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Absence of Binding of Pancreatic and Urinary Kallikreins to α_2-Macroglobulin

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Pancreatic and urinary kallikreins failed to form the typical serine proteinase complex with α_2M (α_2-macroglobulin). Studies were performed to compare this with the binding of trypsin to α_2M at various molar binding ratios, with the use of Sephadex G-200 gel filtration to separate free and α_2M-bound enzyme fractions. The subunit conversion was totally absent with pancreatic kallikrein from which traces of a binding proteinase had been removed. The lack of binding is believed to be the result of the restricted specificity of the kallikreins.

Human α_2M* is a high-molecular-weight plasma protein which functions as an inhibitor of proteolytic enzymes [for reviews, see Bourrillon & Razafimahaleo (1972) and Barrett & Starkey (1973)]. Access to the active site is retained by low-molecular-weight substrates used to determine the activity of the complex-bound enzymes. Virtually all serine proteinases are believed to form an equimolar complex with α_2M (Gentou *et al.*, 1968; Barrett & Starkey, 1973; Hamberg *et al.*, 1973; Werb *et al.*, 1974). The binding of kallikreins (EC 3.4.21.8), which are characterized by a restricted proteolytic specificity on kininogen [for details, see Werle & Fiedler (1969)], has been less well studied. The rapid binding to α_2M of plasmin after activation of plasminogen in human plasma (Hamberg, 1969*a*,*b*) and of plasma kallikrein (Harpel, 1970) is believed to regulate plasma kinin release. According to the hypothesis of Barrett & Starkey (1973), the interaction with α_2M is initiated by a proteolytic attack in a sensitive region of the inhibitor, causing a conformational change and entrapment of the enzyme. The binding is recognized by the production of a specific subunit after reduction (Harpel, 1973). The present investigation deals with the comparison of the affinity for α_2M of some kallikreins with that of trypsin, in view of their different proteinase potentials. A preliminary report of part of this work has been published (Vahtera & Hamberg, 1975).

Experimental

Human α_2M was prepared by the method of Hamberg *et al.* (1973) with minor changes. Mixed blood-bank plasma (The Finnish Red Cross Blood Transfusion Service; 380ml) was precipitated with $(NH_4)_2SO_4$ (2M) and passed through a Sephadex G-200 column (7.8cm×82cm) at a rate of 40ml/h at 5°C (V_0 = 1 litre). The eluates corresponding to the ascending part and peak of the macroglobulin fraction were pooled (350ml), avoiding the inter-α-anti-trypsin fractions (Ganrot, 1966). After extensive dialysis (against water at 5°C), precipitated IgM was removed by centrifugation. The protein was collected by precipitation between 1.6 and 1.92M-$(NH_4)_2SO_4$, and dialysed. Final purification was performed by zone electrophoresis in polyvinyl chloride (block dimensions 18cm×35cm×0.5cm) with sodium diethylbarbiturate buffer, pH8.6, I = 0.1 mol/litre. Coloured (Bromophenol Blue) albumin was applied as tracer, and α_2M was located (as described by Hamberg *et al.*, 1973) relative to the mobility albumin/α_2M = 1.55 (16.5h at 400V, 80–90mA, 13°C; high-voltage-electrophoresis apparatus was from AB Analysteknik, Vallentuna, Sweden).

The binding activity determined from the ratio μg of trypsin/mg of protein (Hamberg *et al.*, 1973) was 0.026 showing that an equimolar complex was formed. To check that the α_2M was functionally intact during experiments, the trypsin binding was occasionally tested by incubating 200μg of α_2M with an excess of trypsin (15μg) for 1min at room temperature (25°C) (pH8.2, 0.1M-Tris/HCl, 0.15M-NaCl). Soya-bean trypsin inhibitor (10μg in 10μl of 2.5mM-HCl) was added to 0.5ml of the incubation mixture, and a 0.5ml sample of the incubation mixture (10μl of distilled water added) was used as a control. After 5min incubation the amidase activity was measured.

