Changes of the Proteinase Binding Properties and Conformation of Bovine α_2-Macroglobulin on Cleavage of the Thio Ester Bonds by Methylamine†

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Received June 8, 1984

ABSTRACT: Cleavage of the thio ester bonds of human α_2-macroglobulin (α_2M) by methylamine leads to an extensive conformational change and to inactivation of the inhibitor. In contrast, cleavage of these bonds in bovine α_2M only minimally perturbs the hydrodynamic volume of the protein [Dangott, L. J., & Cunningham, L. W. (1982) *Biochem. Biophys. Res. Commun. 107*, 1243–1251], as well as its spectroscopic properties, as analyzed by ultraviolet difference spectroscopy, circular dichroism, and fluorescence in this work. A conformational change analogous to that undergone by human α_2M thus does not occur in the bovine inhibitor. However, changes of several functional properties of bovine α_2M are induced by the amine. The apparent stoichiometry of inhibition of trypsin thus is reduced from about 1.2 to about 0.7 mol of enzyme/mol of inhibitor. In spite of this decrease, the interaction with the proteinase induces similar conformational changes in methylamine-treated α_2M as in intact α_2M, as revealed by spectroscopic analyses, indicating that the mode of binding of the proteinase to the inhibitor is essentially unperturbed by thio ester bond cleavage. The reaction with methylamine also greatly increases the sensitivity of bovine α_2M to proteolysis by trypsin at sites other than the "bait" region. Moreover, the second-order rate constant for the reaction with thrombin is reduced by about 10-fold. These results indicate that the thio ester bonds of bovine α_2M, although not required per se for the binding of proteinases, nevertheless are responsible for maintaining certain structural features of the inhibitor that are of importance for full activity.

α_2-Macroglobulin (α_2M)[1] is a high molecular weight (M_r ~700 000), tetrameric plasma proteinase inhibitor of wide specificity that is found in a large number of vertebrate species [see Starkey & Barrett (1982) and references cited therein]. Most knowledge of the mechanism of action of α_2M has been gained from studies of the human protein. The binding of the proteinase to the inhibitor is initiated by restricted proteolysis of a limited portion of the α_2M polypeptide chain, the "bait" region (Harpel, 1973; Swenson & Howard, 1979a; Barrett et al., 1979; Sottrup-Jensen et al., 1981b). This cleavage leads to a conformational change that creates a binding site for the proteinase in each half-molecule of the inhibitor (Barrett et al., 1974, 1979; Björk & Fish, 1982; Gonias et al., 1982; Branegård et al., 1982; Gonias & Pizzo, 1983a; Dangott et al., 1983; Björk et al., 1984). The enzyme binds to this site in a manner that greatly decreases its activity against high molecular weight substrates but largely preserves its activity against low molecular weight substrates (Barrett & Starkey, 1973; Harpel, 1976). The inhibitor may thus physically entrap the proteinase (Barrett & Starkey, 1973). Although each α_2M molecule has the capacity to bind two proteinase molecules, some binding sites may decay before binding the enzyme, resulting in observed binding stoichiometries of less than 2 (Travis & Salvesen, 1983; Howell et al., 1983; Gonias & Pizzo, 1983b; Björk, 1984; Straight & McKee, 1984).

The proteolysis of the bait region also leads to the appearance of a free thiol group and of an activated glutamic acid residue in each α_2M chain. Most evidence indicates that in the intact inhibitor these two groups form a thio ester bond that is cleaved during reaction with the proteinase (Sottrup-Jensen et al., 1980, 1981a; Howard, 1981; Salvesen et al., 1981). The activated glutamic acid can react with amino groups on the proteinase, thereby covalently linking a proportion of the noncovalently "trapped" enzyme molecules to the inhibitor (Salvesen & Barrett, 1980; Sottrup-Jensen et al., 1981c; Wu et al., 1981).

The α_2M thio ester bonds can be cleaved by small primary amines in a nucleophilic substitution reaction (Swenson & Howard, 1979b; Sottrup-Jensen et al., 1980; Howard, 1981; Salvesen et al., 1981; Larsson & Björk, 1984). In human α_2M, this cleavage leads to a conformational change similar to that induced by proteinases (Björk & Fish, 1982; Gonias et al., 1982; Dangott et al., 1983) and to inactivation of the inhibitor (Steinbuch et al., 1968; Barrett et al., 1979). However, recent studies have provided evidence that bovine α_2M, as well as the inhibitor from rat, does not undergo a comparable conformational change on reaction with methylamine, despite cleavage of the thio ester bonds, and also remains at least partially active (Dangott & Cunningham, 1982; Gonias et al., 1983). These findings suggest that in bovine α_2M, the changes of the properties of the inhibitor induced by thio ester bond cleavage alone can be studied separately from the major conformational change induced by subsequent reaction of the thio ester cleaved protein with proteinases. Characterization of these changes may aid in delineating those properties of the α_2M molecule which are directly maintained by the thio ester bonds and may thus further elucidate the role of these bonds

† Supported by grants from the Swedish Medical Research Council (Project 4212), the Swedish Council for Forestry and Agricultural Research (Project A5861), and the Konung Gustaf V:s 80-Årsfond.

[1] Abbreviations: α_2M, α_2-macroglobulin; STI, soybean trypsin inhibitor; PMSF, phenylmethanesulfonyl fluoride; TPCK, N^{α}-*p*-tosyl-L-phenylalanine chloromethyl ketone; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; EDTA, (ethylenedinitrilo)tetraacetate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TNS, 6-(4-toluidino)-2-naphthalenesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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in α_2M structure and function. This work presents the results of such studies.

Materials and Methods

Bovine blood was routinely collected at slaughter (within 5 min after the animals were killed) into $^1/_{10}$th volume of 0.1 M sodium citrate, pH 7.5, containing STI to give a final concentration of 50 mg/L blood. α_2M was purified by precipitation of the plasma with poly(ethylene glycol) (5.5–14% w/v) followed by zinc chelate chromatography (Kurecki et al., 1979), chromatography on Cibacron Blue Sepharose (Virca et al., 1978), and gel chromatography on Sephacryl S-300 (Pharmacia, Uppsala, Sweden). For some preparations, blood was collected by venipuncture into citrate containing, besides STI, also aprotinin and heparin (final concentrations 50 000 kallikrein inhibitory units/L and 20 mg/L blood, respectively). Moreover, in these preparations, PMSF was added to a concentration of 100 μM before precipitation with poly(ethylene glycol) and before zinc chelate chromatography. The yield of α_2M was about 20%.

