Interaction of Urokinase with α_2 -Macroglobulin Investigated by Isoelectric Focusing

EVIDENCE FOR NON-SPECIFIC DISSOCIABLE BINDING

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The binding of urokinase to human α_2 M (α_2 -macroglobulin) was investigated in comparison with the formation of the equimolar trypsin- α_2 M complex. Experiments were performed by molecular-sieving on Sephadex G-200, subunit conversion by sodium dodecyl sulphate-polyacrylamide-gel electrophoresis after reduction and isoelectric focusing in linear sucrose gradients with ampholytes pH 3.5–10.0. Urokinase activity was determined with α -N-acetyl-L-lysine methyl ester and by activation of plasminogen on unheated fibrin plates. α_2 M was determined by single radial immunodiffusion. α_2 M was capable of binding some urokinase by a non-specific type of attachment that could be disrupted by isoelectric focusing but not by gel filtration. The pI of the undissociated trypsin- α_2 M complex was 6.0, and differed from that of the pure α_2 M (5.2–5.4). Likewise the pI of the immunoreactive α_2 M was 5.2 after exposure to urokinase, whereas the dissociated urokinase focused at pI 10.2. This indicated lack of true inhibitor-complex formation, which was also sustained by total absence of subunit conversion. The results are in agreement with our previous findings with pancreatic and urinary kallikreins.

α_2 M (α_2 -macroglobulin) is a high-molecular-weight glycoprotein known to exhibit quite broad specificity in inhibiting essentially all active endopeptidases of animal, plant and bacterial origin (Sasaki *et al.*, 1974). The enzyme binding is believed to occur by a two-stage mechanism involving a primary proteolytic attack on the inhibitor (Barrett & Starkey, 1973; Fine *et al.*, 1975). As a consequence of binding, a subunit of α_2 M (mol.wt. 85000) is formed and can be detected by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis after reduction (Harpel, 1973). However, it was demonstrated that pancreatic and urinary kallikreins (kininogenin, EC 3.4.21.8) failed to form the typical serine proteinase complex with α_2 M (Hamberg *et al.*, 1973; Vahtera & Hamberg, 1976). The subunit conversion was also totally absent. The lack of binding is believed to be the result of the restricted specificity of the kallikreins. Urokinase (EC 3.4.99.26) belongs to the group of proteolytic enzymes with unknown catalytic mechanism. Ong *et al.* (1976) obtained evidence that urokinase belongs to the class of active-site serine enzymes. It also has

a very narrow specificity, and the only known natural substrate is plasminogen (White & Barlow, 1970). Compared with other proteinase inhibitors (see review by Laskowski & Sealock, 1971), the binding mechanism with α_2 M is not well understood, and differs in particular by leaving access to the active site of the enzymes. As shown by Boyde & Pryme (1968) and Boyde (1969), a non-specific, apparently electrostatic, type of binding to α_2 M was found with aspartate aminotransferase (EC 2.6.1.1). The absence of subunit conversion with urokinase (Harpel, 1973) suggested that urokinase apparently belongs to the group of non-binding proteinases. On the other hand, Ogston *et al.* (1973) have indicated that the activation of plasminogen by urokinase is inhibited by α_2 M. In the present investigation the complex-formation with urokinase was investigated by using isoelectric focusing to distinguish between the irreversible and non-specific types of binding.

Materials and Methods

Human α_2 M was prepared from mixed blood-bank plasma (from the Finnish Red Cross Blood Transfusion Service) as previously described (Hamberg *et al.*, 1973; Vahtera & Hamberg, 1976). The preparation used in these experiments was contaminated with traces of haptoglobin, as shown by immunodiffusion analyses (Ouchterlony, 1948)

Abbreviations used: α_2 M, α_2 -macroglobulin; tosyl, toluene-*p*-sulphonyl.

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and immunoelectrophoresis (Grabar & Williams, 1955); α_2 M concentrations were measured either by using $A_{280}^{1\%} = 10$ (Hamberg *et al.*, 1973) or by single radial-immunodiffusion analyses by the method of Mancini *et al.* (1965) with monospecific antiserum against α_2 M raised in the rabbit. The standard curve was prepared with standard human serum (stabilized; from Behringwerke A.G., Marburg/Lahn, Germany). To check the binding capacity of the preparation, α_2 M was incubated with excess trypsin and the unbound enzyme was inhibited with soya-bean trypsin inhibitor (both preparations were from Worthington Biochemical Corp., Freehold, NJ, U.S.A.) as described previously (Vahtera & Hamberg, 1976). The amidase activity was measured by the method of Erlanger *et al.* (1961) with α -N-benzoyl-DL-arginine *p*-nitroanilide hydrochloride (from Fluka A.G., Buchs, Switzerland) as substrate. The binding ratios (μ g of trypsin/ μ g of α_2 M; calculated by single radial-immunodiffusion analysis) were close to the equimolar ratio 0.029:1 (0.032–0.038:1).

