Inhibition of Human Blood Coagulation Factor Xa by α_2-Macroglobulin†

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ABSTRACT: The inactivation of activated factor X (factor Xa) by α_2-macroglobulin (α_2M) was studied. The second-order rate constant for the reaction was 1.4×10^3 M^{-1} s^{-1}. The binding ratio was found to be 2 mol of factor Xa/mol of α_2M. Interaction of factor Xa with α_2M resulted in the appearance of four thiol groups per molecule of α_2M. The apparent second-order rate constants for the appearance of thiol groups were dependent on the factor Xa concentration. Sodium dodecyl sulfate gradient polyacrylamide gel electrophoresis was used to study complex formation between α_2M and factor Xa. Under nonreducing conditions, four factor Xa–α_2M complexes were observed. Reduction of these complexes showed the formation of two new bands. One complex (M_r 225 000) consisted of the heavy chain of the factor Xa molecule covalently bound to a subunit of α_2M, while the second complex (M_r 400 000) consisted of the heavy chain of factor Xa molecule and two subunits of α_2M. Factor Xa was able to form a bridge between two subunits of α_2M, either within one molecule of α_2M or by linking two molecules of α_2M. Complexes involving more than two molecules of α_2M were not formed.

Factor X plays a pivotal role in the clotting cascade because it can be activated by both the intrinsic and the extrinsic pathway. Regulation of the clotting cascade can take place by several plasma proteinase inhibitors which are capable of inhibiting activated clotting factors. Activated factor X (factor Xa) is in vitro mainly inactivated by antithrombin III and α_1-antitrypsin, as was observed in a purified system and in plasma (Ellis et al., 1982; Fuchs & Pizzo, 1983; Gitel et al., 1984; Jesty, 1986a,b). Although α_2-macroglobulin (α_2M)[1] only accounts in vitro for 10–15% of the factor Xa inactivation, Fuchs and Pizzo (1983) demonstrated that 2 min after injection of ^{125}I-labeled factor Xa into a mouse, 90% of the radioactivity was bound to α_2M. This suggests that, in vivo, α_2-macroglobulin is a major inhibitor of factor Xa.

α_2-Macroglobulin is a tetramer of four identical subunits formed by the noncovalent association of two disulfide-linked pairs of subunits (Harpel, 1973). It is capable of forming complexes with different kinds of active proteinases (Barrett & Starkey, 1973). The inactivation process of the proteinases is initiated by the cleavage of a vulnerable region, the bait region, near the middle of the α_2M subunit. This is followed by a conformational change resulting in steric entrapment of the enzyme (Barrett & Starkey, 1973). The active site of the bound proteinase is not involved in maintaining the complex, since activity toward small substrates is usually only partly impaired or not impaired at all (Starkey, 1979). Covalent binding of most enzymes involves an internal thio ester between cysteine and glutamate residues, and this site is required for the activity of human α_2M. Recently, Wang et al. (1983, 1984) provided evidence for the formation of covalent bonds between a single thrombin molecule and two subunits of α_2M. Similar conclusions were drawn from studies of the inhibition of kallikrein by α_2M. Analysis of the reaction mixtures with SDS–polyacrylamide slab gel electrophoresis indicated the formation of complexes that involved the binding of more than one subunit of α_2-macroglobulin per enzyme molecule (van der Graaf et al., 1984).

This study involves the interaction between factor Xa and α_2-macroglobulin in a purified system. The kinetics of inactivation of factor Xa by α_2-macroglobulin have been determined, and the nature of the molecular complexes formed between factor Xa and α_2-macroglobulin has been examined. Evidence is provided for the linking of more than one subunit of α_2-macroglobulin by factor Xa.

Materials and Methods

Human factor X was purified essentially as described by Miletich et al. (1978), with one additional purification step using fast protein liquid chromatography with a Mono Q column (Pharmacia, Uppsala, Sweden). Factor X was dialyzed against a buffer containing 20 mM histidine and 10 mM sodium citrate, pH 6.0. After application to the column, factor X was eluted with a linear gradient (20 mL) of sodium chloride (0–1 M) in the histidine buffer. The flow rate was 1 mL·min^{-1} and the fraction size 1 mL. Analysis of the factor X preparation under nonreducing conditions showed a single band with an approximate molecular weight of 72 000. The specific activity was 75 units/mg, determined with a one-stage clotting assay. Factor X was activated with Russell's viper venom (Kabi) in a factor X:RVV ratio (w/w) of 10:1 in 20 mM Tris, 150 mM NaCl, and 7 mM $CaCl_2$, pH 7.4. The activation was followed with the amidolytic substrate Bz-Ile-Glu-(-OR)-Gly-Arg-pNA (S2222, Kabi). At maximal activation, the mixture was diluted 10-fold in the histidine buffer as described above and immediately applied to the Mono Q column. Factor Xa was eluted in the salt gradient.

