparent that the cleavage event is pseudo first order under these experimental conditions. The second-order rate constant, determined from the slope of a replot of the pseudo-first-order rate constants vs. the methylamine concentration (Figure 3), was determined to be 11.6 ± 0.8 M^{-1} s^{-1}. In this case, the concentration of methylamine in free base form was used, and a pK_a value of 10.43 was used for methylamine (Isenman & Kelb, 1982). The rate of incorporation of [^{14}C]methylamine into α_2M is pH dependent, and this effect has been attributed to deprotonation of methylamine (Salvesen et al., 1981). The hydrolysis of the thiol ester bonds by methylamine was likewise found to be highly dependent upon pH, when examined in the pH range of 7.0–8.5 (Table I). A replot of the observed rate vs. the concentration of methylamine in the free base form,

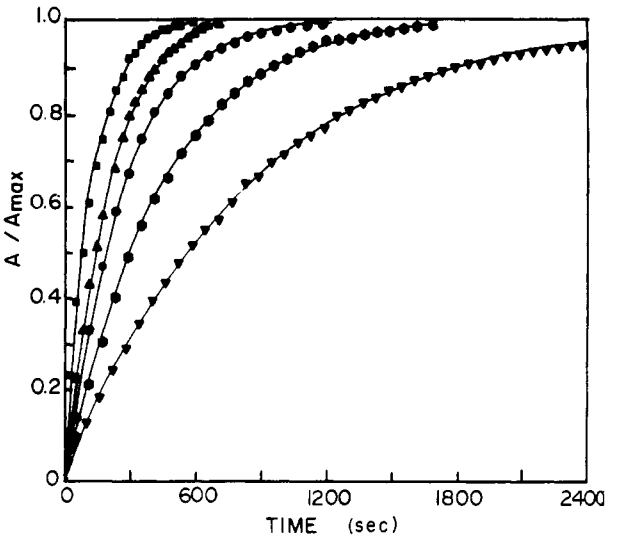

FIGURE 1: Time course of sulfhydryl appearance induced by methylamine treatment of α_2M monitored by reaction of the liberated sulfhydryl groups with 4,4′-dithiodipyridine. Data points were taken at selected time intervals from the trace. A_{max} was determined following extensive incubation and corresponds to 3.7 mol of sulfhydryl/mol of α_2M. The final concentration of α_2M was 1 μM, and the concentration of 4,4′-dithiodipyridine was 2 mM. The methylamine concentrations were (■) 200, (▲) 150, (●) 100, (●) 50, and (▼) 25 mM.

Table I: Effect of pH on the Rate of Sulfhydryl Exposure Induced by Methylamine

pH	k_{app} (s^{-1})
7.0	1.0×10^{-3}
7.5	3.2×10^{-3}
8.0	7.6×10^{-3}
8.5	11.3×10^{-3}

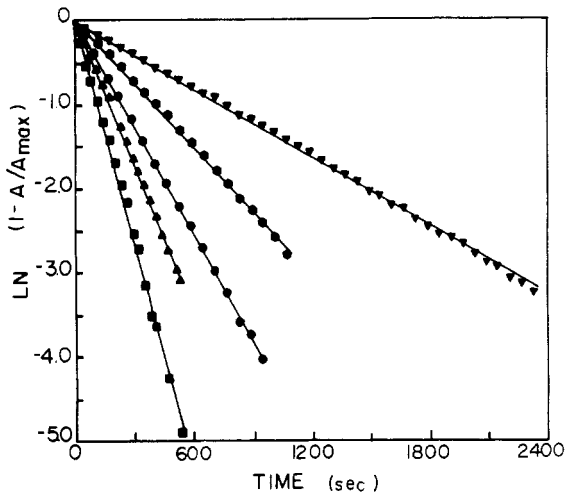

FIGURE 2: First-order plot of the data from Figure 1. Lines represent the best fit determined by the method of least squares. The concentrations of methylamine were (■) 200, (▲) 150, (●) 100, (●) 50, and (▼) 25 mM.

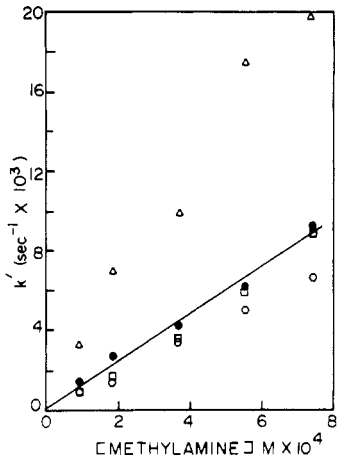

FIGURE 3: Replot of the pseudo-first-order rate constants vs. the concentration of methylamine in free base form, using a pK_a of 10.43 for methylamine. The line represents the best fit to the data from Figure 2 determined by the method of least squares. (●) Data obtained from plots in Figure 2. Rate constants determined from best fit of the TNS fluorescence data to Scheme I (○), Scheme II (□), and model 3 (△).

calculated at each pH value, was linear for the values in the pH range 7.0–8.0, with a value of 10.9 M^{-1} s^{-1} for the second-order rate constant obtained from the slope. At pH 8.5, the rate of thiol ester cleavage was no longer rate limiting. These data indicate that the pH effect can be readily explained by deprotonation of methylamine with increasing pH.

