Biochimica et Biophysica Acta, 429 (1976) 591—599

BBA 67748

INHIBITION OF UROKINASE BY COMPLEX FORMATION WITH HUMAN α_1-ANTITRYPSIN

INGE CLEMMENSEN and FINN CHRISTENSEN
Department of Clinical Chemistry, Hvidovre Hospital, DK-2650 Hvidovre (Denmark)
(Received September 16th, 1975)

Summary

Human α_1-antitrypsin was prepared from fresh human plasma by $(NH_4)_2SO_4$-precipitation, gel filtration, affinity chromatography on concanavalin A, ion exchange chromatography and isotachophoresis. Human urokinase (EC 3.4.99.26) (plasminogen activator from urine) with M_r 46 000 and 36 000 was further purified from Urokinase Leo reagent preparation by gel filtration on Sephadex G-100 Superfine. The hydrolytic activity of urokinase on acetyl-glycyl-L-lysine methyl ester acetate (Ac-Gly-Lys-OMeAc) was inhibited in a strong time-dependent manner by α_1-antitrypsin. Complex formation between enzyme and inhibitor could be demonstrated in crossed immunoelectrophoresis against anti-α_1-antitrypsin and anti-urokinase serum as well as by sodium dodecyl sulphate polyacrylamide gel electrophoresis. The latter method revealed the formation of 1 : 1 and 2 : 1 molar enzyme-inhibitor complexes.

Introduction

The fibrinolytic system plays an important role in the removal of fibrin deposits in the organism. It is activated by activators present or generated in tissues [1—3], blood [4], urine [5] and other body fluids. Activators are probably highly specific serine proteinases [6]; they convert the plasma proenzyme, plasminogen, to the unspecific serine-histidine proteinase, plasmin [7] (EC 3.4.21.7), which catalyses the hydrolysis of fibrin. Activation of plasminogen as well as the effect of plasmin is regulated by inhibitors in tissues, blood and in extravascular body fluids [8]. Among these, the plasma protein, α_2-macroglobulin, appears to be of great physiological importance [9,10]. Inhibitors of plasminogen activation are also present in tissues and in blood, and activator inhibitors have been purified from placenta [11,12] and plasma [13,14]. Recently we have demonstrated that the polyvalent proteinase inhibitor, α_1-antitrypsin, is an inhibitor of the urine activator, urokinase (EC 3.4.99.26) [15].

The object of this paper is to present a study on the mechanism of action of purified human α_1-antitrypsin on purified human urokinase by using different electrophoretic and kinetic methods.

Materials

Buffers. Tris: 0.05 M Tris · HCl, 0.10 M NaCl (pH 7.70 (20°C), I = 0.15 mol/kg). Tris-gelatin: gelatin, mass conc. = 2.5 g/l dissolved in Tris buffer. Ammonium sulphate p.a. (Merck, Darmstadt, W. Germany). Sephadex G-100 Superfine (Pharmacia, Uppsala, Sweden). Acrylamide (Ortec Inc. Eastman Kodac, Rochester, N.Y.), enzyme grade and N,N^1-methylenebisacrylamide (Eastman Kodac). Ammonium persulphate (Merck). Monosodium riboflavine-5^1-phosphate, dihydrate (B.D.H. Poole, England). Tetra methylenediamine (Merck). Sodium dodecyl sulphate (Hopkins and Williams LTD, London, England). Agarose, Indubiose A45 (L'Industrie Biologique, Genevilliers, France). M-Partigen, α_1-antitrypsin (Behringwerke, Marburg Lahn, W. Germany). Specific rabbit immune globulins against human serum and human α_1-antitrypsin, dissolved in 0.1 M NaCl, 0.015 M NaN_3 (Dakopatts, Copenhagen, Denmark). Specific rabbit immune serum against urokinase was prepared by immunization (performed by Dakopatts) with urokinase M_r = 36 000 (UK II), which was purified by gel filtration from Leo Urokinase reagent preparation (see "Methods"). Acetyl-glycyl-L-lysine methyl ester acetate (Ac-Gly-Lys-OMeAc) (Sigma, Chemical Company, Saint Louis, Montana), 10 mM in 0.15 M KCl. α-*N*-Benzoylarginine ethylester (Bz-Arg-OEt) (Sigma, Chemical Company), 10 mM in 0.15 M KCl with 5 mM Tris · HCl adjusted to pH 7.8 with 0.10 M KOH. Urokinase (Leo Pharmaceuticals), reagent grade, containing 9.220 Ploug units/mg. 1 Ploug unit equals 1.31 CTA units [16]. Assuming a M_r of 54 000 and a specific activity of 104 000 CTA units/mg protein for an almost pure preparation [5,17], then 1 mol of urokinase is $5.5 \cdot 10^{12}$ CTA units or $4.2 \cdot 10^{12}$ Ploug units. Trypsin, crystalline, Trypure (Novo, Copenhagen, Denmark). The stock solution, 1 g/l, was prepared in a solution containing 2 mM HCl, 20 mM $CaCl_2$ and 6.8 M Glycerol. The active site concentration was determined to 31.5 μM by titration with *p*-nitrophenyl-*p*-guanidinobenzoate as previously described [18].

