GI, 89556-50-3; [diiodo-Tyr[11]]conotoxin GI, 89556-51-4; [diiodo-Tyr[11],monoiodo-His[10]]conotoxin GI, 89556-52-5.

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Kinetics of Appearance of Sulfhydryl Groups in α_2-Macroglobulin on Reaction of the Inhibitor with Amines†

Lars-Johan Larsson and Ingemar Björk*

ABSTRACT: The mechanism of the appearance of sulfhydryl groups in α_2-macroglobulin in the reaction with amines was characterized by analyses of the kinetics with ammonia and methylamine. All reactions occurred under pseudo-first-order conditions in the range of pH (7.0–8.6) and amine concentration (10–600 mM) investigated. The logarithm of the pseudo-first-order rate constant increased linearly as a function of pH with a slope of unity, indicating that the unprotonated amine is the active species in the reaction. Plots of the observed pseudo-first-order rate constants vs. concentration of unprotonated amine at constant pH were also linear and gave second-order-rate constants of 0.32 and 13.8 M^{-1} s^{-1} for ammonia and methylamine, respectively, at pH 8.0; similar values were obtained at pH 8.6. Activation energies of 85 and 100 kJ mol^{-1} and activation entropies of 10 and 95 J K^{-1} mol^{-1} for ammonia and methylamine, respectively, were estimated from Arrhenius plots, suggesting that the higher reaction rate for methylamine is due primarily to a higher activation entropy. These results are consistent with the release of sulfhydryl groups being caused by a nucleophilic attack of the uncharged amine on a thio ester bond of α_2-macroglobulin in a bimolecular reaction occurring under pseudo-first-order conditions. The characteristics of the reaction suggest that the thio ester in each α_2-macroglobulin subunit reacts independently and equivalently with the amine and also that the thio ester bond cleavage initiates the reaction sequence leading to inactivation of the inhibitor. The α_2-macroglobulin thio ester was further characterized by a comparison of the rates of cleavage of this bond by a series of amines with the corresponding rates of cleavage of a small thio ester, ethyl thioacetate. The protein thio ester was cleaved more rapidly by small primary amines than the model thio ester. The thio ester bond in α_2-macroglobulin thus appears comparatively labile. The reactivity of the different amines with α_2-macroglobulin did not parallel the reactivity with the small thio ester but rather the inverse of the size of the amines, indicating that access of reagents to the protein thio ester is sterically hindered.

The protein α_2-macroglobulin is a plasma proteinase inhibitor with several unique properties. It has a high relative molecular mass (~725 000) and consists of four apparently identical polypeptide chains (Jones et al., 1972; Hall & Roberts, 1978; Swenson & Howard, 1979a; Sottrup-Jensen et al., 1979). It inhibits a wide variety of proteinases of different classes and with different specificities (Barrett & Starkey, 1973; Harpel, 1976). Moreover, it binds the proteinase in a manner that abolishes or markedly decreases the activity of the enzyme against macromolecular substrates but largely preserves the activity against small substrates (Barrett & Starkey, 1973; Harpel, 1976). These properties have led to the suggestion that the inhibitor physically entraps the proteinase (Barrett

† From the Department of Medical and Physiological Chemistry, Faculty of Veterinary Medicine, Swedish University of Agricultural Sciences, Uppsala Biomedical Center, S-751 23 Uppsala, Sweden. *Received October 7, 1983.* Supported by grants from the Swedish Medical Research Council (Projects 4212 and 6688), the Swedish Council for Forestry and Agricultural Research (Project A5861), and Konung Gustaf V:s 80-årsfond.

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& Starkey, 1973). This proposal is supported by the findings that the binding of the enzyme is initiated by proteolytic cleavage of α_2M[1] (Harpel, 1973; Barrett et al., 1979; Swenson & Howard, 1979b; Sottrup-Jensen et al., 1981b) and is associated with a conformational change that alters the shape of the inhibitor (Barrett et al., 1974, 1979; Björk & Fish, 1982; Gonias et al., 1982; Branegård et al., 1982).

