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Effect of Methylamine on the Reaction of α_2-Macroglobulin with Enzymes†

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Received June 22, 1989; Revised Manuscript Received December 6, 1989

ABSTRACT: The kinetics of reaction of α_2-macroglobulin (α_2M) with thrombin and with trypsin were studied in the presence and absence of methylamine. The rate of enzyme-induced thiol release was found to be the same whether or not amine was present. The result suggests that covalent bond formation and enzyme-catalyzed amine incorporation proceed via a common (enzyme-dependent) rate-determining step. The reaction of lysyl-modified enzymes (which show poor covalent binding with α_2M) was similarly unaffected by amine, indicating that enzyme-catalyzed steps were also rate determining for hydrolysis of the thiol ester. The products of the reactions were analyzed by native and denaturing gel electrophoresis. Methylamine did not affect the total binding of enzyme to α_2M but did cause a substantial decrease in covalent binding. Surprisingly, not all covalent complexes were affected by the presence of amine: complexes in which enzyme was covalently bound to one half-molecule increased compared to the reaction with no amine; complexes in which two half-molecules are cross-linked by two bonds to a single enzyme were substantially reduced, however. The results are consistent with a mechanism of reaction in which an enzyme-dependent step is rate determining. This step is accompanied by activation of two thiol esters. One of these reacts immediately with the bound enzyme (or may be hydrolyzed if the enzyme amine groups are blocked). The other activated center is capable of reaction with external nucleophiles such as methylamine.

The reaction of methylamine with the plasma proteinase inhibitor α_2-macroglobulin (α_2M)[1] has provided a useful tool for the understanding of the mode of action of the inhibitor with enzymes. It is now understood that methylamine inactivates α_2M by competing with the lysyl amino groups of enzymes. Binding of enzymes to α_2M is associated with the activation of internal Cys-Glu thiol esters which react with the enzyme amino groups. Although covalent binding is not required for the reaction of enzymes with α_2M, a substantial number of new Glu-Lys cross-links are formed [for reviews, see Roberts (1986) and Sottrup-Jensen (1989)]. The potential for reaction is great, and unusual complexes can form in which more than one subunit of α_2M is bound via Lys–Glu bonds to the enzyme (Wang et al., 1983, 1984). In addition to providing a clue to the identity of the site of nucleophilic reaction, methylamine may provide information about the dynamics of the enzyme reaction since it has been observed that, in the presence of enzymes, the rate of reaction is greatly enhanced. This activation of α_2M by enzymes can, in fact, allow incorporation of other amines and even other proteins (Sottrup-Jensen et al., 1981; Salvesen et al., 1981). Sottrup-Jensen et al. (1981) made the surprising observation, however, that the total enzyme incorporation was not substantially reduced when methylamine was present. They found that covalent binding was about 50% reduced, although it has been reported that covalent binding can be almost completely eliminated by treatment of α_2M with methylamine in the presence of chymotrypsin (Crews et al., 1988). To further investigate this process, we studied the effect of methylamine on the kinetics of appearance of thiols during the reaction of enzymes with α_2M. We found that the rate of thiol generation was the same in the enzyme-catalyzed methylamine incorporation as in normal reaction with enzyme, indicating that the two processes share a rate-determining step. Product distribution indicated that amine competed only with the enzyme amino group that formed the second covalent bond to α_2M.

Materials and Methods

Human α_2M was prepared by a combination of zinc chelate chromatography (Virca et al., 1978) and chromatography on Cibacron Blue–Sepharose (Kurecki et al., 1979) as described previously (Feinman et al., 1985). Human α-thrombin was the generous gift of Dr. John W. Fenton II of the New York State Department of Health, Albany, NY. Trypsin (type II) and sodium dodecyl sulfate ($NaDodSO_4$) were from Sigma. All other reagents were the highest grade commercially available. [^{14}C]Methylamine specially purified by distillation was purchased from New England Nuclear.

