Purification and characterization of human α_2 -macroglobulin conformational variants by non-ideal high performance size-exclusion chromatography

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Human α_2 -macroglobulin (α_2 M) was eluted as a single nondispersed peak from a TSK-G4000SW size exclusion chromatography column equilibrated in 20 mM-sodium phosphate/100 mM-NaCl, pH 7.2 (PBS). The void volume and total accessible volume of the column were 6.08 ml and 14.42 ml. The elution volume (V_e) of native α_2 M was 9.20 ± 0.04 ml. The V_e was altered minimally by changing the ionic strength or adding ethanol to the equilibration buffer. Ternary α_2 M–trypsin, containing 2 mol of proteinase/mol of inhibitor, and α_2 M–methylamine failed to be eluted in well-defined peaks when the column was equilibrated in PBS. The majority of either preparation was recovered slowly at V_e values greater than 14.5 ml, reflecting significant nonideal interactions with the support structure. Addition of 10% ethanol or increased ionic strength in the equilibration buffer independently caused either form of reacted α_2 M to be eluted in a distinct peak at decreased V_e , suggesting that the nonideal interactions included hydrophobic and electrostatic adsorption. When the equilibration buffer was 80 mM-sodium phosphate/320 mM-NaCl, pH 7.2, partial resolution of ternary α_2 M–trypsin and α_2 M–methylamine was obtained with a single column run. The V_e values of ternary α_2 M–trypsin and α_2 M–methylamine in this buffer were 13.15 ± 0.08 ml and 11.94 ± 0.14 ml, respectively. The V_e of native α_2 M was 8.84 ± 0.03 ml. The resolving capacity of TSK-G4000SW was exploited to purify native α_2 M rapidly and efficiently from solutions that contained significant amounts of either ternary α_2 M–trypsin or binary α_2 M–trypsin (1 mol of proteinase/mol of inhibitor). This purification was complete within the limits of sensitivity of denaturing and nondenaturing polyacrylamide-gel electrophoresis. α_2 M–plasmin was well resolved from native α_2 M. The V_e of α_2 M–plasmin was 12.88 ± 0.32 ml in 80 mM-sodium phosphate/320 mM-NaCl, pH 7.2. A number of procedures were used to prepare solutions with up to 90% binary α_2 M–trypsin. The V_e of binary α_2 M–trypsin in these various solutions was intermediate between the values of native α_2 M and ternary α_2 M–trypsin. The conformations of binary and ternary complex, as reflected by mobility in nondenaturing electrophoresis, were identical, confirming previous results. Finally, in the binary α_2 M–trypsin complex, the single trypsin cleaved more than two, and as many as all four α_2 M subunits.

INTRODUCTION

Ideal size exclusion chromatography refers to the resolution of macromolecules based on hydrodynamic volume. Nonideal size exclusion chromatography refers to various interactions of solutes with the support structure that alter elution time. For most of the commercially available supports suitable for high performance chromatography, these nonideal interactions are hydrophobic and electrostatic or ionic in nature (Kopaciewicz & Regnier, 1982; Pfannkoch *et al.*, 1980; Regnier & Gooding, 1980). The TSK-gel-SW columns are chemically modified silica gel based supports that reportedly minimize nonideal interactions (Fukano *et al.*, 1978). This support was chosen for an analysis of the conformational states of the plasma protein α_2 M.

Human α_2 M is an $M_r \sim 718\,000$ glycoprotein composed of four identical subunits of known primary structure (Hall & Roberts, 1978; Swenson & Howard, 1979; Sottrup-Jensen *et al.*, 1984). The subunits are covalently

linked into pairs by disulphide bonds (Harpel, 1973). A single pair of subunits, or a 'half-molecule', is capable of functioning independently in that it binds proteinase irreversibly without involving the enzyme active site (Gonias & Pizzo, 1983a,b, 1984).

The mechanism for inhibition of proteinases by α_2 M is best described by the 'trap hypothesis' (Barrett & Starkey, 1973; Barrett *et al.*, 1979). Reactions are initiated when a proteinase recognizes and cleaves a susceptible peptide bond at the α_2 M 'bait region'. This cleavage causes conformational change in the inhibitor which physically entraps the proteinase. A covalent linkage may be formed between proteinase lysine residues and one of four α_2 M thioesters; however, the complex between α_2 M and proteinase is irreversible regardless of whether this bond is formed (Salvesen & Barrett, 1980; Salvesen *et al.*, 1981; Sottrup-Jensen *et al.*, 1980; Saunders *et al.*, 1971; Gonias & Pizzo, 1983c).