α_2M was purified from human plasma as described by van der Graaf et al. (1984). α_2M bound 1.8 mol of trypsin/mol of α_2M using trypsin assayed by active-site titration with 4-nitrophenyl 4-guanidinobenzoate hydrochloride. Analysis of the α_2-macroglobulin preparation under reducing conditions indicated that approximately 90% of the protein migrated as a single band with an apparent molecular weight of 170 000 (Figure 2A).

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[1] Abbreviations: α_2M, α_2-macroglobulin; RVV, Russell's viper venom; SBTI, soybean trypsin inhibitor; SDS, sodium dodecyl sulfate; dansyl-GGACK, dansyl-L-glutamylglycyl-L-arginine chloromethyl ketone dihydrochloride; PAA, polyacrylamide; Tris, tris(hydroxymethyl)aminomethane; kDa, kilodalton(s).

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Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin as a reference.

Kinetic Assays. The α_2M:factor Xa binding ratio was determined by incubating a constant amount of factor Xa (0.45 μM) with increasing amounts of α_2M in 50 mM Tris and 150 mM NaCl, pH 7.4, in a final volume of 50 μL, at 37 °C. After 2 h, 20-μL samples were withdrawn and mixed with 20 μL of soybean trypsin inhibitor (SBTI) (10 mg/mL) for 30 s. Then the mixture was added to 960 μL of 0.2 mM Bz-Ile-Glu-(-OR)-Gly-Arg-pNA in 50 mM Tris and 150 mM NaCl, pH 7.8. The change in absorbance at 405 nm was followed continuously with a Uvikon double-beam spectrophotometer.

The kinetic constants for the hydrolysis of Bz-Ile-Glu-(-OR)-Gly-Arg-pNA by factor Xa and α_2M-bound factor Xa were determined. α_2M-bound factor Xa was prepared by incubating factor Xa (0.2 μM) with a 25-fold molar excess of α_2M as described above. Ten-microliter samples were taken from either a factor Xa solution or a factor Xa–α_2M mixture and then added to 990 μL of substrate solution in 50 mM Tris and 150 mM NaCl, pH 7.8.

For the kinetic studies of the inactivation of factor Xa by α_2M, a constant amount of factor Xa (0.2 μM) was incubated at 37 °C with increasing amounts of α_2M in 50 mM Tris, 150 mM NaCl, and 4 mM $CaCl_2$, pH 7.4, in a final volume of 300 μL. At various times, two aliquots of 17.5 μL were withdrawn. One aliquot was added to 500 μL of 0.2 mM Bz-Ile-Glu-(-OR)-Gly-Arg-pNA in 50 mM Tris and 150 mM NaCl, pH 7.8, and the other aliquot was added to 50 μL of SBTI (10 mg/mL), incubated for 1 min at room temperature, and then added to the amidolytic substrate. The difference between the two rates of hydrolysis was used as a measure of the free residual factor Xa in the mixture.

Release of Thiol Groups. The kinetics of appearance of free sulfhydryl groups in the reaction of α_2M with factor Xa were measured with the reagent dithiopyridine (Grassetti & Murray, 1967). A constant amount of α_2M (1 μM) in 50 mM Tris and 150 mM NaCl, pH 7.4, was incubated at 37 °C with different concentrations of factor Xa in the presence of 0.5 mM dithiopyridine. The increase in free thiol groups was measured by monitoring the change in absorbance at 324 nm using a Beckman Model 3600 double-beam spectrophotometer.