† Supported, in part, by Grant HL 14992 from the National Heart, Lung and Blood Institute, U.S. Public Health Service.

[1] Abbreviations: α_2M, α_2-macroglobulin; $NaDodSO_4$, sodium dodecyl sulfate; TCA, trichloroacetic acid; DFP, diisopropyl fluorophosphate; *cis*-DDP, *cis*-dichlorodiammineplatinum(II); 4-PDS, 4,4′-dithiodipyridine.

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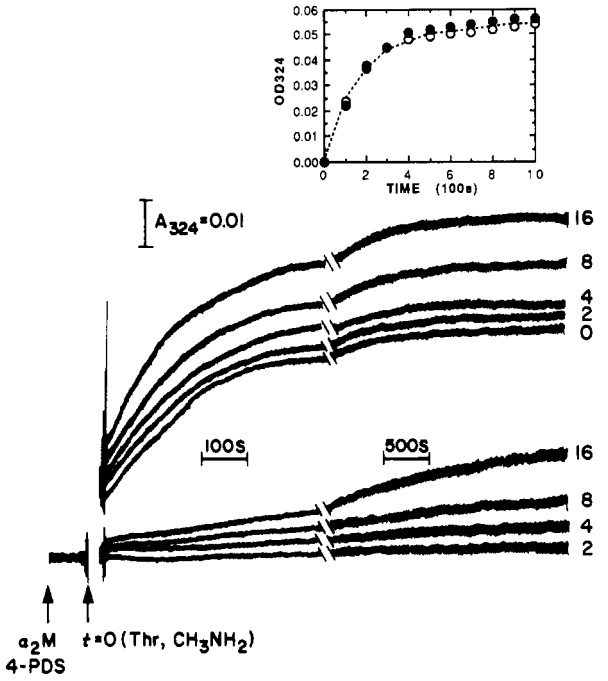

FIGURE 1: Kinetics of the appearance of thiol groups in the reaction of α_2M with thrombin and methylamine. Lower traces: reaction of α_2M (1 μM) with methylamine alone at the indicated concentrations (millimolar). Upper traces: Same conditions except 2 μM thrombin was added with the methylamine. INSET: Data calculated by subtraction of the curves for reaction with methylamine alone from the values for the reaction in the presence of thrombin. Concentration of methylamine was (●) 2 mM, (○) 16 mM, or (dashed line) 0 mM (reaction of thrombin alone, 0 trace in main figure). Reactions were carried out at 25 °C in 0.05 M Tris-HCl buffer, pH 8.0. Thiol release was monitored at 324 nm using 4,4′-dithiodipyridine (4-PDS).

Trypsin and thrombin activity was assayed by active-site titration using *p*-nitrophenol *p*′-guanidinobenzoate hydrochloride obtained from ICN Pharmaceuticals (Chase & Shaw, 1967). Thiols were monitored as described previously (Liu et al., 1987) using the reagent 4,4′-dithiodipyridine (Aldrich Chemical Co.). Iodination of trypsin and thrombin with ^{125}I in the presence of lactoperoxidase was performed according to the procedure of Martin et al. (1976). Reductive methylation of trypsin was performed as described by Wu et al. (1981) using $NaCNBH_4$ as reducing reagent. The number of free amino groups remaining in the methyltrypsin was estimated with 2,4,6-trinitrobenzenesulfonic acid by the method of Satake et al. (1960) as modified by Wang et al. (1976).

Polyacrylamide gel electrophoresis was carried out with slabs on a Gelbond support. The analytical gel used for quantitating total ^{125}I-enzyme bound to α_2M was a gradient of 3.5–10% acrylamide. For quantitating covalent complex formation, a 3.5% gel containing 0.1% $NaDodSO_4$ was used. Enzyme–α_2M reactions were stopped by the addition of either diisopropyl fluorophosphate (DFP) or ice-cold 10% (w/v) trichloroacetic acid (TCA). In the latter case, samples were centrifuged, and the recovered pellet was washed extensively, neutralized with ammonia vapor, and solubilized with 1% (w/v) $NaDodSO_4$/25 mM-Tris-HCl buffer, pH 8.0, before being applied to the gel. Radioactivity on the protein was counted by a γ counter (Beckman).