Numerous physical studies describe the extensive structural changes that occur in α_2 M that is reacted with

proteinase (Gonias *et al.*, 1982a; Bjork & Fish, 1982; Straight & McKee, 1982; Barrett *et al.*, 1979; Cummings *et al.*, 1984; Strickland & Bhattacharya, 1984). In all of the above mentioned studies, except for the calorimetry study of Cummings *et al.* (1984), small primary amines react with α_2 M thioesters causing conformational change that is indistinguishable from the proteinase-induced change. The reacted forms of α_2 M have smaller Stokes radii than the native protein as determined by analytical sedimentation velocity (Gonias *et al.* 1982a; Bjork & Fish, 1982). Attempts to resolve the conformational forms of α_2 M with conventional gel filtration chromatography have been unsuccessful (Barrett *et al.*, 1979).

An important question in α_2 M chemistry is the extent to which bound proteinases are buried within the structure of the inhibitor and out of contact with other solutes. The original kinetic studies of Ganrot (1966, 1967) demonstrated that the active sites of bound proteinases are accessible to small chromogenic substrates. Larger substrates such as fibrinogen and soybean trypsin inhibitor also react with α_2 M-proteinase, albeit at a dramatically decreased rate (Harpel & Mosesson, 1973; Gonias & Pizzo, 1983b; Bieth *et al.*, 1981). The Stokes radius of α_2 M-trypsin complex with 2 mol of bound trypsin/mol is slightly larger than that of α_2 M-methylamine, although still smaller than the radius of native α_2 M (Gonias *et al.*, 1982a). A possible explanation for this is that the proteinase protrudes from the binding site pockets in a manner that increases the Stokes radius. A recent model of α_2 M structure, proposed by this laboratory (Feldman *et al.*, 1985), positions the two proteinase binding sites so that accessibility of solutes to bound proteinases and proteinase protrusion occur at the poles of the long axis of an ellipse. Therefore, the model allows for bound proteinases to affect the Stokes radius of the complex. There are no currently available reports of successful binding of anti-trypsin antibodies to α_2 M-trypsin complex. Cummings & Castellino (1984) utilized monoclonal antibodies to plasmin and demonstrated that a significant fraction of the plasmin heavy chain protrudes from binding sites when bound to α_2 M.

Although human α_2 M binds 2 mol of trypsin/mol (ternary complex), complexes containing 1 mol/mol (binary complex) and lacking further proteinase-binding activity may be formed at low trypsin activity (Howell *et al.*, 1983; Gonias & Pizzo, 1983c). A mathematical model describing random distribution of proteinase molecules into equivalent binding sites adequately predicts quantities of binary and ternary α_2 M-trypsin complex formed under specific reaction conditions (Gonias & Pizzo, 1983c; Pochon & Bieth, 1982; Strickland & Bhattacharya, 1984). The current investigation compares the chromatographic behavior of native α_2 M, α_2 M-methylamine, binary α_2 M-trypsin, ternary α_2 M-trypsin and α_2 M-plasmin, which is a predominantly binary complex. Large differences in the elution profiles of these complexes are demonstrated utilizing a variety of chromatography buffers. Conditions are presented for performing single-step purifications of native α_2 M from preparations containing significant amounts of 'reacted' forms. The differences in elution profiles demonstrated by the various forms of α_2 M reflect nonideal interactions with the TSK-G4000SW support. The α_2 M surface structure responsible for the nonideal chromatography interactions may be important in the biological function of the protein.

EXPERIMENTAL PROCEDURES

Materials

The sources of chemicals are listed elsewhere (Gonias & Pizzo, 1983a). α_2 M was purified by the method of Kurecki *et al.* (1979) as modified by Imber & Pizzo (1981). The $A_{280}^{1\%1\text{ cm}}$ of α_2 M is 8.93 (Hall & Roberts, 1978). Trypsin was purchased from Worthington and further purified by chromatography on Sephadex G-50 (Pharmacia) equilibrated in 1 mM-HCl as previously described (Gonias & Pizzo, 1983c). The final preparation was 80% active as determined by active site titration with *p*-nitrophenyl-*p*'-guanidinobenzoate (Chase & Shaw, 1967). Plasminogen was purified by the method of Deutsch & Mertz (1970) as modified by Brockway & Castellino (1972). Affinity chromatography variant 2 was utilized in all experiments. Streptokinase was purified from Kabikinase (Pharmacia) according to the method of Castellino *et al.* (1976). Plasminogen was activated with streptokinase as previously described (Gonias *et al.*, 1982b). Protein concentrations in purified preparations of plasminogen and streptokinase were determined spectrophotometrically (Gonias *et al.*, 1982b). Active plasmin concentration was determined by active site titration (Chase & Shaw, 1967).

α_2 M complexes.