Bovine β-trypsin (EC 3.4.21.4) and α-thrombin (EC 3.4.21.5) were isolated as described previously (Carlström et al., 1977; Björk et al., 1984). Active-site titrations with 4-nitrophenyl 4-guanidinobenzoate (Chase & Shaw, 1970) gave 0.89 ± 0.02 (SD, n = 5) and 0.85 ± 0.02 (n = 4) mol of active sites/mol of enzyme for trypsin and thrombin, respectively. STI (type IS) was obtained from Sigma Chemical Co. (St. Louis, MO).

Bovine antithrombin was prepared by affinity chromatography on heparin–Sepharose (Miller-Andersson et al., 1974). Pig mucosal heparin (150 B.P. units/mg) was obtained from KabiVitrum, Stockholm, Sweden.

Gradient gel electrophoresis was performed on commercial 4–30% (w/v) gels (Pharmacia). Before analyses of trypsin–α_2M mixtures, the enzyme was inactivated for 15 min at 25 °C with 1 mM PMSF and 2 mM TLCK. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis was done on 5% gels (Weber & Osborn, 1969). The samples were routinely denatured for 45 min at 37 °C in 1% (w/v) sodium dodecyl sulfate with or without 0.3 M β-mercaptoethanol. However, in some experiments, the samples were first incubated for 15 min at 25 °C with 1 mM PMSF, 1 mM TLCK, 1 mM TPCK, 2 mM EDTA, 10 mM iodoacetamide, and 0.2 mg/mL STI. Sodium dodecyl sulfate and β-mercaptoethanol were then added to final concentrations of 2% (w/v) and 0.6 M, respectively., and the samples were immediately incubated for 2 or 5 min at 100 °C or 45 min at 37 °C. Molecular weight standards were obtained from Pharmacia.

The procedures used for amino acid analyses, thin-layer electrofocusing, immunoelectrophoresis, and immunodiffusion have been described previously (Nordenman et al., 1977; Björk & Nordling, 1979). Commercial antisera (Dako-immunoglobulins, Copenhagen, Denmark) against bovine serum and human α_2M were used in the latter analyses.

The kinetics of appearance of sulfhydryl groups in the reaction of α_2M with methylamine were monitored by a continuous spectrophotometric assay in which the liberation of these groups was coupled to their reaction with DTNB (Larsson & Björk, 1984). The number of sulfhydryl groups in α_2M released in the reaction with trypsin was determined by a similar method (Björk et al., 1984).

The stoichiometry of inhibition of trypsin by α_2M was determined by two different methods. One of these involved measurements of the activity of free trypsin by a fibrin gel assay (Björk et al., 1984), while the other was based on the ability of the inhibitor to greatly decrease the rate of reaction of the bound enzyme with STI (Ganrot, 1966; Barrett & Starkey, 1973; Björk et al., 1984). Control experiments showed that the reaction between intact or methylamine-treated bovine α_2M and trypsin was complete within the 5-min period during which the proteins were incubated before free or bound enzyme was measured. The ability of α_2M in plasma to protect trypsin from inactivation by STI was measured with a chromogenic substrate, essentially as in the stoichiometry analyses (Björk et al., 1984).

The amount of trypsin covalently bound to α_2M was analyzed by first saturating the intact or methylamine-treated inhibitor with ^{125}I-labeled trypsin in molar ratios to α_2M of 1.3 and 0.8, respectively (see Results). Unbound trypsin was then removed by gel chromatography on Sepharose 6B (Pharmacia). The proportion of covalently bound enzyme was measured by further gel chromatography on Sepharose 6B in 0.1% (w/v) sodium dodecyl sulfate and 0.02 M sodium phosphate, pH 7.0, after denaturation of the sample in 1% dodecyl sulfate for 45 min at 37 °C. Trypsin was ^{125}I labeled by a procedure based on immobilized glucose oxidase and lactoperoxidase (Enzymobead reagent; Bio-Rad Laboratories, Richmond, CA) with no detectable loss of activity.

The rate of reaction of α_2M-bound trypsin with STI was measured at 25 °C by incubation of α_2M (final concentration 0.12 μM) for 2 min with trypsin in a molar ratio to the inhibitor of 1.5 and subsequent addition of STI to a concentration of 2.5 μM. At successive intervals, trypsin activity was assayed by addition of 50-μL portions of the reaction mixture to a cuvette containing 500 μL of the chromogenic substrate *N*-benzoyl-L-isoleucyl-L-glutamylglycyl-L-arginine *p*-nitroanilide (S2222; KabiVitrum) at a final concentration of 400 μM.

The kinetics of inhibition of thrombin by α_2M were studied at 25 °C. The reaction mixture contained 0.5–6 μM α_2M, 0.05–0.2 μM thrombin, and 0.1% (w/v) poly(ethylene glycol) (to stabilize the enzyme). In all reactions, the concentration of α_2M was at least 10-fold greater than that of thrombin. At different times, portions of 100 μL were removed from the reaction mixture and added to a cuvette at 37 °C containing 2.15 mL of a solution of antithrombin and heparin (final concentrations 0.4 μM and 6 μg/mL, respectively) in buffer with 1% poly(ethylene glycol). After 30 s (sufficient time to inactivate all free thrombin), the activity of the α_2M-bound thrombin was measured by addition of 250 μL of a 1 mM solution of the substrate D-phenylalanyl-L-pipecolyl-L-arginine *p*-nitroanilide (S2238, KabiVitrum). The activity of the bound thrombin at infinite time of reaction was estimated by non-linear least-squares regression (James & Roos, 1975), and pseudo-first-order rate constants were then obtained from semilogarithmic plots in the usual manner (Frost & Pearson, 1961). All reactions were followed to at least 85% completion, as judged from the estimated activity at infinite time.

The ultraviolet difference absorption, tryptophyl fluorescence, and far-ultraviolet circular dichroism changes accompanying the reactions of bovine α_2M with methylamine or trypsin were analyzed essentially as described in detail for the human protein (Björk & Fish, 1982). The major modification was that fluorescence was measured in an SLM 4800S spectrofluorometer (SLM-Aminco, Urbana, IL). The increase in fluorescence due to the binding of TNS (obtained from Serva, Heidelberg, Germany) to α_2M was also used to probe these reactions (McClure & Edelman, 1966; Strickland, 1983).

Most analyses of methylamine-treated α_2M were done with inhibitor that had reacted with 100 mM amine for 2 h at 25 °C, conditions which were sufficient to expose >98% of the

total number of sulfhydryl groups released (see Results). The protein was then diluted so that the final concentration of methylamine in the analyses was <20 mM. However, in the studies of the kinetics of inhibition of thrombin, the time of the reaction with methylamine was extended to 3 h to ensure a complete reaction, and no dilution was done due to the high protein concentrations necessary. Control experiments showed that the activities of the enzymes were unaffected by amine concentrations up to at least 100 mM under all conditions used in the analyses.

