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Kallikrein Inhibitory Capacity of α_2-Macroglobulin Subunits (38463)

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Although human α_2-macroglobulin was first isolated in 1955 (1), its chemical structure and biologic function remain poorly understood. In 1958 Schönenberger found the molecular weight of the protein to be between 820,000 and 950,000 and noted that, on exposure to 5 *M* urea, the molecule would dissociate into two subunits of equal molecular weight which would reassemble when returned to physiologic conditions (2). Since that time a number of investigators have studied the properties and structure of the protein (3–6). Recently, Jones *et al.* have determined a molecular weight of 750,000 and a value of 18.1S for S_{20w} (7). These investigators confirmed the work of Schönenberger and others by demonstrating the dissociation of the protein into two subunits of equal molecular weight having an S_{20w} value of 11S in urea solutions at neutrality and in urea-free aqueous solutions at pH 2.5–4.5. These investigators further showed that limited reduction of the protein with thiols liberated a subunit with a molecular weight approximately one-quarter that of the intact molecule which retained the serologic specificity of the intact protein.

α_2-Macroblobulin inhibits the activities of several enzymes including trypsin, plasmin, thrombin, and plasma kallikrein, and one of its major biologic roles may be that of a proteolytic enzyme inhibitor (8). It is an unusual inhibitor in that it reduces the proteolytic activity of these enzymes to a greater extent than their esterolytic activity. Furthermore, it protects the esterolytic activity of enzymes bound to it from large protease inhibitors such as soybean trypsin inhibitor (SBTI)[1] (8).

The purpose of the present study was to investigate the interaction of the α_2-macroglobulin 11S subunits with plasma kallikrein, one of the enzymes inhibited by the parent molecule (9, 10). The goal was to determine whether the intact molecule is necessary for the retention of kallikrein inhibitory capacity. It will be shown that the 11S subunits do indeed have substantial inhibitory activity; the relevance of this observation to the hypothesis that α_2-macroglobulin is a divalent protease inhibitor will be discussed.

Materials. Synthetic bradykinin and 3× crystallized soybean trypsin inhibitor (SBTI) were obtained from Calbiochem, Los Angeles, CA. Rabbit anti-human α_2-macroglobulin, rabbit anti-human IgG, rabbit anti-whole human serum, and Partigen α_2-macroglobulin radial immunodiffusion plates with standards were obtained from Behring Diagnostics, Inc., Somerville, NJ. Cyanate-free urea was prepared by passing 8 *M* urea through an analytical-grade mixed-bed resin AG 501—X8 (D)—20–50 mesh (Bio Rad Laboratories, Richmond, CA). The resulting solution was used in the preparation of certain of the urea-containing buffers as discussed under Results. *p*-Tosyl-L-arginine-methyl ester (TAME) was obtained from ICN Nutritional Biochemicals Corp., Cleveland, OH.

Immunochemical methods. Ouchterlony plate analysis (11), immunoelectrophoretic analysis (12) and radial immunodiffusion (13) were carried out by standard methods.

Protein purification. Gel filtration was performed with Sepharose 6-B and Sephadex G-200 (Pharmacia Fine Chemicals, Inc., Piscataway, NJ) (14). Conditions of individual runs are given under Results. Protein solutions were concentrated by ultrafiltration (15). All protein purification procedures were carried out at 4°.

Protein concentration was determined by optical density at 280 nm and was periodically checked by the method of Lowry *et al.* (16). The

[1] Abbreviations used in this paper: SBTI, soybean trypsin inhibitor; TAME, *p*-tosyl-L-arginine-methyl ester; ACD, acid-citrate dextrose; PBS, phosphate-buffered saline (0.15 *M* NaCl, 10 m*M* $NaPO_4$ at pH 7.4).

concentration of α_2-macroglobulin subunits was determined by comparing their absorbance at 280 nm with that of standard (native) α_2-macroglobulin preparations.