Ternary α_2 M-trypsin was prepared by reacting α_2 M with trypsin at $r = 2.1$ (r is the molar ratio of active trypsin to α_2 M). In some experiments the trypsin was inactivated with 1.0 mM-phenylmethanesulphonyl fluoride. Mixtures of native α_2 M, binary α_2 M-trypsin and ternary α_2 M-trypsin were obtained with single additions of trypsin to α_2 M at $r < 2$. The relative concentrations of the three components were determined with the equations outlined by Gonias & Pizzo (1983c). Simplified systems for studying binary α_2 M-trypsin were obtained by sequentially adding trypsin to solutions of α_2 M according to the following two procedures. In the first set of experiments, α_2 M was reacted with trypsin at $r = 1.0$. This initial reaction converts 50% of α_2 M into binary α_2 M-trypsin and 25% of the α_2 M into ternary α_2 M-trypsin, both of which lack further proteinase binding activity (Gonias & Pizzo, 1983c). After 10 min, additional trypsin was added to the solution. The concentration of trypsin in the second portion was sufficient to convert all of the remaining native α_2 M into ternary α_2 M-trypsin so that the final preparation consisted of only two components (binary α_2 M-trypsin and ternary α_2 M-trypsin) in equal concentrations. In the second set of experiments, up to ten separate aliquots containing equivalent amounts of trypsin were added to each solution of α_2 M at 10 min intervals. The number of mol of active proteinase in each aliquot was one-tenth of the number of mol of α_2 M, so that after ten successive additions, equal concentrations of trypsin and α_2 M were present in solution. Theoretically, this procedure should yield solutions highly enriched in binary α_2 M-trypsin since small amounts of free trypsin react only with native α_2 M at each step to form predominantly binary complex (Gonias & Pizzo, 1983c). The calculated percentages of native α_2 M, binary α_2 M-trypsin and ternary α_2 M-trypsin after ten sequential trypsin additions are 6.4%, 87.5%, and 6.1% respectively.

α_2 M-plasmin complex was prepared by reacting α_2 M with a 4-fold molar excess of active plasmin for 60 min.

For most experiments, pure α_2 M-plasmin complex was resolved from unreacted plasmin by chromatography on Ultrogel AcA-22 (LKB Instruments). This procedure is described in detail elsewhere (Gonias & Pizzo, 1983b). All α_2 M-proteinase complexes were prepared at 22 °C in 20 mM-sodium phosphate/100 mM-sodium phosphate, pH 7.4. α_2 M-methylamine was prepared as previously described (Gonias *et al.*, 1982a). Unreacted methylamine was removed by dialysis.

H.p.l.c.

The h.p.l.c. system utilized for these experiments consisted of components purchased from LKB, including pump model 2150, h.p.l.c. controller model 2152, an LKB 2140 rapid spectral detector and recording integrator model 2220. A model 7125 syringe loading sample injector with a 100 μ l sample loop was used in all experiments. The loop was filled completely to ensure reproducibility. The size exclusion column was a 7.5 mm $\times$ 300 mm TSK-G4000SW (Toyo Soda Manufacturing Co.; model 01417) equipped with a 7.5 mm $\times$ 7.5 mm precolumn. The flow rate for all experiments was 0.25 ml/min. Data were analysed by using the Wavescan spectral detector program (LKB Instruments). The experiments were performed on two separate columns with equivalent results.

The four chromatography buffers used were: 20 mM-sodium phosphate/100 mM-NaCl, pH 7.2 (PBS); 80 mM-sodium phosphate/320 mM-NaCl/10% (v/v) ethanol, pH 7.2; 80 mM-sodium phosphate/320 mM-NaCl, pH 7.2; 20 mM-sodium phosphate/55 mM-NaCl/10% (v/v) ethanol, pH 7.2. All samples for chromatography, containing at least 75 μ g of protein, were equilibrated in the appropriate buffer before application to the sample loop. All elution volumes represent distance from sample loop to post-column absorbance detector. The void volume (V_0) of the column was 6.08 ml as estimated with Blue Dextran 2000 (Pharmacia). The total accessible volume, defined as the sum of V_0 and the pore volume, was estimated at 14.42 ml with NaN_3 . Each protein elution volume (V_e) represents the average of at least three experiments. The error value accompanying each V_e is the population s.d. Adsorption of solutes to the column support structure resulted in delayed elution and peak dispersion, manifested as a less sharp elution peak and tailing in the downslope of the peak. These parameters were estimated in each experiment so that comparisons could be made between the different protein complexes and chromatography buffers. The bandwidth (σ), was calculated as V_e minus the volume at $1/\sqrt{2}$ of the maximum height on the ascending limb of the peak. Tailing in the elution profile after reaching maximum peak height was estimated with the same calculation on the descending limb of the peak (τ). Column runs lasted 70–85 min unless otherwise specified. All chromatograms presented in this manuscript were obtained at $\lambda = 280$ nm.