SDS–Polyacrylamide Slab Gel Electrophoretic Studies. The complex formation between factor Xa and α_2M was analyzed by using SDS–polyacrylamide slab gel electrophoresis. For this study, 0.19 μM α_2M was incubated with 0.78 μM factor Xa in 50 mM Tris, 150 mM NaCl, and 4 mM $CaCl_2$, pH 7.4 at 37 °C, in a total volume of 2 mL. At different times, two aliquots of 100 μL were taken. One aliquot was added to 12.5 μL of dansyl-GGACK (Calbiochem), 2 mM in 10 mM HCl, and incubated for 15 min at 37 °C. Subsequently, 87.5 μL of a SDS solution (13 mM Tris, 3% w/v SDS, 0.01% w/v bromophenol blue, and 10% v/v glycerol, pH 6.8) was added and incubated for 1 h at 37 °C. The second aliquot of 100 μL was treated in the same way except that the SDS solution contained 5% v/v β-mercaptoethanol. Both mixtures were divided into 30-μL aliquots and frozen at −20 °C until analysis by SDS slab gel electrophoresis. Under these conditions, the SBTI-resistant activity reached a maximum after 20 min and decreased only slightly during 4 h of incubation (data not shown). SDS gradient PAA slab gel electrophoresis was performed on 3–18% gradient PAA slab gels essentially according to the method of Laemmli (1970) under conditions as described by van der Graaf et al. (1984). After electrophoresis, the gels were transilluminated with long wavelength ultraviolet light and photographed by using a green filter and Polaroid type 42 film. After photography, the gels were immunoblotted essentially according to Towbin et al. (1979). The nitrocellulose blots were incubated with a rabbit antiserum against human factor X (kindly donated by Dr. K. Mertens, Central Laboratory of the Red Cross, Amsterdam) overnight at room temperature. A peroxidase-labeled goat anti-rabbit antiserum (Nordic, Tilburg, The Netherlands) was used as second antibody. After immunoblotting, the gels were stained with Coomassie Brilliant Blue R-250.

SDS–agarose electrophoresis was performed as described by Sixma et al. (1984) for the separation of von Willebrand factor multimers. A digest of adenovirus-5 DNA with restriction endonuclease *Hin*dIII (Boehringer Mannheim, FRG) was used as a molecular weight marker, together with high molecular weight markers for polyacrylamide electrophoresis from Pharmacia (Uppsala, Sweden).

Table I: Kinetic Constants for the Hydrolysis of Bz-Ile-Glu-(-OR)-Gly-Arg-pNA by Factor Xa and α_2M-Bound Factor Xa[a]

	K_m (mM)	k_{cat} (s^{-1})
factor Xa	0.62	6848
α_2M–factor Xa	0.59	3546

[a] K_m and k_{cat} values were obtained by using Lineweaver–Burk plots. Amidolytic activities were determined as described under Materials and Methods. The line is a least-squares fit of the means of duplicate determinations. The correlation coefficients were 0.998 or higher.

Results

Kinetics of Inactivation of Factor Xa by α_2-Macroglobulin. Incubation of factor Xa with a 25-fold molar excess of α_2M for 2 h at 37 °C resulted in a decrease of the amidolytic activity to 35% of the original amidolytic activity of factor Xa when Bz-Ile-Glu-(-OR)-Gly-Arg-pNA was used at a concentration of 0.2 mM. The remaining activity was 83% resistant to inhibition by SBTI, when SBTI was used in a thousandfold molar excess over α_2M-bound factor Xa during relatively short incubation times (30 s). After 2 h of incubation of SBTI and the α_2M–factor Xa complex, 22% of the original factor Xa amidolytic activity was present.

The binding ratio of factor Xa and α_2-macroglobulin was determined by measuring the SBTI-resistant activity after 30 s of incubation in aliquots taken from reaction mixtures containing factor Xa (0.45 μM) and different concentrations of α_2M after 2-h incubation at 37 °C. α_2M bound 2.1 mol of factor Xa/mol of α_2M.

To study the kinetics of inactivation of factor Xa by α_2M, factor Xa was incubated with different concentrations of α_2M. At various times, samples were withdrawn and analyzed for SBTI-resistant amidolytic activity (30 s). Irreversible inactivation of factor Xa with time was observed. The second-order rate constant for the inactivation of factor Xa was found to be 1.4×10^3 M^{-1} s^{-1}.

Binding of factor Xa to α_2M resulted in the formation of a complex, which still possessed amidolytic activity. Therefore, it was of interest to determine whether binding of factor Xa to α_2M affected the kinetic parameters K_m and k_{cat} for the hydrolysis of Bz-Ile-Glu-(-OR)-Gly-Arg-pNA by the enzyme. α_2M-bound factor Xa was prepared by incubating factor Xa with a 25-fold molar excess of α_2M for 2 h at 37 °C. The results for the determination of the K_m and the k_{cat} values are listed in Table I. The K_m remained unchanged, but the k_{cat} decreased approximately 2-fold when factor Xa had formed a complex with α_2-macroglobulin.