TNS Fluorescence Studies. Previous studies have reported that the fluorescence of ANS is enhanced in the presence of porcine α_2M or the trypsin–α_2M complex (Jacquot-Armand & Krebs, 1973). In the present study, the interactions of several fluorescent probes with α_2M, methylamine-treated α_2M, and α_2M–protease complexes were investigated. It was found that 6-(*p*-toluidino)-2-naphthalenesulfonic acid (TNS) is a more sensitive probe for monitoring conformational changes in α_2M than ANS or bis(ANS). The uncorrected emission spectra of this dye with α_2M, and several α_2M derivatives, are shown in Figure 4. In the presence of α_2M (curve 4), the fluorescence of TNS was enhanced. Concomitant with the increase was a wavelength shift of the maximal emission from 460 to 430 nm. Upon methylamine treatment (curve 5) or upon complex formation with trypsin or plasmin (curves 6 and 7, respectively), an additional 4-fold increase

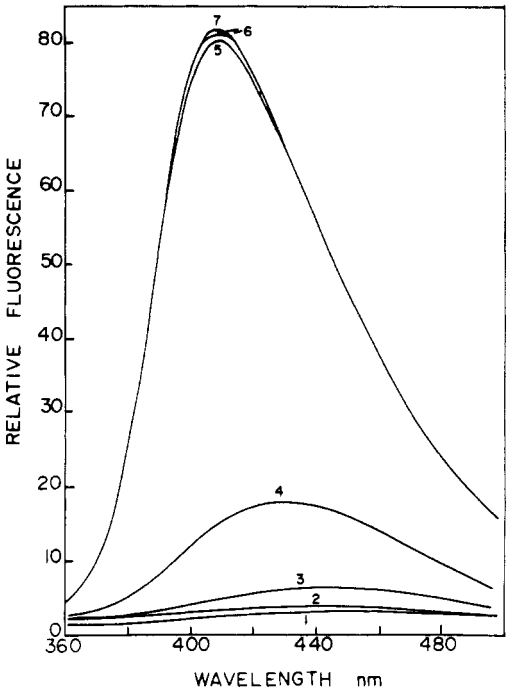

FIGURE 4: Uncorrected emission spectra of TNS in the presence of buffer (1), 2 μM trypsin (2), 1 μM plasmin (3), 1 μM α_2M (4), 1 μM methylamine-treated α_2M (5), α_2M–trypsin complex (1:2 mole ratio of α_2M:trypsin) (6), and α_2M–plasmin complex (1:1 mole ratio of α_2M:plasmin) (7). The final concentration of TNS was 50 μM, and all scans were carried out at 30 °C. Excitation was at 315 nm, and 10-nm band-pass excitation and emission slits were used.

in the fluorescence intensity and a further shift in the maximal emission to 412 nm were observed. The emission spectra of TNS in the presence of trypsin and plasmin are also shown (curves 2 and 3, respectively). The results shown in Figure 4 are consistent with several reports in the literature indicating that a conformational change occurs in α_2M in the presence of small amines and proteases (Gonias et al., 1982; Bjork & Fish, 1982; Straight & McKee, 1982; Barrett et al., 1979). Further, the data suggest that the enhanced TNS fluorescence is due to a binding site on α_2M, since the emission spectra of the protease complex are similar to the spectra of the methylamine derivative, and appears to be independent of the protease used.

Kinetics of Methylamine-Induced TNS Fluorescence Change. The alteration in TNS fluorescence of methylamine-treated α_2M has afforded an opportunity to examine the kinetics associated with this process. In these studies, the fluorescence change was continuously monitored in a Perkin-Elmer fluorometer equipped with a thermostated cell. The results of these experiments, shown in Figure 5, are expressed as F/F_{max} in which F_{max} represents the maximal fluorescence value determined by prolonged incubation with 200 mM methylamine (2 h). The fluorescence reached this maximal value prior to 2 h, and further incubation, for up to 6 h, resulted in no further increase in fluorescence intensity. The data points shown represent the average of duplicate determinations. The rate of conformational change, as measured by TNS fluorescence alterations, is dependent upon the concentration of methylamine. Furthermore, a lag phase, also dependent upon methylamine concentration, is observed. Recent studies have reported an alteration of the intrinsic fluorescence of α_2M upon methylamine treatment (Bjork & Fish, 1982; Straight & McKee, 1982), and it was therefore of interest to determine if the rate of intrinsic fluorescence change correlated with the rate measured by TNS fluores-

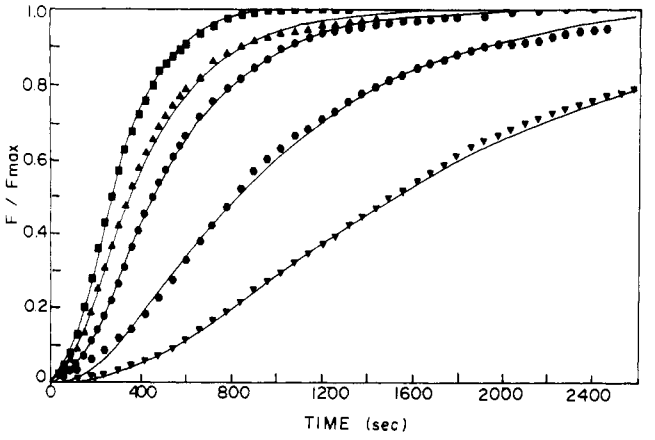

FIGURE 5: Time course of the TNS fluorescence change induced by methylamine treatment of α_2M. The fluorescence was continuously monitored, and the data points were taken from the trace at the selected time intervals. Each data point represents an average of duplicate runs. F_{max} represents the maximal fluorescence change and was determined by extensive incubation with methylamine. The curves are calculated from the fit parameters derived from treatment of the data in terms of Scheme II. The final concentration of α_2M was 1 μM, and the methylamine concentrations were (■) 200, (▲) 150, (●) 100, (●) 50, and (▼) 25 mM.