All analyses, with the exceptions noted below, were done in 50 mM Hepes and 0.1 M NaCl, pH 8.0 (Björk & Fish, 1982). However, 0.2 M Hepes, 0.1 M NaCl, and 2 mM EDTA, pH 8.00, were used in the analyses of the kinetics of sulfhydryl group appearance (Larsson & Björk, 1984). Moreover, because of the far-ultraviolet absorbance of Hepes, 50 mM Tris-HCl and 0.1 M NaCl, pH 8.0, were used instead in the circular dichroism analyses.

Protein concentrations were obtained by absorption measurements at 280 nm. The specific absorption coefficients and molecular weights used in the calculations were determined in this work (see Results) or have been reported previously (Björk & Fish, 1982). All concentrations of proteinases were active-site concentrations.

Results

Purity and General Properties. Most preparations of bovine α_2M showed only one band in gradient gel electrophoresis under native conditions. Occasionally, one or two faint bands (comprising <5% of the staining intensity on the gel) with higher apparent molecular size, presumably representing aggregates, were observed. Like human α_2M (Barrett et al., 1979), the bovine protein was quantitatively converted to a faster migrating form on reaction with trypsin. Immunoelectrophoresis with an antiserum against bovine serum produced only one precipitin arc. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis under nonreducing conditions gave one band, whereas two additional, minor bands with apparent molecular weights of ~105 000 and ~70 000, together accounting for 20–25% of the staining intensity, were seen under reducing conditions (Figure 4), also with methylamine-treated α_2M. These bands were not reduced in amount when blood was collected by venipuncture into a proteinase inhibitor cocktail and PMSF was added at two stages of the purification procedure. They were also unchanged when α_2M was treated with a mixture of proteinase inhibitors before and during denaturation in sodium dodecyl sulfate at 37 or at 100 °C.

The mobility of the major band of bovine α_2M observed in sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions was compared with the mobilities of the subunit of human α_2M and of a series of standards. This analysis consistently gave a molecular weight for the polypeptide chain of bovine α_2M 5000–10 000 lower than that of the chain of human α_2M. Since the molecular weight of the native bovine inhibitor (Nagasawa et al., 1970) indicates a tetrameric structure, analogous to that of human α_2M, a molecular weight of 700 000 was used for the bovine protein (taking the molecular weight of human α_2M to be 725 000; Jones et al., 1972; Hall & Roberts, 1978).

The absorption coefficient at 280 nm of bovine α_2M was estimated by measurements of the absorption and total amino acid content of a solution of the protein in buffer. A carbohydrate content in bovine α_2M of 10% (Nagasawa et al., 1970) was used to calculate the total protein concentration. Two analyses of different preparations gave absorption coefficients of 0.81 and 0.83 mL mg^{-1} cm^{-1}; a value of 0.82 mL mg^{-1} cm^{-1} was thus used throughout the work.

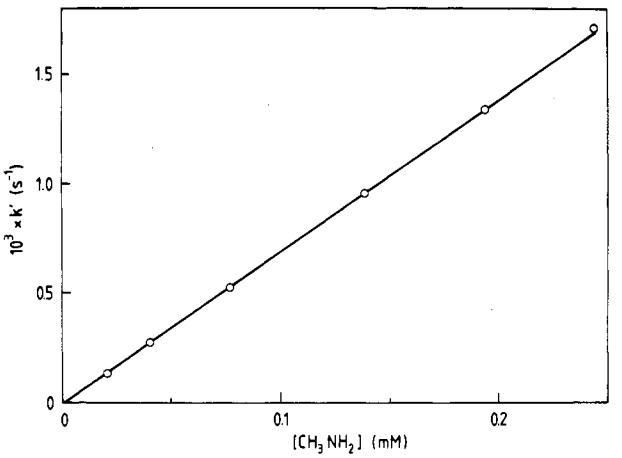

FIGURE 1: Pseudo-first-order rate constants (k') for the appearance of sulfhydryl groups in bovine α_2M at pH 8.00, 25 °C, and at different concentrations of unprotonated methylamine. The analyses were done with a protein concentration of 3.8 μM and a DTNB concentration of 100 μM (Larsson & Björk, 1984). The total concentrations of methylamine were 25, 50, 100, 200, 300, and 400 mM.

The amino acid composition of bovine α_2M was found to be highly similar to that of human α_2M (Dunn & Spiro, 1967). The value of $S\Delta Q$, a parameter used empirically as a measure of the relatedness of two proteins (Marchalonis & Weltman, 1971), was 4. Since values of less than 50 are taken to reflect a high degree of homology (Marchalonis & Weltman, 1971), bovine and human α_2M must be closely related proteins with very similar amino acid sequences. This similarity was also shown by immunodiffusion analyses with an antiserum against human α_2M, in which the two proteins gave a reaction of partial identity.

Kinetics of Reaction with Methylamine. The rate of appearance of sulfhydryl groups, generally considered to reflect thio ester bond cleavage (Sottrup-Jensen et al., 1980; Howard, 1981; Salvesen et al., 1981), in the reaction of bovine α_2M with methylamine was investigated at pH 8.00 and 25 °C with total methylamine concentrations from 25 to 400 mM. Semilogarithmic plots of the data were linear at all amine concentrations, indicating that the reactions occurred under pseudo-first-order conditions. A plot of the observed pseudo-first-order rate constants vs. the concentrations of unprotonated amine, the active species in the reaction (Larsson & Björk, 1984), was linear (Figure 1) and gave a second-order rate constant of 7.0 M^{-1} s^{-1}. The kinetics of sulfhydryl group appearance thus are similar to those of the reaction of methylamine with human α_2M, i.e., consistent with a nucleophilic attack of the uncharged amine on the α_2M thio ester in a bimolecular reaction occurring under pseudo-first-order conditions. However, the rate constant is only about half of that for the reaction of methylamine with human α_2M (Larsson & Björk, 1984).

Methylamine caused the appearance of 3.5 ± 0.1 (SD, n = 6) mol of sulfhydryl groups/mol of protein, i.e., close to one group per α_2M subunit, as has been reported previously for human α_2M (Sottrup-Jensen et al., 1980; Larsson & Björk, 1984). The number of sulfhydryl groups released is consistent with the incorporation of 3.8 mol of methylamine/mol of bovine α_2M reported by Dangott & Cunningham (1982), the difference being due mainly to the use of different absorption coefficients for bovine α_2M.