Release of Thiol Groups. The appearance of free thiol groups as a result of the interaction of α_2M with factor Xa

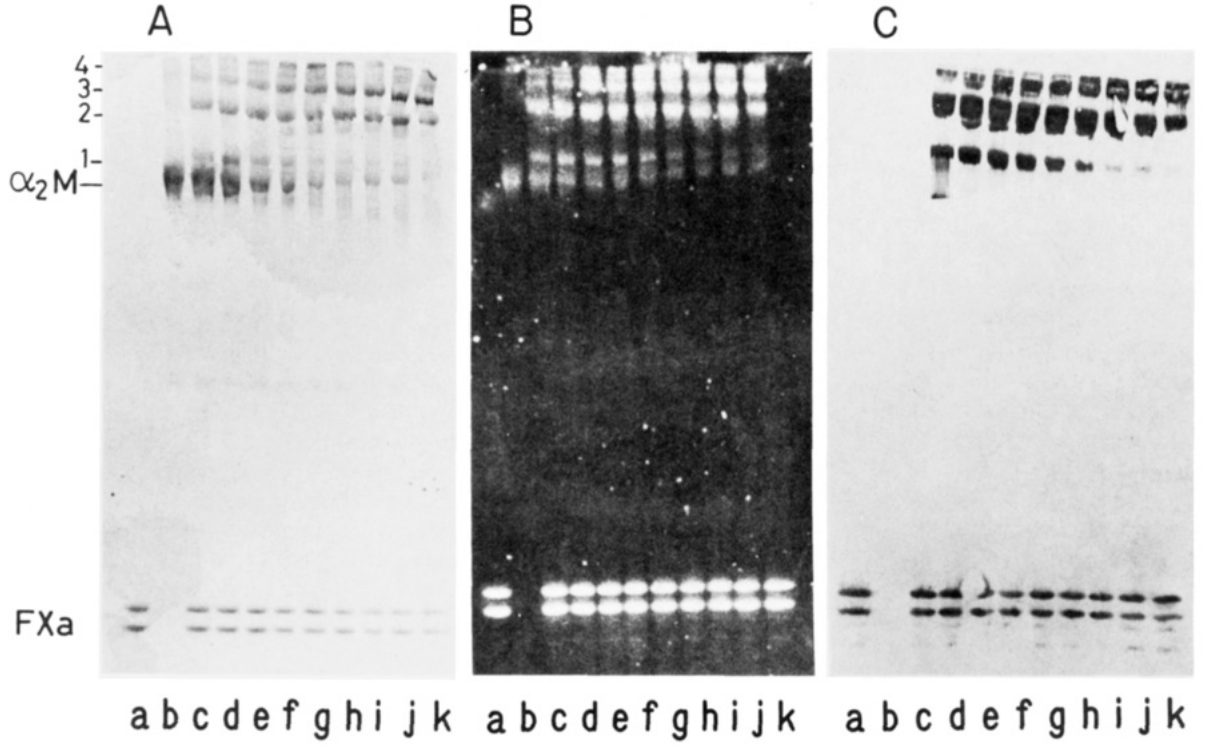

FIGURE 1: SDS 3–18% gradient PAA slab gel electrophoretic analysis of the reaction between factor Xa (0.19 μM) and α_2M (0.78 μM). At different times, aliquots were taken as described under Materials and Methods and analyzed on the gel (nonreduced). (A) Stained gel; aliquots were taken at 0 min (no α_2M present) (a), 0 min (no factor Xa present) (b), 5 min (c), 10 min (d), 20 min (e), 30 min (f), 45 min (g), 60 min (h), 120 min (i), 180 min (j), and 240 min (k). (B) Before staining of gel A, it was photographed while illuminated with ultraviolet light. (C) Immunoblotting using an anti-factor X antiserum. α_2M, factor Xa (FXa), and four new bands are indicated.

Table II: Second-Order Rate Constants for the Thiol Group Release of the Reaction of α_2M (1 μM) with Factor Xa[a]

factor Xa (μM)	$10^2 \times k_{sh}$ (M^{-1} s^{-1})
0.5	6.8
1	4.7
2	3.5
4	2.2

[a] Thiol group release was measured as described under Materials and Methods. Second-order rate constants were determined from the initial phase of the reaction.

was determined spectrophotometrically with dithiopyridine as an indicator. For these experiments, α_2M was incubated with increasing concentrations of factor Xa in the presence of dithiopyridine, and the change in absorbance at 324 nm was recorded. The reactions showed biphasic kinetics with a very slow final phase. The initial phase was found to follow second-order kinetics. Apparent second-order rate constants for the initial phase are listed in Table II. These constants appear to be dependent on the factor Xa concentration.