The α_2M–proteinase reaction is accompanied by the appearance of one free sulfhydryl group per α_2M polypeptide chain (Sottrup-Jensen et al., 1980, 1981a; Howard, 1981; Salvesen et al., 1981). Concurrently, a glutamic acid residue is released in an active form that can react covalently with amino groups, such as lysine residues on the proteinase (Salvesen et al., 1981; Sottrup-Jensen et al., 1981c; Wu et al., 1981; Van Leuven et al., 1981b). Certain primary amines, e.g., methylamine, also cause the appearance of a sulfhydryl group in each α_2M subunit and form an amide with the same glutamic residue that is activated in the reaction with proteinases (Swenson & Howard, 1979b, 1980; Sottrup-Jensen et al., 1980, 1981a, 1982; Salvesen et al., 1981). In human α_2M, but not in the proteins from cow or rat (Dangott & Cunningham, 1982; Gonias et al., 1983), this reaction leads to inactivation of the inhibitor (Steinbuch et al., 1968; Barrett et al., 1979) and to a conformational change similar to that induced by proteinases (Björk & Fish, 1982; Gonias et al., 1982). Although circumstantial, this evidence strongly suggests that a labile thio ester bond exists in each α_2M polypeptide chain (Sottrup-Jensen et al., 1980; Howard, 1981; Salvesen et al., 1981).

The role of the putative thio ester in α_2M structure and function is unclear. Further analyses of the reactions that have led to the proposal of such a bond are required to elucidate this role. To that end, we have characterized the kinetics of the appearance of sulfhydryl groups in α_2M on reaction with amines.

Materials and Methods

DTNB was purchased from Fluka, Buchs, Switzerland, and Hepes from Sigma Chemical Co, St. Louis, MO. Ethyl thioacetate was obtained from ICN Pharmaceuticals, Inc., Plainview, NY. *n*-Pentylamine was from Fluka; all other amines were from E. Merck, Darmstadt, Germany. Ammonia, methylamine, dimethylamine, trimethylamine, ethylamine, and *n*-pentylamine were obtained as hydrochlorides, and the remainder was obtained as free amines.

α_2M was prepared from fresh-frozen human plasma by precipitation with poly(ethylene glycol) (Barrett et al., 1979), zinc-chelate chromatography (Kurecki et al., 1979; Sottrup-Jensen et al., 1980), and gel chromatography on Sephacryl S-300 (Pharmacia, Uppsala, Sweden). The purity of the preparations was comparable to that of material used previously (Björk & Fish, 1982). Concentrations of α_2M were obtained by absorption measurements at 280 nm. The specific absorption coefficient and the relative molecular mass used in the calculations were 0.90 L g^{-1} cm^{-1} (Dunn & Spiro, 1967; Hall & Roberts, 1978) and 725 000 (Jones et al., 1972; Hall & Roberts, 1978), respectively.

The kinetics of appearance of sulfhydryl groups on reaction of α_2M with amines were monitored by a continuous assay, in which the liberation of these groups was coupled to their reaction with DTNB (Ellman, 1959). The assay was done in a Cary 219 spectrophotometer (Varian Associates, Palo Alto, CA) with self-masking, semi-microcuvettes, thermostated to within 0.1 °C of the desired temperature. The sample cell contained 800 μL of α_2M in buffer (0.2 M Hepes and 2 mM EDTA, pH 7.00–8.60, with NaCl added to a total ionic strength of the buffer of 0.25), and the blank contained the same volume of buffer. A volume of 100 μL of 1 mM DTNB in the Hepes buffer was then added to both cells, and the absorbance change was noted. The reaction was started by the addition to both cells of 100 μL of amine, dissolved in the Hepes buffer and carefully adjusted to the appropriate pH. The final concentration of α_2M was 3.3 μM and that of amine 10–600 mM. The progress of the reaction was monitored continuously at 410 nm on the 0.2 absorbance scale and with a bandwidth of 2 nm. The absorbance changes at selected time intervals were corrected for a small amount of reoxidation of the *p*-nitrothiophenol anion (see Results). The absorbance change at infinite time was estimated from these data by nonlinear least-squares regression with a computer program, MINUIT (James & Roos, 1975), adapted for use on a NORD 10 computer (A/S Norsk Data-Elektronikk, Oslo, Norway). Pseudo-first-order rate constants were then obtained from semilogarithmic plots in the usual manner (Price & Dwek, 1974). All but the slowest reactions were followed to at least 90% completion, as judged from the estimated value for the absorbance change at infinite time.