To measure methylamine incorporation, a solution of α_2M in 50 mM Tris-HCl buffer, pH 8.0, was incubated for various time intervals with different molar excesses of [^{14}C]methylamine (48 Ci/mmol) with or without native enzyme or derivatives (the enzyme:α_2M ratio was 2). Samples were centrifuged, and the recovered pellet was washed extensively. Radioactivity on the protein was counted with an LS 100C liquid scintillation counter (Beckman). The results were converted to moles of methylamine bound per mole of α_2M of M_r 725 000.

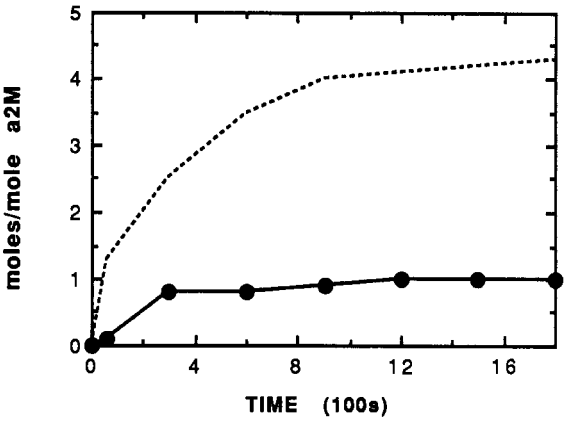

FIGURE 2: Enhanced incorporation of [^{14}C]methylamine into α_2M in the presence of thrombin. Reaction mixtures contained thrombin (2 μM), α_2M (1 μM), and [^{14}C]methylamine (16 mM). Reactions were stopped with 10% cold TCA and centrifuged as described under Materials and Methods, and the washed pellets were counted (●). All data points were averages of triplicate experiments. The time course of thiol appearance under these conditions (---), determined from a parallel experiment (Figure 1), is shown for comparison.

RESULTS

Kinetics of Thiol Release. The reaction of enzymes with α_2M can be followed by monitoring the appearance of new thiols as the thiol ester bond is broken. Figure 1 shows the kinetics of the reaction with thrombin in the presence and absence of methylamine. As shown in the figure, methylamine alone also reacts with α_2M in an uncatalyzed process. This reaction is slow but significant, and for each methylamine concentration studied, the kinetic curve for reaction of amine alone was subtracted from the enzyme curve. The inset to Figure 1 shows that after the subtraction, the kinetics of thiol appearance in the presence of enzyme are essentially the same whether or not amine is present. This suggests that the processes that give rise to the new thiol—covalent enzyme complex formation, methylamine incorporation, and possibly hydrolysis of the thiol ester—share a rate-determining step.

Reaction of Methylamine with α_2M. Figure 1 shows the rather striking effect that even at a 16 000-fold molar excess of methylamine does not alter the kinetics of thiol appearance in the presence of enzyme. It is therefore important to show that, under the conditions of this experiment, there is a change in product distribution; that is, that methylamine is, in fact, incorporated into α_2M. We measured the binding of [^{14}C]-methylamine during the enzyme reaction, and the results, shown in Figure 2, indicate that amine is incorporated and the time course for incorporation is similar to that for thiol release. It is to be expected, then, that this bound methylamine prevented covalent bond formation with the enzyme, and we, therefore, next analyzed the enzyme-containing products.

Effect of Methylamine on Enzyme Complexes. Figure 3 shows the time course of the formation of α_2M–thrombin complexes in the presence and absence of methylamine. It is evident that there is actually little change in the total amount of enzyme bound when amine is added. A similar observation was made by Sottrup-Jensen et al. (1981) for the trypsin reaction. On the other hand, the percent of binding that is covalent has changed substantially—from 80–90% during most of the course of reaction in the absence of amine to less than 50% in its presence. This is what one would expect given the presumed role of amine as a competitor of covalent binding.