Rabbit (male) urinary kallikrein was a purified preparation from freshly collected (0°C) urine with added 0.005% sodium merthiolate. The enzyme was

* Abbreviations: α_2M, α_2-macroglobulin; IgM, immunoglobulin M; Tos-Arg-O[^{3}H]Me, αN-tosyl-L-arginine [^{3}H]methyl ester hydrochloride; KU, kallikrein units as defined by Frey *et al.* (1968), see also Kutzbach & Schmidt-Kastner (1972, 1973).

separated from urokinase by isoelectric focusing (Hamberg & Joutsimo, 1974) and collected at pI4.9 (pool range pH4.5–5.7). The kallikrein activity was 8KU/mg of protein. Pig pancreatic kallikrein preparations were highly purified preparations (Kutzbach & Schmidt-Kastner, 1972) (gifts from Bayer A.G., Werk Elberfeld, Germany) KZC 2/73 (1290KU/mg) and KZC 1/75 (1180KU/mg).

The activity of kallikreins was determined with Tos-Arg-O[^{3}H]Me (125mCi/mmol; Calatomic Inc., Los Angeles, CA, U.S.A.) as substrate, at pH8.2 (Beaven *et al.*, 1971). Liquid-scintillation counting of radioactivity was performed by using an LKB-Wallac 8100 counter and Insta-Gel (Packard Instrument Co., Downers Grove, IL, U.S.A.). Trypsin was determined with α-*N*-benzoyl-DL-arginine *p*-nitroanilide hydrochloride from Fluka A.G., Buchs, Switzerland, as substrate (Erlanger *et al.*, 1961).

Polyacrylamide-gel (5%) electrophoresis was performed by the method of Weber & Osborn (1969) with 20μg of immunologically pure α_2M. For studies of the subunit conversion as described by Harpel (1973), the samples were reduced with dithiothreitol (A grade; Calbiochem, Los Angeles, CA, U.S.A.) in urea (ultrapure; Schwarz/Mann, Orangeburg, NJ, U.S.A.) and sodium dodecyl sulphate (BDH Chemicals, Poole, Dorset, U.K.). Similarly treated α_2M, ovalbumin (Sigma Chemical Co., St. Louis, MO, U.S.A.; grade V) and transferrin (KABI, Stockholm, Sweden) served as molecular-weight markers.

Results

Fig. 1(*a*) illustrates a normal binding experiment using a 10min incubation time with 117μg of trypsin and 2mg of α_2M (2:1 molar ratio). After gel filtration