The rate of aminolysis of a small thio ester, ethyl thioacetate, was studied similarly. The sample cell contained 16 μM ethyl thioacetate (diluted from a stock solution in ethanol) in 0.2 M sodium borate, 0.1 M NaCl, 2 mM EDTA, and 10% (v/v) ethanol, pH 10.00, and the reference cell contained buffer and ethanol only. The analyses were then performed and evaluated as described above.

Results

Validity of the Assay. Analyses of native α_2M before the start of the reaction regularly showed less than 0.1 mol of sulfhydryl groups/mol of protein. The amines caused the appearance of 3.5–3.8 mol of sulfhydryl groups/mol of protein, as calculated from the absorbance at 410 nm extrapolated to infinite time. The reaction thus exposes close to one sulfhydryl group per α_2M subunit, as has been reported previously (Sottrup-Jensen et al., 1980).

Several control experiments were done to ascertain that the assay measures the true rate of appearance of the sulfhydryl groups. Most important was to establish that the reaction of the exposed sulfhydryl groups with DTNB was sufficiently fast not to influence the analyses. Therefore, α_2M (3.3 μM) was incubated with 200 mM methylamine for 60 min at different pH values and was then reacted with DTNB under the same conditions as in the assay. Consistently, maximum absorbance was reached with the dead time of this operation, i.e., 5–10 s. A possible delay of this magnitude could only have affected the observed rates of the fastest reactions studied and to maximally about 5%. In these control experiments, a small amount (<3%/h) of reoxidation of the *p*-nitrothiophenol anion was observed; hence, all values were corrected for this phenomenon. Further controls showed that prolonged incubation of α_2M with DTNB does not cause the appearance of sulfhydryl groups and also that incubation of amines with DTNB does not result in cleavage of the latter.

The marked pH dependence of the reaction of α_2M with the amines (see below) necessitated certain experimental precautions. Hepes was chosen as buffer, since it covers the desired pH range and does not contain primary amino groups. A buffer concentration of 0.2 M was used, as a small decrease

[1] Abbreviations: α_2M, α_2-macroglobulin; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetate.

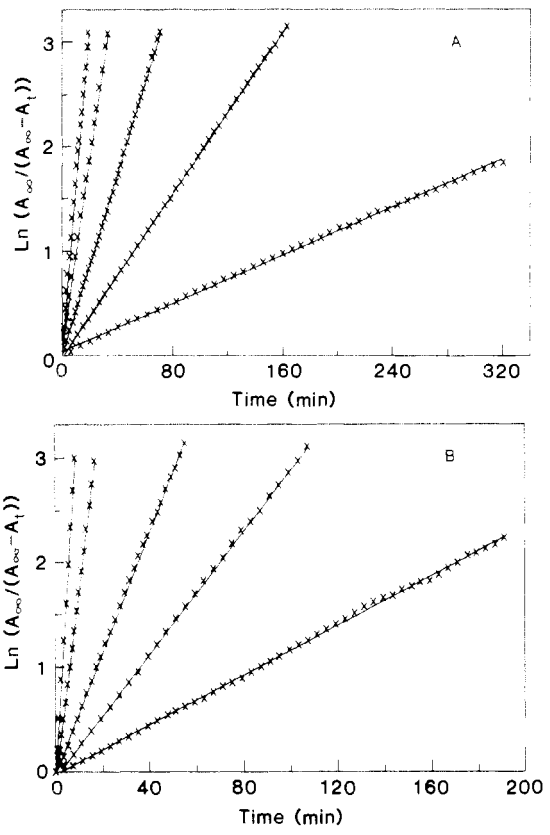

FIGURE 1: First-order plots of the rate of appearance of sulfhydryl groups in α_2M at 25.0 °C and at different pH values: (A) reaction with 200 mM ammonia; (B) reaction with 100 mM methylamine. The pH values for the curves (in order of increasing slope) are 7.0, 7.4, 7.8, 8.2, and 8.6. Concentrations are total amine concentrations (i.e., the sum of the concentrations of the unprotonated and protonated forms). A_t and A_∞ are the absorbances at 410 nm at time t and at infinite time, respectively.