No faster migrating form of bovine α_2M could be detected by gradient gel electrophoresis when the protein was reacted

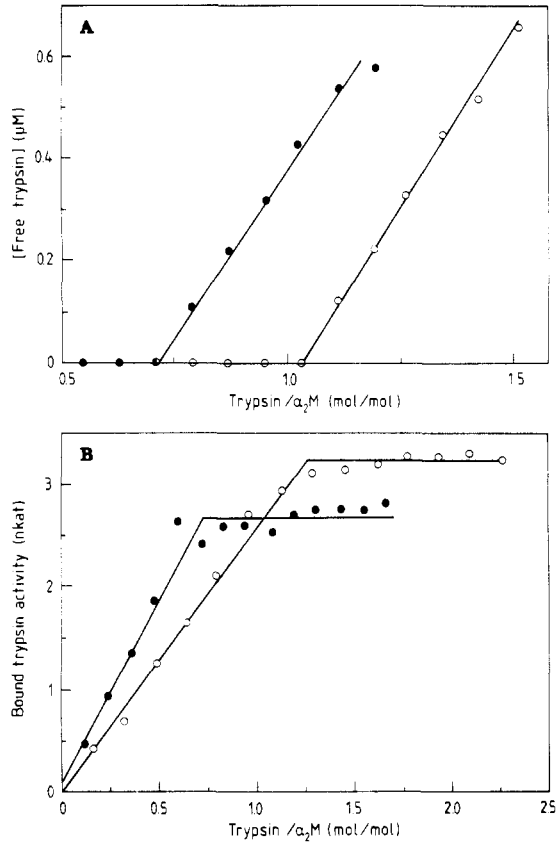

FIGURE 2: Stoichiometry of inhibition of trypsin by intact and methylamine-treated bovine α_2M. (A) Fibrin gel assay; (B) STI assay. (O) Intact α_2M; (●) α_2M treated with methylamine. A series of samples, containing α_2M att a constant concentration (0.95 μM in panel A and 0.17 μM in panel B) and increasing concentrations of trypsin, was incubated for 5 min at 25 °C and was then assayed for free or bound enzyme (Björk et al., 1984).

with methylamine, as shown previously by Dangott & Cunningham (1982). The reaction conditions were extended to 400 mM methylamine for 48 h, making even a slow reaction unlikely. However, reaction with an excess of trypsin quantitatively converted the methylamine-treated protein to the faster form.

Stoichiometry of Inhibition of Trypsin. Both methods used to determine the stoichiometry of inhibition of trypsin showed that 1 mol of intact bovine α_2M inhibited 1.1–1.2 mol of enzyme (Figure 2). The apparent stoichiometry of inhibition thus was lower than that for the human protein, which gave a stoichiometry of about 1.7 with the same methods (Björk et al., 1984). Both methods further showed that cleavage of the thio ester bonds of bovine α_2M with methylamine resulted in a decrease of the apparent stoichiometry of inhibition to about 0.7. These results agree well with analyses by gradient gel electrophoresis, which showed that complete conversion of native and methylamine-treated α_2M from the "slow" to the "fast" form (Barrett et al., 1979) occurred at molar ratios of enzyme to inhibitor of about 1.2 and 0.8, respectively. The decrease of the apparent stoichiometry of inhibition is also in approximate agreement with experiments showing that the ability of α_2M in bovine plasma to protect trypsin from inactivation by STI was reduced to 40–50% by exposure of the plasma to methylamine. In this respect, the purified inhibitor is thus representative of at least the major part of the α_2M present in bovine plasma.

Like the reaction with methylamine, reaction of bovine α_2M with saturating amounts of trypsin resulted in the appearance of 3.5 ± 0.1 (SD, n = 3) mol of sulfhydryl groups/mol of α_2M,

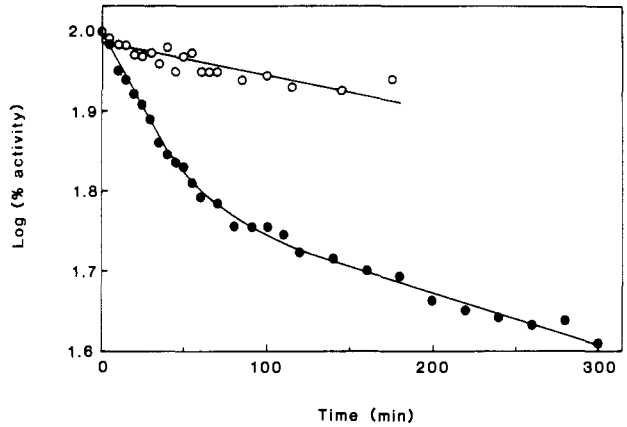

FIGURE 3: First-order plot of the reaction at 25 °C between STI and trypsin bound to intact or methylamine-treated bovine α_2M. (O) Intact α_2M; (●) α_2M treated with methylamine. The reaction was analyzed under pseudo-first-order conditions, i.e., at a 20–30-fold molar ratio of STI to the bound enzyme. The original activity was taken as that measured 1 min after addition of STI to the α_2M–trypsin reaction mixture, i.e., when all free trypsin had been inactivated. This time was also taken as the zero time of the reaction.

i.e., about one group per α_2M subunit, as shown previously for the human protein (Sottrup-Jensen et al., 1980; Björk et al., 1984). Analyses with radioactively labeled trypsin showed that less than 5% of the total amount of enzyme that was bound to either intact or methylamine-treated bovine α_2M was bound covalently. In contrast to what has been demonstrated for human α_2M (Ohlsson & Skude, 1976), the isoelectric point of the bovine inhibitor was not appreciably changed in the reaction with trypsin.

Reaction of Bound Trypsin with STI. Trypsin bound to intact bovine α_2M reacted only very slowly with STI (Figure 3), similar to what has been reported for human α_2M (Bieth et al., 1981; Wang et al., 1981). An approximate second-order rate constant of 5 M^{-1} s^{-1} was calculated from the data. In contrast, trypsin bound to the methylamine-treated protein reacted at an appreciably faster rate (Figure 3). However, the reaction was still slow enough not to have affected the stoichiometry analyses with the STI method. The first-order plot revealed two phases of the pseudo-first-order reaction (Figure 3), although possible additional phases could not be excluded. Analyses of the two discernible phases (Frost & Pearson, 1961) indicated that the faster phase accounted for about 40% of the total inactivation of trypsin and that the apparent second-order rate constants for the two phases were about 200 and 10 M^{-1} s^{-1}. Thus, at least two forms of the complex between methylamine-treated bovine α_2M and trypsin appear to exist in which the bound trypsin reacts with STI with rate constants differing by about 20-fold.

