Complex-formation and inhibition of urokinase by blood plasma proteins

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(Received 25 April 1983/Accepted 1 May 1983)

We have studied the formation of covalent complexes between ^{125}I -urokinase (^{125}I -UK) and proteins in human plasma. Although ^{125}I -UK reacts with many proteinase inhibitors in purified systems, the predominant complexes formed in plasma are with antithrombin III (ATIII) and α_2 -macroglobulin ($\alpha_2\text{M}$). ^{125}I -UK interacts with purified $\alpha_2\text{M}$ or $\alpha_2\text{M}$ in plasma to form a characteristic pattern of multiple complexes whose M_r values by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis are in the range of 380 000–720 000, under non-reducing conditions, and 180 000–430 000 after reduction. We also examined the inhibition of UK amidolytic activity by plasma and by purified ATIII. In the presence of saturating concentrations of ATIII and heparin, an apparent first-order rate constant of $6.8 \times 10^{-1} \text{ s}^{-1}$ was calculated for the inhibition of urokinase. In contrast, the rate constant for the formation of covalent ATIII–UK complexes was lower, suggesting the inhibition of UK proceeds first via the formation of transient non-covalent intermediates that are then transformed more slowly into covalent end products. The observed rate constants for enzyme inhibition or complex-formation with plasma or purified inhibitors are insufficient to account for the reported clearance rate of injected UK *in vivo*.

Plasminogen activators are specific enzymes that activate plasminogen by limited proteolysis. The resulting enzyme, plasmin, is a general, trypsin-like proteinase that catalyses fibrolysis. Owing to the medical importance of intravascular coagulation, considerable effort has been committed to the development of thrombolytic therapy based on plasminogen activation (Bell, 1977; Fletcher *et al.*, 1965; Fletcher & Alkjaersig, 1977). Although it is well established that urokinase (UK) (EC 34.21.31) infusions rapidly increase the fibrinolytic activity of blood, this effect is very short-lived, the limited duration apparently being due to the disappearance, by unknown mechanisms, of UK from the circulation.

In view of the variety and high concentration of proteinase inhibitors in the plasma, it appears reasonable to assume that elimination of UK might occur primarily by way of complex being rapidly removed by some undefined clearance mechanism. This hypothesis is supported by the work of several laboratories who have reported that UK either forms

complexes with, or is inhibited by, ATIII (Clemmensen, 1978), $\alpha_1\text{PI}$ (Clemmensen & Christensen, 1976), $\alpha_2\text{M}$ (Ogston *et al.*, 1973), and α_2 -anti-plasmin ($\alpha_2\text{AP}$) (Moroi & Aoki, 1976). All of these studies made use of purified inhibitors and of reaction conditions that differed from those prevailing in plasma or blood. Gallimore (1980) and Walker & Ogston (1982) tested plasma protein fraction for the ability to inhibit UK, but did not characterize complex-formation.

Murano *et al.* (1980) studied the formation of SDS-resistant complexes between radioiodinated high- and low- M_r UK and inhibitors in plasma. Our work confirms these findings using different methods and provides new information about the kinetics of complex-formation between the enzyme and plasma proteinase inhibitors.

Experimental

Materials

Reagents were obtained as follows; *NNN'*N'-tetramethylethylenediamine and ammonium persulphate (Eastman Kodak Co., Rochester, NY, U.S.A.); Triton X-100, acrylamide, methylene-bisacrylamide and Coomassie Brilliant Blue (Sigma Chemical Co., St. Louis, MO, U.S.A.); urokinase (Leo Pharmaceutical Co., Ballerup, Denmark);

Abbreviations used: UK, urokinase; ATIII, anti-thrombin III; $\alpha_2\text{M}$, α_2 -macroglobulin; SDS, sodium dodecyl sulphate; $\alpha_1\text{PI}$, α_1 -proteinase inhibitor (formerly α_1 -antitrypsin or α_1 -trypsin inhibitor, ' $\alpha_1\text{AT}$ '); $\alpha_2\text{AP}$, α_2 -antiplasmin; <Glu-Gly-Arg-*p*NA, pyroglutamylglycylarginine *p*-nitroanilide (S-2444); IgG, immunoglobulin G.