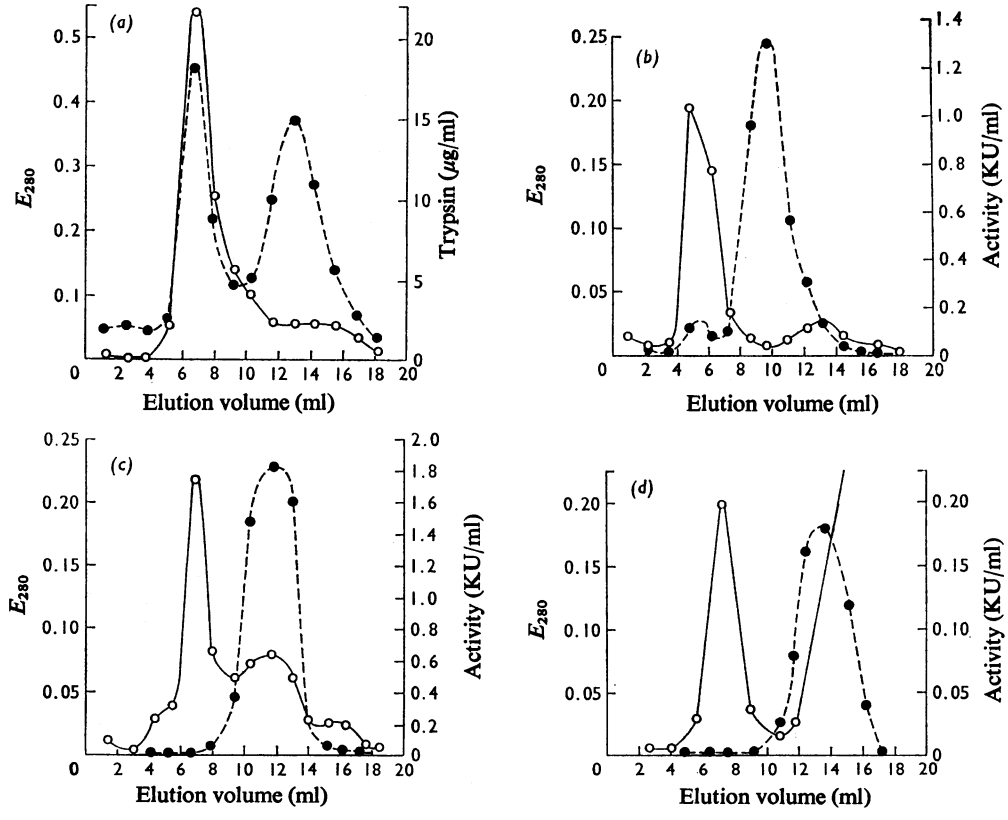
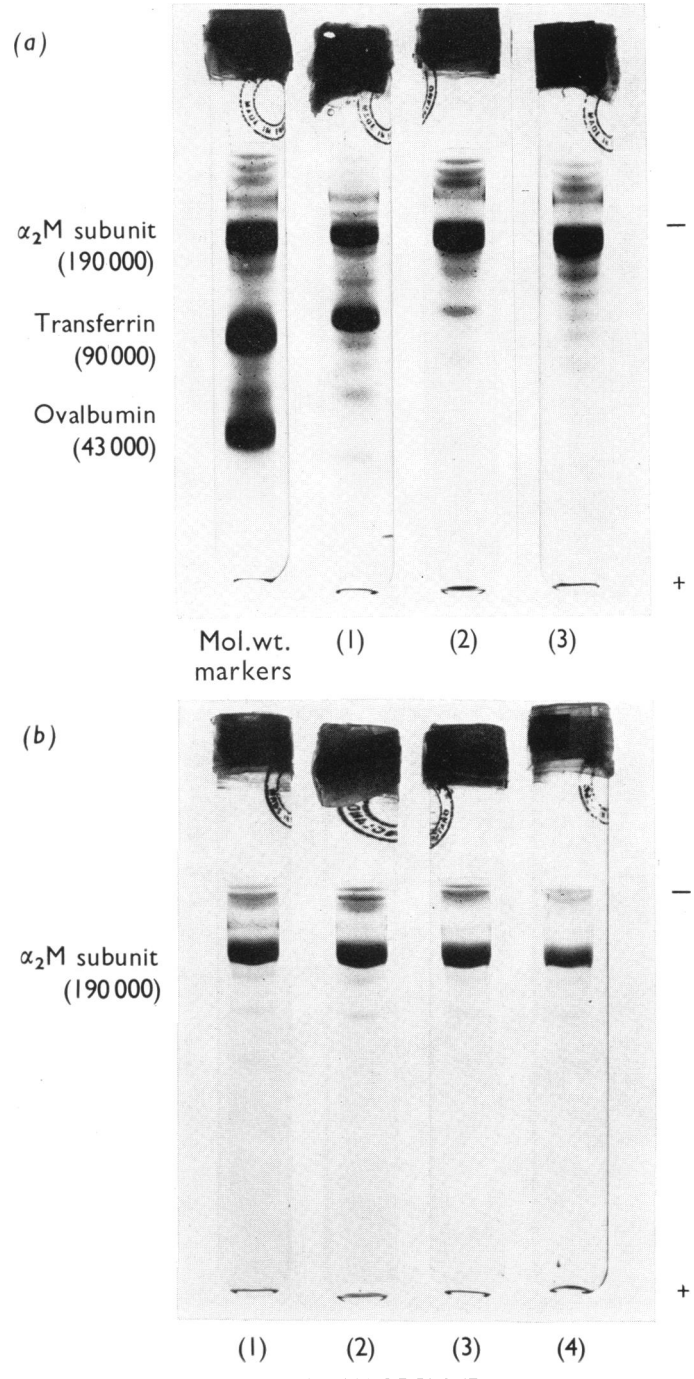


