Binding of proteinases to human α_2 -macroglobulin with its thioester bonds cleaved by methylamine in the presence of a thiol-group-cyanylating reagent

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After cleavage of the thioester bonds of human α_2 -macroglobulin (α_2 M) by methylamine, the inhibitor undergoes an extensive conformational change and loses its ability to bind proteinases. In contrast, similar cleavage in the presence of dinitrophenyl thiocyanate, a reagent that cyanylates the liberated thiol groups, does not change the mobility of α_2 M in gel electrophoresis, and the inhibitor also retains activity [Van Leuven, Marynen, Cassiman & Van den Berghe (1982) *Biochem. J.* **203**, 405–411]. Analyses in this work show that also the spectroscopic properties of α_2 M are essentially unperturbed under these conditions. These observations are consistent with the major change of the conformation of the protein having been arrested by the cyanylation reaction. However, several functional properties of the protein are altered, indicating that a limited conformational change does occur. The apparent stoichiometry of binding of trypsin is thus decreased to about 0.5 mol of enzyme/mol of α_2 M. Nevertheless trypsin induces a similar conformational change in all molecules of the modified inhibitor as that induced in untreated α_2 M. This behaviour indicates a similar mode of binding of the enzyme to the modified α_2 M as to intact α_2 M, but also a high extent of non-productive activation of binding sites in the modified inhibitor. A further difference to untreated α_2 M is that most of the bound trypsin molecules react considerably faster with soya-bean trypsin inhibitor. The rate of inhibition of thrombin is also greatly decreased, and the modified inhibitor is more sensitive than untreated α_2 M to proteolysis at sites outside the 'bait' region. The properties of the cyanylated human α_2 M are thus similar to those of bovine α_2 M in which the thioester bonds have been cleaved by methylamine in the absence of the cyanylating reagent [Björk, Lindblom & Lindahl (1985) *Biochemistry* **24**, 2653–2660]. These results indicate that the thioester bonds of human and bovine α_2 M are not required as such for the stability of the gross conformation of the protein or for the binding of proteinases. Nevertheless they participate directly in maintaining certain structural features, similar in the two inhibitors, that are necessary for full proteinase-binding ability. Disruption of these structures leads to a slower and less efficient trapping of the enzymes.

INTRODUCTION

α_2 -Macroglobulin (α_2 M) is a large (M_r approx. 725000), tetrameric plasma proteinase inhibitor with wide specificity (Jones *et al.*, 1972; Barrett & Starkey, 1973; Harpel, 1976; Hall & Roberts, 1978) and a unique mechanism of action. After an initial cleavage by the proteinase of a peptide bond in a limited region of the α_2 M chain, the 'bait' region, the enzyme is bound to the inhibitor in a manner that abolishes its activity only against macromolecular substrates (Harpel, 1973; Barrett & Starkey, 1973; Barrett *et al.*, 1979; Swenson & Howard, 1979; Sottrup-Jensen *et al.*, 1981b). The binding is associated with a major conformational change that alters both the tertiary structure and the shape of α_2 M (Barrett *et al.*, 1974; Gonias *et al.*, 1982; Björk & Fish, 1982; Branegård *et al.*, 1982; Dangott *et al.*, 1983). The inhibition may thus involve physical 'entrapment' of the proteinase (Barrett & Starkey, 1973). The maximal stoichiometry of binding of enzyme to inhibitor is 2:1, indicating that the α_2 M half-molecule is the functional unit of the inhibitor (Ganrot, 1966; Barrett *et al.*, 1979; Swenson & Howard, 1979; Sottrup-Jensen *et al.*, 1980;

Pochon *et al.*, 1981; Gonias & Pizzo, 1983a; Björk *et al.*, 1984). The proteolytic cleavage of the 'bait' region also leads to the appearance of a free thiol group in each α_2 M chain (Sottrup-Jensen *et al.*, 1980, 1981a; Howard, 1981; Salvesen *et al.*, 1981). Most evidence indicates that in intact α_2 M this group forms a thioester bond with a glutamic acid located three residues towards the C-terminal end of the chain (Sottrup-Jensen *et al.*, 1980, 1981a; Howard, 1981; Salvesen *et al.*, 1981). This bond apparently is destabilized after the 'bait'-region cleavage, allowing it to react rapidly with nucleophiles, such as amino groups on the proteinase. A proportion of the enzyme molecules thereby become covalently linked to the inhibitor by an amide bond with the glutamic acid residue (Salvesen & Barrett, 1980; Salvesen *et al.*, 1981; Sottrup-Jensen *et al.*, 1981c; Wu *et al.*, 1981).