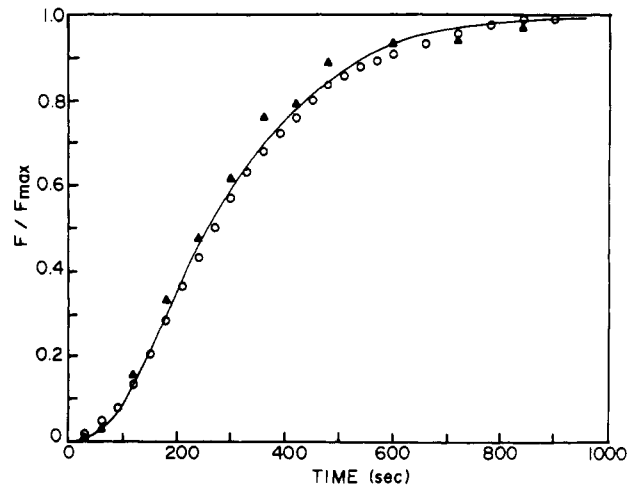

FIGURE 6: Comparison of the change in TNS fluorescence (O) and the change in intrinsic fluorescence (▲) upon incubation of α_2M with 200 mM methylamine. Experimental conditions are as described in Figure 5. Intrinsic fluorescence measurements were made at an excitation wavelength of 280 nm and an emission wavelength of 325 nm. The curve represents the fit obtained from treatment of the data in terms of Scheme II.

cence. At a methylamine concentration of 200 mM, it was found that the rate of change, measured by alteration in the intrinsic fluorescence, was identical with that measured by TNS fluorescence alterations (Figure 6). Trypsin treatment of α_2M abolished the methylamine-induced TNS fluorescence change in α_2M. Further, methylamine treatment of α_2M abolished the trypsin-induced TNS fluorescence change in α_2M.

The data obtained by measuring the change in TNS fluorescence were fitted to three mechanisms. The first mechanism assumes that the four subunits present in α_2M act independently of each other and is shown in Scheme I, where P = the α_2M subunit, PS = the α_2M subunit with free sulfhydryl generated from thiol ester hydrolysis, P*S = the conformationally altered subunit, k = the bimolecular rate constant for thiol ester hydrolysis by methylamine, and k_c = the first-order rate constant for the conformational change occurring in α_2M. The first step represents the hydrolysis of the thiol ester by the nucleophile and does not give rise to an alteration in TNS fluorescence, while the second step represents the conformational change occurring in α_2M.

The second mechanism assumes that α_2M contains two dimeric units, each consisting of two subunits, with the two dimeric units in α_2M acting as independent entities. This mechanism is shown in Scheme II, where P = the α_2M dimeric unit, PS_1 and PS_2 = molecules with equivalent sulfhydryl groups released in each monomer after thiol ester cleavage, PS_1S_2 = a molecule with two thiol ester bonds hydrolyzed, $P^*S_1S_2$ = a molecule with an altered conformation, k = the bimolecular rate constant for hydrolysis of the thiol ester by methylamine, and k_c = the first-order rate constant for the conformational change. In this mechanism, the initial step represents the random hydrolysis of one of two thiol ester bonds present in the dimeric unit of α_2M. The second step generates a molecule in which two thiol ester bonds are hydrolyzed. This step is followed by a unimolecular step representing the conformational change in α_2M.

A third mechanism considered assumes that all four thiol ester bonds in α_2M must be hydrolyzed prior to the conformational change occurring in α_2M (see the Appendix). In this mechanism, the initial step represents the random hydrolysis of one of the four thiol ester bonds in α_2M. Thus, four intermediate species would be present, each with one thiol ester hydrolyzed. In the next step, representing the random hydrolysis of a second thiol ester bond, three possible pathways exist for each intermediate species with one thiol ester hydrolyzed. The third step represents the random hydrolysis of a third thiol ester with two possible pathways for each intermediate species, while the fourth step generates a molecule with four thiol ester bonds hydrolyzed. This step is then followed by a unimolecular step representing the conformational change in α_2M and giving rise to the observed increase in TNS fluorescence.

Experimentally, the reactions were carried out under pseudo-first-order conditions in which the concentration of methylamine was much greater than the concentration of α_2M. The bimolecular rate constant, k, was determined from the pseudo-first-order rate constant by using the relationship $k' = k$[methylamine].

The data were fit to these mechanisms by two methods. In the first method, the best-fit values were determined by using a computer program in which the fitting was done in a series of iterations where the parameters were systematically adjusted until a least-squares solution was reached. The fit was examined with regard to a variety of parameters, indicating goodness of fit, as well as visual examination of the resulting fit by a graph of the predicted and observed values. In the fitting sequence, various initial values of the parameters were used in order to avoid a local minimum in the fitting process. The best-fit values were determined at each concentration of methylamine; in general, the fit of the data to each model was good, and it was virtually impossible to distinguish between the models on the basis of statistical analysis. In order to distinguish between the various models, the best-fit values obtained for k' were compared with the experimentally determined values obtained from measurement of sulfhydryl generation, and the results of this comparison are shown in Figure 3. It appears that Scheme II gives values that are closest to the experimentally observed value.