Isolation of proteins. α_2-Macroglobulin was isolated as follows: Normal human venous blood (four parts) was taken into acid-citrate dextrose (ACD) (one part) in plastic Fenwal bags (Fenwal Div. Travenol Lab., Morton Grove, IL) and centrifuged to separate the cells. The supernatant plasma was removed immediately and stored frozen at −60° until used. It was then thawed and mixed in a plastic vessel with SBTI to give a final concentration of 100 μg SBTI/ml plasma. From this point on, the isolation procedure described by Harpel was used except that barium chloride and barium sulphate adsorptions were omitted (9). All samples of α_2-macroglobulin were subjected to a final starch-block electrophoresis (17) before use and were demonstrated to be free of contaminating protein by Ouchterlony plate and immunoelectrophoretic analysis with anti-whole human serum and to have no detectable esterase activity (for esterase methods, see below). Purified α_2-macroglobulin was stored for up to three weeks at 4° in PBS (0.15 *M* NaCl, 10 m*M* $NaPO_4$ at pH 7.4) with 0.9% glycine added to minimize protein aggregation upon prolonged storage. For longer periods the protein was stored at −60°. α_2-Macroglobulin concentrations were determined by radial immunodiffusion.

Partially purified kallikrein and kininogen were prepared as previously described (10).

Ultracentrifugational analysis. For ultracentrifugational analyses of samples of α_2-macroglobulin and its subunits, 0.3-ml samples were layered over 13.0-ml linear 5–20% sucrose gradients in PBS. The gradients were centrifuged at 35,000 rpm for 16 hr at 5° in an SW 40Ti swinging-bucket rotor in a Beckman Model L2-65B ultracentrifuge. After centrifugation, tubes were punctured, the effluents passed through a Gilford Model 2000 recording spectrophotometer equipped with a flow-through cell (0.5-cm light path), and the absorbance of the effluents monitored continuously at 280 nm. The absorbance scales shown in the figures are modified to correspond to a 1-cm light path.

Measurement of kallikrein activity. (a) Rat uterus bioassay. Kinin activity and kallikrein activity were assayed on the isolated rat uterus as previously described (10). Kallikrein activity was expressed in "kallikrein doses." One kallikrein dose was defined as the amount of kallikrein in 0.1 ml of sample which released an average of 20 ng of bradykinin equivalent when incubated with 0.1 ml of a standard kininogen substrate for 10 min at 37° in PBS (10).

(b) Esterase assay. Kallikrein was assayed for esterase activity using TAME as a substrate as previously described (10, 18).

Results. The initial goal of this study was to isolate the 11S subunits of α_2-macroglobulin in such a manner that the subunits would remain stable under physiologic conditions. It was found that this could be accomplished by dialyzing the protein for 48 hr against 3 *M* urea in PBS followed by dialysis against PBS with 0.9% glycine. Figure 1 shows an ultracentrifugational analysis of subunits prepared in this manner. If one assigns the literature value of 18S to the intact molecule (7), the sedimentation coefficient for the more slowly sedimenting protein is calculated to be very nearly 11S, or almost exactly what one would predict for a subunit having one half the molecular weight of the intact α_2-macroglobulin molecule.

Although in the experiment shown in Fig. 1, complete dissociation of the molecule was obtained, dissociation was not always complete, and in seven separate experiments varied from

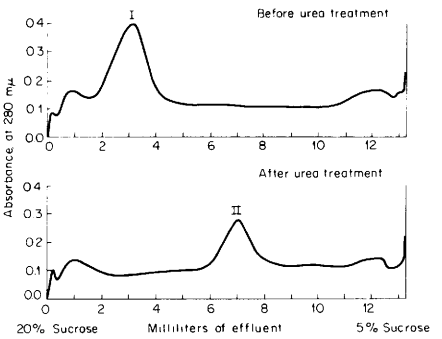

FIG. 1. Sucrose density gradient analysis of α_2-macroglobulin before and after exposure to 3 *M* urea. The top panel shows the sedimentation properties of the native molecule; the bottom panel shows the sedimentation profile obtained after 48 hr of dialysis against 3 *M* urea in PBS followed by dialysis against PBS alone (4°). Treatment with urea results in a loss of the original peak (I) and the appearance of a new peak (II) corresponding to a subunit having one-half the molecular weight of the intact protein. Centrifugation conditions are given under Methods. The total quantity of protein layered onto the gradients shown in the top and bottom panels was 0.81 and 0.53 mg, respectively.