Proteolytic Cleavage by Trypsin. Bovine α_2M was found to be more extensively cleaved by trypsin than the human inhibitor, as revealed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions (Figure 4). Thus, subsaturating amounts of trypsin (<0.8 mol/mol of α_2M) caused complete disappearance of the M_r ~170 000 band and the appearance of several bands of lower apparent molecular weights. An excess of enzyme resulted in even further cleavage. In contrast, essentially only one band is seen with human α_2M even with an excess of trypsin (Harpel, 1973; Swenson & Howard, 1979a; Björk et al., 1984). Cleavage of the thio ester bonds by reaction with methylamine increased the sensitivity of bovine α_2M to proteolysis. Thus, a molar ratio of enzyme to inhibitor of 0.1 was sufficient to cleave all of the α_2M subunits to several smaller fragments. Analyses

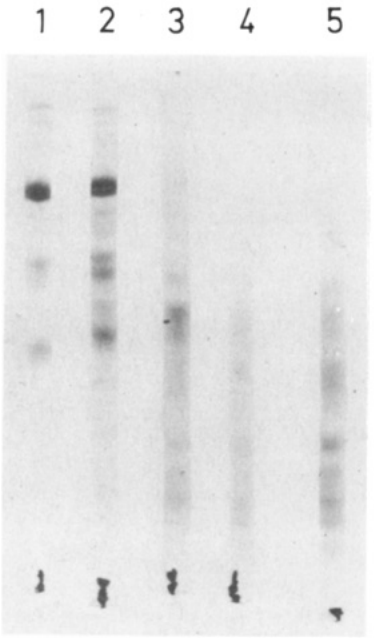

FIGURE 4: Trypsin cleavage of intact and methylamine-treated bovine α_2M, analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions. Gel 1, bovine α_2M alone; gels 2, 3, and 4, bovine α_2M reacted with trypsin in enzyme to inhibitor ratios of 0.25, 0.8, and 2.0, respectively; gel 5, methylamine-treated bovine α_2M, reacted with trypsin in an enzyme to inhibitor ratio of 0.1. The reactions with trypsin were done for 5 min at 25 °C at an α_2M concentration of 1.4 μM. Before addition of sodium dodecyl sulfate, the samples were incubated for 15 min at 25 °C with 1 mM PMSF and 2 mM TLCK to inactivate the enzyme; 12–18 μg of protein was applied to each gel.

without reduction of the samples gave only one band in all cases, indicating that the fragments of both untreated and methylamine-treated α_2M are held together by disulfide bonds.

Kinetics of Inhibition of Thrombin. The effect of thio ester bond cleavage of bovine α_2M on the kinetics of inhibition of proteinases was studied with thrombin, since the reactions with trypsin were too fast to be monitored with manual methods. The reactions were done at varying concentrations of α_2M under pseudo-first-order conditions, i.e., at a 10-fold or higher molar ratio of inhibitor to enzyme. Semilogarithmic plots of the data were all linear within the precision of the analyses, indicating that the reactions followed pseudo-first-order kinetics. A plot of the pseudo-first-order rate constants vs. α_2M concentration was linear and gave a second-order rate constant of 2.5×10^3 M^{-1} s^{-1} (Figure 5). The reaction of methylamine-treated α_2M with thrombin was considerably slower (Figure 5) with an approximate second-order rate constant of 2.0×10^2 M^{-1} s^{-1}. Thus, the reaction of bovine α_2M with methylamine results in about a 10-fold reduction of the rate of inhibition of thrombin.

Spectroscopic Properties. The conformational changes induced in bovine α_2M on reaction with methylamine, and in the intact and methylamine-treated protein on reaction with trypsin, were analyzed by ultraviolet difference spectroscopy, tryptophan fluorescence, extrinsic fluorescence induced by binding of TNS, and circular dichroism (Figures 6–9). All these analyses revealed a similar pattern. The spectroscopic properties of intact bovine α_2M thus were highly similar to those of the human protein (Björk & Fish, 1982; Gonias et al., 1982; Dangott et al., 1983; Strickland, 1983). The reaction between methylamine and bovine α_2M caused only small changes of these properties. In contrast, trypsin induced considerably larger spectroscopic changes, which were similar to those induced by the enzyme in human α_2M (Björk & Fish, 1982; Gonias et al., 1982; Dangott et al., 1983; Strickland, 1983). Moreover, the reaction between trypsin and methylamine-treated bovine α_2M resulted in spectroscopic changes very similar to those caused by the reaction between the enzyme and intact α_2M. The only exception to the latter finding was the TNS fluorescence, which reproducibly was slightly higher in the reaction of methylamine-treated α_2M with trypsin than in the reaction of intact α_2M with the enzyme.

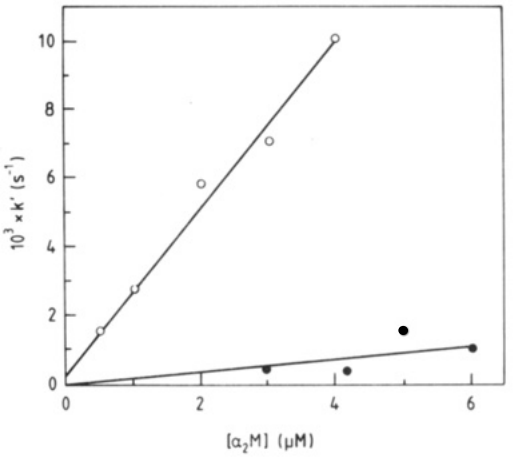

FIGURE 5: Pseudo-first-order rate constants at pH 8.0, 25 °C, for the inhibition of thrombin by intact or methylamine-treated bovine α_2M as a function of α_2M concentration. (○) Intact α_2M; (●) methylamine-treated α_2M.

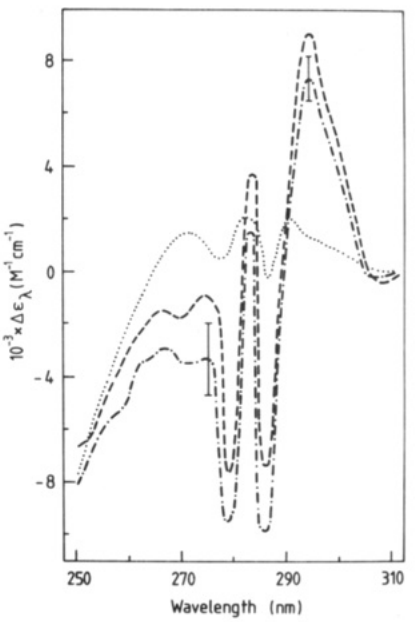

FIGURE 6: Ultraviolet absorption difference spectra measured between bovine α_2M alone and bovine α_2M reacted with methylamine, trypsin, or methylamine followed by trypsin. (···) α_2M reacted with methylamine; (---) α_2M reacted with trypsin; (–·–) α_2M reacted first with methylamine and then with trypsin. The α_2M concentration was 2.1–2.4 μM. The reactions with trypsin were done for 5 min at 25 °C with a molar ratio of enzyme to inhibitor of 1.5. Tandem cells with 1-cm compartments were used, and methylamine, trypsin, or buffer was added to the appropriate reference compartments. The bandwidth was 1 nm. The error bars represent standard deviations calculated from three separate analyses; curves without standard bars show average results from two analyses.