Other techniques

α_2 M was radioiodinated by the solid state lactoperoxidase method (David & Reisfeld, 1974) as previously described (Gonias & Pizzo, 1983a). Nondenaturing polyacrylamide gel electrophoresis was performed using the Hepes/imidazole buffer system described by McLellan (1980). Denaturing polyacrylamide gel electrophoresis

was performed according to the procedure of Neville (1971). All samples were incubated in 1% SDS containing 8 mg of dithiothreitol/ml for 45 min at 37 °C prior to electrophoresis under denaturing conditions. Protein solutions eluted from the h.p.l.c. column were concentrated with Centricon 30 microconcentrators (Amicon) prior to electrophoresis. Up to 50-fold concentration was obtained with less than 30% loss of protein on the membrane.

RESULTS

Chromatography of single protein preparations

U.v. absorption monitoring showed that native α_2 M was eluted in a single, sharp, well demarcated peak from the column equilibrated in PBS. The V_e was 9.20 ml (Table 1). The indices of peak dispersion (σ and τ) were relatively small, indicating minimal interactions with the column support substance. The buffer system was altered in order to diminish either electrostatic or hydrophobic nonideal interactions. These manipulations minimally affected V_e or bandwidth. Tailing of elution after V_e was minimized in high ionic strength solutions without ethanol.

α_2 M-methylamine failed to be eluted in a discernible peak from the column equilibrated in PBS (Table 2). Radioiodinated α_2 M-methylamine was recovered slowly over a period of greater than 2 h (30 ml) starting well past the V_e of native α_2 M. Addition of either salt or ethanol to the chromatography buffer caused α_2 M-methylamine to be eluted in a readily detected peak, suggesting that both hydrophobic and electrostatic interactions caused adsorption of α_2 M-methylamine to TSK-G4000SW in PBS. Peak dispersion remained extensive compared with native α_2 M in all the chromatography buffers; σ and τ

Table 1. Chromatography of native α_2 M

The buffers are: High salt, 80 mM-sodium phosphate/320 mM-NaCl, pH 7.2, high salt plus ethanol, 80 mM-sodium phosphate/320 mM-NaCl/10% (v/v) ethanol, pH 7.2; low salt plus ethanol, 20 mM-sodium phosphate/55 mM-NaCl/10% (v/v) ethanol, pH 7.2. n represents the number of repetitions of the experiment.

Buffer	V_e (ml)	n	σ (ml)	τ (ml)
PBS	9.20 $\pm$ 0.04	5	0.23	0.37
High salt	8.84 $\pm$ 0.03	3	0.22	0.27
High salt + ethanol	9.34 $\pm$ 0.08	3	0.24	0.36
Low salt + ethanol	8.86 $\pm$ 0.01	3	0.25	0.37

Table 2. Chromatography of α_2 M-methylamine

For details see Table 1.

Buffer	V_e (ml)	n	σ (ml)	τ (ml)
PBS	No peak	5	—	—
High salt	11.94 $\pm$ 0.14	3	0.79	1.93
High salt + ethanol	11.90 $\pm$ 0.50	5	1.05	1.79
Low salt + ethanol	10.63 $\pm$ 0.16	4	0.63	1.49

Table 3. Chromatography of ternary α_2 M–trypsin

For details see Table 1.

Buffer	V_e (ml)	n	σ (ml)	τ (ml)
PBS	14.57 ± 0.20	3	1.24	—*
High salt	13.15 ± 0.08	3	0.35	0.96
High salt + ethanol	13.34 ± 0.15	4	0.25	0.36
Low salt + ethanol	10.73 ± 0.15	3	0.76	1.45

* Not calculated; see the text.

values were minimized in low ionic strength solution with ethanol. The V_e was also somewhat lower in the same buffer.

The behavior of ternary α_2 M–trypsin on TSK-G4000SW was similar to that of α_2 M–methylamine in many respects (Table 3). PBS once more proved an unsuitable buffer for recovery of the complex. The V_e was 14.57 ml and the bandwidth extremely large. The descending limb of the elution peak consistently took the form of a slowly declining boundary. Recovery within 75 min (18.75 ml) was not quantitative. High salt or ethanol independently improved elution. The sharpest peaks were obtained with the combined high salt and ethanol buffer [80 mM-sodium phosphate/320 mM-NaCl/10% (v/v) ethanol, pH 7.2]. The V_e of ternary α_2 M–trypsin was significantly greater than that of α_2 M–methylamine in high salt buffers with or without ethanol. The V_e , σ and τ of ternary α_2 M–trypsin in 20 mM-sodium phosphate/55 mM-NaCl/10% (v/v) ethanol, pH 7.2, were equivalent to the values for α_2 M–methylamine. The large decrease in the V_e of ternary α_2 M–trypsin in the low salt/ethanol buffer theoretically represents decreased adsorption to the support or ion exclusion. Ternary α_2 M–trypsin complex that had been inactivated with phenylmethanesulphonyl fluoride was indistinguishable from the active complex on TSK-G4000SW. No alterations in structure were apparent when ternary α_2 M–trypsin was exposed to 10% ethanol prior to electrophoresis under denaturing or non-denaturing conditions.