50% to 100%. Dissociation was favored by longer periods of dialysis against 3 *M* urea and was not appreciably affected by protein concentration. Thus, in one experiment, increasing the time of dialysis against urea from 13 to 48 hr increased the percent dissociation from approximately 20% to 65%. In the same experiment, varying the α_2-macroglobulin concentration between 0.7 and 2.5 mg/ml had no effect upon the degree of dissociation obtained. Dissociation could not be attributed to the effects of cyanate ion, since it was demonstrable even when cyanate-free urea in 0.1 *M* Tris–HCl (pH 7.5) was substituted for commercial urea in PBS-glycine buffer. For routine isolation of the subunits a period of 48 hr of dialysis against freshly prepared 3 *M* urea in PBS was used. Once isolated, the subunits remained stable in PBS for more than 2 hr at 37° and over 3 weeks at 4°. Figure 2 demonstrates that the subunits can also be isolated by Sepharose 6-B gel filtration, and Fig. 3 that the antigenic characteristics of the isolated α_2-macroglobulin subunits remain identical to those of the intact protein as detected by double-diffusion in agar against rabbit anti-human α_2-macroglobulin. During immunoelectrophoretic analysis against rabbit anti-human macroglobulin both the subunits and intact α_2-macroglobulin itself (at equal concentrations) show a similar rate of migration toward the anode (Fig. 4). The intact protein, as would be expected by virtue of its higher molecular weight, does not migrate as far toward the trough.

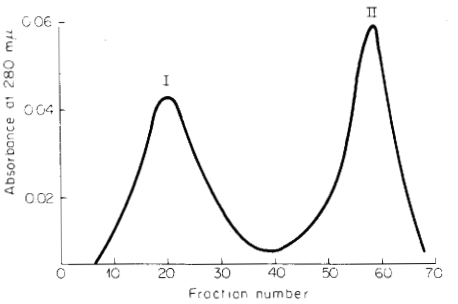

FIG. 2. Sepharose 6-B gel filtration of α_2-macroglobulin treated with 3 *M* urea as described under Results. The α_2-macroglobulin (I) and subunit (II) peaks were identified with specific antiserum against α_2-macroglobulin by double-diffusion in agar. Protein concentration is indicated by the solid line. Sample—0.7 ml of α_2-macroglobulin–subunit mixture (3.0 mg/ml); column—2.5 × 80 cm; flow rate—15 ml/hr; fractions—5 ml/tube; buffer—PBS. The degree of dissociation observed in this particular experiment (one of six) is seen to be approximately 50%.

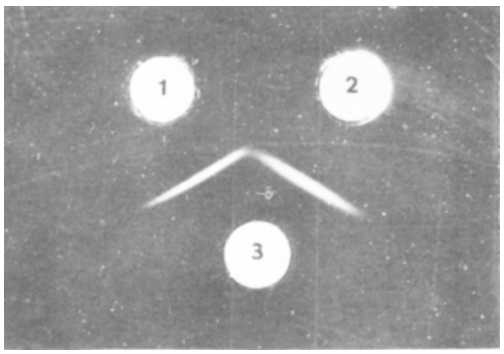

FIG. 3. Ouchterlony plate analysis of α_2-macroglobulin and 11S subunits. (1) α_2-macroglobulin; (2) 11 S subunits; (3) rabbit anti-α_2-macroglobulin.

Once a method had been devised for isolating 11S subunits which would remain stable under physiologic conditions, the subunits were tested for kallikrein inhibitory capacity in both the rat uterus and the esterase assays. Kallikrein inhibitory capacity in both assays was expressed as the potency ratio between the activities of a control and an inhibited preparation of kallikrein.[2] Previous work has shown that the activity of serial dilutions of native kallikrein decreases in parallel in both assays (10). Therefore, if an inhibitor affected kallikrein's esterase and kinin-releasing activity to an equal extent, the potency ratios obtained in both assays would be equal. Figure 5 gives representative results of one of the three separate experiments comparing the kallikrein inhibitory capacity of the α_2-macroglobulin subunits with that of the native protein. It is seen that the α_2-macroglobulin 11S subunits, like the native molecule, were far more effective inhibitors in the rat uterus bioassay than in the esterase assay. In the bioassay, on a weight basis, the inhibitory capacity of the subunits was only 25–30% that of the native molecule, while in the esterase assay the inhibitory capacities appeared similar. In control experiments it was

[2] Rat uterus assay:

$$\text{Potency ratio} = \frac{\text{Kallikrein doses/ml (control)}}{\text{Kallikrein doses/ml (inhibited)}}$$

Esterase assay:

$$\text{Potency ratio} = \frac{\mu\text{moles/ml/hr (control)}}{\mu\text{moles/ml/hr (inhibited)}}$$