Small primary amines inactivate human α_2 M (Steinbuch *et al.*, 1968; Barrett *et al.*, 1979), evidently by a nucleophilic attack on the thioester bonds by the unprotonated amine (Sottrup-Jensen *et al.*, 1980; Howard, 1981; Larsson & Björk, 1984). The thioester-bond cleavage leads to at least two sequential conformational changes of the inhibitor, the final conformational

Abbreviations used: α_2 M, α_2 -macroglobulin; DnpSCN, 2,4-dinitrophenyl thiocyanate; CN α_2 M, α_2 M treated with methylamine in the presence of DnpSCN; STI, soya-bean trypsin inhibitor.

state being very similar to that induced by the reaction of α_2 M with proteinases (Barrett *et al.*, 1979; Björk & Fish, 1982; Gonias *et al.*, 1982; Dangott *et al.*, 1983; Strickland & Bhattacharya, 1984; Larsson *et al.*, 1985). The thioester bonds of bovine and rat α_2 M are also cleaved by amines, but, in contrast with human α_2 M, no major conformational change ensues, and the inhibitor retains activity (Dangott & Cunningham, 1982; Gonias *et al.*, 1983; Feldman *et al.*, 1984; Björk *et al.*, 1985). However, changes of several functional properties are induced by the amine in bovine α_2 M, such as a decreased apparent stoichiometry of binding and a lower rate of inactivation of proteinases (Björk *et al.*, 1985). Studies have shown that reaction of human α_2 M with methylamine in the presence of DnpSCN, a reagent that cyanylates the liberated thiol group, to a large extent prevents the amine-induced change of shape and loss of activity of this protein (Van Leuven *et al.*, 1982). To elucidate further the role of the thioester bonds in α_2 M structure and function, the properties of the form of human α_2 M that is formed under these conditions have been characterized in the present work and compared with those of methylamine-treated bovine α_2 M.

MATERIALS AND METHODS

Methylamine was obtained from E. Merck, Darmstadt, Germany, and DnpSCN from ICN Pharmaceuticals, Plainview, NY, U.S.A. Fresh stock solutions (20–25 mM) of the latter reagent in ethanol were prepared before each experiment. [14 C]Methylamine and Na 125 I were purchased from Amersham International, Amersham, Bucks., U.K.

Human α_2 M was prepared from fresh frozen 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 protein was similar to that of preparations used previously (Björk & Fish, 1982).

Bovine trypsin (type III, twice crystallized; EC 3.4.21.4) and STI (type IS) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). β -Trypsin was isolated by affinity chromatography of the commercial preparation on agarose-linked STI (Robinson *et al.*, 1971; Yung & Trowbridge, 1975). Bovine α -thrombin (EC 3.4.21.5) was isolated by ion-exchange chromatography on SP- (sulphopropyl-)Sephadex (Pharmacia) after activation of crude prothrombin with a bovine brain suspension (Lundblad *et al.*, 1975; Silverberg, 1979). Active-site titrations with 4-nitrophenyl 4-guanidinobenzoate (Chase & Shaw, 1970) gave 0.89 ± 0.02 (mean $\pm$ S.D.; $n = 5$) and 0.95 ± 0.02 ($n = 3$) mol of active sites/mol of enzyme for trypsin and thrombin respectively. The solvent for trypsin was 1 mM-HCl/10 mM-CaCl $_2$, whereas thrombin was dissolved in 0.1 M-NaCl.

CN α_2 M was prepared by treating the protein (10–20 mg in 4–8 ml of 0.1 M-NaCl/50 mM-Hepes/NaOH buffer, pH 8.0) with 100 mM-methylamine/1 mM-DnpSCN for 90 min at 25 °C. Excess reagents were then removed by gel chromatography on a column (2.5 cm $\times$ 20 cm) of Sephadex G-50 (Pharmacia), eluted at a flow rate of 50 ml/h.