In the second method, the value of k' in each scheme was fixed at the experimentally measured value, determined by measurement of sulfhydryl generation, and the best-fit value of k_c determined. The results of this fit are shown in Figure 7, where the fits are superimposed over the actual data. In

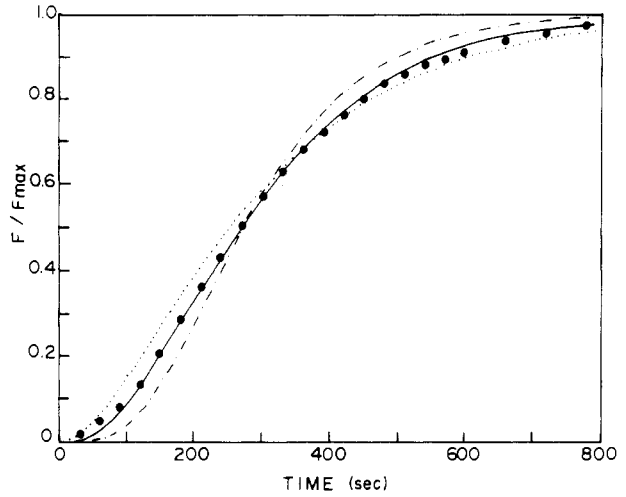

FIGURE 7: Comparison of the fit obtained with Scheme I (···), Scheme II (—), and model 3 (-·-) when k' was fixed at the experimentally obtained value and the best-fit value of k_c was determined. The concentration of methylamine was 200 mM, and a value of 9.3×10^{-3} s^{-1} for k' was used. The actual data, obtained from the experiment described in Figure 5, are also shown (●).

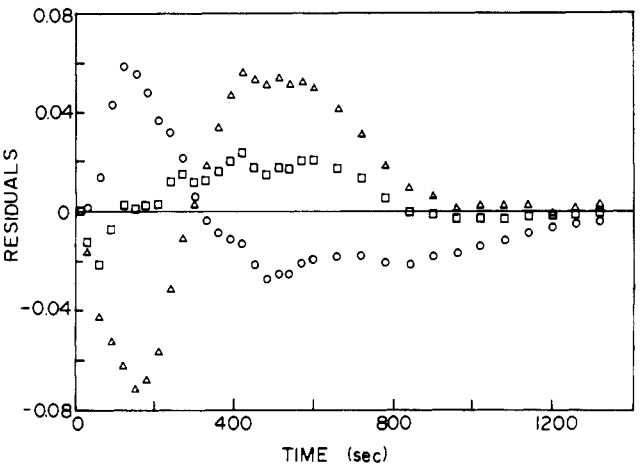

FIGURE 8: Plot of residual values for observed and calculated data vs. time for the fit described in Figure 7. (○) Scheme I; (□) Scheme II; (△) model 3.

this analysis, the concentration of methylamine was 200 mM, a concentration where a slightly more precise estimate of k_c could be obtained. It is obvious from the data in Figure 7 that Scheme II gives the best fit to the data. This is further illustrated in Figure 8, where the residuals of predicted and observed values vs. time are shown.

On the basis of the two fitting methods, it appears that Scheme II gives a slightly better fit than the other models. When the data were fit to this model (Figure 5), the value obtained for the bimolecular rate constant for hydrolysis of the thiol ester bonds was 11.9 ± 0.7 M^{-1} s^{-1}, while the rate constant for the conformation change was $(9.7 \pm 2.0) \times 10^{-3}$ s^{-1}. Several other models were evaluated, but no improvement in the fit was observed.

In order to assess the uniqueness of the parameter values, a parameter-dependency study was performed as described under Materials and Methods (data not shown). At higher concentrations of methylamine, the value of k_c was fairly well determined, while the value of k' could be precisely determined. At lower concentrations of methylamine, k' was precisely determined while only the lower limit of k_c could be determined.

Effect of Methylamine on Protease Binding to α_2M. It was of interest to examine the effect of methylamine on the ability of α_2M to bind proteases and to determine if the activity loss of α_2M correlated with the conformational change measured by TNS fluorescence. In these experiments, α_2M was inactivated with 50 mM CH_3NH_2. At selected time intervals, aliquots were withdrawn and added to a solution containing a 5-fold molar excess of trypsin or plasmin in 50 mM Tris, pH 7.2. The lower pH ensured that the hydrolysis reaction with methylamine was greatly reduced during the incubation with the protease. After 5 min, the amount of protease bound was quantitated by the method of Ganrot (1966) by using a 2-fold molar excess of soybean trypsin inhibitor over protease to inhibit free protease activity. Under these conditions, the inhibition of trypsin or plasmin by SBTI in the α_2M–protease complex was negligible. The results of this experiment are shown in Figure 9. For comparison, the change in TNS fluorescence is also shown (data from Figure 5). These data indicate that the loss of trypsin binding activity is in reasonable accord with the rate of conformational change. In similar experiments, where a large amount of SBTI was added (200-fold molar excess over trypsin), it was determined that significant inhibition of trypsin in the α_2M–trypsin complex was observed. These results, which are in agreement with the report by Wang et al. (1981) providing evidence that SBTI can form a complex with α_2M–trypsin, further emphasize the care that should be taken in examining the trypsin binding ability of α_2M by this method. Additional experiments were performed in which ^{125}I-labeled trypsin was incubated with α_2M previously treated with methylamine. Following incubation, the mixture was fractionated by high-performance gel-permeation chromatography, and it was observed that trypsin did not bind to the modified form of α_2M. In the case of plasmin (Figure 9), the loss of the ability of α_2M to protect plasmin from SBTI inhibition did not correlate with the conformational change measured by TNS and did not parallel the results obtained for trypsin.