Methods

Crossed immuno-electrophoresis was performed in an agarose gel with barbiturate buffer [19]. Disc electrophoresis was done as described by Fønss-Beck [20]. Sodium dodecyl sulphate/polyacrylamide gel electrophoresis was performed as described by McDonagh et al. [21], except that the gel contained acrylamide, 100 g/l and methylenebisacrylamide, 1.35 g/l. A Quickfit PAGE instrument was used. Molecular weights were estimated by using crystalline gamma globulin (M_r = 150 000) (Kabi, Stockholm, Sweden), phosphorylase A (M_r, 94 000) (Boehringer, Mannheim, G.F.R.), transferrin (M_r 74 000) (Novo), bovine albumin (M_r, 69 000) (Armour Pharmaceuticals Company, Eastbourne, England) and lysozyme from egg white (M_r, 14 000) (Sigma) as reference markers. The catalytic concentration of urokinase was determined on Ac-Gly-

Lys-OMeAc as substrate, by means of pH titration (Titrator TTTIC and Titrigraph, Radiometer, Copenhagen) [22]. The concentration of α_1-antitrypsin in μM was determined immunologically by means of the Mancini Technique [23] on M-Partigen plates using Protein-Standard-Plasma (Behringwerke) as reference and M_r = 61 000. It was also determined by the absorbance at 280 nm using $E^{1\,\%\,w/v}_{1\,cm}$ = 6.88 [24] and M_r = 61 000. Furthermore, it was estimated by titration with trypsin. Mixtures containing trypsin (active site), 1.24 nmol and serial dilutions of α_1-antitrypsin in Tris were incubated at 37°C for 10 min and residual trypsin activity was estimated as described [25]. The catalytic concentration of trypsin was a linear function of the α_1-antitrypsin concentration.