Gradient-gel electrophoresis was performed on commercial 4–30% (w/v) polyacrylamide gels (Pharmacia). Before analyses of trypsin/ α_2 M mixtures, the

enzyme was inactivated for 15 min at 25 °C with 1 mM-phenylmethanesulphonyl fluoride and 2 mM-L-7-amino-1-chloro-3-tosylamidoheptan-2-one ('TLCK'). The gels were stained with Amido Black and scanned at 595 nm in a gel scanner (Quick Scan Junior; Helena Laboratories, Beaumont, TX, U.S.A.). Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was done on 5% gels (Weber & Osborn, 1969). After inactivation of any added trypsin, as in the analyses by gradient gel electrophoresis, the samples were denatured for 45 min at 37 °C in 1% (w/v) sodium dodecyl sulphate/10 mM-sodium phosphate buffer, pH 7.0, with or without 0.3 M-2-mercaptoethanol. Thin-layer electrofocusing was done on LKB Ampholine polyacrylamide-gel plates, covering a pH range from 3.5 to 9.5 (LKB-Produkter, Bromma, Sweden). Gel chromatography in 6 M-guanidinium chloride was performed as described by Fish *et al.* (1969).

The incorporation of methylamine into α_2 M was analysed by treating the protein (1 mg in 300 μ l) with 25 mM-[14 C]methylamine (specific radioactivity 92.5 GBq/mol) for 6 h at 25 °C in the absence and in the presence of 1 mM-DnpSCN. The protein was then precipitated and the precipitate extensively washed with cold 10% (w/v) trichloroacetic acid. The precipitated protein was dissolved in 1 ml of 0.5 M-NaOH, and radioactivity was measured by liquid-scintillation counting. Triplicate analyses were done. The amount of methylamine bound was calculated from a standard curve of methylamine measured under the same conditions as the samples. The number of thiol groups in α_2 M released by methylamine or trypsin was determined by reaction with 5,5'-dithiobis-(2-nitrobenzoic acid) (Sottrup-Jensen *et al.*, 1980; Björk *et al.*, 1984).

The stoichiometry of binding of trypsin to α_2 M was analysed by three different methods. One of these involved measurements of the activity of free trypsin by a fibrin-gel assay (Björk *et al.*, 1984), and another was based on the ability of α_2 M to decrease greatly the rate of reaction of the bound enzyme with STI (Ganrot, 1966; Barrett & Starkey, 1973; Björk *et al.*, 1984). The third method directly measured the amount of 125 I-labelled trypsin bound to α_2 M. Trypsin was labelled with 125 I, by a procedure based on immobilized glucose oxidase and lactoperoxidase (Enzymobead Reagent; Bio-Rad Laboratories, Richmond, CA, U.S.A.), with no detectable loss of activity. α_2 M (1.5 mg in 800 μ l) was incubated for 5 min at 25 °C with the radiolabelled trypsin in a molar ratio of enzyme to inhibitor of 2.5:1. In some experiments STI was then added in a molar ratio to trypsin of 2.0:1, and the incubation was continued for another 5 min. Bound and free trypsin were separated by gel chromatography on a column (0.9 cm $\times$ 60 cm) of Sepharose 6B (Pharmacia), and the amount of bound trypsin was calculated from the ratio between the two peaks.

The rate of reaction of α_2 M-bound trypsin with STI, as well as the kinetics of inhibition of thrombin by α_2 M, were analysed essentially as described for the bovine protein (Björk *et al.*, 1985). The changes of u.v. difference absorption, tryptophan fluorescence, extrinsic fluorescence induced by binding of 6-(4-toluidino)naphthalene-2-sulphonic acid and far-u.v. c.d. accompanying the reactions of α_2 M with methylamine or trypsin were measured essentially as in previous investigations (Björk & Fish, 1982; Strickland & Bhattacharya, 1984; Björk *et al.*, 1985). The major modification was that fluorescence

Fig. 1. Stoichiometry of inhibition of trypsin by untreated α_2 M and CN α_2 M

(a) Fibrin gel assay; (b) STI assay. $\circ$, Intact α_2 M; $\bullet$, CN α_2 M. A series of samples, containing α_2 M at a constant concentration [0.80 μ M in (a) and 0.22 μ M in (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).

Fig. 2. First-order plot of the reaction between STI and trypsin bound to untreated α_2 M or CN α_2 M

$\circ$, Untreated α_2 M; $\bullet$, CN α_2 M. α_2 M at a final concentration of 0.20 μ M was incubated for 5 min at 25 °C with trypsin in molar ratios to the inhibitor of 2.0:1 for untreated α_2 M and 0.55:1 for CN α_2 M. STI was then added to a concentration of 7.5 μ M, and trypsin activity was assayed at successive intervals. The original activity was taken as that measured 1 min after addition of STI, i.e. when all free trypsin had been inactivated. This time was also taken as the zero time of the reaction.

was measured in an SLM 4800S spectrofluorimeter (SLM-Aminco, Urbana, IL, U.S.A.).