Co-chromatography of conformation variants

Experiments were performed in which mixtures of native α_2 M, ternary α_2 M–trypsin and α_2 M–methylamine were chromatographed simultaneously (two in each run). Fig. 1 shows the results obtained using high salt buffer. The V_e of each α_2 M variant remained unchanged in the presence of other forms of α_2 M. Resolution of native α_2 M and ternary α_2 M–trypsin was essentially complete, consistent with the data collected with the isolated proteins. A widely used quantitative expression for chromatographic resolution (R) is:

$$R = \frac{2(V_{e2} - V_{e1})}{\tau_1 + \sigma_2}$$

The subscript 1 refers here to native α_2 M and the subscript 2 refers to α_2 M–trypsin. The calculated R of 14.2 is much greater than those values determined for proteins of markedly different M_r on TSK-G4000SW (Kato *et al.*, 1980). The R value for native α_2 M and ternary α_2 M–trypsin in high salt/ethanol buffer was 12.6

Fig. 1. Chromatography of conformational variants of α_2 M in 80 mM-sodium phosphate/320 mM-NaCl, pH 7.2

Chromatogram *a* shows native α_2 M (75 μ g) and ternary α_2 M–trypsin (110 μ g). Chromatogram *b* shows native α_2 M (75 μ g) and α_2 M–methylamine (100 μ g). Chromatogram *c* shows α_2 M–methylamine (80 μ g) and ternary α_2 M–trypsin (80 μ g). Absorbance is in relative units that are not standardized for the three chromatograms.

(chromatogram not shown). As expected, less complete resolution was obtained in experiments with native α_2 M and α_2 M–methylamine or α_2 M–methylamine and ternary α_2 M–trypsin. The presence of a clear doublet peak in the chromatogram of α_2 M–methylamine and ternary α_2 M–trypsin confirms that the two forms of α_2 M interact differently with the support matrix.

Ternary α_2 M–trypsin inactivated with phenylmethanesulphonyl fluoride and native α_2 M were mixed at a molar ratio of approx. 1:2 and chromatographed in PBS. The starting material was compared with the protein that was eluted in the first chromatography peak by SDS- and non-denaturing polyacrylamide gel electrophoresis (Fig. 2). well-described bands on SDS electrophoresis include the $M_r \sim 180000$ subunit and the $M_r \sim 90000$ subunit fragments resulting from tryptic cleavage of the 'bait region' (Barrett *et al.*, 1979; Swenson & Howard, 1979). The bands seen in SDS with mobility less than that of the uncleaved subunit probably represent covalent adducts between a proteinase molecule and more than one α_2 M subunit cleavage fragment (Barrett *et al.*, 1979; Salvesen & Barrett, 1980). The starting material before chromatography contained significant amounts of cleaved and noncleaved α_2 M subunits.

In nondenaturing gel electrophoresis, the mobility of α_2 M increases when the protein undergoes conformational change after reaction with trypsin. This technique also demonstrated the heterogeneity in the α_2 M population before chromatography. Fig. 2 shows that within the limits of sensitivity of the two electrophoresis systems, the first peak recovered during chromatography contained pure native α_2 M. The time required for recovery of the purified preparation was less than 45 min.

Chromatography of α_2 M–plasmin

The V_e of purified α_2 M–plasmin was 12.88 ± 0.32 ml ($n = 3$) in 80 mM-sodium phosphate/320 mM-NaCl, pH 7.2. Significant peak dispersion was present ($\sigma = 1.36$, $\tau = 1.91$) consistent with extensive adsorption effects. Attempts to purify α_2 M–plasmin from unreacted plasmin by h.p.l.c. were not successful, since plasmin was

Fig. 2. Polyacrylamide-gel electrophoresis of native α_2 M purified from a solution containing ternary α_2 M-trypsin

Lanes (a) and (e) show native α_2 M. Lanes (b) and (f) show ternary α_2 M-trypsin. The solution subjected to chromatography is shown in lanes (c) and (g). This solution contained 360 μ g of native α_2 M and 190 μ g of ternary α_2 M-trypsin. The equilibration buffer was PBS. The first protein peak eluting at $V_e \sim 9.2$ ml was pooled and is shown in lanes (d) and (h). Lanes (a)–(d) and (e)–(h) show electrophoresis under denaturing (SDS) and non-denaturing (native) conditions, respectively.