Sephadex G-100, Sephacryl S-200, DEAE-Sephadex G-50, heparin-Sepharose CL-6B, Protein A-Sepharose CL-6B, DEAE-Sephacel, high-molecular-weight and low-molecular-weight standard proteins (Pharmacia, Uppsala, Sweden); purified agar (Difco Laboratories, Detroit, MI, U.S.A.); SDS (BDH Chemicals, Poole, Dorset, U.K.); Na¹²⁵I (New England Nuclear, Boston, MA, U.S.A.); 1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycoluril was purchased from Pierce Chemical Co., Rockford, IL, U.S.A.; Aquacide III from Calbiochem, La Jolla, CA, U.S.A. Goat antisera to rabbit IgG was obtained from Cappel Laboratories, Cochranville, PA, U.S.A.; and rabbit antisera against α_2 -MA from Behringwerke, Marburg an der Lahn, Germany. Human α_1 PI and C1-inactivator were generously provided by Dr. N. Heimbürger (Behringwerke). The synthetic substrate for UK, <Glu-Gly-Arg-pNA, was obtained from KABI Diagnostica, Stockholm, Sweden. Human plasminogen was prepared from outdated human plasma by two cycles of affinity chromatography (Deutsch & Mertz, 1970). Outdated EDTA-treated human plasma was obtained from the New York Blood Center. All other chemicals were of reagent grade.

Purification of proteinases and proteinase inhibitors

Protein concentrations were determined by the method of Hartree (1972). The enzymic activity of UK preparations was determined by measuring the rate of hydrolysis of the chromogenic substrate <Glu-Gly-Arg-pNA, using the procedure described by KABI Diagnostica and included with the product. UK was purified by affinity chromatography employing an 6-aminohexanoic acid-benzamidine-Sepharose column (Holmberg *et al.*, 1970). The bound enzyme was eluted with distilled water and freeze-dried; it was homogeneous as determined by SDS/polyacrylamide-gel electrophoresis and staining with Coomassie Blue G250. Solutions of UK were acidified to pH 3 with 1 mM-HCl for storage at 4°C.

Human ATIII was purified by the method of Miller-Anderson *et al.* (1974). ATIII from pooled frozen plasma was absorbed to a heparin-Sepharose CL-6B column; bound material was eluted employing a NaCl gradient from 0.1 to 1.5M. Further purification was achieved by ion-exchange chromatography on DEAE-Sephadex, and by successive gel-permeation chromatography on Sephacryl S-200 and Sephadex G-100. The protein was homogeneous as assessed by SDS/polyacrylamide-gel electrophoresis and staining with Coomassie Blue G250. A 0.12 μ M solution of the purified protein inhibited 50% of the amidolytic activity of a solution of thrombin containing 6 NIH (National Institutes of Health) units/ml (Blomback *et al.*, 1974).

α_2 M was prepared either by the methods of Harpel (1973) or Virca *et al.* (1978) and was generously provided by Dr. Winnie Wan of this University. After SDS/polyacrylamide-gel electrophoresis and staining with Coomassie Blue G-250, the protein appeared homogeneous.

Iodination of UK

UK (a mixture of S1, the 33 000- M_r and S2, the 53 000- M_r form) was iodinated by using the solid-phase chloramide 1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycoluril (Fraker & Speck, 1971); 100 μ g of protein was iodinated in each case. Free ¹²⁵I was removed from the reaction by absorption of the ¹²⁵I-UK to a benzamidine-Sepharose column, the column being washed with 0.145 M-NaCl buffered with 6.25 mM-sodium borate, pH 8.2, and the active enzyme was eluted with distilled water. The enzymic activity of the iodinated urokinase was determined by measuring the rate of hydrolysis of the chromogenic substrate <Glu-Gly-Arg-pNA. The elution of ¹²⁵I-UK from benzamidine-Sepharose was qualitatively identical with that of unlabelled enzyme. Water alone eluted 13% of the initial enzyme activity. An additional 20% of enzyme activity was eluted with 0.1 M-glycine/HCl, pH 3.5, containing 0.5% Triton X-100. When analysed by electrophoresis the ¹²⁵I-UK was resolved as two proteins with apparent M_r 53 000 and 33 000 in non-reducing polyacrylamide gels, or 33 000 and 20 000 in the presence of reducing agents. At least 71% of the ¹²⁵I-UK retains enzymic activity (Fig. 5 below), and the specific radioactivities were approximately 10 μ Ci/mg.