Purification and characterization of urokinase. Urokinase Leo reagent preparation, 0.128 μmol in 2 ml 0.05 M Tris · HCl, 0.60 M NaCl (pH 7.70 (20°C), I = 0.65 mol/kg) was applied on a Sephadex G-100 Superfine column (Pharmacia K 16/70, 0.70 × 200 mm) equilibrated and subsequently eluted with the same buffer. The bed height was 0.64 m, the total volume was 127 ml and the void volume, V_o, estimated by Blue Dextran 2000 (Pharmacia) was 50 ml. A constant upstream flow with a flow rate of 3.5 ml/h was obtained by means of a peristaltic pump (LKB, Sweden). Fractions were collected at intervals of 20 min and eluted protein was measured as absorbance at 280 nm. Protein fractions were indicated by their partition coefficient (K_{AV}) = (V_e − V_o)/(V_t − V_o) where V_e was the elution volume of protein fractions, V_o was the void volume and V_t was the total volume of the gel bed. The active enzyme was eluted in two peaks (Fig. 1) with K_{AV} values of 0.27 and 0.39. The peak fraction eluted with K_{AV} = 0.27, designated UK I, had a M_r of around 53 000, when estimated from K_{AV} value [26]. It showed two bands on disc electrophoresis (not shown) and on sodium dodecyl sulphate/polyacrylamide gel electrophoresis (Fig. 3) three bands with M_r = 60 000, 46 000 and 36 000. The catalytic concentration of urokinase was estimated to 6.30 μM, using Leo reagent preparation of urokinase as reference. The peak fraction eluted with K_{AV} = 0.39, designated UK II, had a M_r of around 31 000 when estimated from the K_{AV} value. It showed only one protein on disc electrophoresis (not shown) and on sodium dodecyl sulphate/polyacrylamide gel electrophoresis one band with an M_r of 36 000 (Fig. 4). The catalytic concentration of urokinase was estimated to 7.5 μM. Examination of all eluted fractions on plasminogen-free fibrin plates [27] gave no lysis of fibrin.

Purification of α_1-antitrypsin was prepared from fresh human plasma by $(NH_4)_2SO_4$-precipitation, gel filtration on Sephadex G-200, affinity chromatography on concanavalin A, ion exchange chromatography and isotachophoresis as previously described [15]. The all-over yield of α_1-antitrypsin was 0.2 and crossed immunoelectrophoresis against immuno globulin against human serum proteins, showed only traces of one or two other proteins. An M_r of 61 000 was found by sodium dodecyl sulphate/polyacrylamide gel electrophoresis. The concentration (a) of α_1-antitrypsin in the stock solution was 400 μM, when estimated by the Mancini technique. The concentration (b) was 420 μM, when determined by absorbance at 280 nm. The concentration (c) was 260 μM, when determined by titration with trypsin, assuming that trypsin and α_1-antitrypsin form 1 : 1 molar complex [28]. The ratios b/a and c/a were 1.05 and

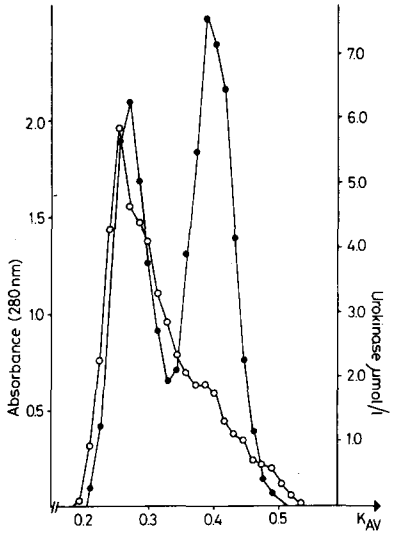

Fig. 1. Gel filtration by Sephadex G-100 Superfine of Leo Urokinase reagent preparation. Material: 0.128 µmol Leo Urokinase dissolved in 2 ml Tris. The column (1.6 × 70 cm, bed height 64 cm) was equilibrated and eluted with the same buffer at 10° C. Fractions of 1.2 ml were collected at 20-min intervals. The protein concentration was estimated as the absorbance at 280 nm and a light path of 1 cm (○). The catalytic amount of eluted urokinase (●) was estimated with Ac-Gly-Lys-OMeAc as substrate by means of pH titration. Maximum of activity occurred with partition coefficient (K_{AV}) 0.27 and 0.39 called UK I and UK II, respectively.

0.65 respectively. α_1-Antitrypsin concentrations used in experiments are based on active inhibitory site titration.