All analyses except the c.d. studies were done in 0.1 M-NaCl/50 mM-Hepes/NaOH buffer, pH 8.0 (Björk & Fish, 1982). Owing to the far-u.v. absorbance of Hepes, 0.1 M-NaCl/50 mM-Tris/HCl buffer, pH 8.0, was used instead in the c.d. analyses.

Protein concentrations were obtained by absorption measurements at 280 nm. The specific absorption coefficients and M_r values used in the calculations have been reported previously (Björk & Fish, 1982; Larsson *et al.*, 1985). All concentrations of proteinases were active-site concentrations.

RESULTS

Preparation of CN α_2 M

Previous kinetic studies have shown that the cleavage of the thioester bonds of human α_2 M by 100 mM-methylamine at pH 8.0 at 25 °C is > 98% complete in 90 min (Larsson & Björk, 1984). Analyses in this work showed that these conditions led to the appearance of 3.7 mol of thiol groups/mol of protein and to the incorporation of 3.5 mol of methylamine/mol of protein. Concurrently > 95% of the protein was converted into the form that migrates faster in gradient gel electrophoresis. Treatment of α_2 M under the same conditions, but in the presence of 1 mM-DnpSCN, led to the incorporation of 3.2 mol of methylamine/mol of protein, indicating that the thioester bonds are also cleaved in the presence of the cyanylating reagent. However, only < 10% of the protein was converted into the faster-migrating form, in agreement with previous analyses by Van Leuven *et al.* (1982). These conditions thus were suitable for the preparation of CN α_2 M. The reaction was terminated by removal of excess reagents by gel chromatography.

No free DnpSCN was detected in the preparations of CN α_2 M by comparison of the u.v.-absorption spectrum of the modified inhibitor with that of untreated α_2 M. In these analyses, the free reagent would have given an absorbance increase around 325 nm due to the dinitro-phenyl group, which is not bound to the modified protein (Degani & Degani, 1980). Gel chromatography of CN α_2 M in 6 M-guanidinium chloride, analysed by absorption measurements at 280 nm, also gave no evidence for any free DnpSCN. Addition of an excess of trypsin or 200 mM-methylamine to CN α_2 M gave no detectable thiol groups, a further indication that all thioester bonds had been cleaved in the modified inhibitor. A slow increase, occurring during the course of several days, of the form of the protein migrating faster in gradient gel electrophoresis was observed. The modified protein also aggregated on storage in solution or during freezing and thawing, as evidenced by an

Fig. 3. Trypsin cleavage of $\text{CN}\alpha_2\text{M}$, analysed by dodecyl sulphate/polyacrylamide-gel electrophoresis under reducing conditions

The reactions with trypsin were done for 5 min at 25 °C at the molar ratios of enzyme to inhibitor indicated. The concentration of $\text{CN}\alpha_2\text{M}$ was 0.8 μM . A 15–40 μg portion of protein was applied to each gel.

increase of the absorbance at 340 nm. All analyses therefore were done within 24 h.

Stoichiometry of binding of trypsin

Both methods used to determine the apparent stoichiometry of inhibition of trypsin showed that 1 mol of untreated $\alpha_2\text{M}$ inhibited 1.8 mol of enzyme (Fig. 1). In contrast, the apparent stoichiometry of inhibition of $\text{CN}\alpha_2\text{M}$ was found to be about 0.5:1 with the same methods. Control experiments excluded that this finding was an experimental artifact due to a slower reaction. Similarly, untreated $\alpha_2\text{M}$ and $\text{CN}\alpha_2\text{M}$ bound 1.7 and 0.8 mol of ^{125}I -labelled trypsin/mol of protein respectively. Treatment of the trypsin- $\alpha_2\text{M}$ complexes with STI did not appreciably change these binding ratios, indicating that all the enzyme was tightly bound. The low binding of trypsin to $\text{CN}\alpha_2\text{M}$ is in agreement with analyses by gradient gel electrophoresis, which showed that complete conversion of the modified protein from the 'slow' into the 'fast' form (Barrett *et al.*, 1979) occurred at a

