Studies on human pregnancy-associated plasma protein A

Purification by affinity chromatography and structural comparisons with α_2 -macroglobulin

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Pregnancy-associated plasma protein-A (PAPP-A) has been purified by a combination of methods including antibody-affinity chromatography. The resultant protein, obtained in 16% yield from maternal serum, appeared as a single major component on non-denaturing polyacrylamide and SDS/polyacrylamide gel electrophoresis. The protein showed a single component when analysed by isoelectric focusing under denaturing conditions in the presence and absence of reduction and had a pI of 4.34 and 4.42 respectively. These pI values were indistinguishable from those of α_2 -macroglobulin (α_2 M). The molecular weight of the PAPP-A polypeptide as shown by SDS/polyacrylamide-gel electrophoresis was 187 000, with a minor component of mol.wt. 82 500 that was attributed to proteolysis. Since native PAPP-A had a molecular weight on gel chromatography very similar to that of α_2 M (620 000–820 000), it was concluded that PAPP-A was a homotetramer. In the absence of reduction, a high-molecular-weight (420 000) protomer of PAPP-A was found. It was deduced that PAPP-A, like α_2 M, is a dimer, whose protomers are composed of disulphide-linked polypeptide chains. It was found that the molecular weight of the PAPP-A polypeptide exceeded that of α_2 M by 3.3%, but that the total carbohydrate content of PAPP-A exceeded that of α_2 M by 10% and that its neutral carbohydrate content exceeded that of α_2 M by between 7.4 and 9.0%. The significance of the estimated molecular weights of α_2 M (181 000) and its major tryptic fragments is discussed in the light of published values. A tryptic fragment of α_2 M (82 500 mol.wt.) was apparently the same size as the major tryptic fragment of PAPP-A.

Lin, Halbert and colleagues (Lin *et al.*, 1974; Halbert & Lin, 1979) have described a large protein antigen that they called 'pregnancy-associated plasma protein A' (PAPP-A). PAPP-A is macroglobulin with an α_2 -mobility in agarose gels, which was detectable by antibody antigen crossed electrophoresis in the serum of pregnant women from approx. 15 weeks of gestation onwards (see Lin & Halbert, 1976, for review) and whose concentration in maternal serum increases 5–10-fold during the course of the second half of pregnancy. Preliminary evidence from immunofluorescence microscopy indicated the presence of PAPP-A in placental trophoblast cells (Lin & Halbert, 1976), suggesting that the placenta is a source of PAPP-A and that the

increase in placental size during pregnancy accounted for the increasing concentration of PAPP-A in maternal serum from 15 weeks onwards. The protein is not detectable in umbilical-cord serum.

Independently, Sutcliffe (1976) and Sutcliffe *et al.* (1979) raised an antiserum against PAPP-A that cross-reacted with a sample of anti-PAPP-A that was kindly provided by Dr. T. M. Lin and Dr. S. P. Halbert and had been described by them (Lin & Halbert, 1976). The present paper describes the purification of PAPP-A with a combination of methods including antibody-affinity chromatography. Since it emerged that PAPP-A is of similar overall molecular weight and electrophoretic mobility to α_2 M, the latter was also purified, and an initial structural comparison between the two serum proteins is presented. This comparison was motivated in part by the knowledge that α_2 M is an

Abbreviations used: α_2 M, α_2 -macroglobulin; PAPP-A, pregnancy-associated plasma protein A; IgG, immunoglobulin G; SDS, sodium dodecyl sulphate.

inhibitor of active endopeptidases (see Barrett & Starkey, 1973), so that an indication of homology between the two proteins might provide testable hypotheses about the function of PAPP-A.

Experimental

Materials

Normal human serum was obtained from adult male volunteers and normal pregnancy serum was obtained from 25 women at delivery with their informed consent. The blood was collected through the vagina at delivery, NaN_3 was added to a final concentration of 0.01% (w/v), and the blood was allowed to clot at 4°C. The blood was centrifuged at 9000g for 20 min and the supernatant of serum was stored at -20°C.

Sephacrose of types 4B, 6B and DEAE-CL6B was obtained from Pharmacia (G.B.) Ltd. Ultragel AcA 34 was obtained from LKB. Chemicals and other materials were obtained either from sources described below or from BDH.

Cross-linked protein for molecular-weight standards was obtained from BDH (cat. no. 44230 R) and from the laboratory of J. R. C. Neuraminidase from *Clostridium perfringens* was obtained from Sigma Chemical Co. (cat. no. N9130). Endoglycosidase H from *Streptomyces griseus* was obtained from Sikagutu Kogyo Co., Chuo-Ku, Tokyo, Japan (30 units/mg of protein).

Sheep anti-PAPP-A was raised as described by Sutcliffe *et al.* (1979). Rabbit anti- $\alpha_2\text{M}$ was obtained from Behring Diagnostics (a division of Hoechst Pharmaceuticals).

