Rat α_2 Acute-Phase Macroglobulin

ISOLATION AND PHYSICOCHEMICAL PROPERTIES

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1. Rat α_2 acute-phase macroglobulin was isolated from turpentine-injected rats by Sephadex G-200 chromatography and ion-exchange chromatography on DEAE-cellulose. This method, since it does not include $(\text{NH}_4)_2\text{SO}_4$ treatment, allows the study of the physicochemical as well as the biological properties of the molecule. 2. The purity of the preparation was demonstrated by ultracentrifugation, polyacrylamide-gel electrophoresis, fused 'rocket' immunoelectrophoresis as well as double immunodiffusion. 3. The rat α_2 acute-phase macroglobulin was characterized in terms of its main physical and chemical properties. Its isoelectric point was determined by isoelectrofocusing to be 4.55; $s_{20,w}^0$ was 18.4S and $E_{1\text{cm}}^{1\%}$ at 278nm was 6.8. The mol.wt. was determined by light-scattering to be 770000. 4. The amino acid content was compared with that of rat α_1 macroglobulin and was found very similar. The carbohydrate composition of α_2 acute-phase macroglobulin was determined to be: hexose, 4.25%; glucosamine, 3.4%; sialic acid, 2%; fucose, 0.2%. From these results it was concluded that α_2 acute-phase macroglobulin, although a typical acute-phase reactant, possesses the characteristic physicochemical properties of α macroglobulins.

Rat α_2 acute-phase macroglobulin has been demonstrated under a variety of physiological and pathological conditions (Darcy, 1955; Beaton *et al.*, 1961; Heim, 1962, 1964; Fine *et al.*, 1962). Later, this protein was found in normal adult rats (Eddington *et al.*, 1972; Ganrot, 1973a) and its concentration determined by means of electroimmunoassay.

Evidence for hepatic synthesis of α_2 acute-phase macroglobulin was presented by Sarcione & Bogden (1966); isolation was first performed by Menninger *et al.* (1970), and Jamieson *et al.* (1972) determined some of its physicochemical properties. Interaction with trypsin and plasmin was described by Ganrot (1973b). The comparative study of the enzyme binding capacity of rat α_2 acute-phase macroglobulin and rat α_1 macroglobulin (present in the serum of normal adult rat) has given very similar results (Gauthier & Mouray, 1975b), suggesting an identical molecular structure of the two proteins around the binding site. However, their antigenic properties as well as their physiological variations are unrelated. A further investigation of the physicochemical properties of α_2 acute-phase macroglobulin was necessary to try to explain the metabolism of this protein.

The purpose of this work was to isolate rat α_2 acute-phase macroglobulin and determine its main physicochemical constants as well as amino acid and carbohydrate content.

Experimental

Isolation procedure

Blood collection. Two normal adult male rats (Wistar C.F., 300–500g body wt.) were injected subcutaneously with turpentine (0.5ml/100g body wt.). Blood was collected 48h later by cardiac puncture under light diethyl ether anaesthesia. After clot formation and centrifugation about 15–20ml of serum was obtained and subjected to the first step of the isolation procedure.

Gel filtration on Sephadex G-200. Initially, the serum was subjected to chromatography on Sephadex G-200; the column (2.5cm $\times$ 100cm) was equilibrated in 0.1M-Tris/HCl (pH7.4)/0.15M-NaCl. One portion of the first eluted peak (Fig. 1a) containing mainly α_1 macroglobulin and α_2 acute-phase macroglobulin was collected and subjected to the second step of isolation.

Ion-exchange chromatography on DEAE-cellulose. The collected fractions (40–50ml) were dialysed in 0.05M-Tris/HCl (pH8)/0.03M-NaCl and subdivided in two parts of about 25ml each; ion-exchange chromatography was performed on one of the portions with a linear concentration gradient by using a modification of Ganrot's (1973a) method; for this experiment 250ml of equilibrating buffer and 250ml of 0.05M-Tris/HCl (pH8)/0.20M-NaCl were used. The elution diagram (Fig. 1b) shows two main peaks.

Fig. 1. Purification of rat α_2 acute-phase macroglobulin

(a) Elution pattern after the first stage of the preparation with Sephadex G-200 chromatography; (b) the fraction indicated by hatching in (a) was further separated by DEAE-cellulose ion-exchange chromatography.