DISCUSSION

All preparations of bovine α_2M were homogeneous by most criteria but showed two minor bands in sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions. The apparent molecular weights of these bands were

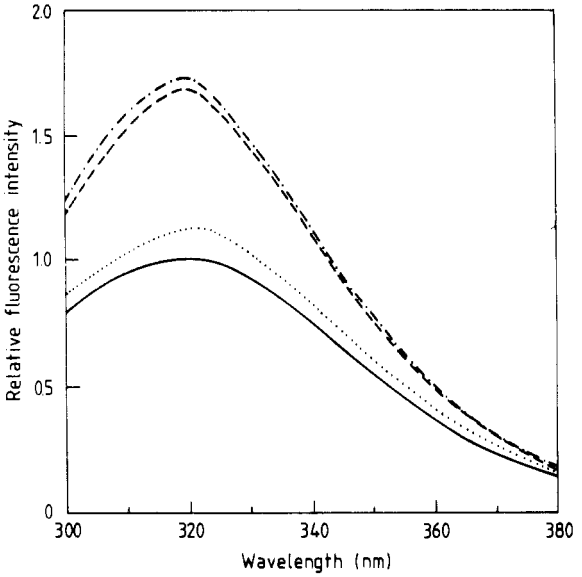

FIGURE 7: Corrected fluorescence spectra of bovine α_2M alone and bovine α_2M reacted with methylamine, trypsin, or methylamine followed by trypsin. (—) α_2M alone; (···) α_2M reacted with methylamine; (---) α_2M reacted with trypsin; (-·-) α_2M reacted first with methylamine and then with trypsin. The conditions of the reactions with trypsin were as in Figure 6. The excitation wavelength was 279 nm, and the bandwidth was 4 nm in both channels. The α_2M concentration was 0.12 μM. Inner filter corrections were done as described earlier (Björk & Fish, 1982). The spectrum for bovine α_2M alone has been normalized to 1.0.

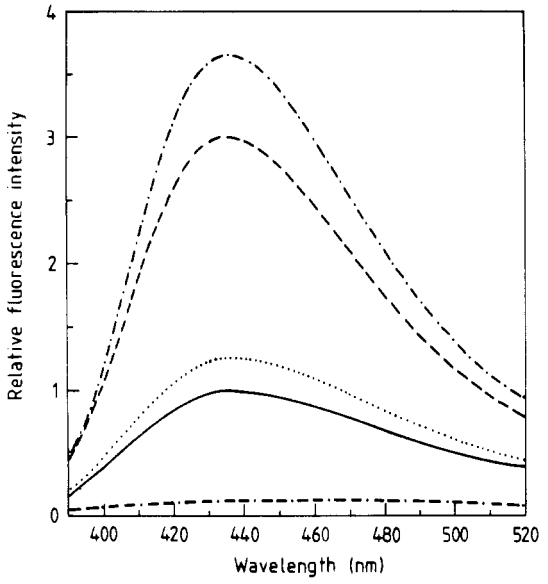

FIGURE 8: Corrected fluorescence spectra of TNS bound to bovine α_2M alone and to bovine α_2M reacted with methylamine, trypsin, or methylamine followed by trypsin. (—) α_2M alone; (···) α_2M reacted with methylamine; (---) α_2M reacted with trypsin; (-·-) α_2M reacted first with methylamine and then with trypsin; (-·-·-) trypsin alone. The conditions of the reactions with trypsin were as in Figure 6. The α_2M concentration was 1.2 μM and the TNS concentration 100 μM. The excitation wavelength was 375 nm, and the bandwidth was 4 nm in both channels. The spectrum for TNS bound to α_2M alone has been normalized to 1.0.

similar to those of the bands obtained by heat-induced fragmentation of human α_2M (Harpel et al., 1979; Howard et al., 1980). However, such fragmentation has been shown to require high temperature and an intact thio ester bond, whereas the bands of the bovine preparation were obtained at 37 °C and also with methylamine-treated α_2M. Heat-induced fragmentation thus appears an unlikely cause of the two minor

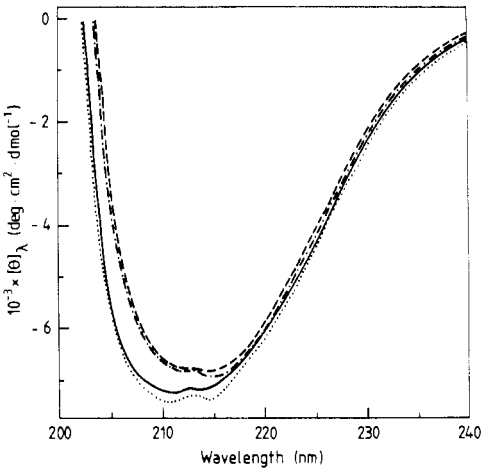

FIGURE 9: Far-ultraviolet circular dichroism spectra of bovine α_2M alone and bovine α_2M reacted with methylamine, trypsin, or methylamine followed by trypsin. (—) α_2M alone; (···) α_2M reacted with methylamine; (---) α_2M reacted with trypsin; (-·-) α_2M reacted first with methylamine and then with trypsin. The conditions of the reactions with trypsin were as in Figure 6. Cells with 0.1-cm path length and an α_2M concentration of 0.43 μM were used. Methylamine and/or trypsin of the same concentrations as in the samples were included in the blanks. The unit on the ordinate is mean residue ellipticity. The mean residue weight for bovine α_2M was calculated to be 112 from the amino acid analyses.

bands. Alternatively, the bands may represent proteolysis products, formed either in vivo or in vitro. This possibility is indicated by the high sensitivity of bovine α_2M to proteolysis by added trypsin. However, the amount of the bands was unaffected by extensive efforts to reduce proteolytic degradation during isolation of the protein and treatment of the samples before electrophoresis. Regardless of the origin of the minor bands, it is apparent that the form of α_2M that gives rise to them still must be active, since all the protein was in the active, slow form (Barrett et al., 1979) in gradient gel electrophoresis and all of it was converted to the inactive, fast form on reaction with trypsin. A possible proteolytic cleavage thus must have occurred outside the bait region and cannot have triggered the conformational change associated with the binding of a proteinase (Barrett & Starkey, 1973; Barrett et al., 1979). A fully active conformation of bovine α_2M is also indicated by the finding that the spectroscopic properties of the bovine protein, and the changes of these properties on reaction with trypsin, were very similar to the corresponding properties of human α_2M. It is therefore unlikely that the presence in the preparations of a small amount of the form of bovine α_2M that gives rise to the two minor bands can have affected the results of the analyses of the protein to any significant extent.