Results

Inactivation of urokinase by α_1-antitrypsin. Purified α_1-antitrypsin 130 µM and urokinase 1.2 µM were incubated at 37°C. At different time intervals 0.12 nmol urokinase and 13 nmol α_1-antitrypsin were withdrawn and the hydrolytic activity of urokinase was estimated on Ac-Gly-Lys-OMeAc. The hydrolytic activity of urokinase decreased linearly with time in the first 1.5 h of incubation with α_1-antitrypsin and after 5 h only 0.16 of the initial activity was present. Urokinase incubated with buffer retained its hydrolytic activity. The same pattern of results was obtained with UK I and UK II (3 experiments with Leo Urokinase reagent preparation and 4 experiments with UK I and UK II).

Demonstration of the existence of a complex between urokinase and α_1-antitrypsin, by crossed immunoelectrophoresis against anti-human-α_1-antitrypsin and antihuman-urokinase. Crossed immunoelectrophoresis of α_1-antitrypsin against anti-α_1-antitrypsin revealed one precipitate in the serum α_1-globulin region. After incubation for 2 h at 37°C with Leo Urokinase reagent preparation, two additional immunoprecipitates appeared in the serum β_1- and α_2-globulin region (Fig. 2A). Incubation of UK I with α_1-antitrypsin for 2 h entailed the same pattern in the β_1- and α_2-globulin region as observed with the Leo reagent preparation; however the α_2-globulin precipitate was relatively small (Fig. 2C). Incubation of UK II with α_1-antitrypsin resulted in only one

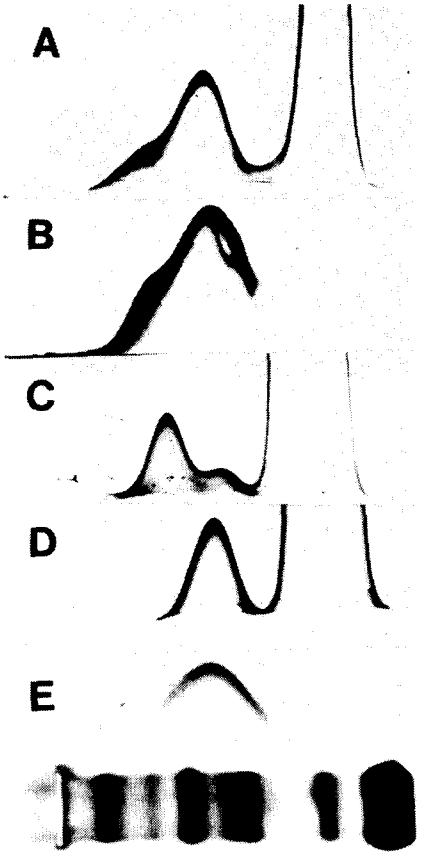

Fig. 2. The pattern of immunoprecipitates obtained by a crossed immunoelectrophoresis at pH 8.6 of a mixture of human α_1-antitrypsin and urokinase after incubation for 2 h at 37°C. The second dimension electrophoresis (anode at top) was run into agarose gel containing immunoglobulins against α_1-antitrypsin (volume fraction in gel 0.04 (A), 0.015 (C and D)) and immunoserum against urokinase (volume fraction in gel 0.08 (B and E)). The incubation mixture applied in the slit in the first dimension electrophoresis (anode to the right) contained 0.24 nmol Urokinase Leo reagent preparation and 0.85 nmol α_1-antitrypsin (A and B), 0.11 nmol UK I and 0.85 nmol α_1-antitrypsin (C) and 0.13 nmol UK II and 0.85 nmol α_1-antitrypsin (D and E). The bottom panel shows protein fractions obtained by a first dimension electrophoresis of normal plasma.

additional precipitate in the α_2-globulin region (Fig. 2D). The precipitates in the α_2- and β_1-globulin region were also recognized in crossed immunoelectrophoresis against anti-urokinase (Fig. 2B and 2E). In absence of inhibitor, urokinase precipitated in the γ-globulin region against anti-urokinase. Crossed immunoelectrophoresis of urokinase and crossed immunoelectrophoresis of α_1-antitrypsin did not precipitate with anti-α_1-antitrypsin and anti-urokinase respectively. Crossed immunoelectrophoresis of UK I against antibody raised in rabbit with UK I revealed two antigenic different precipitates, and only one of the precipitates changed electrophoretic mobility in crossed immunoelectrophoresis after addition of α_1-antitrypsin. The other one was regarded as an impurity without enzymatic activity.