Treatment of maternal serum samples for affinity chromatography

All procedures were carried out at 4°C. Normal pregnancy serum (1.7 litres) was adjusted to a final concentration of 1.2M-(NH_4)₂SO₄ with 4M-(NH_4)₂SO₄, pH 7.0. After being left for 1 h, followed by centrifugation at 5500g for 20 min, the supernatant was taken (the precipitate contained no detectable PAPP-A) and adjusted to 2M-(NH_4)₂SO₄ with 4M-(NH_4)₂SO₄, pH 7.0. After 18 h the preparation was centrifuged again and the supernatant contained no detectable PAPP-A. The precipitate was resuspended in 75 ml of phosphate-buffered saline (0.16M-NaCl/5 mM-potassium phosphate, pH 7.5) and dialysed against three changes of 2 litres of phosphate-buffered saline at 4°C. The non-diffusible material was then repeatedly passed over a column (2.5 × 10 cm) of Sepharose 4B to which had been coupled 450 mg of control IgG of serum from a non-immunized sheep (see below for details). The flow rate was 100 ml/h and the material was recycled through the column for 18 h, after which the material termed 'filtered maternal serum protein' was

washed out of the column in phosphate-buffered saline. The object of this step was to remove clot-like material that otherwise accumulated on the top of the antibody-affinity column in the next step. Accumulation of this material could not be prevented by recycling the material over pure Sepharose 4B or by the addition of 3.25 mg of sodium citrate/ml to inhibit further clotting. (The same problem was encountered when normal human serum was used instead of normal pregnancy serum).

Preparation of affinity-chromatography medium

All protein samples were coupled to Sepharose 4B by using the method of Porath *et al.* (1967) [see Sutcliffe *et al.* (1978) for details]. Before use, the material was packed into a column and extensively washed with 2M-KI in phosphate-buffered saline at 4°C, and then equilibrated with phosphate-buffered saline.

Sheep anti-PAPP-A (160 ml) was treated to remove residual antibody specificities for adult serum proteins by passing it over a column (2.5 cm × 9 cm) of Sepharose 4B to which had been coupled 390 mg of normal human serum proteins. The antiserum was eluted with phosphate-buffered saline at 50 ml/h and the column was regenerated by passing through 75 ml of 0.5M-acetic acid at 100 ml/h, followed by 300 ml of phosphate-buffered saline. The extent of adsorption was tested by two-dimensional antibody-antigen crossed electrophoresis using 1 μl of normal human serum as test antigen and 10 mg of antiserum protein/ml of agarose in the second-dimension gel. After the adsorption was complete, the IgG fraction (0.98 g of protein) was precipitated with Na₂SO₄ by the method of Kekwick *et al.* (1940). Control IgG was also prepared by this method and the respective antibody preparations were dialysed against 1000 vol. of 0.1M-NaHCO₃ and coupled to Sepharose.

Purification of PAPP-A

A sample of 'filtered maternal serum protein' (2.5 g of protein in 50 ml) was applied to a column (2.5 cm × 18 cm) of Sepharose 4B to which had been coupled 0.98 g of the IgG fraction from the sheep anti-PAPP-A serum (treated as described above). The column was equilibrated with phosphate-buffered saline. The sample was eluted with the same buffer at a flow rate of 100 ml/h at 4°C. The eluate from this column was passed through a Uvicord II analyser (LKB Instruments) and the A_{280} was recorded. When the A_{280} had begun to fall, the column was washed with 150 ml of 1.0M-NaCl/10 mM-potassium phosphate, pH 7.5. Phosphate-buffered saline was then applied to the column until the $A_{1\text{cm}, 280}$ in the effluent fell to less than 0.02.

Bound PAPP-A was eluted with 150 ml of 1.5 M-KI in phosphate-buffered saline at a flow rate of 100 ml/h at 4°C, followed by 500 ml of phosphate-buffered saline. The adsorbance, due to both KI and protein, was monitored and the protein peak (approx. 150 ml) was collected into a stirred beaker containing 150 ml of 10 mM-potassium phosphate, pH 7.5 (buffer A) at 4°C. Conductometric analyses of the eluate showed that the peak concentration of KI did not exceed 0.9 M in the eluate before the dilution with buffer A.

The eluted PAPP-A fraction was dialysed at 4°C against four changes of a 15-fold excess of buffer A to decrease the concentration of KI in the solution (measured by conductance) to below 2 mM-KI. The material was then applied (at 100 ml/h) to a column (1.5 cm × 35 cm) of DEAE-Sepharose CL6B equilibrated with buffer A. The column was then washed with 100 ml of buffer A before the bound protein was eluted at 56 ml/h with a linear gradient of 900 ml of 0.05 M-NaCl in buffer A and 900 ml of 0.4 M-NaCl/10 mM-potassium phosphate, pH 6.5 (buffer B). The elution of protein was monitored by A_{280} and that of PAPP-A by antibody-antigen crossed electrophoresis. The fractions enriched in PAPP-A were pooled and concentrated by ultrafiltration by using a 7.6 cm-diameter stirred ultrafiltration cell and an XM 100A ultrafiltration membrane (Amicon). The concentrated material was stored at -20°C before analysis.