Interaction between Factor Xa and α_2M: Analysis by SDS Gradient PAA Slab Gel Electrophoresis. Incubation of α_2M with a 4-fold molar excess of factor Xa results in the formation of four new bands on SDS gradient PAA slab gel electrophoresis under nonreducing conditions (Figure 1A). The α_2M band at 360 kDa, a half-molecule of α_2M, disappears in time. All the new bands are of higher molecular weight, indicating that at least one half-molecule of α_2M is present. To obtain information if the new bands contain factor Xa, the incubation of factor Xa and α_2M was terminated in the presence of a synthetic fluorescent inhibitor specific for factor Xa, dansyl-GGACK. Illuminating the gels with ultraviolet light shows fluorescence of the excess factor Xa and of the four new bands (Figure 1B). α_2M alone also showed some fluorescence, probably due to aspecific binding of the fluorescence group. Further evidence that the bands contained factor Xa was obtained by immunoblotting with a specific antiserum against factor X. All new bands reacted positively with the antiserum, indicating that these bands contain at least one molecule of factor Xa (Figure 1C). Bands 1 and 2 are formed immediately in the reaction of factor Xa with α_2M, while bands 3 and 4 only appeared after a lag phase. Band 1 reached a maximum with time and then decayed. Bands 2, 3, and 4 increased in time to a maximum value.

Analyzing the same samples under reducing conditions showed the formation of at least three new bands, two of higher molecular weight, 225K and 400K, and one of lower molecular weight, 85K, compared to the subunit band of α_2M of 170K (Figure 2A). There is an apparent decrease in the 170-kDa band in time, but it does not disappear. Fluorescence is present in the excess (heavy chain) of factor Xa and in the 225- and 400-kDa bands (Figure 2B). A faint fluorescence is observed in the 85-kDa band and in an area around 140 kDa. Aspecific binding of the fluorescence group to the intact subunit of α_2M does not occur under reducing conditions, since no fluorescence can be seen in the 170-kDa band. This indicates that the bands at 225 and 400 kDa contain factor Xa. Further evidence was obtained from immunoblotting experiments using a specific antiserum against factor X (Figure 2C). Both bands reacted positively with the antiserum.

To examine if complexes between factor Xa and α_2M are formed, that cannot enter the unreduced slab gel, SDS–agarose electrophoresis was performed. With this technique, molecular weights of up to 20×10^6 can be detected. Upon incubation with factor Xa, the band of α_2M decreases in time. However, only one new band can be observed, both with Coomassie brilliant blue staining (Figure 3A) and by fluorescence (Figure 3B). This band has an apparent molecular weight of 740K–940K.

Discussion

The aim of this study was to elucidate the mechanism of inactivation of factor Xa by α_2-macroglobulin. In vitro,