Demonstration of complexes between urokinase and α_1-antitrypsin by sodi-

um dodecyl sulphate/polyacrylamide gel electrophoresis. Sodium dodecyl sulphate/polyacrylamide gel electrophoresis revealed that incubation for 5 h at 37°C of a solution of UK I containing molar excess of α_1-antitrypsin (M_r, 61 000) resulted in the appearance of 4 bands with M_r values 96 000 (not reproduced on the photograph), 115 000, 140 000 and 150 000 (Fig. 3C). This was associated with a disappearance or decrease in intensity of UK I protein bands with M_r = 36 000 and 46 000. The change in UK I band of M_r 60 000 was difficult to evaluate, because it coincided with the α_1-antitrypsin band (Fig. 3C). Incubation for 5 h at 37°C of a solution of UK II containing molar excess of α_1-antitrypsin resulted in the appearence of 2 bands with M_r values of 96 000 and 140 000 and in a decrease in intensity of the UK II band possessing a M_r of 36 000 (Fig. 4C). An identical pattern of results was obtained in 4 experiments

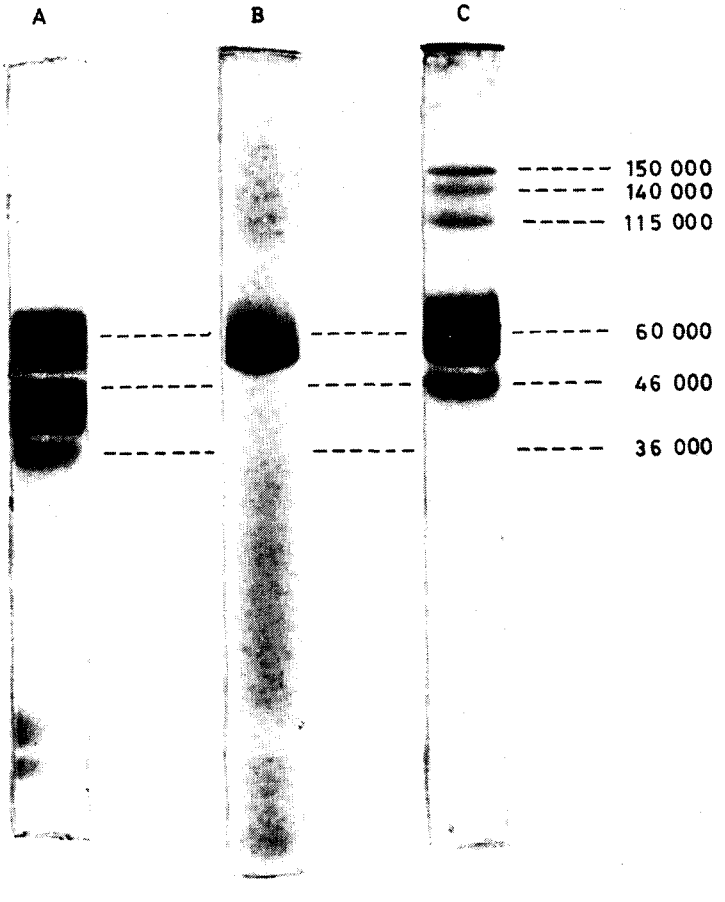

Fig. 3. The pattern of protein bands observed after sodium dodecyl sulphate/polyacrylamide gel electrophoresis in 10% w/v polyacrylamide of UK I, α_1-antitrypsin and a mixture of UK I and α_1-antitrypsin after incubation for 5 h at 37°C. A, 0.13 nmol UK I, B, 1.1 nmol α_1-antitrypsin and C: 0.13 nmol UK I and 1.1 nmol α_1-antitrypsin.