Purification of α_2 M

Two preparations of human α_2 M were made, one from 10 ml of pooled normal human serum, the other from 10 ml of fresh normal human plasma. All procedures were carried out at 4°C. In both cases the sample was applied to a column (2.5 cm × 85 cm) of Ultragel Aca 34 gel-filtration medium equilibrated with phosphate-buffered saline. The protein was eluted at 40 ml/h and the adsorbance of the eluate was monitored. Antibody-antigen crossed electrophoresis was used to detect the peak fractions of α_2 M that were present in the protein eluted first from the column. The peak fractions were pooled (46–50 ml in total), dialysed against two changes of 2 litres each of buffer A and then applied to a column (1.5 cm × 33 cm) of DEAE-Sepharose CL6B. The unbound protein was eluted with buffer A at 160 ml/h and α_2 M was eluted with a linear gradient formed between 900 ml of buffer A and 900 ml of buffer B. α_2 M eluted at 0.13 M-NaCl; the peak fractions were pooled (20–40 ml in total) and concentrated to 6 ml by ultrafiltration with an XM-100A membrane. The concentrated protein was then applied to a column (2.5 cm × 30 cm) of Sepharose CL6B equilibrated with phosphate-buffered saline and was eluted in the same buffer at a

flow rate of 56 ml/h. In both preparations, the single major protein peak eluting from the column contained α_2 M. Immunoelectrophoresis with sheep anti-(whole human serum) antibody showed that α_2 M was the only major protein present in these preparations. The recoveries of α_2 M from human serum and plasma in these experiments were 36 and 56% respectively.

Cross-linking

α_2 M was cross-linked by using a modification of the method of Jones *et al.* (1978). α_2 M at 0.75 mg/ml in 0.1 M-triethanolamine/HCl, pH 8.0 (buffer C) was dialysed against 1000 vol. of buffer C. To 4 vol. of the dialysed protein was added 1 vol. of a solution containing 27.3 mg of dimethyl suberimidate freshly dissolved in 1 ml of buffer C adjusted to 0.2 M with respect to NaOH, adjusted to pH 8.0. The reaction time was 1 h at 20°C and the reaction was terminated by the addition of sample preparation buffer for SDS/polyacrylamide-gel electrophoresis.

Immunoelectrophoretic methods

Double immunodiffusion was performed as described by Ouchterlony (1967). One- and two-dimensional antibody-antigen crossed electrophoreses were performed as described by Laurell (1966), with the modification that 100 mM-Tris was present in the gel and tank buffers.

Polyacrylamide-gel electrophoresis

Non-dissociating gel electrophoresis, with 4% (w/v) polyacrylamide gels, was carried out as described by Ryrie (1975) except that the Triton X-100 was omitted.

SDS/Tris/polyacrylamide-gel electrophoresis was carried out in slab gels as described by Laemmli (1970), with the modification that the tank buffer was 52 mM-Tris/53 mM-glycine, containing 0.1% (w/v) SDS, as described by Marsden *et al.* (1976). SDS/phosphate/polyacrylamide-gel electrophoresis was carried out as described by Maizel (1971) and used in the analysis of cross-linked protein. Samples for electrophoresis were made up by one of two methods.

Method 1. Protein (5–20 μ g) was made up with urea to a final (urea) concentration of 5 M. When reduction was required, dithiothreitol was added to 0.14 M. Samples were incubated at 37°C for 45 min, then made up with SDS to 0.5% (w/v) and loaded on the gel immediately.

Method 2. Protein (2–20 μ g) was made up in 8% (w/v) β -mercaptoethanol/0.8% (w/v) SDS/23% (v/v) glycerol. The sample was then boiled for 3 min and applied to the gel. After electrophoresis the gels were stained for 30 min in 0.2% (w/v) Coomassie

Brilliant Blue in 50% (v/v) methanol/10% (v/v) acetic acid at 60°C. They were destained in 25% (v/v) methanol/10% (v/v) acetic acid at 60°C and stored in 10% (v/v) acetic acid.

Determination of polypeptide molecular weights

The following molecular-weight marker proteins were used: myosin (mol.wt. 220 000; kindly given by Dr. J. W. Dow, Department of Biochemistry, University of Glasgow); the *arom* multienzyme complex (mol.wt. 165 000; Lumsden & Coggins, 1977); RNA polymerase from *Escherichia coli* (mol.wt. of β -subunit 150 000; of β' -subunit, 160 000; and of α -subunit, 40 000; Coggins *et al.*, 1977); bovine serum albumin (mol.wt. 68 000); pyruvate dehydrogenase of *E. coli* (mol.wt. of its chains 100 000, 80 000 and 56 000 respectively; Coggins *et al.*, 1976); ovalbumin (mol.wt. 43 000) and β -lactalbumin (mol.wt. 18 400). Cross-linked reference proteins were used as specified under 'Materials' (mol.wt. range 53 000–424 000).

Isoelectric focusing

Isoelectric focusing was performed in 6% (w/v) polyacrylamide tube gels containing 6 M-urea with 1.2% (v/v) of Ampholine of pH range 4–6 and 0.4% (v/v) of ampholine of pH range 3.5–10. The cathodal and anodal buffers were 0.4% ethanolamine and 0.2% H₂SO₄ respectively. Samples for analysis were prepared in a final concentration of 6 M-urea and 0.1% (v/v) of ampholine of pH range 4–6, with 14 mM-dithiothreitol when reduction was required. Samples were loaded at the cathodal end under a protective layer of 5.0% (v/v) glycerol and 0.1% (v/v) of ampholine of pH range 4–6, and were run for 15 h at 0.5 mA/tube. After focusing, the gels were washed in 12% (w/v) trichloroacetic acid at 60°C for 40 min and stained as for the SDS/polyacrylamide gels.