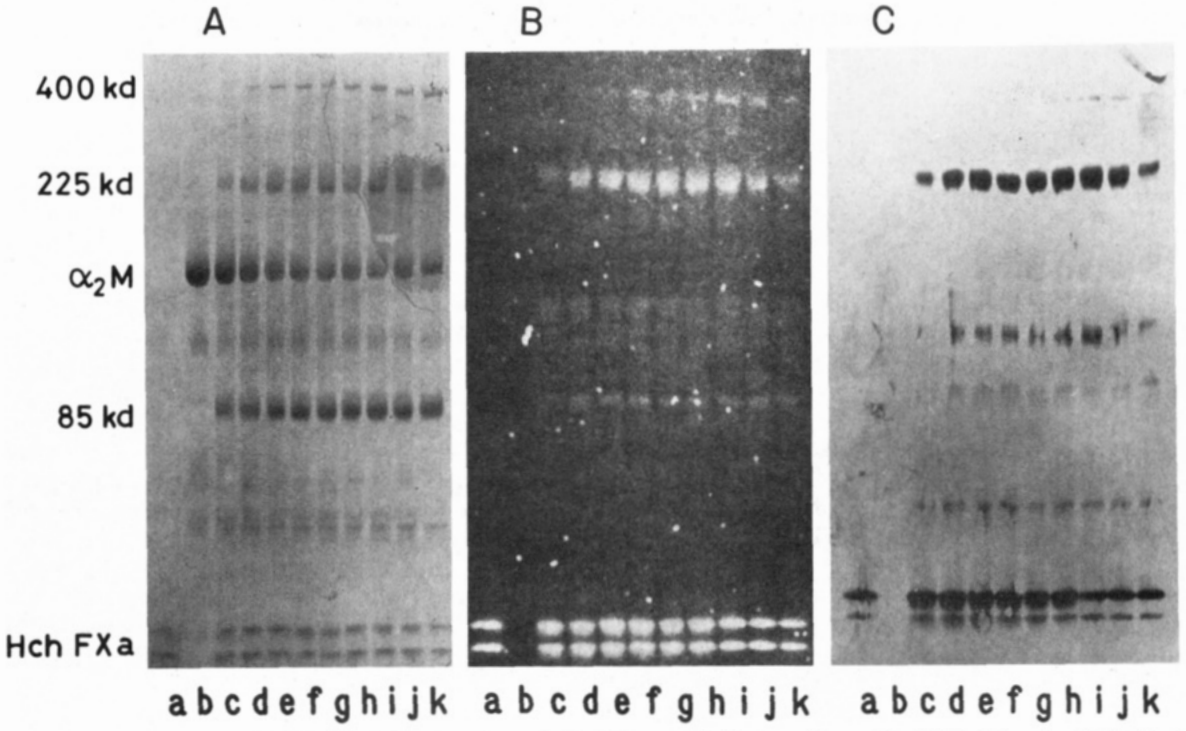

FIGURE 2: SDS 3–18% gradient PAA slab gel electrophoretic analysis of the reaction between factor Xa (0.19 μM) and α_2M (0.78 μM). The incubation mixtures are the same as described in the legend to Figure 1 and were reduced prior to analysis on the gel. α_2M and the heavy chain of factor Xa (Hch FXa) are indicated.

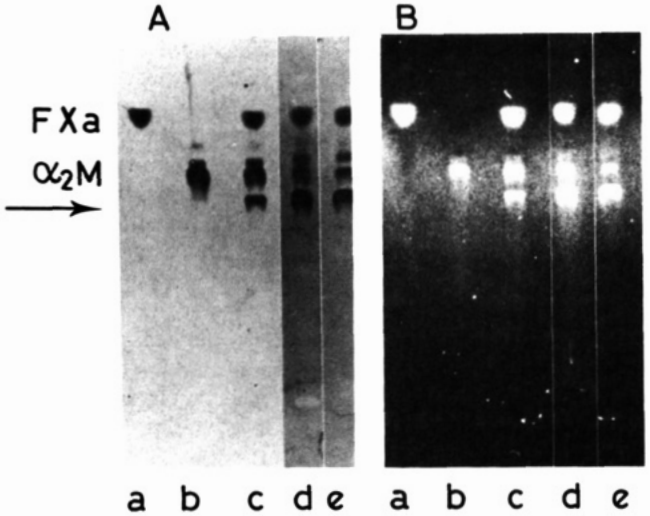

FIGURE 3: SDS–agarose electrophoretic analysis of the reaction of factor Xa (0.19 μM) and α_2M (0.78 μM). At different times, aliquots were taken as described under Materials and Methods and analyzed on the gel. (A) Stained gel; aliquots were taken at 0 min (no α_2M present) (a), 0 min (no factor Xa present) (b), 5 min (c), 60 min (d), and 240 min (e). (B) Before staining of gel A, it was photographed while illuminated with ultraviolet light. The arrow indicates a new band.

α_1-antitrypsin and antithrombin III account for nearly 90% of the inactivation capacity of factor Xa (Ellis et al., 1982; Fuchs & Pizzo, 1983; Gitel et al., 1984; Jesty, 1986a,b). In vivo, however, α_2M is responsible for most of the inactivation of factor Xa (Fuchs & Pizzo, 1983).

The molecular mechanism of the interaction of α_2M with different proteinases involves the formation of an α_2M–proteinase complex that still exhibits considerable reactivity toward low molecular weight substrates (Starkey, 1979). When complexed to α_2M, factor Xa retains 35% of its amidolytic activity on Bz-Ile-Glu-(-OR)-Gly-Arg-pNA. In the presence of a thousandfold molar excess and during longer incubation times, SBTI gave a minor but significant decrease of the amidolytic activity of the α_2M–factor Xa complex. This is in agreement with the observation that SBTI can react with α_2M-bound trypsin, resulting in a ternary complex (Bieth et al., 1981; Wang et al., 1981). The reaction of α_2M-bound factor Xa with SBTI also implies that high molecular weight substrates have access to the active site of α_2M-bound factor Xa.

It is generally assumed that the reduced enzymatic activity of the α_2M–proteinase complex is a reflection of steric hindrance. In addition, changes in kinetic parameters may contribute to the changes in enzymatic activity observed for α_2M-bound enzymes. Binding of factor Xa to α_2M is associated with a decrease in k_{cat}, but the K_m for the hydrolysis of Bz-Ile-Glu-(-OR)-Gly-Arg-pNA remains the same. Binding of kallikrein (van der Graaf et al., 1984) or trypsin (Rinderknecht et al., 1975) to α_2M resulted in a lower apparent K_m for the hydrolysis of H-D-Pro-Phe-Arg-pNA and Z-Gly-Gly-Arg-2-naphthylamide hydrochloride, respectively, by α_2M-bound enzyme as compared to the free enzyme. Van Leuven et al. (1981) also demonstrated differences, dependent on the substrate, for the K_m and V_{max} of native trypsin and of the α_2M–trypsin complex. The kinetic effects of entrapment of an enzyme within α_2M are likely to be complex and probably depend to a large degree on the altered microenvironment.