α_2 acute-phase macroglobulin has been shown to be eluted in the first peak, since it reacts with anti-(inflammatory rat serum), but not anti-(normal rat serum) antiserum. The NaCl concentration at the α_2 acute-phase macroglobulin elution volume was approx. 78 mM.

Methods

Electrophoresis. Fused 'rocket' immunoelectrophoresis was used as described by Svendsen & Rose (1970); the experiments were performed in 1% (w/v) agarose/0.075 M-barbital (pH 8.6)/2% (w/v) calcium lactate with a voltage gradient of 3 V/cm for 15 h. Anti- α_2 acute-phase macroglobulin antiserum was prepared in rabbits (strain Fauve de Bourgogne) by injecting 1.8 ml of a 1:1 (v/v) emulsion of antigen (1 mg/ml) and complete Freund's adjuvant. Intradermal injections were made dorsally at 10 to 15 different points and were repeated 15 days later. The blood was collected by cardiac puncture at least 8 days after the second injection. Anti-(normal rat serum) antiserum was from Behringwerke A.-G., Marburg, Germany.

Polyacrylamide-disc-gel electrophoresis was performed by the method of Davis (1964) in 5.5% (w/v) gel. The experiments were performed in Tris/glycine buffer, pH 8.3, with a current of 4 mA/gel. Staining was done with 1% Amido Black.

Physical measurements. Determination of the isoelectric point was by isoelectrofocusing in a sucrose gradient and with carrier ampholites (pH range 4–6) in a LKB 8100 column (Vesterberg & Svensson,

1966). Approx. 25 mg of α_2 acute-phase macroglobulin was initially desalted by gel filtration on a Sephadex G-25 column (1 cm $\times$ 10 cm) and then applied to the column. A voltage not exceeding 400 V was applied during the experiment, which lasted 48 h at 4°C. Measurements of E_{278} and the pH were made on eluted fractions of 2 ml each.

Sedimentation coefficients were obtained by using the Beckman Spinco ultracentrifuge at 44000 rev./min and 20°C. The α_2 acute-phase macroglobulin solutions were equilibrated in 0.05 M-Tris/HCl (pH 8)/0.07 M-NaCl, at concentrations of 2.36, 5.15 and 8.46 mg/ml. The sedimentation values were determined by the method of Schachman (1957) and converted into s values in water at 20°C ($s = s_{\text{obs.}} \times 1.019$).

The molecular weight of α_2 acute-phase macroglobulin was calculated by light-scattering by using the Wippler and Scheibling photogoniometer; α_2 acute-phase macroglobulin was equilibrated in 0.05 M-Tris/HCl (pH 8)/0.15 M-NaCl and filtered twice through a 0.45 μ m Millipore membrane. Molecular weight was determined by means of the following equation:

$$\frac{1}{M} = KI_B \left(\frac{dn}{dc} \right)^2 \left(\frac{c}{\Delta i} \right)_{c=0, \theta=0}$$

where M is the molecular weight, K is a constant depending on the wavelength of the incident light (546 nm) and on the Rayleigh ratio of the standard solution (benzene), and c is the concentration. The refractive-index increment dn/dc was found to be 0.18 ml/g. Δi and I_B (light scattered by the sample and the standard respectively at an angle of 90°) were determined experimentally.

Chemical analysis. Nitrogen determination was performed by the micro Kjeldahl procedure. Proteins were determined both with the biuret reagent method and by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Total hexose content was measured by the orcinol/sulphuric acid method (Tillmans & Philippi, 1929) adapted to the Technicon autoanalyser by Demaille *et al.* (1965). An equimolar solution of D-galactose and D-mannose was used as a standard. The minor degree of fucose interference was not deducted.

Approx. 1.5 mg of protein was dissolved in 4 M-HCl and hydrolysed in a sealed tube for 4 h at 100°C to determine the hexosamine content. Hexosamines were measured by the method of Neuhaus & Letzring (1957) and were identified during the amino acid analysis by using the Technicon autoanalyser.

Fucose was determined by the Dische & Shettles (1948) reaction. Sialic acid was measured as described by Aminoff (1961); hydrolysis was performed in 0.05 M-H₂SO₄ for 30 min at 80°C.

For the amino acid analysis approx. 1.5 mg of α_2 acute-phase macroglobulin was hydrolysed for periods of 24 and 48 h in 1 ml of 5.6 M-HCl at 105°C.