Fig. 4. Pseudo-first-order rate constants at pH 8.0 at 25 °C for the inhibition of thrombin by untreated $\alpha_2\text{M}$ or $\text{CN}\alpha_2\text{M}$ as a function of inhibitor concentration

○, Untreated $\alpha_2\text{M}$; ●, $\text{CN}\alpha_2\text{M}$. The thrombin concentration was 0.025–0.2 μM and the concentration of $\alpha_2\text{M}$ at least 10-fold higher. Note different scales on the ordinate for untreated $\alpha_2\text{M}$ and $\text{CN}\alpha_2\text{M}$.

trypsin/ $\alpha_2\text{M}$ molar ratio of about 0.5:1. Cleavage of the thioester bonds of $\alpha_2\text{M}$ by methylamine in the presence of DnpSCN thus markedly lowers the apparent stoichiometry of binding of trypsin.

Reaction of bound trypsin with STI

The rate of reaction with STI of trypsin bound to untreated $\alpha_2\text{M}$ or $\text{CN}\alpha_2\text{M}$ was analysed under pseudo-first-order conditions, i.e. at a 20- to 40-fold molar ratio of STI to the bound enzyme (Fig. 2). Trypsin bound to untreated $\alpha_2\text{M}$ reacted very slowly with STI, as has been described previously (Bieth *et al.*, 1981; Wang *et al.*, 1981). A highly approximate second-order rate constant of $2 \text{ M}^{-1} \cdot \text{s}^{-1}$ was obtained. In contrast, trypsin bound to $\text{CN}\alpha_2\text{M}$ reacted at an appreciably faster rate. The semi-logarithmic plot was curved, suggesting at least two phases of the reaction. Analyses of the two discernible phases (Frost & Pearson, 1961) indicated that the faster phase accounted for about 80% of the reaction and that the apparent second-order rate constants for the two phases were about 6 and $110 \text{ M}^{-1} \cdot \text{s}^{-1}$. These data are thus compatible with at least two forms of the complex between $\text{CN}\alpha_2\text{M}$ and trypsin, in which the bound enzyme reacts with STI with rate constants differing by about 20-fold.

Proteolytic cleavage by trypsin

Untreated human $\alpha_2\text{M}$ is cleaved by trypsin at the 'bait' region into two fragments of similar size, giving essentially only one band of apparent M_r approx. 85000 in conventional dodecyl sulphate/polyacrylamide-gel electrophoresis (Harpel, 1973; Barrett *et al.*, 1974; Swenson & Howard, 1979; Howell *et al.*, 1983; Björk *et al.*, 1984), or two closely spaced bands in some systems (Barrett *et al.*, 1979). $\text{CN}\alpha_2\text{M}$ was found to be more sensitive to proteolysis (Fig. 3). Thus even amounts of

Fig. 5. Corrected fluorescence spectra of untreated α_2 M and $\text{CN}\alpha_2$ M in the absence and in the presence of trypsin

—, α_2 M; ----, α_2 M + trypsin; ····, $\text{CN}\alpha_2$ M; - · - · -, $\text{CN}\alpha_2$ M + trypsin. The α_2 M concentration was $0.12 \mu\text{M}$. The reactions with trypsin were done for 5 min at 25°C with a molar ratio of enzyme to α_2 M or $\text{CN}\alpha_2$ M of 2.5:1 and 1.0:1 respectively. The excitation wavelength was 278 nm and the excitation and emission bandwidths were 4 and 2 nm respectively. Inner-filter corrections were done as described earlier (Björk & Fish, 1982). The spectrum for α_2 M has been normalized to 1.0.

Fig. 6. Corrected fluorescence spectra of 6-(4-toluidino)naphthalene-2-sulphonic acid bound to untreated α_2 M and $\text{CN}\alpha_2$ M in the absence and in the presence of trypsin

—, α_2 M; ----, α_2 M + trypsin; ····, $\text{CN}\alpha_2$ M; - · - · -, $\text{CN}\alpha_2$ M + trypsin. The α_2 M concentration was $1.4 \mu\text{M}$, and the conditions of the reactions with trypsin were as indicated in Fig. 5 legend. A 6-(4-toluidino)naphthalene-2-sulphonic acid concentration of $50 \mu\text{M}$ and cells with 0.3 cm pathlengths were used. The excitation wavelength was 315 nm and the bandwidth was 4 nm in both channels. Blank spectra of 6-(4-toluidino)naphthalene-2-sulphonic acid in buffer with or without trypsin have been subtracted. The spectrum for 6-(4-toluidino)naphthalene-2-sulphonic acid bound to α_2 M has been normalized to 1.0.

trypsin lower than those required to saturate the modified inhibitor caused additional cleavages, and slightly higher than saturating amounts resulted in the disappearance of most of the initial cleavage product, the band with M_r approx. 85000. Analyses without reducing agent gave essentially only one band, indicating that most of the fragments are linked by disulphide bonds.