Protein precipitation

Trichloroacetic acid (12.5%, w/v, at 4°C) was added to an equal volume of protein solution. After standing on ice for 15 min, the precipitate was recovered by centrifugation at 2500 g for 15 min. For precipitation with phosphotungstic acid, the protein solution (1 ml) contained 6% (v/v) final concentration of trichloroacetic acid. To this was added 0.2 ml of 5% (w/v) phosphotungstic acid in 2 M-HCl at 4°C. After 10 min at 4°C the pellet was recovered by centrifugation as described above.

For acetone precipitation, 1 ml of protein solution was added to 5 ml of acetone and, after 15 min at room temperature, the pellet was recovered by centrifugation.

Determination of carbohydrate

Total carbohydrate was estimated by the anthrone method (Koehler, 1952). Neutral carbohydrate were assayed by the phenol/H₂SO₄ method of Dubois *et al.* (1956), using galactose (BDH) as standard. The protein for analysis (400–600 μ g/ml) was either in phosphate-buffered saline or had been resuspended in water after precipitation in trichloroacetic acid, trichloroacetic acid/phosphotungstic acid, or acetone. As judged from the standard curve and control proteins, these precipitation procedures did not interfere with the carbohydrate assay.

Carbohydrate digestion

To 8 μ g of protein (PAPP-A or α_2 M) in phosphate-buffered saline was added 0.12 unit of neuraminidase in a total volume of 35 μ l containing 30 mM-sodium citrate buffer, pH 5.5. The mixture was incubated for 0.5 h at 37°C. For endoglycosidase H digestion, 8 μ g of protein was added to 1 munit of endoglycosidase H (Tarentino *et al.*, 1974) in a total volume of 40 μ l in 11 mM-sodium citrate/27 mM-Na₂HPO₄, pH 5.4. The enzyme had previously been examined for proteinase activity by using controls of bovine serum albumin and denatured bovine serum albumin that had been reduced and alkylated. No proteolytic activity was observed. In this experiment, reduction was carried out by adding bovine serum albumin (to a final concentration of 5.5 mg/ml) to a solution of 36 mM-dithiothreitol/0.8 M-urea/0.24 M-Tris/HCl, pH 8.4. This was incubated at 37°C for 0.5 h and then made up to 0.1 M with respect to iodoacetamide. Incubation was continued for 0.25 h and terminated by the addition of 5 vol. of acetone. The reduced and alkylated bovine serum albumin was recovered as a precipitate after centrifugation at 5000 g and was resuspended in 50 mM-sodium phosphate buffer, pH 7.5.

Trypsin digestion

To 5–10 μ g of PAPP-A or α_2 M (0.2–0.6 mg/ml in phosphate-buffered saline) was added 2 μ l of trypsin (5–500 μ g/ml) and the mixture was kept at 18°C for 3 min before the reaction was stopped by making up the sample for SDS/polyacrylamide gel electrophoresis by Method 1 (above). For acid-denaturation experiments, 2 μ l of 1 M-HCl was added to the protein, followed after 1 min by 1 M-NaOH and 2 M-Tris/HCl, pH 8.4.

Determination of protein

Protein was assayed by the folin method (Lowry *et al.*, 1951), with bovine serum albumin as standard. Acid-precipitated samples were first neutralized.

Results and discussion

Specificity of antisera

Sheep and rabbit anti-PAPP-A antisera were adsorbed with male human serum protein coupled to Sepharose. The resultant adsorbed antisera were tested by antibody-antigen crossed electrophoresis. No reactions were found with non-pregnant-human serum as a source of antigen, but one α_2 -mobility precipitation arc was obtained with maternal serum (at term) as antigen (Fig. 1a). The antigen eluted from gel-filtration columns (see below) as a macro-globulin, much as described by Halbert & Lin (1979); it cross-reacted with an anti-PAPP-A serum kindly supplied by Dr. Halbert. As shown in Fig. 1(b), sheep anti-PAPP-A and rabbit anti- α_2 M do not reveal any cross-reactivity between pure PAPP-A and α_2 M.

Purification of PAPP-A

As Table 1 shows, only 4% of the PAPP-A was lost in the fractionation steps before the affinity chromatography. The quantity of filtered maternal serum protein applied to the affinity column was adjusted so that all the PAPP-A was bound to the column and thus the fraction of PAPP-A subjected to affinity chromatography was representative of a major fraction of PAPP-A in serum. Three peaks of material adsorbing at 280nm were eluted from the affinity column (see Fig. 2). NaCl (1M) eluted approx. three times as much protein from the column as did the subsequent elution with KI (see Table 1). Some of the adsorbance in the third peak in Fig. 2 is due to the adsorbance of KI at 280nm. No PAPP-A was detected in the effluent before the elution with KI and, since PAPP-A is stable in 1M-NaCl (results not shown), it was concluded that 1M-NaCl did not elute PAPP-A from the column.