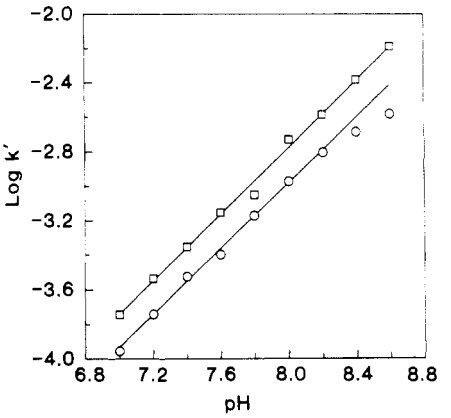

FIGURE 2: Logarithm of the pseudo-first-order rate constant (k') for the appearance of sulfhydryl groups in α_2M at 25.0 °C as a function of pH: (○) reaction with 200 mM ammonia; (□) reaction with 100 mM methylamine. Concentrations are total amine concentrations. The rate constants were computed from the plots in Figure 1 and from additional plots for the intermediate pH values.

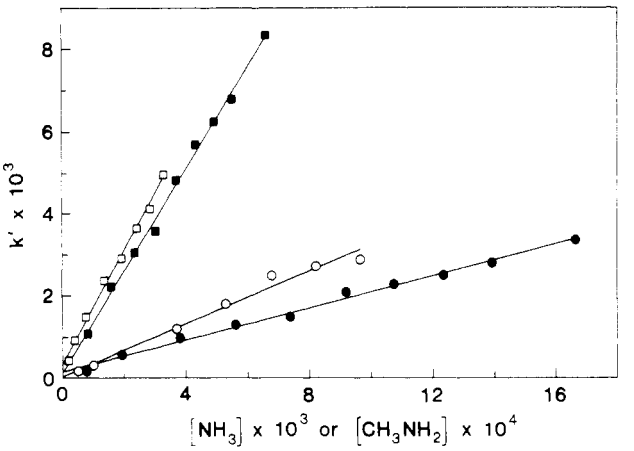

FIGURE 3: Pseudo-first-order rate constants (k') for the appearance of sulfhydryl groups in α_2M at 25.0 °C and at different concentrations of unprotonated amine: (○ and ●) reaction with ammonia at pH 8.0 and 8.6, respectively; (□ and ■) reaction with methylamine at pH 8.0 and 8.6, respectively.

of pH was observed during the reaction at lower concentrations. The pH of all solutions was carefully adjusted before the start of the experiment, and the pH of the reaction mixture was checked after the reaction. In all experiments, pH was constant to ±0.01 unit.

pH Dependence. The pH dependence of the rate of appearance of sulfhydryl groups in α_2M was studied at 25.0 °C in the pH range 7.00–8.60 with ammonia and methylamine at a total amine concentration (i.e., the sum of the protonated and unprotonated forms) of 200 and 100 mM, respectively. Semilogarithmic plots of the data were linear at all pH values investigated (see Figure 1 for representative plots), indicating that the reactions occurred under pseudo-first-order conditions. A marked increase of the reaction rate with pH was evident. Plots of the logarithm of the pseudo-first-order rate constants vs. pH (Figure 2) gave straight lines up to pH 8.2 for ammonia and throughout the pH range for methylamine. The slopes of these lines were 0.95 and 0.97 for the two amines, respectively, i.e., essentially unity for both. This behavior, at pH values lower than about 1 unit below the pK_a values of the amines, shows that the unprotonated amine is the active species in the reaction [see e.g., Price & Dwek (1974)] and that the dominating effect of pH on the reaction rate is to change the concentration of this species.

Concentration Dependence. The dependence of the rate of liberation of sulfhydryl groups on the concentration of amine was studied with ammonia and methylamine at 25.0 °C and at pH 8.00 and 8.60. A range of total amine concentration of 10–600 mM was covered. All semilogarithmic plots were linear also in these experiments, reflecting pseudo-first-order conditions. Since the pH-dependence studies showed that the unprotonated amine is the active form, the observed pseudo-first-order rate constants were plotted against the concentration of this species (Figure 3). In the calculations of these concentrations, the variation of the apparent dissociation constant of the amines with total amine concentration was approximated by the experimentally determined ionic strength dependence of this constant (Harned & Owen, 1930), also taking into account the ionic strength of the buffer. Straight lines were obtained for both amines at both pH values. Second-order rate constants (based on the concentration of unprotonated amine) of 0.32 and 0.20 M^{-1} s^{-1} for ammonia and 13.8 and 12.4 M^{-1} s^{-1} for methylamine at 25.0 °C and at pH 8.00 and 8.60, respectively, were calculated from these plots. The rate constants measured at the two pH values are similar for each amine, in spite of the fact that the observed reaction rate at the same total concentration of amine was about 4 times higher at the higher pH. This similarity further supports the conclusion that the major effect of pH on the reaction rate is to change the concentration of unprotonated amine. The moderate difference between the two rate constants obtained for ammonia probably reflects primarily the greater error of the experiments with this amine at pH 8.6. Thus, evaporation of ammonia at the high concentrations present at this pH may have appreciably affected the measurements. Moreover, the