Kinetics of inhibition of thrombin

The reactions between trypsin and either untreated α_2 M or $\text{CN}\alpha_2$ M were too fast to be monitored with manual methods. Instead, the kinetics of inhibition of proteinases was studied with thrombin, which reacts more slowly with the inhibitor. All reactions were done at a 10-fold or higher inhibitor/enzyme molar ratio. Semi-logarithmic plots were all linear within experimental error, consistent with the reactions occurring under pseudo-first-order conditions. A plot of the pseudo-first-order rate constants versus the concentration of untreated α_2 M was linear (Fig. 4) and gave a second-order rate constant of $7.1 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$. This value is higher than that of about $2.5 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ determined previously under second-order conditions (Straight & McKee, 1982). The reaction of $\text{CN}\alpha_2$ M with thrombin was considerably slower and was only studied at a limited range of inhibitor concentrations (Fig. 4). These analyses

gave an approximate second-order rate constant of $25 \text{ M}^{-1} \cdot \text{s}^{-1}$, i.e. about a 300-fold lower value than that of untreated α_2 M.

Isoelectric point

Untreated α_2 M and $\text{CN}\alpha_2$ M gave an identical pattern in thin-layer isoelectric focusing with four major bands in the pH range 5.3–5.6 (results not shown). In keeping with the finding by Ohlsson & Skude (1976), reaction of untreated α_2 M with trypsin increased the isoelectric point of the protein, the bands instead focusing between pH 5.6 and 5.9. A similar increase in isoelectric point on reaction with trypsin was observed also for $\text{CN}\alpha_2$ M.

Spectroscopic properties

The conformations of untreated α_2 M and $\text{CN}\alpha_2$ M before and after reaction with trypsin were compared by analyses of tryptophan fluorescence, extrinsic fluorescence induced by binding of 6-(4-toluidino)naphthalene-2-sulphonic acid and c.d. U.v. difference spectroscopy was also attempted, but gave no meaningful results for $\text{CN}\alpha_2$ M because of the development of turbidity, a phenomenon to which this method is uniquely sensitive. The three spectroscopic analyses that were performed gave similar results (Figs. 5–7). The spectroscopic

Fig. 7. Far-u.v. c.d. spectra of untreated α_2 M and $\text{CN}\alpha_2$ M in the absence and in the presence of trypsin

—, α_2 M; ----, α_2 M + trypsin; ····, $\text{CN}\alpha_2$ M; - · - ·, $\text{CN}\alpha_2$ M + trypsin. The α_2 M concentration was $0.48 \mu\text{M}$ and the conditions of the reactions with trypsin were as indicated in Fig. 5 legend. Cells with 0.1 cm pathlengths were used. Trypsin of the same concentration as in the samples was included in the blanks. The unit on the ordinate is mean-residue ellipticity.

properties of $\text{CN}\alpha_2$ M thus were found to be similar to those of untreated α_2 M. In contrast, reaction of α_2 M with methylamine in the absence of DnpSCN resulted in marked spectroscopic changes of the inhibitor of the same magnitude as those induced by trypsin (results not shown), as has been reported previously (Gonias *et al.*, 1982; Björk & Fish, 1982; Dangott *et al.*, 1983; Strickland & Bhattacharya, 1984). The differences between untreated α_2 M and $\text{CN}\alpha_2$ M that were observed (Figs. 5–7) can be largely accounted for by the small amount ($< 10\%$) of $\text{CN}\alpha_2$ M that has undergone the methylamine-induced major conformational change, as indicated by gradient-gel electrophoresis. The analyses further showed that reaction of $\text{CN}\alpha_2$ M with trypsin produced spectroscopic changes similar to, although not identical with, the changes induced in untreated α_2 M on reaction with the enzyme (Figs. 5–7).