To obtain an estimate of recovery of PAPP-A and protein from the affinity-chromatography step, the PAPP-A fraction (see Fig. 2) was dialysed (see the Experimental section) and then concentrated by ultrafiltration to 46ml (see Table 1, upper panel), yielding a recovery of PAPP-A of 21% and a purification of 27.8-fold. However, this procedure repeatedly resulted in precipitation of protein as the same was concentrated. This occurred not only in the present experiments, but also in similar experiments on the purification of human α -uterine protein (Sutcliffe *et al.*, 1980a) and of α -fetoprotein (R. G. Sutcliffe, unpublished work). Thus interpretation of recovery data from the affinity-chromatography step is difficult, and the dialysed fraction of PAPP-A from the affinity column was not concentrated routinely before ion-exchange chromatography. Because of the small amount of protein present in the PAPP-A fraction, nine fractions were pooled before the ion-exchange step (Table 2b). In

Fig. 1. Serological characteristics of anti-PAPP-A (a) Antibody-antigen crossed electrophoresis using 2% sheep anti-PAPP-A and 1.5% rabbit anti- α_2 M was used to examine these proteins. PAPP-A precipitin arcs are observed when 10 μ l of maternal serum (track 2) but not when 10 μ l of non-pregnant serum (track 1) is used. Both antigens together in the same well (track 3) yield a precipitin arc of similar size to track 2. (b) Purified PAPP-A (1 μ g; the present paper) and 1.0 μ g of purified α_2 M (the present paper; each in 5 μ l of phosphate-buffered saline) were subjected to antibody-antigen crossed electrophoresis by using a mixed bed of 1.5% rabbit anti- α_2 M (m) and 2% sheep anti-PAPP-A (p). When the proteins were applied to antigen wells close together, no reaction of identity is observed between the overlapping precipitin arcs.

three ion-exchange experiments, PAPP-A eluted from DEAE-Sepharose as a single unimodal peak, with the maximum concentration of PAPP-A eluting at 0.25M-NaCl (see Fig. 3). When 'filtered maternal serum protein' was fractionated in parallel experiments on the same ion-exchange column, PAPP-A eluted at the same NaCl concentration. For comparison, the two preparations of α_2 M (see

Table 1. *Purification of PAPP-A from maternal serum*

(a) Shows the details from the $(\text{NH}_4)_2\text{SO}_4$ precipitation and filtration of a single sample of delivery blood, followed by the antibody-affinity chromatography step. (b) Shows details of the PAPP-A and protein contents firstly for the pooled eluates after a total of nine portions of filtered maternal serum protein had been applied in succession to the affinity column and, secondly, for the protein in the single subsequent ion-exchange step. One unit of PAPP-A activity is defined as that quantity of PAPP-A present in 1 ml of a large pool of pregnancy sera used as a reference stock. The pregnancy sera were obtained from women at term. The quantity of PAPP-A was determined by antibody-antigen crossed electrophoresis.

	Volume (ml)	Protein (mg)	PAPP-A (units)	Specific activity of PAPP-A (units/mg of protein)	PAPP-A (% recovery)
(a) Initial treatments					
Delivery serum	100	6400	112	0.018	100
2 M- $(\text{NH}_4)_2\text{SO}_4$ fraction	50	3110	110	0.035	98
Filtered maternal serum protein	50	2950	107	0.036	96
Affinity chromatography					
Not retained on column in phosphate-buffered saline	540	2760	0	0	0
Eluted with 1 M-NaCl	230	144	0	0	0
Eluted with 1.5 M-KI/phosphate-buffered saline	285	46	23	0.500	21
(b) Filtered maternal serum protein (nine cycles of 50 ml each)					
DEAE/Sephacrose CL6B fraction after nine cycles of affinity chromatography	23	17.4	158	9.100	16

Fig. 2. *Affinity chromatography of 50 ml of filtered maternal serum protein for PAPP-A*

The Figure shows transmission at 280 nm of the effluent from the antibody affinity column (see the text for details). Filtered maternal serum was applied at 'zero' volume. The vertical arrows show the points of application of 1 M-NaCl (NaCl), 1.5 M-KI/phosphate-buffered saline (KI) and phosphate-buffered saline (P). The horizontal bar indicates the fractions enriched in PAPP-A, detected by antibody-antigen crossed electrophoresis.

Fig. 3. *Ion-exchange chromatography of the dialysed fractions containing PAPP-A from antibody-affinity chromatography*

KI effluents from the affinity chromatography were dialysed and pooled, applied to a Sepharose DEAE-CL6B column and eluted with a salt gradient (see the text). $A_{1\text{cm},280}$ (O) and the concentration of NaCl (----) were measured. The horizontal bar indicates the fractions containing PAPP-A, detected by antibody-antigen crossed electrophoresis.

the Experimental section) eluted as unimodal peaks, but the maximum concentration of $\alpha_2\text{M}$ eluted at 0.13 M-NaCl in both cases.