EXPLANATION OF PLATE I

Fused 'rocket' immunoelectrophoresis of fractions obtained after the second stage of separation

Anti-(normal rat serum) antiserum (0.3ml) and 0.3ml of anti-(rat α_2 acute-phase macroglobulin) antiserum were mixed with the antibody-containing gel; 5 μ l of each eluted fraction was applied to the wells of antibody-free gel and analysed as described in the Experimental section.

EXPLANATION OF PLATE 2

Ouchterlony double immunodiffusion

Pooled fractions ($10\mu\text{l}$) of α_2 acute-phase macroglobulin (α_2) following the second stage of the purification reacted with anti-(normal rat serum) antiserum (N) and anti-(α_2 acute-phase macroglobulin) antiserum ($A\alpha_2$). The other well contained rat α_1 macroglobulin.

EXPLANATION OF PLATE 3

Disc-gel electrophoresis

(a) Normal rat serum, (b) inflammatory rat serum, (c) α_2 acute-phase macroglobulin after the second stage of purification.

EXPLANATION OF PLATE 4

Ultracentrifugation pattern of purified α_2 acute-phase macroglobulin

The protein concentration was 5.15 mg/ml; the photograph was taken at a schlieren angle of 70° 44 min after top speed was reached. Sedimentation is from left to right.

Table 1. Physical properties of rat and human α -macroglobulinsReferences: ^a, Gauthier *et al.* (1974); ^b, Schönenberger *et al.* (1958); ^c, Jones *et al.* (1972).

	Macroglobulin properties		
	Rat α_2 acute phase	Rat α_1	Human α_2
$E_{1\text{cm}}^{1\%}$ (278 nm)	6.8	8.5 ^a	8.1 ^b
pI	4.55	4.4 ^a	5.4 ^b
$s_{20,w}^0$ (S)	18.4	17.8 ^a	19.6 ^b
Mol.wt.	770000	760000 ^a	725000 ^c

Excess of HCl was removed by desiccation over NaOH pellets *in vacuo*. Residues were dissolved in citrate buffer/12.5% (w/v) sucrose, pH 2.2, containing leucine standard. α_1 Macroglobulin was studied by using the same procedure; purification of this protein has been previously described (Gauthier *et al.*, 1974).

Results

Purity criteria

The purity of the material obtained after the two stages of preparation was investigated by electrophoresis, double-immunodiffusion and ultracentrifugation techniques. Fused 'rocket' immunoelectrophoresis (Plate 1) shows the distribution of proteins after the ion-exchange chromatography. The first eluted fractions containing α_2 acute-phase macroglobulin were pooled and purity was further checked by double immunodiffusion (Plate 2).

Engler *et al.* (1972) mentioned the difficulty in distinguishing between α_1 macroglobulin and α_2 acute-phase macroglobulin with disc electrophoresis. A good separation was obtained when electrophoresis was performed in 5.5% gels (Plates 3a and 3b). α_2 Acute-phase macroglobulin appears as a double band (Plate 3c). Only one band with a slightly increased mobility was observed after incubation with chymotrypsin. The same result has been previously obtained with rat α_1 macroglobulin (Gauthier & Mouray, 1975a).

The ultracentrifugation of purified α_2 acute-phase macroglobulin showed no traces of impurity (Plate 4).

Physical properties

The results are summarized in Table 1 and compared with those obtained for rat α_1 macroglobulin and human α_2 macroglobulin.

Spectrophotometry. The spectrum of ultraviolet absorption shows the characteristic profile of proteins with maximum absorption at 278 nm. No significant absorption was found above 330 nm. The extinction

Fig. 2. Isoelectric focusing of α_2 acute-phase macroglobulin

Fractions (2 ml each) were collected after 48 h under a maximal voltage of 400 V. ∇ , pH; $\bullet$, E_{278} .

Fig. 3. Molecular weight of α_2 acute-phase macroglobulin

$c/(I - I_0)$ was determined by extrapolation from experimental values to zero concentration.

coefficient was measured with the Beckman DB spectrophotometer at 278 nm. The α_2 acute-phase macroglobulin solution was desiccated at 60°C under an increasing vacuum. A value of $E_{1\text{cm}}^{1\%}$ at 278 nm of 6.8 was obtained in 0.1 M-Tris/HCl, pH 8.

Isoelectric point. After isoelectric focusing with carrier ampholites, the purified α_2 acute-phase macroglobulin was focused as a single peak at pH 4.55 (Fig. 2).