DISCUSSION

Previous studies have shown that cleavage of the thioester bonds of human α_2 M by methylamine in the presence of DnpSCN, which presumably rapidly cyanylates the liberated thiol groups, does not affect the mobility in gradient gel electrophoresis of most α_2 M molecules (Van Leuven *et al.*, 1982). This observation indicates that no appreciable change of shape of these molecules has occurred. The present work similarly shows that also the spectroscopic properties and the isoelectric point of the inhibitor are essentially unchanged under

these conditions, consistent with no major change also of the secondary and tertiary structure of $\text{CN}\alpha_2$ M. Moreover, similar changes of the hydrodynamic and spectroscopic properties, as well as of the isoelectric point, are induced by trypsin in $\text{CN}\alpha_2$ M as in the untreated inhibitor. This finding indicates that similar changes of the conformation of the two forms of the inhibitor occur on trypsin binding and that the enzyme therefore is bound in essentially the same manner to the two forms. Thus it is apparent that the conformation of human α_2 M, which normally is highly unstable on cleavage of the thioester bonds, can be stabilized in a near-native state by rapid reaction of the liberated thiol groups with appropriate ligands.

Although no major conformational change has occurred in $\text{CN}\alpha_2$ M, therefore, several functional properties of this form of α_2 M are different from those of the untreated inhibitor. The apparent stoichiometry of binding of trypsin is thus markedly decreased. However, also, those amounts of trypsin that are just sufficient to saturate $\text{CN}\alpha_2$ M, i.e. less than 1 mol/mol of α_2 M, cleave the 'bait' region and induce the conformational change in all molecules of the modified inhibitor. This behaviour indicates that the decreased stoichiometry of binding is due to a high extent of non-productive activation of binding sites, i.e. in many molecules the 'bait' region is cleaved and the conformational change normally leading to trapping of the proteinase occurs, but in such a manner that no enzyme is bound (Travis & Salvesen, 1983; Howell *et al.*, 1983; Gonias & Pizzo, 1983b; Björk, 1984). A further difference to untreated α_2 M is that those molecules that are trapped are done so in a way that allows at least most of them to react faster with STI than does trypsin bound to untreated α_2 M. This observation is in agreement with previous results obtained by Van Leuven *et al.* (1982), and suggests a less efficient trapping mechanism in $\text{CN}\alpha_2$ M. However, the active site of the proteinase is still protected from access to large substrates, such as fibrin. $\text{CN}\alpha_2$ M also inactivates thrombin, and by inference possibly also other proteinases, at a considerably lower rate than does untreated α_2 M, perhaps because of a less accessible 'bait' region. Finally, $\text{CN}\alpha_2$ M is more sensitive to proteolytic cleavage by trypsin at sites outside the 'bait' region. Together, these observations suggest that a limited conformational change, possibly restricted to the vicinity of the thioester bond and not detected by the hydrodynamic and spectroscopic analyses, has occurred in $\text{CN}\alpha_2$ M.

The properties of $\text{CN}\alpha_2$ M are highly similar to those of bovine α_2 M in which the thioester bonds have been cleaved by methylamine in the absence of a cyanylating reagent. This form of bovine α_2 M thus also has essentially the same hydrodynamic and spectroscopic properties as the untreated inhibitor, and thus has not undergone a major conformational change (Dangott & Cunningham, 1982; Björk *et al.*, 1985). Nevertheless, like $\text{CN}\alpha_2$ M it shows a decreased stoichiometry of inhibition of trypsin, a greater accessibility of the bound trypsin to STI, a much lower rate of inhibition of thrombin and an increased sensitivity to proteolysis, compared with the untreated inhibitor (Björk *et al.*, 1985). It is thus apparent that similar functional changes, probably reflecting a similar limited conformational change, are induced in human α_2 M and bovine α_2 M as an immediate consequence of cleavage of the thioester bonds. This finding indicates that these bonds, although not required as such for the

stability of the gross conformation of α_2 M or for the binding of proteinases, nevertheless are directly responsible for maintaining certain structural features, which are similar in the two inhibitors, and which are necessary for full proteinase-binding ability. Disruption of these structures leads to a slower and less efficient trapping of the enzymes.

This research was supported by grants from the Swedish Medical Research Council (Project no. 4212), the Swedish Council for Forestry and Agricultural Research (Project no. 600/83) and Konung Gustaf V:s 80-års-fond. I thank Miss Tua Lindblom for excellent technical assistance.

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Received 8 March 1985/11 June 1985; accepted 1 July 1985