The ion-exchange fractions of protein that contained PAPP-A (see horizontal bar in Fig. 3) were pooled into four consecutive pools of equal volume and were analysed by electrophoresis. A single major band of protein was observed on non-dissociating and SDS/polyacrylamide tube gels (Figs. 4a and 4b). On SDS/polyacrylamide gels a

minor component was observed of mol.wt. 82 500. On immunoelectrophoresis there was a single arc with anti-PAPP-A and no arcs with anti-(normal human serum) antiserum.

Isoelectric focusing

When PAPP-A and $\alpha_2\text{M}$ were subjected to isoelectric focusing in urea they focused as single protein bands with indistinguishable isoelectric points (pI 4.42). When the proteins were reduced, their isoelectric points were indistinguishable at

Fig. 4. Analysis of PAPP-A

(a) Non-denaturing 3%-polyacrylamide-tube-gel electrophoresis of 15 µg of PAPP-A. (b) PAPP-A (15 µg) run on SDS/3.5% polyacrylamide tube gels, after treatment with (1) and without (2) dithiothreitol. See 'Method 1' under 'Polyacrylamide-gel electrophoresis' in the Experimental section. The molecular-weight standards were calculated from the R_m values of standard proteins. (c) Analysis of PAPP-A (tracks 1-3) and α_2 M (tracks 4-7) on a 5.5% polyacrylamide/SDS slab gel. The protein in tracks 1, 2, 6 and 7 had been treated with endoglycosidase H (e) and that in tracks 2, 5 and 7

pI4.34. A mixture of PAPP-A and α_2 M yielded a single focused band. Amino acid analyses by Bischof (1979) indicated that α_2 M and PAPP-A have similar amino acid compositions and this may explain their similar isoelectric points.

Quantity of PAPP-A in maternal serum

As it appeared that the PAPP-A was of high purity, it was possible to calculate the quantity of PAPP-A in maternal serum at term by means of antibody-antigen crossed electrophoresis. A pool of pregnancy sera was made from 22 mothers at term. The PAPP-A concentration in this pool was 0.105 mg/ml. The concentration of α_2 M in serum lies between 2.0 and 4.0 mg/ml.

Quaternary structure

In the present experiments, the apparent molecular weight of the α_2 M polypeptide was 181000. In parallel experiments using the same standard proteins for calibration, the apparent molecular weight of the PAPP-A polypeptide was consistently about 6000 larger. The value obtained for α_2 M differs from that of 185000 published by Harpel (1973), Hall & Roberts (1978) and Swenson & Howard (1979). This may reflect the use of different standard proteins for calibrating the gel, but does not invalidate the comparison of α_2 M and PAPP-A polypeptide sizes in the present paper.

α_2 M is a homotetramer (see Swenson & Howard, 1979) whose native molecular weight has been reported as between 620000 and 820000 (see Hall & Roberts, 1978). It can be divided into two 370000-mol.wt. protomers, each consisting of two disulphide-linked polypeptides of 185000 mol.wt. (Frénay & Bourrillon, 1974). On interaction with a variety of endopeptidases, the polypeptides of α_2 M are cleaved to two 85000-mol.wt. fragments (Harpel, 1973; Swenson & Howard, 1979).

In the present study, α_2 M could be dissociated with urea and dithiothreitol into a subunit of 181000 mol.wt. and with urea alone into a subunit of 320000 mol.wt. When α_2 M was cross-linked with dimethyl suberimidate and subjected to reduction in the presence of SDS, three bands were observed on the gel, the smallest corresponding to 182000 mol.wt.; the middle-sized one being estimated to correspond to 340000 mol.wt. and the largest corresponding to 750000 mol.wt. (by extrapolation). These bands were interpreted as monomer, dimer and tetramer respectively. PAPP-A treated with urea under reducing and non-reducing

with neuraminidase (n) by using the protocol described in the Experimental section. Protein was prepared for electrophoresis by 'Method 2' (see the Experimental section).

conditions gave molecular weights somewhat greater than the monomeric and dimeric α_2M species respectively (see Figs. 4*b* and 4*c*).

The estimated molecular weight of the non-reduced PAPP-A was 420 000. Since the native PAPP-A molecule co-elutes with α_2M on an Ultragel AcA 34 column (Fig. 5) and on a Sepharose CL4B column (results not shown), and since the latter has a native mol.wt. of approx. 620 000–820 000, PAPP-A is most probably a tetrameric protein composed, like α_2M , of dimers of disulphide-bonded polypeptide chains.

Degradation products

Although there was a single major electrophoretic component in reduced α_2M (mol.wt. 181 000), some low-molecular-weight components were also observed (provided the sample was reduced). Of these components, the predominant one had a mol.wt. of 90 000, though a minor one of mol.wt. 82 500 was also observed. When purified α_2M was treated with an equimolar amount of bovine trypsin and was then reduced, SDS/polyacrylamide gels (run at 40 mA for 4 h) revealed an increase in the relative amount of the 90 000-mol.wt. band and a decrease in the quantity of the 181 000-mol.wt. band (Fig. 6, tracks 2–5). If the protein was not reduced before electrophoresis, the 90 000-mol.wt. band was not visible. This suggested that the 90 000-mol.wt. polypeptide in the present work corresponds to that seen as an 85 000-mol.wt. component by Harpel (1973) and by Swenson &

Howard (1979) and that the difference in apparent molecular weights in these three studies is probably due to the use of different molecular-weight standards. However, when similar slab gels were run at a lower current (9 mA for 17 h) the 90 000-mol.wt. band resolved into two bands corresponding to mol.wts. 91 000 and 85 000 respectively. Bearing in mind the presence of the 82 500-mol.wt. components as well, this seemed a somewhat similar result to that of Harpel (1973), who found that chymotrypsin cleaved α_2M to yield three bands that migrated very closely together on SDS/polyacrylamide gels, the middle band corresponding to apparent mol.wt. of 85 000.