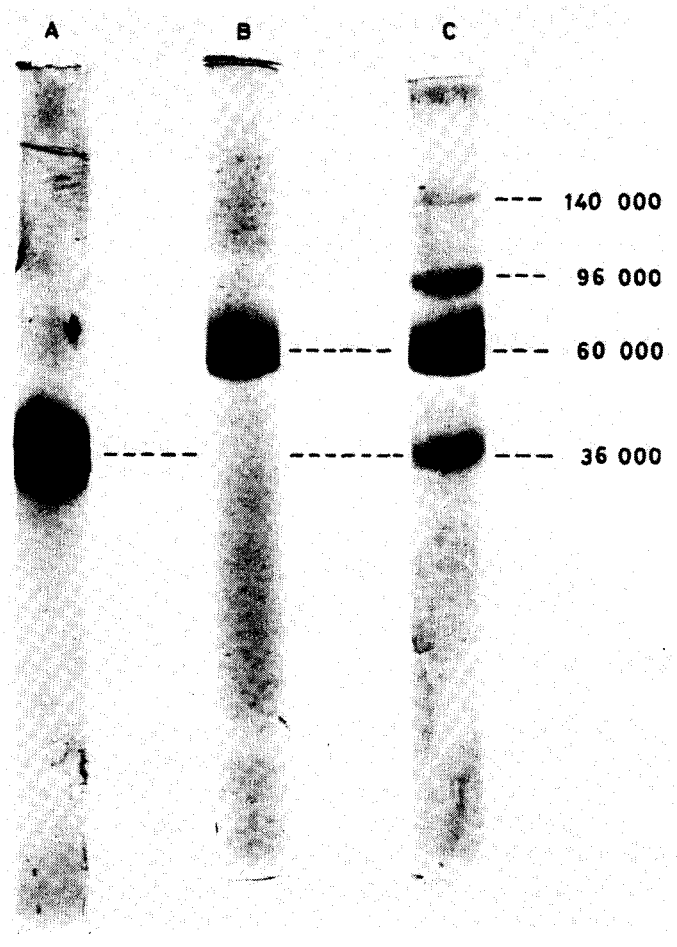

Fig. 4. The pattern of protein bands observed after sodium dodecyl sulphate/polyacrylamide gel electrophoresis in 10% w/v polyacrylamide of UK II, α_1-antitrypsin and a mixture of UK II and α_1-antitrypsin after incubation for 5 h at 37°C. A, 0.16 nmol UK II, B, 1.1 nmol α_1-antitrypsin and C, 0.16 nmol UK II and 1.1 nmol α_1-antitrypsin.

with Leo reagent preparation (not shown) and 5 experiments with UK I and UK II.

Discussion

The data obtained in the present study indicate that urokinase is inhibited by α_1-antitrypsin in a time-dependent manner as a result of the formation of enzyme-inhibitor complexes. Results obtained by sodium dodecyl sulphate gel electrophoresis (Figs. 3 and 4) as well as by crossed immunoelectrophoresis (Fig. 2) indicate that α_1-antitrypsin forms complexes with two molecular variants of urokinase. One variant with a M_r of 46 000 was prevailing in UK I (Fig. 3) and migrated as an β_1-globulin after complex formation with α_1-antitrypsin (Fig. 2C). The other variant with an M_r of 36 000 constituted UK II (Fig. 4)

and migrated as an α_2-globulin after complex formation with inhibitor (Fig. 2D and E). The impurity found in UK I by crossed immunoelectrophoresis of UK I against anti-UK I is probably the protein band of M_r 60 000, since the UK I band with an M_r of 36 000 disappeared and the UK I band with an M_r of 46 000 decreased in intensity upon incubation with α_1-antitrypsin (Fig. 3). The existence of two molecular species of urokinase in sodium dodecyl sulphate gel electrophoresis agrees with the Leo Urokinase reagent preparation separated into two activity peaks with K_{AV} values corresponding to an M_r of around 31 000 and 53 000. The presence of molecular weights as indicated above is consistent with results obtained by others [5,16].