Analysis of UK-inhibitor complexes by polyacrylamide-gel electrophoresis

UK was incubated with proteinase inhibitors or whole plasma at 37°C for various times. Fresh plasma was obtained from healthy volunteers by venipuncture, anticoagulated with 14 mM-EDTA or heparin (33 units/ml), and used immediately. The reaction between UK and the proteinase inhibitors was stopped by the addition of twice-concentrated electrophoresis sample buffer to produce final concentrations of 2.5% SDS. Reduced samples were treated with 2.5% SDS and 5% β -mercaptoethanol and heated for 30 min at 60°C.

Polyacrylamide slab gels were prepared and stained (Laemmli, 1970). A range of protein M_r standards (Pharmacia) was used in a reduced or non-reduced form. The α_2 M dimer (360 000, non-reduced) and monomer (180 000, reduced) were used as internal M_r standards. Autoradiographs were made by using Kodak SB-5 X-ray film.

Immunoprecipitation of α_2 M-¹²⁵I-UK complexes

Rabbit antisera to α_2 M were passed over a 400 μ l protein A-Sepharose column, washed with 1.2 ml of

phosphate-buffered saline (140 mM-NaCl/8.1 mM- Na_2HPO_4 /2.7 mM-KCl/1.5 mM- KH_2PO_4 , pH 7.25) and eluted with 1.2 ml of 3 M-potassium thiocyanate. The eluted IgG was dialysed twice against phosphate-buffered saline and the dialysis residue concentrated with Aquacide III to a final IgG concentration of 2.6 mg/ml. The titre of the anti- $\alpha_2\text{M}$ was determined by double immunodiffusion to be 4 mg/ml (Oudin, 1980). The IgG fraction of goat antisera to rabbit IgG was prepared by $(\text{NH}_4)_2\text{SO}_4$ precipitation and chromatography on DEAE-Sephacel. The titre of the goat anti-rabbit sera was 0.6 mg/ml. ^{125}I -UK was incubated at 37°C with EDTA-treated plasma containing 100 nM-Trasylol or with 250 μg of $\alpha_2\text{M}$ /ml. After 100 min a

5-fold excess of antibody to $\alpha_2\text{M}$ was added and the mixtures kept at 4°C for 48 h. One equivalent of goat anti-rabbit IgG was added, and samples were incubated at 4°C for an additional 24 h. The immunoprecipitates and supernatants were separated by centrifugation and both were dissolved in twice-concentrated electrophoresis sample buffer and incubated for 6 h at 22°C.

Results

We studied the interaction of UK with plasma rather than serum because the concentration of proteinase inhibitors is significantly lower in serum (Arnesen & Fagerhol, 1972), and we used a UK pre-

Fig. 1. Time course for the formation of SDS-resistant complexes between ^{125}I -UK and plasma components. ^{125}I -UK (5 μl) was incubated with 5 μl of fresh plasma anticoagulated with 14 mM-EDTA and 2 μl of phosphate-buffered saline (a) or plasma and phosphate-buffered saline containing 33 units of heparin/ml (b). The final concentration of UK was 708 Ploug units/ml, which correspond to a molar concentration of 0.08 μM . (assuming a catalytic activity of 200 000 Ploug units per mg of an equal mixture of 33 000- and 53 000- M_r enzyme). After 5, 15, 45, 90 and 180 min, samples were treated with 2 $\times$ -concentrated electrophoresis sample buffer to produce final concentrations of 2.5% SDS and 5% β -mercaptoethanol, heated to 60°C for 30 min, and electrophoresed on an SDS/4–12.5% polyacrylamide gel. The gel was stained, dried and exposed for 30 h to Kodak SB-5 film.

paration of the type employed in therapy that contained both 33 000- M_r and 53 000- M_r components.