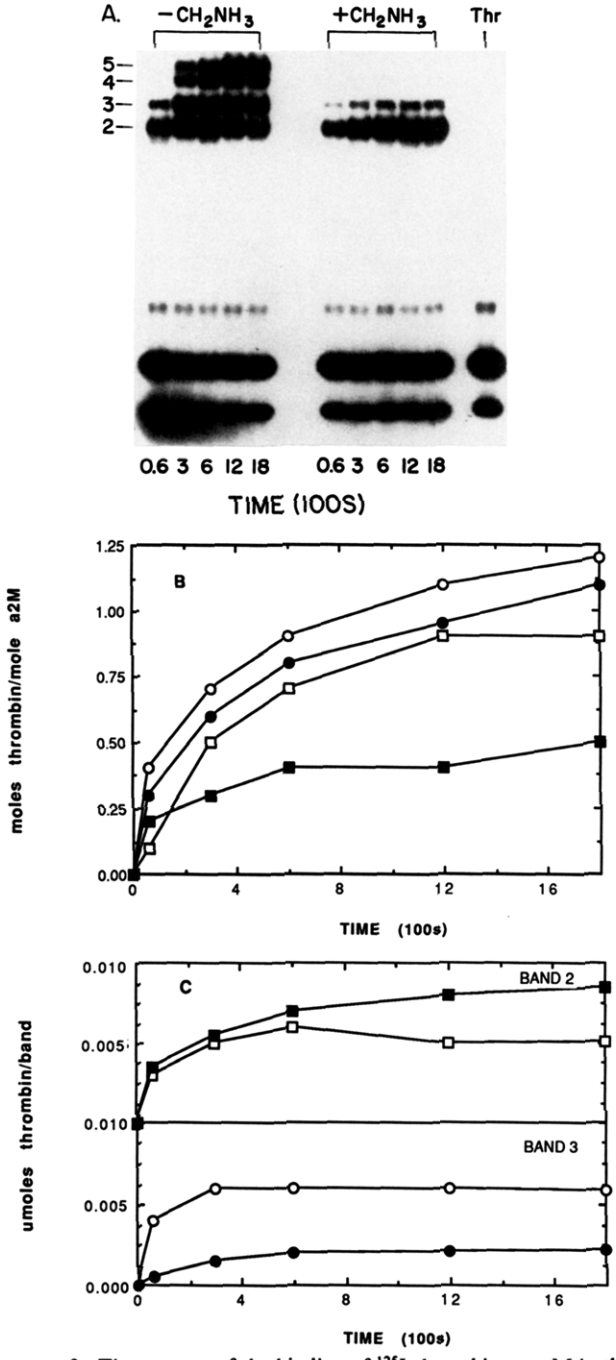

FIGURE 3: Time course of the binding of ^{125}I-thrombin to α_2M in the presence or absence of methylamine. Reaction mixtures contained ^{125}I-thrombin and α_2M at a 2:1 ratio with either no methylamine or 16 mM methylamine. Reactions were stopped at the indicated time points by the addition of DFP and subjected to gel electrophoresis as described under Materials and Methods. (A) Autoradiography of gel electrophoretogram under denaturing ($NaDodSO_4$) conditions. Last lane, radiolabeled thrombin alone. (B) Time course of total and covalent binding of ^{125}I-thrombin to α_2M in the presence and absence of methylamine. Total binding, circles; covalent binding, squares; no methylamine, open symbols; +methylamine, filled symbols. (C) Time course of appearance of band 2 (1 enzyme:1 half-molecule) and band 3 (1 enzyme:2 half-molecules) in the presence (filled symbols) and absence (open symbols) of methylamine. Each point is the average of at least three determinations.