Binding of Plasmin and Trypsin to α_2M Measured by TNS Fluorescence. In order to further characterize the nature of the fluorescence change associated with protease binding to α_2M, a titration was performed in which the TNS fluorescence was measured following addition of various amounts of trypsin (Figure 10A) or plasmin (Figure 10B). Titration of α_2M with DFP-inhibited trypsin or plasmin did not produce any change in TNS fluorescence. In the case of trypsin (Figure 10A), preliminary studies indicated that the fluorescence change was virtually complete after mixing (10 s), and identical results were obtained if the incubations were carried out for 5 or 120 min. The curve shown in Figure 10A represents the sum of the calculated concentrations of the binary (MT) and ternary (MT_1T_2) complexes of α_2M with trypsin obtained from

$$[MT] + [MT_1T_2] = \frac{2[T_0](2[M_0] - [T_0]) + [T_0]^2}{4[M_0]}$$

where $[T_0]$ = the total trypsin concentration, $[M_0]$ = the total α_2M concentration, and [MT] = the concentration of the binary complex and equals $[MT_1] + [MT_2]$, where MT_1 represents binding of trypsin at site 1 and MT_2 represents binding at site 2. This equation was derived as previously described (Pochan & Bieth, 1982) and assumes that the trypsin binding sites on α_2M are equivalent and independent. The excellent fit of the data to the theoretical curve confirms that the trypsin binding sites on α_2M are equivalent and independent and further indicates that formation of the binary complex (either MT_1 or MT_2) is sufficient to cause a conformational change resulting in increased TNS fluorescence. This is consistent with studies reporting the appearance of four

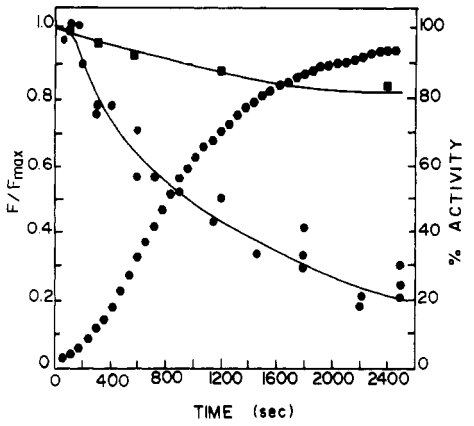

FIGURE 9: Time course of the TNS fluorescence change induced by methylamine treatment of α_2M (●) and inactivation of α_2M measured by loss of trypsin (●) and plasmin (■) binding ability. The final concentrations of methylamine and α_2M were 50 mM and 1 μM, respectively.

free sulfhydryl groups in the molecule upon interaction with one molecule of protease (Sottrup-Jensen et al., 1980) and with recent studies by Howell et al. (1983), who found binding ratios of trypsin–α_2M to be less than 2.0 if serial additions of nonsaturating amounts of trypsin were added. In the case of plasmin (Figure 10B), kinetic experiments showed that an initial rapid interaction with α_2M occurred followed by a much slower phase. Consequently, measurements were made after incubation for 6, 12, and 24 h and were found to be identical for all these time periods. In this case, a binding stoichiometry of 1.2:1 was obtained, in agreement with previous reports in the literature (Howell et al., 1983; Jacquot-Arnard & Guirard, 1976; Straight & McKee, 1982). In several experiments, using various preparations of plasmin, the ratio obtained by using this titration method was always greater than 1.0, suggesting the possibility that some molecules with 2 mol of plasmin/mol of α_2M may be present. Experiments are currently under way to examine this in more detail.

Discussion

Several reports in the literature have documented that upon proteolysis, or upon incubation with small amines, the conformation of α_2M is altered. This conformational change has been measured by alterations in circular dichoism spectra (Gonias et al., 1982), ultraviolet difference spectra (Gonias et al., 1982; Bjork & Fish, 1982), the sedimentation coefficient (Gonias et al., 1982; Bjork & Fish, 1982), and the intrinsic fluorescence (Bjork & Fish, 1982; Straight & McKee, 1983) as well as altered electrophoretic mobility (Barrett et al., 1979). In the present study, a fluorescence probe, TNS, was used to examine the kinetics of the conformational alterations in α_2M induced by methylamine treatment.

At this time, the interaction of TNS with α_2M has not been fully characterized. Previous studies on the interaction of ANS with α_2M and the trypsin–α_2M complex indicated a slight increase in the quantum yield of the dye when bound to the complex vs. that bound to free α_2M (Jacquot-Armand & Krebs, 1973). Although the exact nature of the conformational change occurring in methylamine-treated α_2M and in the α_2M–protease complex that causes an increased TNS fluorescence is not known, it does appear that the changes in TNS fluorescence are reflecting alterations in α_2M conformations. In the first place, all proteases examined interact only weakly with the dye. Further, both methylamine-treated α_2M and several α_2M–protease complexes display a similar increase in fluorescence intensity as well as an identical 18-nm shift in the wavelength of maximal emission (Figure 4). This blue shift and the fluorescent intensity increase are consistent with transfer of the dye to a less polar environment and thus imply a change in the hydrophobicity near the TNS binding region(s) of α_2M. However, the possible contribution of an altered conformation of the protease in the complex, to the TNS fluorescence, cannot be excluded. Finally, the conformational change, measured by this probe, appears to reflect alterations in the functional activity of α_2M and correlates with the loss of trypsin binding activity of the molecule.