Formation of complexes between ^{125}I -UK and plasma components

A small volume of solution containing ^{125}I -UK was added to plasma and, after incubation for the desired time period, the products were analysed by SDS/polyacrylamide-gel electrophoresis and autoradiography. Fig. 1(a) shows the result of a typical experiment in which samples of a reaction mixture were analysed under reducing conditions after different periods of incubation. The principal radioactive bands, which appeared slowly and progressively during the 3 h of incubation, correspond to apparent M_r 430 000, 300 000, 170 000 and 145 000, of which the bands of M_r 300 000 and 170 000 are the most prominent. In reduced gels such as these, uncomplexed ^{125}I -UK is resolved into two bands of M_r 33 000 and 20 000. There is, in addition, a small amount of label diffusely distributed in the zone between 20 000 and 30 000 M_r ; this is probably due to proteolytic degradation occurring in plasma, since it was present neither in the starting material nor in reactions with purified inhibitors. After autoradiography the lanes were sliced and assayed for radioactivity to obtain a more quantitative estimate of the distribution of complexed enzyme; after 180 min of incubation with

Table 1. Time course for the formation of ^{125}I -UK- $\alpha_2\text{M}$ complexes

^{125}I -UK (708 Ploug units/ml; $0.082\ \mu\text{M}$) was incubated at 37°C with EDTA-treated plasma diluted to 42% (v/v) with phosphate-buffered saline, pH 7.25 (see the text for composition) or, at a concentration of 850 Ploug units/ml ($0.1\ \mu\text{M}$), with phosphate-buffered saline containing 0.65 mg of $\alpha_2\text{M}$ /ml ($0.9\ \mu\text{M}$) for the times as shown. Samples at each time point were treated with electrophoresis sample buffer (2.5% SDS/5% (v/v) β -mercaptoethanol), heated to 60°C for 30 min and electrophoresed on an SDS/4–12.5%-polyacrylamide gradient slab gel. On the basis of an autoradiograph, the regions of the polyacrylamide gel containing the $\alpha_2\text{M}$ - ^{125}I -UK complexes and native 33 000- M_r ^{125}I -UK were cut out and counted for radioactivity in a gamma counter. The total radioactivity in each lane was $30\,000 \pm 3000$ (S.E.M.) c.p.m. The percentage of the 33 000- M_r ^{125}I -UK subunit complexed to $\alpha_2\text{M}$ in each case was determined.

Incubation time (min) ...	UK complexed to $\alpha_2\text{M}$ (%)				
	5	15	45	90	180
Plasma	5	10	15	23	34
$\alpha_2\text{M}$	6	7	15	—	38

Fig. 2. Immunoprecipitation of $\alpha_2\text{M}$ - ^{125}I -UK complex by antisera against $\alpha_2\text{M}$

^{125}I -UK (2000 Ploug units/ml; $0.22\ \mu\text{M}$) was incubated with EDTA-treated plasma diluted to 66% (v/v) with phosphate-buffered saline or $\alpha_2\text{M}$ ($250\ \mu\text{g}/\text{ml}$; $0.34\ \mu\text{M}$) in a phosphate-buffered saline solution. The $\alpha_2\text{M}$ was immunoprecipitated as described in the Experimental section. Immunoprecipitates and respective supernatants were electrophoresed on an SDS/4–12.5%-polyacrylamide gradient gel under non-reducing conditions; the dried gel was developed for autoradiography. Lane 1, the immunoprecipitate of ^{125}I -UK and plasma; lane 2, the immunoprecipitate of ^{125}I -UK and $\alpha_2\text{M}$; lane 3, the supernatant of the ^{125}I -UK and plasma reaction; lane 4, the supernatant of the ^{125}I -UK and $\alpha_2\text{M}$ reaction.

plasma, 43% of the ^{125}I associated with 33 000- M_r chain of UK was present in high-molecular-weight form, and four-fifths of this material was incorporated into the four species in the range 170 000–430 000.