These results are different than those of Crews et al. (1987), who found 34 and 13% covalent binding in the absence and presence of amine. We attribute the discrepancy to their use of soybean trypsin inhibitor instead of DFP. We found that soybean trypsin inhibitor does not prevent proteolysis of α_2M complexes during gel electrophoresis (Wang et al., unpublished results). To further explore this question, we measured the time course of appearance of the different enzyme-containing species as seen on a denaturing gel of the reaction mixture.

Thrombin–α_2M complexes usually show five bands on $NaDodSO_4$ gel electrophoresis. Band 1 is the native half-molecule. Bands 2–5 comprise enzyme-containing species that can be seen on an autoradiogram from reaction mixtures containing labeled enzyme (Figure 3). The two slowest migrating bands (4 and 5) have not been characterized and, although dependent on enzyme, may be an artifact of the gel procedures since they are not seen on gel permeation chromatography of native samples. Band 2 is assumed to be a monovalent enzyme complex (one enzyme bound to the half-molecule) whereas band 3 is a bivalent enzyme complex formed from the monovalent complex by reaction of lysyl groups of the bound enzyme with a glutamyl site on a second half-molecule. In brief, the evidence supporting this attribution of band 3 is that the band only appears in enzyme-containing species (Wang et al., 1983, 1984; Feinman et al., 1985), it is lost in preparations involving reactions of half-molecules (Liu et al., 1987), and it is reduced in reactions of lysyl-blocked enzymes (Wu et al., 1981). Although the apparent molecular weight is less than predicted, results of two-dimensional gel electrophoresis are consistent with the attribution (Wang et al., 1983). Figure 3 shows that, in the absence of methylamine, band 2 reaches a maximum with time and then decays to a final value; band 3, on the other hand, reaches a plateau. This is consistent with the idea that band 2 is an intermediate (one Lys–Glu bond) from which band 3 arises by formation of a second bond (Feinman et al., 1985). In the presence of methylamine, however, band 2 does not decay, and the formation of band 3 is substantially inhibited. This is a surprising result and suggests that amines do not compete with enzyme for the first site (band 2 formation) but only with the formation of the second covalent bond (band 3). The accelerated incorporation of methylamine is then apparently due to the activation of a second thiol ester caused by the enzyme reaction. The uncatalyzed reaction would be expected to proceed normally, however. This predicts that, for a given level of enzyme, there is a maximum level of inhibition of covalent binding regardless of the concentration of methylamine. This prediction is borne out by the results of the experiment shown in Figure 4 assessing the effect of amine concentration. As the methylamine concentration is increased, there is a drastic reduction in band 3 but little change in band 2. The gels in Figure 3 also show that bands 4 and 5 are almost completely abolished in reactions run in the presence of methylamine. These bands have not been characterized, but their formation is known to be enzyme-dependent, and it has been suggested that they arise from the species in band 3 (Wang et al., 1984; Feinman et al., 1985). The current results are consistent with this idea or indicate, at least, that if they are an artifact of electrophoresis, their formation requires the bivalently cross-linked species.

Effect of Methylamine on Trypsin Reaction. A unique characteristic of α_2M is its broad specificity. It inhibits a variety of enzymes although the rates of reaction vary greatly. The thrombin reaction is convenient because of the accessible time scale although α_2M is usually assayed with trypsin. To determine if the effect of enzyme on the kinetics of enzyme–α_2M–methylamine reaction is specific for thrombin or is a general characteristic of α_2M, the reactions with trypsin were also studied. Because of the rapid rate for the trypsin reaction, it was necessary to carry out the reaction at low pH and low temperature. The reactions of trypsin with α_2M in the