When similar experiments were carried out on PAPP-A, the trypsin treatment resulted in an increase in the 82 500-mol.wt. component (Fig. 6) that was present as a faint band in pure PAPP-A (Fig. 4*b*) and was only observed when the protein was reduced before electrophoresis. When acid-denatured PAPP-A or α_2M (see the Experimental section) were buffered at pH 7.5 and treated with either an equimolar amount of trypsin, or 1/10 or 1/100 of that quantity of trypsin, effectively no protein bands were observed on SDS/polyacrylamide gels under reducing conditions. It was concluded that the generation of the 82 500–91 000 mol.wt. components from PAPP-A and α_2M is dependent on these proteins being in their natural conformations.

Carbohydrate content

The content of neutral sugars was investigated by the phenol/ H_2SO_4 method of Dubois *et al.* (1956) and compared with the results for α_2M . The precipitation steps (see Table 2) with trichloroacetic acid, phosphotungstic acid and acetone were included in order to distinguish between free and protein-bound carbohydrate in the expectation that some free carbohydrate might exist in the protein samples. However, no evidence for free carbohydrate was found for PAPP-A, and a value of 12.9% was obtained for its neutral carbohydrate content. In the present work the mean value of neutral carbohydrate in α_2M without precipitation compared closely with the published values of 3.08–4.5% (see Hall & Roberts, 1978). However, the effect of precipitation on α_2M was to increase its apparent carbohydrate content. The cause of this increase is unclear. It is not due to effect of residual acid on the folin determination, since the resuspended precipitate was neutralized before the protein was assayed. Such an effect of acid would also be expected for PAPP-A, and this was not found.

The content of total carbohydrate in PAPP-A and α_2M was assayed by the anthrone method (Table 2). The mean value for α_2M (8.6%) can be compared

Fig. 5. Gel filtration of filtered maternal serum protein with analyses of PAPP-A and α_2M .

Filtered maternal serum protein (5 ml) was applied to a column (35 cm $\times$ 2.5 cm) of Ultragel AcA 34 equilibrated in phosphate-buffered saline. The protein was eluted at 50 ml/h in phosphate-buffered saline and monitored via transmission at 280 nm (—). The concentrations of PAPP-A (Δ) and α_2M (O) were measured by antigen-antibody crossed electrophoresis and the results expressed as area (mm²) under each precipitation arc.

Fig. 6. Analysis of PAPP-A and α_2 M and their tryptic cleavage products SDS/4.5–12.5% polyacrylamide slab gels were used. Tracks 2–5, α_2 M; tracks 7–10, PAPP-A. Tracks 1 and 6, pyruvate dehydrogenase standard (10 μ g) (a, b and c are its chains, of 100 000, 80 000 and 56 000 mol.wt. respectively); tracks 4 and 5, α_2 M treated with 5 M-urea in the presence (track 4) and absence (track 5) of dithiothreitol; track 3, as track 4, except the α_2 M has been treated with 2:1 molar ratio of trypsin/ α_2 M; tracks 7 and 8, PAPP-A pretreated with 1:1 molar ratio of trypsin and the protein prepared for electrophoresis as tracks 5 and 4 respectively; track 9, PAPP-A treated as for track 5; track 10, PAPP-A treated as for track 4.

Table 2. Carbohydrate content of PAPP-A and α_2 M

Neutral and total carbohydrate (assayed by the phenol/H₂SO₄ and anthrone methods respectively) was measured in samples of protein, some of which were first subjected to precipitation. Each value was obtained as the mean of at least two determinations. For proteins not precipitated, the values in parentheses refer to the number of independent determinations (each in duplicate) and the values in square brackets represent the range of values observed. The final column shows the difference between the mean values of carbohydrate for PAPP-A and α_2 M. Abbreviations used: TCA, trichloroacetic acid; PTA, phosphotungstic acid.

Assay	Method of precipitation	Mean carbohydrate content (g/100 g of protein)		
		PAPP-A	α_2 M	PAPP-A minus α_2 M
Phenol/H ₂ SO ₄	(a) TCA	10.0	4.7	—
	(b) TCA plus PTA	12.9	6.2	—
	(c) Acetone	15.8	5.6	—
	Mean of (a)–(c)	12.9	5.5	7.4
	(d) No precipitation	12.9 (4) [9–15.7]	3.9 (3) [3.8–4.0]	9.0 —
Anthrone	(d) No precipitation	19.2 (3) [17.7–21.5]	8.6 (3) [7.3–9.6]	10.6 —

Table 3. *Precipitation of protein in trichloroacetic acid (2 ml; 6.25% final concentration at 4°C)*

	Total Protein (μ g)	Protein precipitated (μ g)	Percentage precipitated
α_2 M	910	440	48.4
	910	540	59.3
PAPP-A	420	30	7.1
	420	20	4.8
	500	10	2.4

with those of 6.8–10.3% (mean 8.4%) summarized by Hall & Roberts (1978). The mean value for PAPP-A was 19.2%.