α_1-Antitrypsin is known to form complexes with serine-proteinases. However, conflicting results exist as to whether one mol α_1-antitrypsin can bind one or two mol enzyme [24,28—30]. This may be due to differences in the source of enzyme and to difficulties in the determination of degree of denaturation of enzyme and inhibitor used in stoichiometric measurements. Active inhibitory sites titration by trypsin indicated that 0.65 of our α_1-antitrypsin was active, when it was assumed that enzyme and inhibitor form a 1 : 1 molar complex. If one mol α_1-antitrypsin binds 2 mol of trypsin, only 0.33 of our preparation was active. Partial denaturation of α_1-antitrypsin, did not influence the evaluation of the complex formation between urokinase and inhibitor in our experiments, because the inhibitor was present in great molar excess of urokinase (6 times or more).

The molecular weights in Figs. 3 and 4 suggest that α_1-antitrypsin may form 1 : 1 and 1 : 2 inhibitor-enzyme complexes. The values of the protein bands generated upon incubation of UK I and UK II with α_1-antitrypsin did not exclude the possibility that 2 mol α_1-antitrypsin bind 1 mol urokinase, but 2 inhibitory sites on α_1-antitrypsin has been reported [30] and there have been no reports so far on 1 mol enzyme binding 2 mol α_1-antitrypsin. The possible combinations of urokinase and α_1-antitrypsin are illustrated in Table I. The binding of urokinase to α_1-antitrypsin appears to involve the formation of covalent bonds, since the complexes resist exposure to sodium dodecyl sulphate.

Conflicting results exist on the inhibition of urokinase by α_1-antitrypsin [31—33]. This may be related to the use of too low concentration of α_1-antitrypsin and to the use of too short incubation time. Inhibition of urokinase-induced fibrinolysis by α_1-antitrypsin is very dependent of the molar concentration of the enzyme and the inhibitor in a fibrin clot lysis assay [15]. Inhibition

TABLE I

Possible molar combinations of α_1-antitrypsin (α_1-AT, M_r 61 000) and urokinase (UK) variants with M_r 46 000 and 36 000 in enzyme-inhibitor complexes generated in incubation mixtures of α_1-antitrypsin and UK I or UK II. The values are based on the results in Figs. 3 and 4.

Complex M_r	α_1-AT (M_r = 61 000)	UK (M_r = 46 000)	UK (M_r = 36 000)
96 000	1		1
115 000	1	1	
140 000	1	1	1
140 000	1		2
150 000	1	2	

of activator and the plasmin formed in this assay, has the character of an "all or nothing" reaction, as far as there was no lysis at all or total lysis in a few minutes, depending on the concentration of α_1-antitrypsin and the activator used in the assay [15].

The importance of α_1-antitrypsin in regulating fibrinolysis has to be proven. The protein belongs to the acute phase reactants and the concentration increases 2—3 times during infections, chronic diseases and pregnancy [34]. This may be important when considering the risk of thrombo-embolic episodes as complication to these conditions.

Acknowledgements

Mrs. Anne Preisler, Mrs. Ulla Magdal and Miss Lykke Lindemann are thanked for their excellent technical assistance. Dr. S. Thorsen is thanked for his advice concerning the manuscript. Dr. Jørgen Ploug, Leo Pharmaceuticals, Denmark, kindly supplied the human urokinase.