Fig. 1. *Attempted complex-formation between pancreatic and urinary kallikreins and α_2M analysed by gel filtration of the incubated proteins in comparison with* 1:1 *complex-formation with trypsin*

For details, see the text. (*a*) Trypsin incubated at a molar ratio of 2:1; (*b*) pancreatic kallikrein at a molar ratio of 0.14:1; (*c*) urinary kallikrein pI4.9; (*d*) urine, 10min incubation with α_2M followed by chromatography on a column (0.8cm×30cm) of Sephadex G-200 [flow rate 0.15ml/min; $V_e - V_0$ with unbound enzymes: (*a*) 1.98, (*b*) 1.83, (*c*) 2.0, (*d*) 1.84 (V_0 is the elution volume of α_2M)]. Protein elution was determined at E_{280} (○); enzyme activity was measured with α-*N*-benzoyl-DL-arginine *p*-nitroanilide (μg of trypsin/ml) and Tos-Arg-O[^{3}H]Me (●) (KU/ml).



EXPLANATION OF PLATE 1

Analyses of subunit conversion of α_2M after attempted slow complex-formation with pancreatic kallikrein

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis with 20μg of reduced protein after incubation at a molar ratio of 1:1 with the respective enzymes: (*a*) after 24h interaction time between α_2M and (1) trypsin, (2) purified pancreatic kallikrein, (3) control with α_2M alone; mol.wt. markers indicating the position of the 85000 subunit; (*b*) same analysis but with trypsin-free pancreatic kallikrein at a molar ratio of 1:1 for (1) 10min, (2) 1h, (3) 4h, (4) 24h. Gels were stained with 0.25% Coomassie Brilliant Blue.

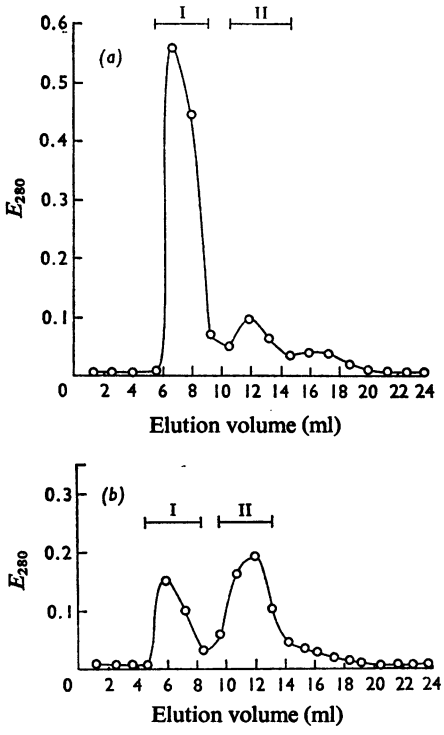


Fig. 2. *Removal of small amounts of α_2M-binding proteinase from purified pancreatic kallikrein: preparation of trypsin-free kallikrein*

Gel filtration after short (10min) incubations with (*a*) 200KU of kallikrein and 2mg of α_2M (2:1 molar ratio) and (*b*) 500KU of kallikrein and 0.5mg of α_2M (20:1 molar ratio). Sephadex G-200 gel filtration on a column of 0.8cm×30cm was carried out; protein was determined at E_{280} (○). The α_2M pool was rejected; the unbound enzyme was collected in pool II (trypsin-free kallikrein) and submitted to binding tests with α_2M.

on Sephadex G-200, 60% of the enzyme was obtained unbound. The reaction between trypsin and α_2M was also rapid at low molar ratio with 3μg of trypsin and 0.7mg of α_2M. After 10min interaction time, 85% (uncorrected) of the enzyme was complex-bound. By using the soya-bean method, the inhibitory effect of α_2M on trypsin activity was tested during a 10min incubation of 5, 10, 20 and 40μg of trypsin with 0.54, 1.08, 2.16 and 4.32mg of α_2M respectively. Corrected for inhibition (average value 25.8%) the trypsin/α_2M binding ratio was 0.035 (Fig. 1*a*), in agreement with earlier findings (Hamberg *et al.*, 1973).