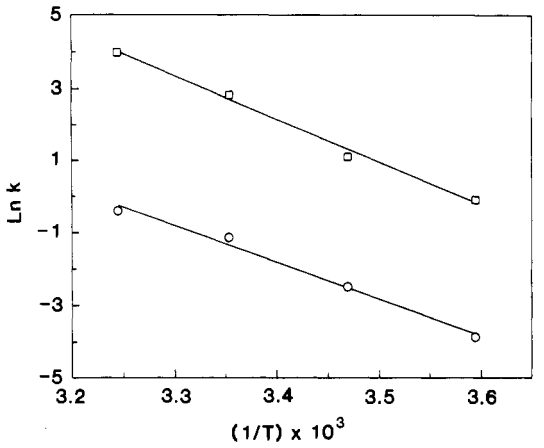

FIGURE 4: Logarithm of the second-order rate constants (k, calculated from the concentration of unprotonated amine) for the appearance of sulfhydryl groups in α_2M as a function of the inverse of the absolute temperature (T): (○) reaction with ammonia; (□) reaction with methylamine. The reactions were done at pH 8.00 with a total amine concentration of 200 mM.

correction for the concentration dependence of the apparent dissociation constant is more critical for ammonia than for methylamine at pH 8.6, since this pH is closer to the pK_a of ammonia.

Temperature Dependence. Analysis of the influence of temperature on the reaction rate was done in the range 5–35 °C at pH 8.00 and with a total amine concentration of 200 mM. Second-order rate constants, on the basis of the concentration of unprotonated amine, were computed from the observed pseudo-first-order constants; the temperature dependence of the intrinsic dissociation constants of ammonia and methylamine (Grau, 1960) was assumed also for the apparent dissociation constants in these calculations. Activation energies of 85 and 100 kJ mol^{-1} for ammonia and methylamine, respectively, were calculated from slopes of Arrhenius plots of the data (Figure 4), while entropies of activation at 25 °C of 10 and 95 J K^{-1} mol^{-1} were computed for the respective amines from the intercepts of the plots at $1/T = 0$. However, the latter values are somewhat uncertain due to the long extrapolations involved.

Rate of the Reaction with Different Amines. The rate of appearance of sulfhydryl groups in α_2M caused by different amines was compared to the rate of the reaction of these amines with a small thio ester, ethyl thioacetate. The reactions were done at 25.0 °C with a total amine concentration of 200 mM. The pH in the reactions with α_2M was 8.60, while pH was increased to 10.00 in the reactions with ethyl thioacetate. The higher pH served to increase the concentration of unprotonated amine and thus to give measurable rates also with the more stable small thio ester. The rate of hydrolysis of ethyl thioacetate at pH 10.0 in the absence of amines was negligible. Appropriate control experiments, as described above for α_2M, were performed also with the small thio ester. All reactions, both with α_2M and the model thio ester, followed pseudo-first-order kinetics. However, the slowest reactions could not be followed to near completion, and the results for these, therefore, are somewhat uncertain. Second-order rate constants were calculated from the concentrations of uncharged amine (Table I). The intrinsic dissociation constants of the amines were taken from Grau (1960), and the apparent dissociation constants at the ionic strength of the analyses were computed from the intrinsic constants with the use of the Debye–Hückel limiting law (Moore, 1972). This procedure results in only approximate apparent dissociation constants;

Table I: Second-Order Rate Constants (k) for the Appearance of Sulfhydryl Groups in the Reaction of Different Amines with α_2M or Ethyl Thioacetate at 25.0 °C[a]