In the case of plasmin, a much slower loss of binding activity was observed. These data suggest a different mode of interaction of plasmin with α_2M than is observed for trypsin and indicate that the structural requirements on α_2M for the interaction with trypsin are disrupted more rapidly in the presence of methylamine than are the requirements for plasmin binding. An understanding of this phenomenon will require further characterization of the interaction of plasmin with α_2M. The titration experiments with plasmin and trypsin are consistent with other observations (Howell et al., 1983; Sottrup-Jensen et al., 1980) that formation of the binary complex of protease with α_2M is sufficient to induce the conformational change in α_2M.

The conformational change does not occur simultaneously with cleavage of the thiol ester bonds by the nucleophile. For example, at 50 mM methylamine, the time required for hydrolysis of 50% of the thiol ester bonds was 5 min, while the time required for 50% of the TNS fluorescence change was 14 min. The data are consistent with a model in which α_2M contains two dimeric units, each consisting of two thiol ester bonds. The random hydrolysis of the two thiol ester bonds present in this dimeric unit is a necessary prerequisite prior to the conformational change measured by TNS fluorescent alterations. In this model, the two dimeric units present in α_2M would act independently of each other. It is interesting to note that this model is consistent with recent data reported by Gonias & Pizzo (1983). These investigators have prepared α_2M "half-molecules", each containing two subunits, from limited reduction and alkylation of human α_2M. These half-molecules appear to contain the functional properties of the intact molecule. The model (Scheme II) predicts the appearance of sulfhydryl groups, which is consistent with the experimental data, as well as explains the lag phase observed in the conformational change measured by TNS fluorescence alterations. It should be emphasized, however, that other models involving reversible steps and more complex processes are certainly feasible. Scheme II appears to be the simplest model that gives the best fit with the experimental observations.

Results somewhat different from those obtained in this study have been recently reported (Straight & McKee, 1982). These authors reported that the hydrolysis of the thiol ester bonds occurred simultaneously with the conformational change measured by alterations in the intrinsic fluorescence of α_2M. At this time, the reason for this discrepancy is not readily apparent. It probably does not reflect differences in the methods used since changes in intrinsic fluorescence appear to correlate with the alterations in TNS fluorescence. Van Leuven et al. (1982) have reported that the incorporation of [^{14}C]methylamine and the resulting conformational change in α_2M, as measured by alterations in electrophoretic mobility, are sequential events in time. In their study, it was found that the incorporation of [^{14}C]methylamine into α_2M followed biphasic kinetics. No evidence of biphasic kinetics for the generation of free sulfhydryl groups was found in this study. It is possible that the apparent discrepancy in the data could be explained by the chemistry of the thiol ester site in α_2M.

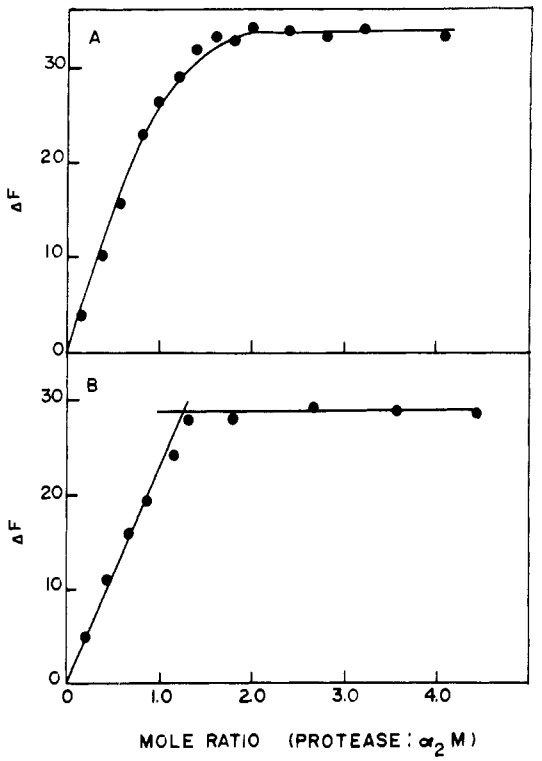

FIGURE 10: (A) Titration of α_2M with increasing concentrations of trypsin as measured by alterations in TNS fluorescence. The final α_2M concentration was 1 μM, and the temperature was maintained at 30 °C. The curve represents the sum of the concentrations of the binary (α_2M–trypsin) and ternary (α_2M–trypsin$_2$) complexes. (B) Titration of α_2M with increasing amounts of plasmin.

Recently, Khan & Erickson (1982) have used a 15-membered thiolactone and the isomeric 5-membered lactam as model compounds of the thiol ester regions in α_2M, C3, and C4. They found that a dynamic equilibrium existed between these two isomeric hexapeptides. Further, it was proposed that the unactivated proteins contain the thiolactone isomer, in which the ring is effectively buried within the interior of the protein and is unable to equilibrate with the lactam isomer. If, upon initial nucleophilic attack of the thiol ester, a conformational change occurs and significant amounts of the lactam isomer are formed, a lower incorporation of [^{14}C]methylamine into α_2M could reflect the rate of conversion of the lactam to the lactone isomer. Since the lactam has a free sulfhydryl group, it is likely that this conversion would not be detected when the appearance of sulfhydryl groups is monitored by using sulfhydryl reagents.