When heparin was present in the reaction mixture, an additional complex of apparent M_r 80 000 was seen (Fig. 1b). Under these conditions the fraction of bound ^{125}I –33 000- M_r -UK rose to 50% of the total, three-fifths of which was in the 80 000- M_r form, the remainder being in the 145 000–430 000- M_r region.

We presumptively identified the predominant complexes seen in Fig. 1 as those between ^{125}I -UK and $\alpha_2\text{M}$ and ATIII, that were experimentally verified (see below). Additionally, we found that both high- and low- M_r forms complexes with $\alpha_1\text{PI}$ (Clemensen & Christensen, 1976), $\alpha_2\text{AP}$ (Moroi & Aoki, 1976) and C1-esterase inhibitor, although these complexes are not seen when ^{125}I -UK is incubated with plasma.

Formation of complexes between UK and $\alpha_2\text{M}$

By using an approach different from that used by Ogston *et al.* (1973) and Murano *et al.* (1980) we have investigated in more detail the pattern of

complexes formed between ^{125}I -UK and $\alpha_2\text{M}$ and the rate of complex-formation.

When the ^{125}I -UK was incubated with physiological concentrations of purified $\alpha_2\text{M}$ and the complexes that formed were reduced and analysed by SDS/polyacrylamide-gel electrophoresis, complexes with apparent M_r 430 000, 300 000 and 170 000 were found, identical with those found in plasma and shown in Fig. 1. The rate of formation of these complexes was also similar under the two reactions (Table 1). The faint radioactive band at M_r = 145 000 that appeared in the reaction of ^{125}I -UK with plasma was not present in reactions with purified $\alpha_2\text{M}$. We compared the products formed in a reaction mixture containing only highly purified inhibitor and ^{125}I -UK with those formed in plasma under comparable conditions. After the completion of incubation, free and complexed $\alpha_2\text{M}$ were immunoprecipitated and the reaction mixtures analysed by SDS/polyacrylamide-gel electrophoresis in a non-reducing gel. The results can be seen in Fig. 2: (i) in both reaction mixtures (containing either plasma or purified α_2 -macroglobulin) and immunoprecipitate contained most of the high M_r radioactive complexes and little or no free ^{125}I -UK; (ii) both reactions gave essentially

Fig. 3. Quantification of complex-formation between ^{125}I -UK and ATIII

(a) ^{125}I -UK (566 Ploug units/ml; $0.066\mu\text{M}$) was incubated at 37°C with ATIII (0.63mg/ml , $9.6\mu\text{M}$) in a phosphate-buffered saline solution with (●—●) or without (●—●) 33 units of added heparin/ml. (b) ^{125}I -UK (708 Ploug units/ml; $0.082\mu\text{M}$) was incubated at 37°C with a solution of EDTA-treated plasma that had been diluted to 42% (v/v) with phosphate-buffered saline in the presence (●—●) or absence (●—●) of 42 units of heparin/ml. At the times shown, portions of these reaction mixtures were treated with sample buffer (final concns. 2.5% SDS, 5% β -mercaptoethanol), heated at 60°C for 30 min and electrophoresed on a SDS/4–12.5%-polyacrylamide slab gel. The gel was stained, dried, and an autoradiograph was developed. On the basis of the autoradiograph, the regions of the acrylamide gel containing the ^{125}I -UK–ATIII complex and the region of the acrylamide gel containing the 33 000- M_r ^{125}I -UK that had not reacted were cut out from each lane and counted for radioactivity in a gamma counter. The total radioactivities in each lane were $19\,500 \pm 1500$ (s.e.m.) (a) and $49\,000 \pm 4000$ (s.e.m.) (b) c.p.m. For each time point, the percentage of the 33 000- M_r ^{125}I -UK bound to ATIII was determined.

identical patterns with respect to α_2 M complexes: the four bands that were present in the plasma sample also appeared in the reaction with the purified inhibitor (i.e. corresponding to M_r 380 000, 440 000, 630 000 and 720 000); (iii) all α_2 M- 125 I-UK complexes are appreciably larger than the 360 000- M_r α_2 M.