	α_2M		ethyl thioacetate		
amine	k (M^{-1} s^{-1})	rel rate	k (M^{-1} s^{-1})	rel rate	k_{α_2M}/k_{ETA}[c]
ammonia	0.43	5.3	6.1×10^{-4}	0.62	700
methylamine	8.1	100	9.9×10^{-2}	100	82
dimethylamine	6.8×10^{-2}	0.84	5.7×10^{-2}	58	1.2
trimethylamine	[b]		1.4×10^{-2}	14	
ethylamine	1.2	15	3.2×10^{-2}	32	37
n-propylamine	1.1	14	6.8×10^{-2}	69	16
isopropylamine	6.0×10^{-3}	0.074	3.5×10^{-2}	35	0.17
n-butylamine	5.4×10^{-2}	0.67	7.9×10^{-2}	80	0.68
sec-butylamine	4.7×10^{-3}	0.058	3.1×10^{-2}	31	0.15
isobutylamine	2.2×10^{-2}	0.27	5.9×10^{-2}	60	0.37
tert-butylamine	[b]		2.6×10^{-2}	26	
n-pentylamine	[b]		[b]		
hydrazine	8.6×10^{-2}	1.1	7.1×10^{-2}	72	1.2
allylamine	1.2	15	1.6×10^{-2}	16	75
benzylamine	[b]		8.1×10^{-3}	8.2	

[a] k values are calculated from the concentration of unprotonated amines. The reactions were done with a total amine concentration of 200 mM at pH 8.60 and 10.00 for α_2M and ethyl thioacetate, respectively. Relative rates were obtained by setting the second-order rate constant for the reaction of methylamine with either α_2M or ethyl thioacetate to 100. [b] Reaction too slow to be measured. [c] k_{ETA}, k for ethyl thioacetate.

consequently, all rate constants are approximate, and the values for ammonia and methylamine, therefore, differ somewhat from those given earlier. Relative rates for the reactions of the different amines with either α_2M or ethyl thioacetate were calculated from the second-order rate constants (Table I). For each amine, the ratio between the rate constants for the reactions with α_2M and with the model thio ester is also given in Table I.

Two major results of these analyses are apparent. First, the relative reaction rates of the amines with α_2M do not parallel the relative rates of reactions with the small thio ester. Thus, the secondary and tertiary amines, dimethylamine and trimethylamine, and primary amines larger than propylamine, which all cleave ethyl thioacetate with second-order rate constants comparable to that of methylamine, react with α_2M with rate constants that are considerably lower than that for methylamine. Moreover, relative to methylamine, ammonia reacts much faster with α_2M than with ethyl thioacetate. Second, the smaller amines react with α_2M with rate constants that are up to 700-fold higher than the corresponding rate constants for the reaction with ethyl thioacetate, while the rate constants for the reaction of the larger amines with α_2M and the small thio ester are comparable.

Discussion

In this work we have used a continuous assay, allowing precise measurements, of the rate of appearance of sulfhydryl groups in α_2M on reaction with different amines. Detailed studies were first done with ammonia and methylamine to characterize the general mechanism of the reaction. The results show that the kinetics are those of a single, bimolecular reaction occurring under pseudo-first-order conditions at all pH values (7.0–8.6) and amine concentrations (10–600 mM) investigated. No evidence of any lag phase or biphasic reaction or of a concentration dependence of the second-order rate constants derived from the analyses was detected. These findings suggest that the four sulfhydryl groups of α_2M are released by the amine independently of each other at identical or highly similar rates. The data are also consistent with the

sulfhydryl group release (i.e., presumably the cleavage of the protein thio ester) being the reaction that initiates the sequence leading to inactivation of the inhibitor.

The pseudo-first-order kinetics of sulfhydryl group release by amines are in agreement with a limited analysis by Straight & McKee (1982) using a similar technique as in this investigation. However, the result is in contrast to the report by Van Leuven et al. (1981a, 1982) that the incorporation of radioactivity in α_2M on reaction with [^{14}C]methylamine is biphasic. This discrepancy may be due to the method used by Van Leuven et al. (1981a, 1982). This method, being discontinuous and involving electrophoretic separation of the labeled α_2M from excess reagent, must have been prone to large experimental error. Nonspecific binding of the radioactive amine to the protein may also have influenced the results. Alternatively, the appearance of sulfhydryl groups may follow different kinetics from the binding of the amine to the activated glutamic residue, although both reactions are considered to reflect the cleavage of the thio ester bonds of α_2M (Sottrup-Jensen et al., 1980; Howard, 1981; Salvesen et al., 1981).