Urokinase was from Leo Laboratories, Ballerup, Denmark [5000 Ploug units (Kjeldgaard & Ploug, 1957)/ampoule]. Urokinase activity is expressed in CTA units (Committee on Thrombolytic Agents of the United States Public Health Service, National Heart Institute); 1 CTA unit = 0.75 Ploug unit (Johnson & Newman, 1964). The esterase activity was measured by a modified method of Hamberg & Savolainen (1968), with α -N-acetyl-L-lysine methyl ester hydrochloride (from Cyclo Chemical Corp., Los Angeles, CA, U.S.A.) as substrate, in 67mM-sodium phosphate buffer (pH 7.8)/0.1M-NaCl.

The esterase activity of the α_2 M preparation was tested with the very sensitive method described by Beaven *et al.* (1971), with α -N-tosyl-L-arginine [3 H]methyl ester hydrochloride (from Calatomic, Los Angeles, CA, U.S.A.) as substrate (Vahtera & Hamberg, 1976). Sodium dodecyl sulphate/5% (w/v) polyacrylamide-gel electrophoresis (Weber & Osborn, 1969) was performed for the study of subunit conversion (Harpel, 1973), as described in detail by Vahtera & Hamberg (1976).

Isoelectric focusing was carried out for 46–48 h at +5°C in the LKB 8100 Ampholine electrofocusing column (110 ml) with 1% ampholyte (LKB Products, Bromma, Sweden), pH 3.5–10.0, in a linear sucrose concentration gradient (Vesterberg & Svensson, 1966). Fractions were collected at a flow rate of 1 ml/min and the pH of the fractions was measured immediately (+5°C). The fibrinolytic activity was determined on unheated fibrin plates (bovine fibrinogen grade B1 or human fibrinogen grade L; from Kabi A.B., Stockholm, Sweden) as described by Hamberg & Savolainen (1966) or by using 0.1% human fibrinogen in 0.8% agarose (0.01 M-sodium phosphate buffer, pH 7.8) as described by Norén

et al. (1975). α_2 M was located and the concentration determined by single radial-immunodiffusion analyses (Mancini *et al.*, 1965). For further analyses the fractions were pooled and dialysed for 24 h against 0.06 M-NaCl and then another 24 h against 0.1 M-Tris/HCl buffer (pH 8.2) / 0.15 M-NaCl or (for fractions containing urokinase) 67 mM-sodium phosphate buffer (pH 7.8)/0.1 M-NaCl.

Results and Discussion

When α_2 M was incubated with urokinase (444 CTA units/mg), all enzyme activity was recovered in the α_2 M fractions after gel filtration (Fig. 1a). The enzyme recovery was 89%. When the amount of enzyme was increased (to 667 CTA units/mg), only 34% of the recovered activity was found with α_2 M.

Fig. 1. Attempted complex-formation between α_2 M and urokinase

(a) 0.75 mg of α_2 M incubated for 1 h at room temperature with 333 CTA units of urokinase; (b) 1 mg of α_2 M incubated for 30 min at 6°C with 667 CTA units of urokinase. The incubation mixtures were gel-filtered through a column (0.8 cm $\times$ 30 cm) of Sephadex G-200 at 6°C in 67 mM-sodium phosphate buffer (pH 7.8)/0.1 M-NaCl; the flow rate was 0.1 ml/min. $\circ$, Protein; $\bullet$, enzyme activity on α -N-acetyl-L-lysine methyl ester.

The total enzyme recovery was 93% (Fig. 1b). Prolonging the incubation time (conditions as in Fig. 1b) to 24h at room temperature (25°C) did not cause increased enzyme binding, since 39% of the recovered activity was found with the α_2 M fractions (total recovery 88%). With 20 μ g of α_2 M from the two peak fractions (Fig. 1a) there was no subunit conversion when tested, after reduction, by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The same result was obtained after 24h incubation. Traces of subunit conversion could be detected after 24h incubation at room temperature with 3333 CTA units of urokinase/mg of protein. This clearly indicates lack of true complex-formation.