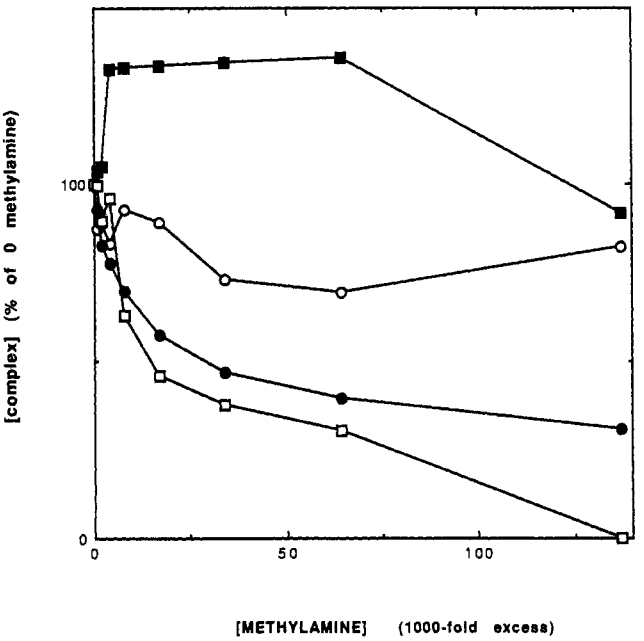

FIGURE 4: Dependence of complex formation on methylamine concentration. The ratio of ^{125}I-thrombin to α_2M was 2:1. Reaction mixtures were incubated for 30 min. The reactions were stopped by the addition of DFP and applied to native or $NaDodSO_4$ gels (see legend to Figure 3). Radioactive bands were cut out and counted. (○) Total complexes; (●) covalent complexes; (■) band 2; (□) band 3.

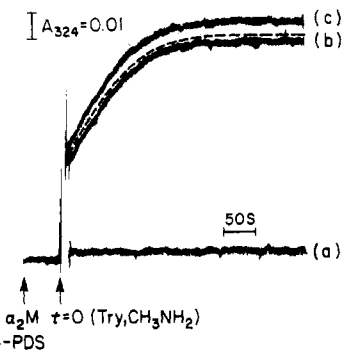

FIGURE 5: Appearance of thiol groups during the reaction of α_2M with trypsin in the presence or absence of methylamine. Reactions were carried out at −4 °C in 0.05 M Tris-HCl buffer, pH 6.8. The concentration of trypsin was 1.5 μM, [α_2M] was 1 μM, and [methylamine] was 8 mM. Thiols were detected at 324 nm using 0.34 mM 4-PDS. (A) Methylamine alone; (b) trypsin alone; (c) trypsin and methylamine. The dashed line indicates the subtraction of curve a from curve c.

presence or absence of methylamine were carried out at −4 °C, pH 6.8 (Figure 5). It is clear that the results were quantitatively similar to those with thrombin: when the curve for thiol released by amine alone was subtracted from that for thiol released in the presence of trypsin, the remainder is essentially the same as the thiol release for trypsin with no added amine (dashed line in Figure 5).

Reaction with Methyltrypsin. Enzyme derivatives in which the lysyl amino groups have been blocked by reductive methylation are capable of reaction with α_2M: new thiol groups appear, but no covalent complexes are formed (Wu et al., 1981; van Leuven et al., 1981). In this case, thiol release is presumably due to simple hydrolysis of the thiol ester. This gave us an opportunity to compare the process of thiol ester hydrolysis with that of amine incorporation. We prepared samples of methyltrypsin with at least 50% lysyl amino groups blocked. This material reacts with α_2M, but almost no covalent bonds are formed (data not shown). Figure 6 indicates