References

1 Bachmann, F., Fletcher, A.P., Alkaersig, N. and Sherry, S. (1964) Biochemistry 3, 1578—1585
2 Astrup, T. (1966), Fed. Proc. 25, 42—51
3 Bernik, M.B. and Kwaan, H.C. (1967) J. Lab. Clin. Med. 70, 650—661
4 Sawjer, W.D., Fletcher, A.P., Alkaersig, N. (1960), J. Clin. Invest. 39, 426—434
5 White, W.F., Barlow, G.H. and Mozen, M.M. (1966) Biochemistry 5, 2160—2169
6 Landmann, H. and Markwardt, F. (1970) Experientia 26, 145—147
7 Robbins, K.C., Bernabe, P., Arzadan, L. and Summaria, L. (1973) J. Biol. Chem. 248, 7242—7246
8 Fritz, H., Tschesche, H., Greene, L.J. and Truscheit, E. (1974) Proceedings of the 2nd International Research Conference, Springer Verlag, Berlin
9 Nilehn, J.E. and Ganrot, P.O. (1967) Scand, J. Clin. Lab. Invest. 20, 113—121
10 Müllertz, S. (1974), Biochem. J., 143, 273—283
11 Kawano, T., Morimoto, K. and Uemura, Y. (1970) Biochemistry 67, 333—342
12 Uszynsky, M. and Abildgaard, U. (1971) Thromb. Diathesis Haemorrh. 24, 580—589
13 Aoki, N. and van Kaulla, K.N. (1971) Am. J. Physiology 220, 1137—1145
14 Hedner, U. (1973) Thrombosis et Diathesis Haemorrhagica 30, 414—424
15 Clemmensen, I., Thorsen, S. and Müllertz, S. (1976) Submitted for publication
16 Kok, P. and Astrup, T. (1969) Biochemistry 8, 79—86
17 Lesuk, A., Terminello, L. and Traver, J.H. (1965) Science 147, 880—881
18 Chase, Jr., T. and Shaw, E. (1969) Biochemistry 8, 2212—2234
19 Ganrot, P.O. (1972) Scand. J. Clin. Lab. Invest. Suppl. 124, 39—41
20 Fønns-Beck, P. and Schmidt, K.D. (1966) Int. J. Protein Res. 1, 85—92
21 McDonagh, J., Messel, H., McDonagh, Jr., R.P. Murano, G. and Blomback, B. (1972) Biochim. Biophys. Acta 257, 135—142
22 Walton, P.L. (1967) Biochim. Biophys. Acta 132, 104—114
23 Mancini, G., Carbonara, P.O. and Heremans, J.F. (1965) Immunochemistry 2, 235—254
24 Johnson, D.A., Pannel, R.N. and Travis, J. (1974) Biochem. Biophys. Res. Commun. 57, 584—589
25 Worning, H. and Mullertz, S. (1966) Scand. J. Gastro. Ent. 1, 268—283
26 Andrews, P. (1964) Biochem. J. 91, 222—233
27 Brakman, P. and Astrup, T., inBang, N.U., Beller, F.K., Deutsch, E. and Mammen. E.F. (1971) Thrombosis and Bleeding Disorders, pp. 332—336, Academic Press, New York
28 Moroi, M. and Yamasaki, M. (1974) Biochim. Biophys. Acta 359, 130—141
29 Crawford, I.P. (1973) Arch. of Biochem. Biophys. 156, 215—222
30 Cohen, A.B. (1973) J. Biol. Chemistry 248, 7055—7059
31 Aoki, N. and Moroi, M. (1974) Proc. Soc. Exp. Biol. Med. 146, 567—570
32 Binder, B., Doleschel, W. and Auserwald, W., (1973) in Protides of Biological Fluids (Peeters, H., ed.) pp. 335—338, vol. 20, Pergamon Press
33 Crawford, G.P.M. and Ogston, D. (1974) Biochim. Biophys. Acta 354, 107—113
34 Ganrot, P.O. and Bjerre, B. (1967) Acta Obstet. Gynec. Scand. 46, 126—137