The results further show that the unprotonated amine is the active species in the reaction, consistent with the release of sulfhydryl groups occurring by a nucleophilic attack of the uncharged amine on the α_2M thio ester. The large increase in reaction rate with pH is thus due primarily to deprotonation of the positively charged amine. However, an auxiliary effect of pH also on the reactivity of α_2M, e.g., by deprotonation of a critical residue or by a conformational change, cannot be excluded. It should be noted that in the experiments with methylamine, pseudo-first-order conditions were found to prevail also at concentrations of active, unprotonated amine that were lower than or comparable to that of α_2M subunit. This is due to the fact that the uncharged methylamine that is consumed during the reaction by being incorporated into α_2M (Swenson & Howard, 1979; Sottrup-Jensen et al., 1980) is replaced by deprotonation of the charged species, thus keeping the concentration of uncharged amine constant.

The activation energy for the release of sulfhydryl groups in α_2M by either ammonia or methylamine is compatible with a nucleophilic substitution reaction. The values for the two amines are similar and of the same magnitude as that for the spontaneous cleavage of the thio ester bond in a cyclic model peptide comprising the proposed thio ester region in α_2M (Khan & Erickson, 1982). The analyses suggest that the higher reaction rate of methylamine than that of ammonia is due primarily to a higher entropy of activation, rather than to a lower activation energy. The positive entropy of activation for both amines indicates an increase in disorder on formation of the transition-state complex. The higher activation entropy for methylamine is compatible with this amine being a more complex molecule than ammonia, thereby allowing increased degrees of freedom in the activated complex.

An attempt to characterize the thio ester in α_2M was done by comparing the rates of cleavage of this bond by a series of amines with the corresponding rates of cleavage of a simple thio ester, ethyl thioacetate. The basis of this comparison was the assumption that the relative reactivities of the amines with a thio ester bond in general (due to, e.g., the nucleophilicity of the amine and the nature of the transition-state complex) would be approximately reflected in the relative reaction rates with the model thio ester, irrespective of the absolute rates of the reactions. Specific properties of the protein thio ester might thus be deduced. The results suggest that the reactivity of the α_2M thio ester is atypical. The reaction rates of the different amines with the α_2M thio ester thus do not parallel the rates with the small thio ester but rather the inverse of the size of the amines. This behavior indicates that access of reagents to the bond in α_2M is sterically hindered. The α_2M thio ester may thus be located in a pocket of the protein, into which only small reagents can penetrate with ease, in agreement with the suggestion by Barrett et al. (1979). However, cleavage of the thio ester renders its sulfhydryl moiety more accessible, since the latter reacts rapidly with a rather bulky reagent, DTNB, and also binds to thiopropyl-Sepharose (Pochon et al., 1983). A further result is that the α_2M thio ester is cleaved much more rapidly by small primary amines than the model thio ester. Although the absolute reaction rates of the amines with other small thio esters have not been investigated, this finding nevertheless indicates that the thio ester bond in α_2M is comparatively labile. This suggestion is in agreement with the lower stability of the cyclic peptide model of the thio ester region of α_2M, compared with that of an acyclic thio ester (Khan & Erickson, 1982). The lability of the thio ester bond in α_2M thus may be induced by the conformation of the polypeptide chain around the bond.

Registry No. Ammonia, 7664-41-7; methylamine, 74-89-5; dimethylamine, 124-40-3; trimethylamine, 75-50-3; ethylamine, 75-04-7; *n*-propylamine, 107-10-8; isopropylamine, 75-31-0; *n*-butylamine, 109-73-9; *sec*-butylamine, 13952-84-6; isobutylamine, 78-81-9; *tert*-butylamine, 75-64-9; *n*-pentylamine, 110-58-7; hydrazine, 302-01-2; allylamine, 107-11-9; benzylamine, 100-46-9; ethyl thioacetate, 625-60-5.

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

Dalton Wang, Anna I. Yuan, and Richard D. Feinman*

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

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

† From the Department of Biochemistry, State University of New York Downstate Medical Center, Brooklyn, New York 11203. *Received September 22, 1983.* This work was supported by U.S. Public Health Service Grant HL 14992 and Award HL 00406 from the National Heart, Lung, and Blood Institute.

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

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