The inhibition process of factor Xa and α_2M involves the formation of a reversible enzyme–inhibitor complex, followed by the formation of an irreversible complex (data not shown). The apparent second-order rate constant is 1.4×10^3 M^{-1} s^{-1}. This is twice as high as the second-order rate constant described by Ellis et al. (1982) for the inactivation of factor Xa by α_2M.

The binding sites for the inactivation of factor Xa by α_2M were determined to be 2 mol of factor Xa/mol of α_2M. This is the ratio in which most proteinases become bound to α_2M (Howell et al., 1983).

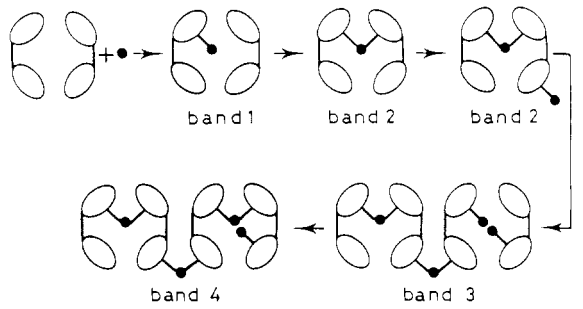

FIGURE 4: Model for the reaction of factor Xa and α_2M. Ellipses and dots indicate subunits of α_2M and factor Xa molecules, respectively. Bands 1–4 refer to the bands on the nonreduced slab gel in Figure 1.

The α_2M molecule contains an alkylamine reactive site which was identified as a Glx residue bound as a γ-glutamyl thio ester to the SH group of a Cys residue (Swenson & Howard, 1979; Sottrup-Jensen et al., 1980; Howard, 1981). The appearance of thiol groups in the reaction of α_2M with factor Xa showed biphasic kinetics with a very slow final phase. After the reaction, four free thiol groups per molecule of α_2M were detectable (data not shown). This is in agreement with determinations of free thiol groups in the reaction of α_2M with trypsin and elastase (Sottrup-Jensen et al., 1980; Salvesen et al., 1981). The initial phase of the appearance of thiol groups was found to follow second-order kinetics. The apparent second-order rate constants are dependent on the factor Xa concentration (Table II). This may be a reflection of different dependences on enzyme concentrations of thio ester centers, because several reactions can take place, e.g., hydrolysis and formation of covalent bonds with enzymes (Feinman et al., 1985).

The formation of factor Xa–α_2M complexes was studied with SDS gradient PAA slab gel electrophoresis. Analysis of the nonreduced samples indicated the formation of four new complexes, with approximate molecular weights of 400K–1000K. The presence of α_2M and factor Xa in all four complexes was demonstrated by Western blotting using a polyclonal antiserum to α_2M (data not shown) or factor X (Figure 1C). The high molecular weight of the new complexes indicates that more than one α_2M molecule is present, linked together by a factor Xa molecule. The fact that the interactions are stable in SDS implies that the formed bonds possess a stability characteristic of covalent bonds.

The reaction of a proteinase with α_2M results in cleavage in the central region of a subunit, generating two disulfide-linked 85-kDa fragments (Harpel, 1973; Starkey, 1979). Analysis of the reduced α_2M–factor Xa mixtures shows the generation of the 85-kDa fragments. In addition to the subunit of α_2M, two new bands of molecular weight 225K and 400K are detected. Both bands contain α_2M (data not shown) and factor Xa, as demonstrated by fluorescence and Western blotting (Figure 2B,C). This suggests that the 225-kDa band consists of the heavy chain of factor Xa linked to an α_2M subunit whereas the 400-kDa band might consist of two α_2M subunits bridged by the heavy chain of factor Xa. A "protein bridge" between subunits of α_2M and proteinases has previously been suggested for trypsin (Krebs et al., 1978) and thrombin (Wang et al., 1983). Experiments performed with dissociated half-molecules of α_2M did not show high molecular weight complexes (Gonias & Pizzo, 1983a,b), confirming the idea that the whole molecule is necessary before a proteinase can form a "protein bridge". Blocking the lysine residue amino groups decreased the formation of these complexes (Wang et al., 1984), suggesting that the linkages are an amide of the type involved in univalent complexes (Sottrup-Jensen et al., 1983).

A faint fluorescence and staining with the anti-factor X antiserum was observed at about 140 kDa. This may be due to a complex of the heavy chain of factor Xa with an 85-kDa fragment of α_2M. Binding to the 85-kDa fragment has been observed before for both the light and heavy chain of kallikrein (van der Graaf et al., 1984).