Although many properties of human and bovine α_2M are similar, as shown here and in earlier work (Nagasawa et al., 1970; Dangott & Cunningham, 1982), several differences are apparent. The thio ester bonds of bovine α_2M thus were shown here to be cleaved by methylamine at about half the rate of those of the human inhibitor, possibly reflecting a more sterically hindered access of the amine to the bonds of bovine α_2M (Larsson & Björk, 1984). Moreover, the apparent stoichiometry of inhibition of trypsin was found to be lower for the bovine protein. This behavior may be due to the structure of bovine α_2M promoting a higher extent of nonproductive activation of the inhibitor, i.e., a faster decay of some proteinase binding sites after activation by cleavage of the bait region, but before binding of the proteinase. Such nonproductive activation, leading to nonintegral apparent stoichiom-

etries of less than 2, has been demonstrated previously for human α_2M (Travis & Salvesen, 1983; Howell, 1983; Gonias & Pizzo, 1983b; Björk, 1984; Straight & McKee, 1984). Human α_2M and bovine α_2M further differ in their sensitivity to proteolysis by trypsin. Whereas human α_2M is cleaved essentially only in the bait region into two fragments of similar molecular weight (Harpel, 1973; Swenson & Howard, 1979a; Barrett et al., 1979; Sottrup-Jensen et al., 1981b), a number of sites are cleaved in the bovine inhibitor. Most of these cleaveages must occur outside the bait region, since the size of the fragments that are produced varies widely. Moreover, most of the cleavages do not lead to inactivation of α_2M, since all the polypeptide chains are cleaved to smaller fragments by amounts of trypsin appreciably lower than those which can be inhibited. However, some inactivation, which may have contributed to the lower apparent stoichiometry of inhibition of trypsin compared to that of human α_2M, cannot be excluded. It is apparent that this extensive proteolysis complicates the identification of the bait region in bovine α_2M.

The major difference between human and bovine α_2M is the behavior of the proteins on reaction with amines. Following cleavage of the thio ester bonds by methylamine, human α_2M undergoes an extensive conformational change that alters both the secondary and tertiary structure as well as the shape of the protein and leads to a total loss of activity (Steinbuch et al., 1968; Barrett et al., 1979; Björk & Fish, 1982; Gonias et al., 1982; Dangott et al., 1983). In contrast, the sedimentation coefficient and the mobility in gradient gel electrophoresis of methylamine-treated bovine α_2M have been shown to be indistinguishable from those of the intact protein, indicating that no appreciable change of shape has occurred (Dangott & Cunningham, 1982). The present work similarly shows that the spectroscopic properties of bovine α_2M are altered only minimally by methylamine, consistent with no major change also of the secondary and tertiary structure. The protein also remains partially active. Thus, all analyses suggest that a gross conformational change analogous to that undergone by human α_2M when the thio ester bonds are cleaved does not occur at a detectable rate in the bovine inhibitor.

The similarity of the conformations of bovine α_2M with intact or cleaved thio ester bonds is further supported by the behavior of the two forms of the inhibitor on binding of trypsin. The changes caused by the proteinase of the hydrodynamic (Dangott & Cunningham, 1982) and spectroscopic properties of the protein are thus virtually identical in the untreated and methylamine-treated inhibitor, suggesting that similar conformational changes are induced in the two forms. The physicochemical changes are also similar to those induced by trypsin in human α_2M, indicating that the proteolytic cleavages occurring in bovine α_2M do not significantly affect the conformation of either the intact or the methylamine-treated protein that has reacted with the proteinase. The fragments, therefore, must remain associated in essentially the same manner as if the polypeptide chain were intact. These results thus show that trypsin binds in a similar manner to bovine α_2M, regardless of whether or not the thio ester bonds of the protein are intact. However, the binding is apparently not identical, since at least some trypsin molecules are bound to the methylamine-treated inhibitor in a mode that allows them to react faster with STI. Nevertheless, it is apparent that the conformational change of bovine α_2M associated with trapping of the proteinase can be studied with a high degree of fidelity in the methylamine-treated protein, e.g., by methods involving labeling of the liberated thiol group. Moreover, the results of such studies can be extrapolated to human α_2M with reasonable accuracy, because of the similarity of the conformational changes induced by trypsin in human and bovine α_2M.

Although no gross conformational change of bovine α_2M thus occurs when the thio ester bonds of the protein are cleaved by amines, definite changes of several functional properties of the inhibitor are induced. The apparent stoichiometry of inhibition of trypsin thus is significantly decreased, possibly as a result of a higher nonproductive activation of proteinase binding sites. An increased inactivation of the inhibitor by the increased proteolysis accompanying the reaction with the enzyme (see below) may also have occurred. However, inactivation of a fraction of the molecules by the amine is less probable, since all the methylamine-treated protein was in the active, slow form in gradient gel electrophoresis and was completely converted to the inactive, fast form by trypsin. An analogous decrease of the stoichiometry of inhibition, without an apparent gross conformational change, has been demonstrated for methylamine-treated rat α_2M (Gonias et al., 1983). Reaction of bovine α_2M with methylamine also greatly increases the sensitivity of the protein to proteolytic cleavage by trypsin. As in native bovine α_2M, the cleavages occur primarily outside the bait region, and most of them do not affect the activity of the inhibitor, since extensively cleaved methylamine-treated α_2M also retains the ability to inactivate the enzyme. Finally, cleavage of the thio ester bonds also greatly decreases the rates by which thrombin and, by inference, possibly also other proteinases are inactivated. This decrease may be due to a lower rate of proteolytic cleavage of the bait region, which thus may have been rendered less accessible to the enzyme. Together, these observations provide evidence that a conformational change of bovine α_2M does occur when the thio ester bonds are cleaved by amines. However, the similar physicochemical properties of the intact and methylamine-treated protein suggest that this change is limited and possibly is restricted mainly to the vicinity of the thio ester bond.

Together, these and earlier studies (Barrett et al., 1974; Sottrup-Jensen et al., 1980; Howard, 1981; Björk & Fish, 1982; Gonias et al., 1982; Dangott & Cunningham, 1982; Dangott et al., 1983) indicate that the thio ester bonds of α_2M are required for the integrity of the conformation of both the human and the bovine inhibitor. In the absence of these bonds, conformational changes of both inhibitors occur. However, the conformation of bovine α_2M is more stable than that of the human inhibitor, due to some unknown structural feature of the protein. The conformational change of bovine α_2M therefore is limited and only decreases but does not abolish the activity of the inhibitor. It is thus evident that an intact thio ester bond per se is not an absolute requirement for the ability of α_2M to bind proteinases. Nevertheless, the thio ester bonds appear to be directly involved in maintaining certain structural features of the inhibitor that are of importance for full activity. A sequel to the studies of bovine α_2M is that the spectroscopic changes of human α_2M observed on reaction of the inhibitor with amines (Björk & Fish, 1982; Gonias et al., 1982; Dangott et al., 1983) presumably arise predominantly from the major conformational change that follows thio ester bond cleavage rather than from the cleavage of the thio ester bonds themselves.

Registry No. Trypsin, 9002-07-7; thrombin, 9002-04-4.