Formation of complexes 125 I-UK and ATIII

The finding in plasma of 125 I-UK complexes with ATIII (Murano *et al.*, 1980) suggested that this inhibitor might contribute to the inhibition of urokinase *in vivo* and promoted us to investigate the interaction in a more quantitative manner. We confirmed Clemmensen's (1978) observation that ATIII inhibited UK and, since its inhibitory potency is greatly enhanced by heparin, we established the optimum levels of heparin required for a particular

concentration of ATIII. This was accomplished by using heparin over the range 0.08–333 units/ml, for ATIII and 125 I-UK concentrations of $9.5 \mu\text{M}$ and $0.080 \mu\text{M}$ respectively. The fraction of UK bound to inhibitor after 45 min incubation at 37°C was determined as in the legend to Fig. 3. The fraction of UK bound to ATIII was linearly related to the logarithm of heparin in concentrations over the range of 1.3–21 units/ml, and reached a plateau at higher concentrations of heparin. The heparin-dependence and time course of the reactions with purified ATIII and plasma were monitored by measuring the formation of complexes using SDS/polyacrylamide-gel electrophoresis, and the results are given in Fig. 3. Extrapolation of the 'heparin-stimulated' curve in Fig. 3(a) gave a value of 75% for the 125 I-UK bound at infinite time. Heparin increased the initial rate of complex-formation between purified ATIII and UK

Fig. 4. Inhibition of the amidolytic activity of UK by ATIII

UK (180 Ploug units/ml; $0.02 \mu\text{M}$) in 0.1 M-Tris buffer, pH 8.0, with 0.5 mg of bovine serum albumin and 100 units of heparin/ml added, was incubated at 37°C with ATIII at 0 mg/ml (—○—), 0.19 mg/ml ($2.9 \mu\text{M}$) (—●—), 0.38 mg/ml ($5.8 \mu\text{M}$) (—●—) or 0.57 mg/ml ($8.8 \mu\text{M}$) (—●—). After the reaction mixtures at 37°C had been incubated for various times, the amidolytic activity of each reaction mixture was determined by removing a $20 \mu\text{l}$ portion, diluting it to 1 ml in 0.1 M-Tris buffer, pH 8.0, containing 0.11 mM ($1.2 \times K_m$) of the substrate <Glu-Gly-Arg-pNA, and recording the rate of change in the A_{405} over a period of 3 min; 100% amidolytic activity = 0.0097 A/min , the mean of the initial measurements of activity. The inset shows the amidolytic activities from the three samples containing ATIII replotted on a semilogarithmic basis.

approx. 100-fold. An apparent first-order rate constant of $3.7 \times 10^{-4} \text{ s}^{-1}$ was derived from the initial rate of ^{125}I -UK-ATIII complex-formation in the presence of heparin. A qualitatively similar pattern was observed when ^{125}I -UK was allowed to react with plasma. In the presence of heparin the rate of complex-formation slowed progressively over 3 h, at which time 30% of the total UK was bound to ATIII and 50% remained unbound. Thus, in comparison with free inhibitor, the reaction with ATIII in plasma is both slower, and is arrested at a lower level of complex-formation, for reasons that have not yet been defined.