To study the possibilities that complexes were formed between α_2M subunits and factor Xa with molecular weights that cannot enter the unreduced slab gel, SDS–agarose electrophoresis was performed (Figure 3). It is shown that the protein bridging cannot continue indefinitely. Upon incubation with factor Xa, a new band was formed with an approximate molecular weight of 750K–940K. Bands 2–4 of the unreduced slab gel probably all contribute to the new band of the agarose gel, but they are not separated by using this technique. For the same reason, band 1 cannot be resolved from the α_2M band.

Our results confirm the model proposed by Feinman et al. (1985) for the reaction of thrombin with α_2M. In Figure 4, a schematic representation is drawn, consistent with our data, for the reaction of factor Xa with α_2-macroglobulin. At first, a covalent bond between a factor Xa molecule and a subunit of α_2M is formed, resulting in band 1 in nonreduced conditions and a 225-kDa band in reduced conditions. Band 2 is formed by linking of the α_2M subunits by this factor Xa molecule. Reduction of this complex results in the formation of the 400-kDa band. Another factor Xa molecule can be bound to band 2, but this complex is not separated from the one with one factor Xa molecule per α_2M. Linking of this second factor Xa molecule with another α_2M molecule results in the formation of band 3 and after internal cross-linking in band 4. Reduction of bands 3 and 4 results in the formation of 225- and 400-kDa bands. The bands 2, 3, and 4 appear to be stable in time and, therefore, will consist of two molecules of factor Xa per α_2M, which was found to be the binding ratio of factor Xa and α_2M. Further cross-linking of factor Xa and α_2M does not occur, since no higher molecular weight complexes could be detected with SDS–agarose electrophoresis.

It remains unknown to what extent the heavy and light chains of factor Xa contribute to the formation of the high molecular weight complexes. In the reaction of kallikrein and α_2M, both the light and heavy chain of kallikrein bound to the 85-kDa fragment (van der Graaf et al., 1984). Further studies are in progress to determine the influence of the light chain of factor Xa in the interaction of the heavy chain of factor Xa with α_2M.

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Tetrahymena pyriformis Cells Are Deficient in All Mannose-P-dolichol-Dependent Mannosyltransferases but Not in Mannose-P-dolichol Synthesis†

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ABSTRACT: Cells of the ciliated protozoan *Tetrahymena pyriformis* incubated with [^{14}C]glucose were found to synthesize Man-P-dolichol and Glc-P-dolichol, as well as $Glc_3Man_5GlcNAc_2$-P-P-dolichol, the latter being the main and largest lipid derivative formed. The missing mannose residues were those known to be transferred from Man-P-dolichol in other systems. Formation of Man-P-dolichol and of dolichol-P-P-oligosaccharides containing up to five mannose units was detected in cell-free assays containing protozoan membranes, rat liver dolichol-P, unlabeled $Man_{4-9}GlcNAc_2$-P-P-dolichol from pig liver, and GDP-[^{14}C]Man. Under exactly the same conditions but with UDP-[^{14}C]Glc instead of GDP-[^{14}C]Man, Glc-P-dolichol and dolichol-P-P-oligosaccharides containing five mannose and one to three glucose residues were formed in the absence of the pig liver compounds. In the presence of the latter, dolichol-P-P derivatives containing nine mannose and one to three glucose units were also synthesized. It is concluded that *T. pyriformis* cells are deficient in all Man-P-dolichol-dependent mannosyltransferases but not in Man-P-dolichol synthesis. The role of the latter compound in this microorganism is unknown.

N-Glycosylation in wild-type mammalian, plant, insect, and fungal cells is initiated by the transfer of an oligosaccharide ($Glc_3Man_9GlcNAc_2$, Figure 1) from a dolichol-P-P derivative to asparagine residues in proteins (Hubbard & Ivatt, 1981; Kornfeld & Kornfeld, 1985). GDP-Man appeared to be the donor of the first five mannose residues (c, d, e, f, and g, Figure 1) added in the assembly of the oligosaccharide, whereas the other four units (h, i, j, and k, Figure 1) were found to be transferred from Man-P-dolichol (Rearick et al., 1980b). On the other hand, Glc-P-dolichol appeared to be the donor of the three glucose residues (Parodi, 1979; Staneloni et al., 1980). Assays performed with yeast and mammalian membranes have shown that removal of the glucose residues from the lipid-linked oligosaccharide drastically reduces the rate of transfer of the latter to protein (Parodi, 1981; Turco et al., 1977; Spiro et al., 1979). On the other hand, removal of mannose residues h–k did not affect the rate of transfer of oligosaccharides containing three glucose residues (Spiro et al., 1979; Staneloni et al., 1981). The successive addition of

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