Fig. 3. Chromatography of native α_2 with purified α_2 M-plasmin complex

The α_2 M-plasmin was present in 2.2-fold molar excess to the native α_2 M.

eluted in a peak that was superimposed on the descending limb of the α_2 M-plasmin peak. The α_2 M-plasmin complex purified by conventional chromatography was significantly resolved from native α_2 M when the two variants were co-chromatographed in high salt buffer (Fig. 3). The resolution was less complete ($R = 5.6$) than that obtained with native α_2 M and ternary α_2 M-trypsin the same buffer, reflecting predominantly the large σ of α_2 M-plasmin.

Chromatography of binary α_2 M-trypsin

α_2 M was reacted with trypsin at $r \leq 1.0$ and the mixture of components subjected to chromatography. The data are summarized in Table 4 and a representative chromatogram ($r = 1.0$) is shown in Fig. 4. Two peaks

Table 4. Chromatography of mixtures of native α_2 M, binary α_2 M-trypsin and ternary α_2 -trypsin in 80mM-sodium phosphate/320 mM-NaCl, pH 7.2

<i>r</i>	Calculated products of reaction (%) [*]				Experimental		
	Native	Binary	Ternary	Binary + ternary	Peak 1 (%)	Peak 2 (%)	V_e (ml) of peak 2
0.35 [†]	68	29	3	32	62	38	– [‡]
0.50	56	38	6	44	54	46	12.08
0.75	39	47	14	61	31	69	12.31
1.00	25	50	25	75	26	74	11.81
1.00	25	50	25	75	28	72	11.69
1.00	25	50	25	75	24	76	11.91

^{*} Based on equations presented in Gonias & Pizzo (1983c).

[†] In each experiment 120–200 μ g of protein was chromatographed.

[‡] Insufficient protein for calculation of V_e .

Fig. 4. Chromatography of the products of reaction between α_2 M and trypsin at $r = 1$

The equilibration buffer was 80 mM-sodium phosphate/320 mM-NaCl, pH 7.2.

Fig. 5. Chromatography of the products of ten sequential reactions of trypsin with α_2 M

The column was equilibrated in 80 mM-sodium phosphate/320 mM-NaCl, pH 7.2. The sample applied to the column contained 500 μ g of protein.

were consistently observed. The first peak demonstrated V_e , σ and τ values equivalent to those of native α_2 M chromatographed alone. This was followed by a relatively broad peak which occasionally included a small shoulder on the descending limb. The area under the two major peaks was measured as an index of the amount of protein eluted at each position. The percentage of protein in the second peak clearly correlated with the predicted sum of the percentage of binary and ternary α_2 M-trypsin at each r value. The percentage of protein in the first peak corresponded to the predicted percentage of native α_2 M alone. The V_e of the second peak (calculated at maximum peak height) was consistently smaller than the V_e of purified ternary α_2 M-trypsin complex. These data suggest that peak two represents the summation of two populations of α_2 M with different V_e values eluting in overlapping peaks.

α_2 M was reacted with trypsin at $r = 1.0$ and then with excess trypsin to generate a preparation with equal amount of binary α_2 M-trypsin and ternary α_2 M-trypsin and no native α_2 M. The preparation was eluted in a single peak with a V_e of 12.68 ml ($\sigma = 0.87$ ml, $\tau = 1.42$ ml). No protein was present in the V_e range 8.7–9.0 ml characteristic of native α_2 M.

Trypsin was added to a solution of α_2 M in ten equal portions at 10 min intervals. The final concentration of trypsin was equal to that of the α_2 M; however, far less protein was detected at the V_e of native α_2 M compared with a single addition of trypsin at $r = 1.0$ (Fig. 5). The theoretical yields of native α_2 M and binary plus ternary α_2 M-trypsin are 6.5% and 93.5%, respectively. The areas under the early ($V_e = 8.9$ ml) and late ($V_e = 12.2$ ml) eluting peaks were 10% and 90% of the total, respectively. Fractions eluting from the column were pooled as shown in Fig. 5 for analysis by polyacrylamide gel electrophoresis. Pool A consisted entirely of native

Fig. 6. Polyacrylamide-gel electrophoresis of fractions recovered from the chromatography of α_2 M reacted with trypsin in ten sequential steps.

Lanes (a) and (e) show native α_2 M. Lanes (b) and (f) are ternary α_2 M-trypsin. Lanes (c) and (g) are pool A from Fig. 5; lanes (d) and (h) are pool B from Fig. 5. Lanes (a)–(d) and (e)–(h) show electrophoresis under non-denaturing (native) and denaturing (SDS) conditions, respectively.