It is interesting to note that two complement proteins, C3 and C4, both of which contain thiol ester bonds (Thomas et al., 1982; Pangburn, 1980; Janatova et al., 1980; Tack et al., 1980), have recently been examined with regard to the events occurring upon incubation with methylamine (Isenman & Kelb, 1982; Isenman et al., 1980). These proteins were similar to α_2M in that the cleavage event did not occur simultaneously with the conformational change, measured by changes in ANS fluorescence and circular dichroism. It appears that the rates of cleavage of the thiol esters present on α_2M are intermediate with those reported for C3 and C4 (5.8 and 24.4 M^{-1} s^{-1}, respectively). The overall rate of conformational change in α_2M, induced by hydrolysis of the thiol ester bonds with methylamine, is rate limited by the cleavage event at lower methylamine concentrations, while at higher methylamine concentrations, the conformational change in C3 and C4 appears to be rate limiting, even at fairly low concentrations of methylamine.

It appears that, under certain circumstances, the integrity of the thiol ester bonds in α_2M, like complement components C3 and C4 (Isenman & Kells, 1982), is important in maintaining the conformation of the protein. In the absence of proteolysis, this conformational change is fairly slow and depends on hydrolysis of the thiol ester bonds. In the presence of certain proteases such as trypsin, however, the conformational change is extremely rapid (Dangott et al., 1983). The mechanism of the conformational change in α_2M induced by proteolysis, and the function of the thiol ester bonds in this process, remains unresolved at this time. Measuring conformational alterations in α_2M by monitoring changes in the properties of TNS fluorescence will be an excellent method to complement other techniques in order to examine the sequence of events occurring in α_2M upon interaction with proteases.

Acknowledgments

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Registry No. Methylamine, 74-89-5; plasmin, 9001-90-5; trypsin, 9002-07-7.

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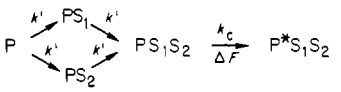
Appendix: Solution of Differential Rate Equations

The integrated rate equations are derived for the following mechanism:



where k' is a pseudo-first-order rate constant and is related to the bimolecular rate constant k by the relationship $k' = k$[nucleophile], P = the dimeric unit of α_2M, S_1 and S_2 = equivalent sulfhydryl groups released in each monomer after thiol ester cleavage, and k_c= the first-order rate constant for the conformational change. The differential equation for species P:

$$-\frac{d[P]}{dt} = k'[P] + k'[P] = 2k'[P] \quad (A1)$$

after separation of variables can be integrated to give

$$[P] = [P_0]e^{-2k't} \quad (A2)$$

where $[P_0]$ = the initial concentration of P. For species PS_1:

$$\frac{d[PS_1]}{dt} = k'[P] - k'[PS_1] \quad (A3)$$

Substituting for [P], multiplying both sides by dt, and rearranging give

$$d[PS_1] + k'[PS_1]dt = k'[P_0]e^{-2k't}\,dt \quad (A4)$$

Multiplying both sides by the integrating factor $e^{k't}$ makes the left-hand side an exact differential allowing integration:

$$\int_0^t d([PS_1]e^{k't}) = \int_0^t k'[P_0]e^{-k't}\,dt \quad (A5)$$

which yields

$$[PS_1] = [P_0]e^{-k't} - [P_0]e^{-2k't} \quad (A6)$$

By symmetry

$$[PS_2] = [P_0]e^{-k't} - [P_0]e^{-2k't} \quad (A7)$$

The differential rate equation for species PS_1S_2 is

$$\frac{d[PS_1S_2]}{dt} = k'[PS_1] + k'[PS_2] - k_c[PS_1S_2] \quad (A8)$$

Proceeding as before, substitute for $[PS_1]$ and $[PS_2]$ from above, multiply both sides by dt, and rearrange to give

$$d[PS_1S_2] + k_c[PS_1S_2]dt = 2k'[P_0](e^{-k't} - e^{-2k't})dt \quad (A9)$$

Multiplying both sides by the integrating factor e^{k_ct} then allows integration:

$$\int_0^t d([PS_1S_2]e^{k_ct}) = 2k'[P_0]\left[\int_0^t e^{(k_c-k')t}\,dt - \int_0^t e^{(k_c-2k')t}\,dt\right] \quad (A10)$$

which evaluates to

$$[PS_1S_2] = \frac{2k'[P_0]}{k_c - k'}e^{-k't} - \frac{2k'[P_0]}{k_c - 2k'}e^{-2k't} + \frac{2k'^2[P_0]}{(k_c - k')(k_c - 2k')}e^{-k_ct} \quad (A11)$$

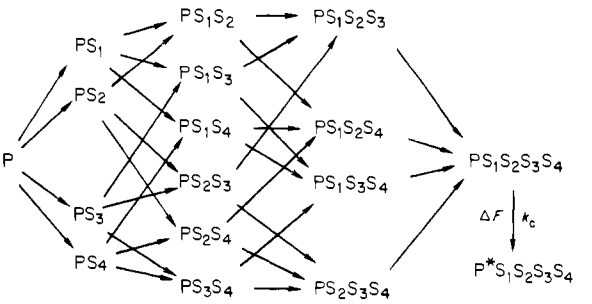
Finally, for species $P^*S_1S_2$:

$$\frac{d[P^*S_1S_2]}{dt} = k_c[PS_1S_2] \quad (A12)$$

Substitution for $[PS_1S_2]$ from above and integration yield the desired final result:

$$[P^*S_1S_2] = \frac{-2k_c[P_0]}{k_c - k'}e^{-k't} + \frac{k_c[P_0]}{k_c - 2k'}e^{-2k't} - \frac{2k'^2[P_0]}{(k_c - k')(k_c - 2k')}e^{-k_ct} + [P_0] \quad (A13)$$

Model 3 of the text involves random cleavage of four identical thiol esters of the tetrameric protein followed by the conformational change:



Unless indicated otherwise, all steps proceed with rate constant k'.