It was further found that the urokinase- α_2 M complex was still capable of binding trypsin. After incubation of 2.0mg of α_2 M with 1777 CTA units of urokinase for 1h at room temperature (25°C), the α_2 M fractions contained 28% of the urokinase activity (total recovery 83%; Fig. 1b). A portion (104 μ g of protein) of the urokinase- α_2 M complex fractions was further incubated with an excess of trypsin (5 μ g) for 10min at room temperature. After the unbound trypsin was inhibited with soya-bean trypsin inhibitor, the complex-bound trypsin corresponded to a trypsin/ α_2 M binding ratio of 0.032:1 (w/w), showing formation of a normal equimolar complex with trypsin. The typical subunit

was now detected by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis after reduction. These results suggested that urokinase was bound differently from the normal proteinase-inhibitor binding. This non-specific binding was further investigated by isoelectric focusing.

When 13333 CTA units of urokinase were focused with 1% ampholyte, pH 3.5–10.0, and the fibrinolytic activity was measured on fibrin plates, urokinase focused at pI 10.2 (72%). A second fibrinolytically active component (28%) was obtained at pI 8.4. This is in agreement with previous findings by Schönebeck *et al.* (1971) with human urokinase, and by Hamberg & Joutsimo (1974), who found two isoelectric variants in this focusing range with urokinase from collected rabbit urine. Andrassy & Ritz (1977) have also shown on disc electrophoresis that pure human urokinase still gave two or three bands, suggesting the presence of isoenzymes. Ohlsson & Skude (1976) have shown that complex-formation with trypsin, chymotrypsin, plasmin, elastase and collagenase changed the pI of α_2 M from 5.1 to 6.0 in plasma. After exposure to urokinase (2667 CTA units of urokinase/6mg of α_2 M protein; same preparation as in Fig. 1a), α_2 M focused at pI 5.2. However, 96% of the recovered urokinase activity focused at pI 10.2, showing that the urokinase complex was dissociated. The α_2 M with pI 5.2 contained only 4% of the activity.

Fig. 2. pI of trypsin- α_2 M complex (1:1)

Incubation mixture of 6mg of α_2 M and 180 μ g of trypsin (10min at room temperature) was focused (47h at +5°C, with 1% ampholyte) between pH 3.5 and pH 10.0 (—). Protein elution was determined at A_{280} (○) (the flow rate was 1 ml/min; the fraction size was 2–3 ml); immunoreactive α_2 M was determined by single radial immunodiffusion (●).

This was further sustained by the total lack of subunit conversion with the α_2 M collected after focusing. Similar results were found with non-binding pancreatic kallikrein (for details see Vahtera & Hamberg, 1976). The true undissociated complex between α_2 M and a serine proteinase focused at pH 6.0, as shown in Fig. 2. This confirms the report by Ohlsson & Skude (1976). For unknown reasons a fraction of non-complex-bound α_2 M was obtained at pI 5.2. The α_2 M preparation applied in these experiments had a low activity towards α -N-tosyl-L-arginine [3 H]methyl ester, apparently due to pre-bound blood plasma enzyme. These anomalous α_2 M fractions were not studied further.

The present findings indicate that α_2 M can form a non-specific complex with urokinase disrupted by isoelectric focusing. This type of binding differs from a true complex and is clearly shown by the dissociation of bound urokinase and by no change of pI after incubation of α_2 M with urokinase.

This binding of a limited amount of urokinase to α_2 M resembles the electrostatic attachment reported with cationic aspartate aminotransferase by Boyde & Pryme (1968). This complex also differed in many respects from the normal α_2 M-plasmin complex. The enzyme could be replaced by trypsin and chymotrypsin and the binding was shown to depend on ionic strength. In all cases so far studied the formation of the stable inhibitor complex appears to involve a proteolytic action on α_2 M that leads to subunit formation after reduction. The electrostatic binding alone must be considered non-specific as an inhibitor reaction, since it can occur with many different compounds and can be dissociated. The lack of subunit conversion with urokinase is in keeping with our previous findings with pancreatic and urinary kallikreins (Vahtera & Hamberg, 1976), suggesting that enzymes with limited proteolytic actions are unable to form the true inhibitor complex. In pathological states the disposal by complex-formation with α_2 M of activated extravascular proteinases may be important, as was suggested in our recent study of the kinin system in synovial fluid (Hamberg *et al.*, 1978).

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