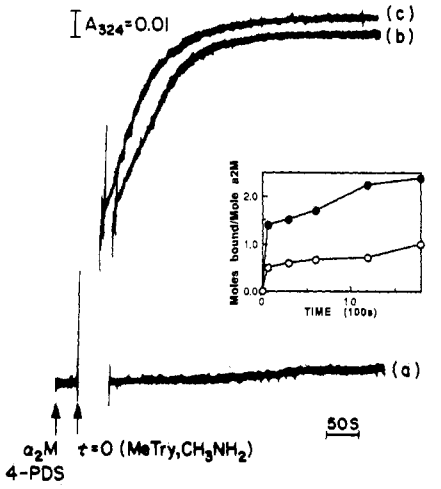

FIGURE 6: Reaction of α_2M with methyltrypsin in the presence or absence of methylamine. Reaction conditions were as in Figure 5. The concentration of methyltrypsin was 2 μM, [α_2M] was 1 μM, and [methylamine] was 8 mM. MeTry: methyltrypsin. INSET: The enhanced incorporation of [^{14}C]methylamine into α_2M in the presence of methyltrypsin under the same conditions. Reactions were run in parallel with those in the kinetic traces. Reactions were stopped with 10% cold TCA and centrifuged as described under Materials and Methods, and the washed pellets were counted. (●) In the presence of methyltrypsin; (○) in the absence of methyltrypsin.

that for methyltrypsin, as for the native enzyme, the rate of thiol release from α_2M in the absence of the amine is the same as that for conditions under which amine is incorporated. Thus, at least for this derivative, hydrolysis of thiol ester appears with the same kinetics as amine incorporation.

DISCUSSION

Covalent bond formation between α_2M and enzymes or external nucleophiles is believed to involve an intermediate activated complex referred to as "metastable" or "nascent" α_2M. The original observation was made in the study of complement C3 and C4 which has a reactive thiol ester similar to the one in α_2M (Law et al., 1980). It is reasonable to assume that the intermediate whose formation is rate determining in the studies described here is the "metastable" α_2M. The molecular nature of this species is not known although Pizzo's group (Roche et al., 1988) has proposed that it can be trapped by reacting trypsin with α_2M treated with *cis*-dichlorodiammineplatinum(II) (*cis*-DDP): the trapped intermediate has undergone proteolytic cleavage but has intact thiol esters and has not changed conformation. Thiols are released and conformational changes occur if the *cis*-DDP is removed. On the other hand, there has been suggestive evidence that an intermediate with free thiols appears before the new bonds are formed (Sottrup-Jensen et al., 1980, 1983; Feinman et al., 1985). Model studies show that an internal pyroglutamate could be such a reactive intermediate (Khan & Erickson, 1982; Erickson & Khan, 1983). In our studies, the formation of the intermediate is rate determining, and it is therefore not possible to tell if thiols are released during this step or the later bond-forming steps.

Proximity of Thiol Ester and Bait Region. The product distribution in the reaction of amines in the presence of enzymes is somewhat surprising. Our results suggest that, in the reactive state, the enzyme is not symmetrically arranged with relation to the activated Glu residues. Thus, the fact that the monovalent complex is not affected by the presence of amine means that the reactive lysyl groups are favorably oriented toward one Glu residue during the activation step. Additional Glu residues must be less favorably arranged and react more slowly than the first (although still faster than the

rate-determining generation of the metastable intermediate). Thus, the sequence of reaction is activation and then covalent bond formation at one residue followed by reaction of additional residues with enzyme, amine, or water. The fact that nucleophiles failed to react with the first residue of the inhibitor in the presence of proteases suggests that the "bait" region is close to the thiol ester site. This is supported by an elegant study of Pizzo et al. (1986) in which covalent bond formation of α_2M with factor Xa was shown to be only at lysines on the heavy chain which contains the active serine. The results are also consistent with distance measurements made by NMR methods. Gettins et al. (1988) showed that the distance between the thiol ester and the bait region is only about 1.1–1.7 nm. Distances between thiol groups are not known, but paramagnetic broadening methods show a maximum distance of 3.8 nm. In the case of small enzymes such as chymotrypsin where the longest axis may only be 4 nm (Winzor & Wills, 1986), the competition by methylamine seems highly favorable although for thrombin with an approximate diameter of 5.7 nm (Tsernoglou et al., 1974), it seems that orientation rather than spatial separation alone must be important in the competition between amine and (bound)-enzyme lysyl groups.

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