REFERENCES

Barrett, A. J., & Starkey, P. M. (1973) *Biochem. J. 133*, 709–724.

Barrett, A. J., Starkey, P. M., & Munn, E. A. (1974) in *Proteinase Inhibitors* (Fritz, H., Tschesche, H., Greene, L.

J., & Truscheit, E., Eds.) pp 574–580, Springer-Verlag, Berlin.
Barrett, A. J., Brown, M. A., & Sayers, C. A. (1979) *Biochem. J. 181*, 401–418.
Bieth, J. G., Tourbez-Perrin, M., & Pochon, F. (1981) *J. Biol. Chem. 256*, 7954–7957.
Björk, I. (1984) *Biochem. Biophys. Res. Commun. 118*, 691–695.
Björk, I., & Nordling, K. (1979) *Eur. J. Biochem. 102*, 497–502.
Björk, I., & Fish, W. W. (1982) *Biochem. J. 207*, 347–356.
Björk, I., Larsson, L.-J., Lindblom, T., & Raub, E. (1984) *Biochem. J. 217*, 303–308.
Branegård, B., Österberg, R., & Sjöberg, B. (1982) *Eur. J. Biochem. 122*, 663–666.
Carlström, A.-S., Liedén, K., & Björk, I. (1977) *Thromb. Res. 11*, 785–797.
Chase, T., & Shaw, E. (1970) *Methods Enzymol. 19*, 20–27.
Dangott, L. J., & Cunningham, L. W. (1982) *Biochem. Biophys. Res. Commun. 107*, 1243–1251.
Dangott, L. J., Puett, D., & Cunningham, L. W. (1983) *Biochemistry 22*, 3647–3653.
Dunn, J. T., & Spiro, R. G. (1967) *J. Biol. Chem. 242*, 5549–5555.
Frost, A. A., & Pearson, R. G. (1961) *Kinetics and Mechanism*, 2nd ed., pp 27–40, 162–164, Wiley, New York.
Ganrot, P. O. (1966) *Acta Chem. Scand. 20*, 2299–2300.
Gonias, S. L., & Pizzo, S. V. (1983a) *Biochemistry 22*, 536–546.
Gonias, S. L., & Pizzo, S. V. (1983b) *J. Biol. Chem. 258*, 14682–14685.
Gonias, S. L., Reynolds, J. A., & Pizzo, S. V. (1982) *Biochim. Biophys. Acta 705*, 306–314.
Gonias, S. L., Balber, A. E., Hubbard, W. J., & Pizzo, S. V. (1983) *Biochem. J. 209*, 99–105.
Hall, P. K., & Roberts, R. C. (1978) *Biochem. J. 171*, 27–38.
Harpel, P. (1973) *J. Exp. Med. 138*, 508–521.
Harpel, P. (1976) *Methods Enzymol. 45*, 639–652.
Harpel, P. C., Hayes, M. B., & Hugli, T. E. (1979) *J. Biol. Chem. 254*, 8669–8678.
Howard, J. B. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2235–2239.
Howard, J. B., Vermeulen, M., & Swenson, R. P. (1980) *J. Biol. Chem. 255*, 3820–3823.
Howell, J. B., Beck, T., Bates, B., & Hunter, M. J. (1983) *Arch. Biochem. Biophys. 221*, 261–270.
James, F., & Roos, M. (1975) *Comput. Phys. Commun. 10*, 343–367.
Jones, J. M., Creeth, J. M., & Kekwick, R. A. (1972) *Biochem. J. 127*, 187–197.
Kurecki, T., Kress, L. F., & Laskowski, M., Sr. (1979) *Anal. Biochem. 99*, 415–420.
Larsson, L.-J., & Björk, I. (1984) *Biochemistry 23*, 2802–2807.
Marchalonis, J. J., & Weltman, J. K. (1971) *Comp. Biochem. Physiol., B: Comp. Biochem. 38B*, 609–625.
McClure, W. O., & Edelman, G. M. (1966) *Biochemistry 5*, 1908–1918.
Miller-Andersson, M., Borg, H., & Andersson, L.-O. (1974) *Thromb. Res. 5*, 439–452.
Nagasawa, S., Sugihara, H., Han, B. H., & Suzuki, T. (1970) *J. Biochem. (Tokyo) 67*, 809–832.
Nordenman, B., Nyström, C., & Björk, I. (1977) *Eur. J. Biochem. 78*, 195–203.
Ohlsson, K., & Skude, G. (1976) *Clin. Chim. Acta 66*, 1–7.
Robinson, N. C., Tye, R. W., Neurath, H., & Walsh, K. A. (1971) *Biochemistry 10*, 2743–2747.
Salvesen, G. S., & Barrett, A. J. (1980) *Biochem. J. 187*, 695–701.
Salvesen, G. S., Sayers, C. A., & Barrett, A. J. (1981) *Biochem. J. 195*, 453–461.
Sottrup-Jensen, L., Petersen, T. E., & Magnusson, S. (1980) *FEBS Lett. 121*, 275–279.
Sottrup-Jensen, L., Hansen, H. F., Mortensen, S. B., Petersen, T. E., & Magnusson, S. (1981a) *FEBS Lett. 123*, 145–148.
Sottrup-Jensen, L., Lønblad, P. B., Stepanik, T. M., Petersen, T. E., Magnusson, S., & Jörnvall, H. (1981b) *FEBS Lett. 127*, 167–173.
Sottrup-Jensen, L., Petersen, T. E., & Magnusson, S. (1981c) *FEBS Lett. 128*, 123–126.
Starkey, P. M., & Barrett, A. J. (1982) *Biochem. J. 205*, 91–95.
Steinbuch, M., Pejaudier, L., Quentin, M., & Martin, V. (1968) *Biochim. Biophys. Acta 154*, 228–231.
Straight, D. L., & McKee, P. A. (1984) *J. Biol. Chem. 259*, 1272–1278.
Strickland, D. K. (1983) *Thromb. Haemostasis 50*, 232.
Swenson, R. P., & Howard, J. B. (1979a) *J. Biol. Chem. 254*, 4452–4456.
Swenson, R. P., & Howard, J. B. (1979b) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4313–4316.
Travis, J., & Salvesen, G. S. (1983) *Annu. Rev. Biochem. 52*, 655–709.
Virca, G. D., Travis, J., Hall, P. K., & Roberts, R. C. (1978) *Anal. Biochem. 89*, 274–278.
Wang, D., Wu, K., & Feinman, R. D. (1981) *J. Biol. Chem. 256*, 10934–10940.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.
Wu, K., Wang, D., & Feinman, R. D. (1981) *J. Biol. Chem. 256*, 10409–10414.
Yung, B. Y., & Trowbridge, C. G. (1975) *Biochem. Biophys. Res. Commun. 65*, 927–930.