Inhibition of amidolytic activity of UK by purified ATIII and by plasma

The inhibitory effect of ATIII and of plasma on the amidolytic activity of UK was assessed by measuring the rate of hydrolysis of the chromogenic peptide substrate <Glu-Gly-Arg-pNA. As shown by the data in Fig. 4, ATIII, in the presence of heparin, inhibited UK progressively as a function of inhibitor concentration and time. The loss of amidolytic activity occurred with pseudo-first-order kinetics and proceeded to essentially complete inhibition. An apparent first-order rate constant of

$6.8 \times 10^{-4} \text{ s}^{-1}$ was determined for the highest concentration of inhibitor. An analysis of the apparent first-order rate constants yielded a second-order rate constant of $93 \text{ M}^{-1} \cdot \text{s}^{-1}$, a value of 10^5 -fold lower than the rate constant for the inhibition of thrombin by ATIII in the presence of heparin (Jordan *et al.*, 1980). The inhibitory effect of heparinized plasma (Fig. 5) qualitatively resembles the results obtained in the heparin-stimulated reaction with purified ATIII. After prolonged incubation with heparinized plasma, 20% of the original UK activity remained. This residual level may be due to enzyme that is complexed with $\alpha_2\text{M}$, since the intact active sites of such enzymes are known to be accessible to low- M_r substrates while excluding macromolecular inhibitors such as ATIII. In the absence of heparin, plasma inhibits UK only very weakly (Fig. 5b). It is noted that the amidolytic activity of the UK solution incubated at 37°C increased with time in the absence of plasma or proteinase inhibitors, and we are unable to account for this small but reproducible effect. Control experiments excluded the possibilities that conversion of 53 000- M_r urokinase to 33 000- M_r UK or conversion of a pro-enzyme into an active form during the incubation at 37°C were responsible for the observed increase in amidolytic activity.

Fig. 5. *Inhibition of amidolytic activity of urokinase by plasma*

UK (160 Ploug units/ml; $0.02 \mu\text{M}$) was added to whole plasma (---●---), plasma diluted to 50% with phosphate-buffered saline (---●---), or 0.1M-Tris/10mM-NaCl, pH 8.0 + $200 \mu\text{g}$ of bovine serum albumin/ml (—●—). (a) Reaction mixtures contained 100 units of heparin/ml. (b) No added heparin. After various times, $20 \mu\text{l}$ portions were removed and their initial amidolytic activity measured as described in Fig. 4; 100% amidolytic activity = 0.0099 A/min , the mean of the initial measurements of activity.

Discussion

Whereas previous reports have documented the ability of several plasma proteinase inhibitors to interact with human urinary UK (Ogston *et al.*, 1973; Clemmensen & Christensen, 1976; Moroi & Aoki, 1976; Clemmensen, 1978; Murano *et al.*, 1980), we have here presented a detailed study of the formation of complexes between UK and proteins in whole plasma. With the reservation that our procedures would have detected only SDS-resistant complexes, UK appears to form only two major kinds of covalent interactions with human plasma.

One of the two main classes of complexes produced by UK-plasma interaction is that formed with α_2 M. This complex appeared slowly and reached a maximum, under our conditions, after about 3 h of incubation; in the absence of heparin it represented the predominant UK complex in plasma. Two aspects of the α_2 M-UK complex are noteworthy: firstly, its relatively slow rate of formation indicates that it is unlikely to play a major role in the rapid elimination of injected UK from the circulation; secondly, the multiplicity of UK- α_2 M complexes and their apparent M_r values are not easily rationalized by current concepts of α_2 M structure.

The general molecular structure of α_2 M has been defined by the work of several laboratories (Blomback *et al.*, 1974; Jones *et al.*, 1972; Roberts *et al.*, 1974): the intact molecule ($M_r = 720\,000$) is known to consist of four identical polypeptide chains (M_r 180 000), which are linked in pairs by disulphide bonds, with two such dimer pairs (M_r 360 000) associating by non-covalent interactions to form the native tetramer. The stoichiometry of proteinase- α_2 M interactions has also been defined for a number of enzymes and has always been reported to be in the range of 1-2 proteinase molecules inhibited per α_2 M tetramer (M_r 720 000). With this background, and with the M_r of UK in mind, the interaction of α_2 M with UK should yield complexes on SDS/polyacrylamide-gel electrophoresis of M_r not exceeding approx. 215 000 (180 000 + 33 000) under reducing conditions.