α_2 M that had not reacted with trypsin. The mobility of the protein on native polyacrylamide gel electrophoresis was low, indicating the active conformation (Fig. 6). SDS gel electrophoresis demonstrated that essentially none of the α_2 M subunits were cleaved. Pool B consisted almost entirely of protein that had undergone conformational change as indicated by increased mobility on native gel electrophoresis. Theoretically, 93.5% of pool B is binary α_2 M-trypsin; however, the mobility of the binary and ternary complexes are equivalent in this system (Gonias & Pizzo, 1983c). The small component of pool B migrating at the slow position most likely corresponds to the tail of the native α_2 M elution peak. In SDS/polyacrylamide gel electrophoresis, the predominant component of pool B was the $M_r \sim 90000$ subunit cleavage fragment. This suggests that, in binary α_2 M-trypsin, greater than two and as many as four α_2 M subunits are cleaved. The uncleaved $M_r \sim 180000$ α_2 M subunits present in pool B may represent the tail of the native α_2 M peak or a small percentage of the subunits in binary α_2 M-trypsin that remain uncleaved. This question is being studied in detail by other techniques.

DISCUSSION

Nonideal interactions with size exclusion columns alter the V_e of a protein compared with the V_e predicted by hydrodynamic parameters alone. For the most part, nonideal interactions result in adsorption of the protein to the surface of the support and an increased V_e . On TSK-GSW columns, solvophobic interactions represent a significant form of nonideal behavior (Pfannkoch *et al.*, 1980; Kopaciewicz & Regnier, 1982). A second form results from underivatized silanol groups that carry negative charges and act as weak cation exchangers. In certain buffers, these underivatized silanol groups can cause repulsion of anionic solutes as well (Pfannkoch *et al.*, 1980). This form of nonideal interaction may decrease the V_e of a protein and produce a somewhat sharper peak.

In this investigation, native α_2 M, α_2 M-methylamine and ternary α_2 M-trypsin were compared by chromatography in a number of different buffers. While native α_2 M demonstrated distinct behavior on the TSK-G4000SW column fundamental similarities existed between α_2 M-methylamine and α_2 M-trypsin. This was best demonstrated in PBS. Native α_2 M was eluted in a single sharp peak at a V_e that was not altered extensively by manipulation of equilibration buffer. By contrast, both α_2 M-methylamine and ternary α_2 M-trypsin were eluted very slowly without forming an identifiable peak. The majority of either preparation was eluted in a volume greater than the total accessible volume of the column. These data indicate that both α_2 M-methylamine and α_2 M-trypsin contain surface regions with significant affinity for the TSK-G4000SW structure. In native α_2 M, these regions are either buried or not oriented properly for similar interaction. Adsorption was decreased and protein peaks were obtained when either α_2 M-trypsin or α_2 M-methylamine were chromatographed in high salt buffer or low salt buffer containing ethanol. The high ionic strength of 80 mM-sodium phosphate/320 mM-NaCl, pH 7.2, should neutralize ionic interactions although solvophobic interactions may be somewhat enhanced. In contrast, 20 mM-sodium phosphate/55 mM-NaCl/10% (v/v) ethanol, pH 7.2, should minimize solute-support

interactions based on hydrophobicity. The relative efficacy of both high salt buffer and low salt buffer containing ethanol compared with PBS in the elution of α_2 M-methylamine and α_2 M-trypsin suggests that both hydrophobic and electrostatic interactions are involved in the adsorption of these proteins to the column support. The buffer consisting of 80 mM-sodium phosphate/320 mM-NaCl/10% (v/v) ethanol, pH 7.2, was similar to that described by Hefti (1982) for minimizing all forms of nonideal interactions. Peak dispersion was decreased significantly when α_2 M-trypsin was chromatographed in this buffer; however, considerably less sharp peaks were obtained with α_2 M-methylamine.

The contribution of electrostatic interactions to the retention of α_2 M-methylamine and α_2 M-trypsin on TSK-G4000SW is somewhat surprising, since α_2 M is anionic near neutral pH (Van Leuven *et al.*, 1981). Reaction with methylamine or trypsin increases the pI of α_2 M, but not sufficiently to change the net charge of the protein to positive at pH 7.2. Since electrostatic adsorption results predominantly from cation exchange, the α_2 M-'reacted form' surface structure must include focal collections of basic amino acids. Isoelectric focusing of α_2 M-trypsin and α_2 M-methylamine shows similar complex patterns not observed with native α_2 M (Van Leuven *et al.*, 1981). This heterogeneity in charge may contribute to the peak dispersion seen when nonideal electrostatic interactions are maximized by using low ionic strength buffer containing ethanol.