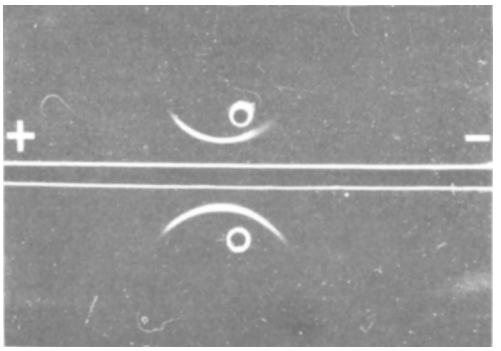

FIG. 4. Immunoelectrophoretic analysis of α_2-macroglobulin (top) and the 11S subunits (bottom).

shown that any α_2-macroglobulin remaining undissociated following urea treatment retained full inhibitory capacity in both assays.

A final series of experiments was performed to investigate the kallikrein binding ability of the 11S subunits. Plasma kallikrein was preincubated with the subunits as indicated in the legend to Fig. 6 and the mixture then subjected to Sepharose 6-B gel filtration. As can be seen in Fig. 6, the kinin-releasing activity of free kallikrein eluted later than the subunits, and there was no kinin-releasing activity associated with the subunit peak. The subunit fractions and kallikrein fractions were then pooled and concentrated as indicated in the legend. When the concentrated pools were assayed for esterase activity, the total activity of the free kallikrein pool was 5.45 mmoles/hr and that of the subunit pool 1.47 mmoles/hr. The free kallikrein pool had a total of 13,200 kallikrein doses of kinin-releasing activity, and the subunit pool had none. Solutions containing subunits not exposed to kallikrein had no esterase or kinin-releasing activity. Repeat chromatography of the complexes on Sephadex G-200 was carried out to determine whether the bound kallikrein (MW 100,000) could be dissociated from either native α_2-macroglobulin (MW 750,000) or the subunits (MW 375,000). Three-milliliter samples containing either the subunits (370 μmoles/hr bound esterase activity) or the native protein (340 μmoles/hr bound esterase activity) were applied to a 2.5 × 80-cm Sephadex G-200 column. The column was eluted with PBS at 15 ml/hr, and 5.0-ml fractions were collected. It was found that no kinin-releasing activity dissociated from either the subunits or the native protein; both species eluted in the void-volume of the G-200 column.

Discussion. A number of investigators have shown that α_2-macroglobulin can be separated into subunits of equal molecular weight by exposing the molecule to acid pH or urea solutions (2–7). This study was designed to investigate some of the properties of these dissociated subunits under physiologic conditions. Although Schönenberger *et al.* showed that the 11S subunits reassembled under physiologic conditions (2), the present study demonstrates that prolonged dialysis against 3 *M* urea produces subunits that will remain dissociated. This effect of prolonged urea dialysis is probably a result of irreversible changes induced in the subunit structure but cannot be attributed to cyanate ion.

The characteristics of these isolated subunits provide some insight into the nature of the intact α_2-macroglobulin molecule. It is evident that the subunits are, in fact, half-molecules of equal molecular weight linked in the whole protein by noncovalent bonds. Their antigenic characteristics as detected by Ouchterlony plate analysis are

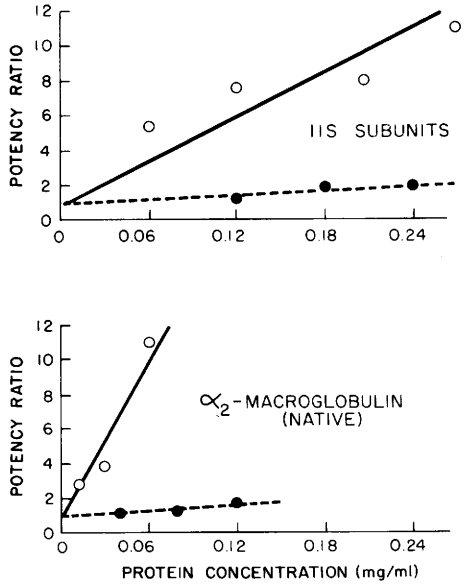

FIG. 5. Inhibition of kallikrein by varying concentrations of α_2-macroglobulin and its 11S subunits. Kallikrein was preincubated with the inhibitors for 1 hr at 37° before testing for activity. Inhibition was measured by determining the potency ratios between the activities of control and inhibited kallikrein preparations as described under Results (10). Progressively higher numbers indicate progressively greater inhibition. Upper graph—effect of the 11S α_2-macroglobulin subunits. Lower graph—effect of the native protein. Open circles—rat uterus assay (minimum of six assays per point); closed circles—esterase assay (minimum of three assays per point).