Yet, as Figs. 1(a) and 1(b) show, at least two much larger components (at M_r approx. 300 000 and 430 000), which necessarily consist of more than a single α_2 M monomer associated with a single UK molecule, were observed in plasma samples. These complexes persisted even when exposed to additional rigorous denaturing agents such as urea and acid pH (results not shown). Similar results are obtained when non-reducing gels were used. Four UK- α_2 M complexes, all larger than the 360 000- M_r dimer, are observed in non-reducing gels after incubating 125 I-UK with plasma or purified α_2 M (Fig. 2). After Coomassie Blue staining only the

360 000- M_r dimer could be seen in the lane containing the purified inhibitor. The addition of one or two bound UK molecules to the 360 000- M_r dimer might have generated a family of complexes within the range 390 000-465 000. The 630 000- and 720 000- M_r complexes must represent an as-yet-undescribed molecular arrangement of α_2 M subunits. One possible mechanism that could account for this phenomenon may be the formation of intramolecular cross-links linking subunits within the α_2 M tetramer accompanying the covalent binding of a proteinase.

The multiplicity of excessively-high- M_r bands is not limited to the UK- α_2 M interaction; qualitatively similar patterns have been observed with complexes of trypsin, plasmin and elastase (W. Wan & E. Reich, unpublished work).

The second major class of UK complexes formed in plasma involved ATIII. We found that the first-order rate constant for the inhibition of UK by ATIII was approximately twice the rate constant for complex-formation, suggesting that the mechanism of inhibition of urokinase may occur via a non-covalent complex that is enzymically inactive and is dissociated by SDS. Although the capacity of ATIII, given the presence of heparin, to bind UK and inhibit its catalytic activity is established, its physiological significance is open to doubt because the heparin-stimulated reaction is slow relative to the disappearance rate of infused UK *in vivo*, and the heparin concentrations required to maintain even this rate of UK binding do not exist in the plasma of untreated individuals. It follows that none of the UK complexes whose formation we have observed in plasma can be important contributors, either singly or together, to the clearance of UK from plasma *in vivo*. This conclusion can be stated even more emphatically for other inhibitors, notably α_1 PI, C1 esterase inhibitor, and α_2 AP, which, although normal constituents of plasma and able to react with UK, apparently bind the enzyme with an affinity below that required for the formation of detectable levels of complexes at their circulating concentrations.

A previous report by Murano and co-workers has reached similar conclusions by different methods (Murano *et al.*, 1980). In their work, UK activity was assayed by measuring the lysis time of a clot formed from UK, plasminogen, thrombin and fibrinogen.

In addition, those authors demonstrated the formation of complexes between high- M_r 125 I-UK and proteinase inhibitors in plasma by gel filtration, a technique that did not allow the resolution of the multiple complexes formed between UK and α_2 M. Complexes formed in plasma between 125 I-UK and α_2 M, ATIII and α_1 PI were identified by immunoprecipitation, using antibodies directed against the

respective inhibitors. The formation of complexes between ATIII and high- and low- M_r UK was monitored by SDS/polyacrylamide-gel electrophoresis. In contrast with the work of Murano *et al.* (1980), we have measured the inhibition of UK activity directly by using chromogenic substrates. Our work confirms their findings and provides new data about the kinetics of inhibition of UK by ATIII and the rate of complex-formation between UK proteinase inhibitors in whole plasma. Our methods resolved the multiple complexes which formed between UK and α_2M , results not reported by Murano *et al.* (1980). Taken together, all of these observations, our results and the fact that plasma only sluggishly inhibits UK, suggest that specific inhibitors of UK, if they exist in plasma, are present either at very low concentrations or as inactive precursor forms. The mechanism by which UK is normally and rapidly inactivated or removed from the circulation remains to be established.

We thank Dr. Dominique Belin and Dr. Jean Dominique Vassalli for their careful reading of the manuscript, and Miss Donna Anderson for assistance in the purification of ATIII.

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