Many experimental techniques have been utilized in previous studies to demonstrate equivalence of the α_2 M conformations after reaction with proteinase or primary amine. Most of these techniques were sensitive to overall secondary and tertiary structure or hydrodynamic properties. More recent studies have focused on the surface structure of α_2 M. Strickland & Bhattacharya (1984) utilized a fluorescence probe, 6-(*p*-toluidino)-2-naphthalenesulphonic acid, and demonstrated an equivalent change in the surface structure of α_2 M after reaction with proteinase or methylamine. The change most likely consisted of a shift of hydrophobic amino acids to the surface near the binding site. Another recent study focused on the interaction of α_2 M with histone (Gonias *et al.*, 1984). α_2 M-methylamine and α_2 M-trypsin bound histone H3 in a manner that resulted in precipitation of the complex. Native α_2 M did not undergo a similar reaction. As was the case in the fluorescence studies, no differences in the surface interactions of H3 with α_2 M-proteinase or α_2 M-methylamine were identified. Despite many similarities in chromatographic behavior, ternary α_2 M-trypsin and α_2 M-methylamine were resolved by h.p.l.c. in some of the buffers studied. This result must reflect differences in the surface structure of α_2 M-proteinase complex compared with α_2 M-methylamine. The most consistent explanation is that the trypsin moieties contribute to the accessible external surface of the α_2 M complex so that they are available for nonideal interactions with the column support. The data would then suggest that models of α_2 M structure that position bound proteinase very deep in the core of the inhibitor are incorrect. Alternatively, we cannot rule out subtle differences in the structure of α_2 M after reaction with proteinase or methylamine. Such differences that might alter the V_e of the protein would be consistent with calorimetry data presented elsewhere (Cummings *et al.*, 1984).

A number of methods for preparing solutions with high concentrations of binary α_2 M-trypsin were used for the first time to demonstrate that the V_e of the binary complex is intermediate between the values of native α_2 M and ternary α_2 M-trypsin. The best approximation of V_e was probably obtained from the experiments in which serial additions of trypsin were used to obtain a preparation with nearly 90% binary complex ($V_e = 12.2$ ml). This V_e is slightly greater than the V_e of α_2 M-methylamine; however, the difference is within the margin of error and may not be significant.

Maximum stoichiometries of proteinase binding to α_2 M range from 1 to 2 mol/mol depending on the proteinase studied. Under physiological conditions, it is probably extremely rare that ternary α_2 M-proteinase complexes are formed due to the high concentration of α_2 M and the unlikelihood that proteinases capable of binding at stoichiometries near 2.0 would be present in sufficient concentration to do so. The binary complex with a vacant proteinase binding site probably represents the more commonly generated product. Therefore, characterization of the binary complex is important. As previously demonstrated, the mobilities of α_2 M with 1 or 2 mol of trypsin bound/mol are equivalent in non-denaturing electrophoresis (Gonias & Pizzo, 1983c). In the present investigation, it is shown that the number of α_2 M subunits cleaved by a single trypsin in binary complex is significantly greater than two and quite likely four. It is suggested that the second pair of α_2 M subunits are cleaved by the bound trypsin, since excess free trypsin was not available to react with the complex. Bovine α_2 M binds 1 mol trypsin/mol maximally and all four of the α_2 M subunits are cleaved (Feldman *et al.*, 1984). In addition, the subunits in human α_2 M-methylamine are cleaved by trypsin extremely slowly (Wang *et al.*, 1981; Gonias & Pizzo, 1983a; Eccleston & Howard, 1985), suggesting that forms of α_2 M other than native are relatively nonreactive with free proteinases.

From a practical standpoint, the chromatograms presented here showed excellent resolution of native α_2 M from ternary α_2 M-trypsin, binary α_2 M-trypsin and α_2 M-plasmin. Electrophoresis experiments demonstrated that purified native α_2 M can be recovered from solutions that contain forms of the nonreactive inhibitor relatively easily and rapidly. Thus, high-performance size exclusion chromatography on TSK-G4000SW represents the first reported procedure that may be applied to the preparative isolation of native α_2 M that is 100% active. This is most important, for biological studies that have frequently been confounded in the past by significant contamination of native α_2 M with the reacted conformational forms of the inhibitor.

Chromatography has frequently been used to determine protein Stokes radius. Such determinations rely upon minimizing nonideal interactions. This study demonstrates that extensive nonideal interactions may occur on TSK-G4000SW, independent of equilibration buffer. The extent of nonideal interactions is not readily predicted by and simple physicochemical parameters. It is concluded, therefore, that while TSK-G4000SW can be an excellent support for resolving proteins, it is probably not advisable to use this column for studying macromolecular hydrodynamics, at least with high- M_r proteins.

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