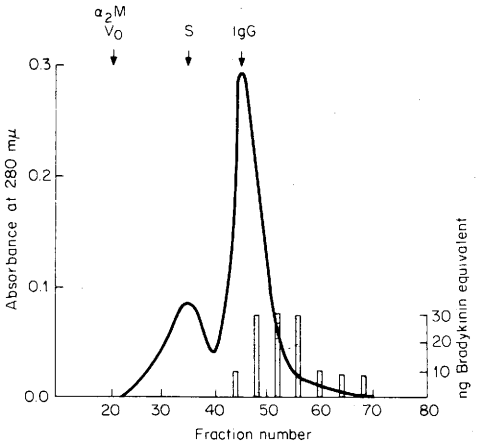

FIG. 6. Sepharose 6-B gel filtration of a solution containing α_2-macroglobulin subunits and plasma kallikrein. The sample was prepared by mixing 3 ml of α_2-macroglobulin subunits (5.0 mg/ml) with 15 ml of kallikrein solution (esterase activity 7.8 mmoles/hr; 18,700 kallikrein doses) and concentrating this solution to 3.9 ml. A 3-ml sample was applied to a 5.0 × 55-cm column. Flow rate—34 ml/hr; fractions—15 ml/tube; buffer—PBS. The effluent was monitored for protein concentration, and kallikrein activity was expressed as ng of bradykinin equivalent released (bars). V_o and the elution positions of native α_2-macroglobulin (α_2M), the subunits (S), and IgG (identified with specific antiserum) are indicated. The fractions containing the subunits (31–39) and free kallikrein (44–75) were pooled, concentrated, and assayed for total kallikrein activity. Subunit pool: 1.5 mmoles/hr esterase activity; no kinin-releasing activity. Free kallikrein pool: 5.5 mmoles/hr esterase activity; 13,200 kallikrein doses of kinin-releasing activity.

similar to those of the native protein. Furthermore, the two subunits cannot be distinguished from each other by ultracentrifugation, Sepharose gel filtration, or immunoelectrophoresis. While further studies will be necessary to determine whether the two subunits are in fact identical, they would appear, by the methods employed in this study, to be similar in size, shape, charge, and antigenic characteristics.

It is evident from the present study that the 11S subunits retain appreciable kallikrein inhibitory capacity. Two explanations could account for the finding that, when compared on a weight basis with the intact molecule, the retention of inhibitory capacity was greater in the esterase assay than in the bioassay: Either the intact molecule is required for full inhibitory capacity, or urea treatment produces irreversible changes that not only prevent reassembly of the molecule but also alter the inhibitory capacity of the subunits.

There is conflicting evidence concerning the number of moles of enzyme which can be bound by 1 mole of α_2-macroglobulin. Thus, while several authors have reported that 1 mole of α_2-macroglobulin binds 2 moles of trypsin (19, 20), and recently Schreiber *et al.* have reported that 1 mole of α_2-macroglobulin binds 2 moles of plasmin (21), others have reported that only 1 mole of plasmin (22) or trypsin (23) can be bound per mole of α_2-macroglobulin. While this problem remains unresolved, the finding that the 11S subunits retain significant inhibitory capacity favors the hypothesis that two moles of enzyme are bound by the native protein. Consistent too with this conclusion is the observation of Pepper that horse α_2-macroglobulin exists naturally in a monomeric form which is readily interconvertible with a dimeric one (24). Proof of the hypothesis, however, must await both precise stoichiometric studies on the binding of enzyme to α_2-macroglobulin and further studies on the chemical structure of the inhibitor.

Summary. Human α_2-macroglobulin can be dissociated by dialysis against 3 *M* urea into two 11S subunits which remain stable under physiologic conditions. These subunits retain kallikrein inhibitory capacity albeit at a reduced level when compared with the native protein. They furthermore protect bound kallikrein from further inhibition by native α_2-macroglobulin. Bound kallikrein can not be dissociated from either the subunits or the native protein by Sephadex G-200 gel filtration.

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