Given the symmetry of the above scheme whereby all species in any column are equivalent, the differential equations can be integrated similarly to Scheme II to yield the solution given in the text:

$$\frac{[P^*S_1S_2S_3S_4]}{[P_0]} = 1 - \frac{4k_c}{k_c - k'}e^{-k't} + \frac{6k_c}{k_c - 2k'}e^{-2k't} - \frac{4k_c}{k_c - 3k'}e^{-3k't} + \frac{k_c}{k_c - 4k'}e^{-4k't} - \frac{24k'^4}{(k_c - k')(k_c - 2k')(k_c - 3k')(k_c - 4k')}e^{-k_ct}$$

It is instructive to consider the more general case of a protein unit with n subunits each having an equivalent thiol ester that must be cleaved in order to trigger a conformational change in the protein unit. Integration of the differential equations yields

$$\frac{[P^*S_1S_2...S_n]}{[P_0]} = 1 - \frac{nk_c}{k_c - k'}e^{-k't} + \frac{n(n-1)k_c}{2!(k_c - 2k')}e^{-2k't} - \frac{n(n-1)(n-2)k_c}{3!(k_c - 3k')}e^{-3k't} + ... + (-1)^{n-1}\frac{nk_ce^{-(n-1)k't}}{k_c - (n-1)k'} + (-1)^n\frac{k_ce^{-nk't}}{k_c - nk'} + (-1)^{n+1}\frac{n!k'^ne^{-k_ct}}{(k_c - k')(k_c - 2k')...(k_c - nk')}$$

The first $(n + 1)$ terms contain the binomial coefficients as do the number of species in each column of the mechanism [see model 2 (Scheme II) and model 3 above]. For all mechanisms, summing all sulfhydryl-containing species gives

$$\frac{[S]_{total}}{n[P_0]} = 1 - e^{-k't}$$

verifying their kinetic equivalence, as assumed.

Nuclear Magnetic Resonance Studies on Calmodulin: Ca^{2+}-Dependent Spectral Change of Proteolytic Fragments†

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ABSTRACT: Proton magnetic resonance spectroscopy was performed in order to study the effect of calcium on the solution conformation of calmodulin with four proteolytic fragments. Two characteristic high field shifted phenylalanines of Ca^{2+}-free calmodulin were found to be located in domain I and/or domain II. In the Ca^{2+}-saturated state, these two phenylalanines also give resonances at higher fields. Another high field shifted phenylalanine appears and is found to be located in domain III or domain IV. It was demonstrated that the interaction between domains I and II and the interaction between domains III and IV are of importance for stabilization of the native structure but the interaction between the N-terminal-half region (domains I and II) and the C-terminal-half region (domains III and IV) was not clarified. Ca^{2+}-dependent slow-exchange behavior of ϵ and δ protons of tyrosine-138 [Ikura, M., Hiraoki, T., Hikichi, K., Mikuni, T., Yazawa, M., & Kagi, K. (1983) *Biochemistry 22*, 2573] was confirmed. The conformational transition between the Ca^{2+}-free and -bound states occurs at a rate of more than 300 s^{-1} in the N-terminal-half fragment, while the transition of the C-terminal-half fragment is slower than 50 s^{-1}.

Calmodulin (CaM)[1] and troponin C (TnC) are homologous Ca^{2+} binding proteins. The former serves as a Ca^{2+}-dependent activator or modulator of numerous enzymes, and the latter regulates muscle contraction in a Ca^{2+}-dependent manner [for a review, see Klee & Vanaman (1982)]. Both proteins consist of four Ca^{2+}-binding domains, numbered as I, II, III, and IV starting from the N terminal. Each domain is made of about 35 amino acid residues, and the sequences of each domain are homologous to one another. The structure of each domain has been considered to be similar to the EF-hand structure of parvalbumin crystal reported by Kretsinger & Barry (1975).

A number of spectroscopic studies show that binding of Ca^{2+} to CaM and to TnC causes considerable conformational changes [for a review, see Klee & Vanaman (1982)]. Because the difference in function between CaM and TnC should be related to the difference in structure, it is of interest to study the conformation of CaM and the conformational change induced by Ca^{2+} binding in detail as compared to TnC.

In earlier proton NMR studies on CaM (Seamon, 1980; Krebs & Carafoli, 1982) and also in our succeeding studies (Ikura et al., 1983a,b), assignments of resonances appearing in the aromatic and high-field methyl regions were made, and the Ca^{2+}-dependence of the spectra was investigated. Because the resonances were assignable only to residues in the C-terminal-half region (domains III and IV), little information about the N-terminal-half region (domains I and II) was obtained.

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[1] Abbreviations: CaM, calmodulin; TnC, troponin C; NMR, nuclear magnetic resonance; CD, circular dichroism; F1, cyanogen bromide fragment containing domain I (residues 1–36); F4, tryptic fragment containing domain IV (residues 107–148); F12, tryptic fragment containing domains I and II (residues 1–75); F34, tryptic fragment containing domains III and IV (residues 78–148); TCA, trichloroacetic acid; TSP, (trimethylsilyl)propionic-d_4 acid; Tml, N^{ϵ},N^{ϵ},N^{ϵ}-trimethyllysine; NOE, nuclear Overhauser enhancement.

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