The binding ratio of pancreatic kallikrein was calculated assuming a mol.wt. of 26000 (Kutzbach & Schmidt-Kastner, 1972). After 10min of incubation with 4.8KU of kallikrein and 0.7mg of α_2M, only a small amount of the enzyme active with Tos-Arg-O[^{3}H]Me was obtained as a complex (Fig. 1*b*). Use of prolonged incubation times and various molar ratios did not seem to influence the binding. When tested at molar ratios of 0.14:1, 1:1 and 1.5:1 and comparing interaction times of 10min, 4h and 24h, most of the enzyme emerged unbound in all cases after gel filtration. After 24h (1:1 molar ratio) a low degree of subunit conversion was obtained (Plate 1*a*), comparable with the corresponding reaction at a molar ratio of 0.14:1 with trypsin.

The gel-filtration pattern after incubation with 4KU of urinary kallikrein (pI4.9) and 0.6mg of α_2M is shown in Fig. 1(*c*). Owing to the focusing of several urinary proteins in the same range as urinary kallikrein (Hamberg & Joutsimo, 1974), a second protein peak coincided with the enzyme. A 24h sample of rabbit urine (102ml) was collected as described by Hamberg & Joutsimo (1974). A 3ml sample was desalted on Sephadex G-25 and concentrated to 4ml by ultrafiltration at 5°C; 1ml was incubated with 0.6mg of α_2M. As seen in the gel-filtration diagrams (Figs. 1*c* and 1*d*), no esterase activity was complex-bound.

Results obtained so far have suggested that the low degree of complex-formation with α_2M may be due to the presence of small amounts of contaminating proteinase with the purified kallikrein preparations. According to Kutzbach & Schmidt-Kastner (1972) the commercial pancreatic kallikrein may contain about 1% trypsin. The method of choice for removing contaminating trypsin seemed, on the basis of the present results, to be by complexing with α_2M. Two different molar ratios were chosen for incubation and gel filtration, as illustrated in Fig. 2. After being pooled as indicated, the complex fractions contained 0.6% (*a*) and 1.8% (*b*) of the original activity; 54 and 62% of unbound kallikrein was collected respectively, which is henceforth referred to as trypsin-free kallikrein. Incubation was performed at a molar ratio of 1:1 with α_2M comparing the interaction at 0, 1, 4 and 24h at room temperature. After reduction with dithiothreitol and gel electrophoresis there was no indication of subunit conversion (Plate 1*b*).

Discussion

As pointed out by Laskowski & Sealock (1971) the tabulation of enzymes inhibited and not inhibited by various proteinase inhibitors is important for the understanding of the physiological function of the inhibitor and as a means of classifying new enzymes. The lack of binding to α_2M shown by the kallikreins used in this study is parallelled by their lack of inhibition by soya-bean inhibitor (Habermann & Klett, 1966; Moriya *et al.*, 1973). A limited degree of subunit conversion, characteristic of the binding reaction and detected by gel electrophoresis (Harpel, 1973),

disappeared after removal of a contaminating proteinase, presumably trypsin. As shown in this study, trypsin binds rapidly to α_2M regardless of temperature of incubation or molar ratio. In contrast with the binding of collagenase (Werb *et al.*, 1974), prolongation of the incubation time to 24h did not cause binding of the pancreatic kallikrein. Although evidence is still conflicting with respect to the proteinase specificity and the catalytic mechanisms involved, our results support the hypothesis that binding to α_2M may depend on the cleavage of a peptide bond in the reactive site of α_2M (Barrett & Starkey, 1973).

The reactive-site cleavage of a peptide bond and the production of two fragments after reduction and carboxymethylation is known to occur with the soyabean inhibitor modified after encounter with trypsin (for details see Laskowski & Sealock, 1971). Present results further sustain that the 85000-mol.wt. product is only formed after binding of a proteinase and is not a true subunit of α_2M (cf. Frénoy *et al.*, 1972; Roberts *et al.*, 1974).

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