Unlike α_2 M, it was found that PAPP-A precipitated to only a small extent in 6.25% (w/v) trichloroacetic acid (see Table 3) and that precipitation was increased by adding phosphotungstic acid to the trichloroacetic acid or by using acetone instead. Similar observations have been made for a range of human serum glycoproteins (Putnam, 1965) and for bovine fetuin, whose high carbohydrate content (25%; Spiro, 1960) presumably increases its acid solubility through its relatively high surface density of hydroxy groups.

In an attempt to obtain corroborative evidence for the carbohydrate content of the proteins, α_2 M and PAPP-A were pretreated with neuraminidase and endoglycosidase H before analyses on SDS/polyacrylamide gels (see Fig. 4c). The quantity of enzymes used was adjusted so that mobility changes observed on the gels were maximal. Neuraminidase decreased the apparent size of both proteins by approximately the same proportion (about 2%). Endoglycosidase H had no detectable effect on α_2 M, but decreased the apparent size of PAPP-A by about 1.5%. A greater effect on mobility was observed when both enzymes were added to PAPP-A, but the subsequent addition of endoglycosidase H to α_2 M after it had been treated with neuraminidase caused no further increase in mobility.

Extent of similarity between PAPP-A and α_2 M

These results provide further support for the notion (Sutcliffe *et al.*, 1980b; Bischof, 1979, 1980) that PAPP-A and α_2 M are similar proteins with a similar quaternary structure. The electrophoresis results indicate that the molecular weight of the PAPP-A polypeptide exceeds that of α_2 M by about 6000 or 3.3%. Since the total carbohydrate contents of the proteins differ by about 10.6%, this suggests that the PAPP-A polypeptide is smaller than that of α_2 M (at a rough approximation 151 000 compared with 165 000 respectively).

Bischof (1979) has provided an indication that PAPP-A may inhibit plasmin or urokinase. In

addition, his work suggests that PAPP-A may inhibit the process of complement-induced haemolysis. However, lack of data on stoichiometry and specificity makes these interesting results difficult to evaluate at present. The suggested inhibition of plasmin by PAPP-A would have a clear functional parallel with the effect of α_2 M on plasmin (see Barrett & Starkey, 1973).

Subunit size of α_2 M

Since there are no proteins of known sequence that are large enough to be used as SDS/polyacrylamide-gel standards in the molecular-weight range 150 000–190 000, estimates of subunit molecular weights (never precise) must be interpreted with caution. Our estimate of the subunit size of α_2 M (181 000) is somewhat smaller than the value of 185 000 found by Harpel (1973) and Hall & Roberts (1978); it is, however, comparable with one quarter of the native mol.wts. of 718 000 and 725 000 (i.e. 179 500 and 181 250 respectively) estimated by sedimentation equilibrium by Hall & Roberts (1978) and Jones *et al.* (1972).

It is difficult to gain precise information about the molecular weight of the whole α_2 M polypeptide from the molecular weights of degradation products, since it is not clear how many fragments are generated. There are now three reported large fragments (91 000, 85 000 and 82 500 mol.wt.) and one small (3500–4000 mol.wt.) glycopeptide (Pan *et al.*, 1980). If the 91 000-mol.wt. component were the product of the initial tryptic cleavage, then the 85 000- and 82 500-mol.wt. components could be further degradation products [perhaps yielding the 3500–4000 mol.wt. glycopeptide of Pan *et al.* (1980)]. Alternatively the 85 000-mol.wt. component could result from an original tryptic cleavage just off the centre of α_2 M, yielding a 91 000-mol.wt. component as well. It does, however, seem unlikely that the molecular weight of the native α_2 M polypeptide exceeds $2 \times 91 000$ (182 000), since 91 000 is the molecular weight of the largest tryptic fragment.

When α_2 M is digested with increasing molar ratios of trypsin (4:1), lower-molecular-weight fragments are observed (Hall & Roberts, 1978). We have consistently observed a band corresponding to a mol.wt. of 82 500. This size corresponds closely to the size of the major fragment when PAPP-A is digested with trypsin.

Possible origins of PAPP-A

At present, although there is no genetic information, it seems probable that PAPP-A is a protein of placental origin that is structurally related to α_2 M and that is expressed in the placenta from a structural gene distinct from that of α_2 M. However, it is also possible that PAPP-A is a modified form of α_2 M resulting from the expression, by the structural gene

for α_2 M in the placenta, of a product that is modified after transcription in a way different from that occurring when α_2 M is synthesized in other tissues. Another possibility is that some α_2 M from blood is taken up and is converted into small quantities of PAPP-A. The evidence from immunofluorescence that PAPP-A is detectable in placental trophoblast (Lin & Halbert, 1976) does not rule out any of these possibilities. The lack of serological cross-reactivity between PAPP-A and α_2 M will only bear on this question when the nature of their antigenic determinants are known.

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