Sedimentation coefficient. The values of $s_{20,w}$ for α_2 acute-phase macroglobulin concentrations of 2.36, 5.15 and 8.46 mg/ml were 17.83, 16.67 and 15.90 S respectively. Extrapolation to zero concentration gave a value of $s_{20,w}^0 = 18.4$ S.

Molecular weight. The scattered light was measured at seven α_2 acute-phase macroglobulin dilutions (0.048–0.217 mg/ml) in order to extrapolate to zero concentration (Fig. 3). The mol.wt. found was 770000 ± 40000 .

Table 2. Amino acid composition of rat α_2 acute-phase macroglobulin and rat α_1 macroglobulin (two analyses)

Amino acid	Composition (mol/100mol)	
	Rat α_2 acute-phase macroglobulin	Rat α_1 macroglobulin
Lys	5.69 $\pm$ 0.16	6.22 $\pm$ 0.21
His	1.58 $\pm$ 0.05	2.14 $\pm$ 0.11
Arg	3.63 $\pm$ 0.12	3.09 $\pm$ 0.13
Asp	8.67 $\pm$ 0.09	9.63 $\pm$ 0.3
Thr	6.97 $\pm$ 0.13	6.72 $\pm$ 0.09
Ser	7.28 $\pm$ 0.01	7.55 $\pm$ 0.25
Glu	12.32 $\pm$ 0.23	12.32 $\pm$ 0.12
Pro	5.02 $\pm$ 0.09	5.16 $\pm$ 0.21
Gly	6.46 $\pm$ 0.19	6.10 $\pm$ 0.05
Ala	7.12 $\pm$ 0.06	6.83 $\pm$ 0.19
Cys (half)	1.28 $\pm$ 0.2	1.68 $\pm$ 0.07
Val	8.67 $\pm$ 0.04	8.29 $\pm$ 0.22
Met	2.06 $\pm$ 0.15	1.92 $\pm$ 0.14
Ile	4.33 $\pm$ 0.06	5.18 $\pm$ 0.2
Leu	11.05 $\pm$ 0.10	9.11 $\pm$ 0.08
Tyr	3.40 $\pm$ 0.2	3.85 $\pm$ 0.03
Phe	4.46 $\pm$ 0.03	4.13 $\pm$ 0.03

Table 3. Carbohydrate composition

Results are expressed as percentage of dry weight.

	Composition (%)	
	α_2 acute-phase macroglobulin	α_1 macroglobulin
Hexose	4.25	3.2
Hexosamine	3.4	3.4
Fucose	0.20	0.45
Sialic acid	2.0	2.1

Chemical analysis

Rat α_2 acute-phase macroglobulin contains 85% protein (mean value). The nitrogen content of the protein has an average value of 12.3%.

Amino acid analysis. The results of the quantitative amino acid determination are summarized in Table 2, which also shows for comparison those values obtained for rat α_1 macroglobulin. Most of the amino acids were released after 24 h of hydrolysis.

Carbohydrate composition. The values obtained (Table 3) are in good agreement with those found for other species, but differ from those reported for rat α_2 acute-phase macroglobulin by Jamieson *et al.* (1972). Hexosamines were identified as being essentially glucosamine. No significant difference was found between the two rat macroglobulins.

Discussion

Since this method of preparation of α_2 acute-phase macroglobulin does not include $(\text{NH}_4)_2\text{SO}_4$ treatment, it permits the study of the structural and physicochemical properties of the molecule as well as of its biological activity, particularly its enzyme binding capacity. Mehl *et al.* (1964) underlined the disadvantage encountered in using $(\text{NH}_4)_2\text{SO}_4$ during human α_2 macroglobulin isolation in experiments designed to study its interaction with trypsin.

Comparison of α_1 macroglobulin and α_2 acute-phase-macroglobulin physical constants shows many similarities between these two proteins. This explains the difficulties encountered during the second step of the separation procedure. However, the use of a very slowly increasing NaCl gradient during ion-exchange chromatography gave a yield of 20–25% of α_2 acute-phase macroglobulin. This value was estimated by reference to the α_2 acute-phase macroglobulin concentration in inflammatory serum by using the electroimmunoassay; α_2 acute-phase macroglobulin content was determined as varying between 2 and 9 g/litre 48 h after a turpentine injection.

Ultracentrifugation, as well as immunological criteria, does not permit demonstration of any contaminants.

Only α_2 acute-phase macroglobulin was purified by this present procedure, as can be seen in Plate 1. Isolation of α_1 macroglobulin from injured rats can be realized by using preparative isotachopheresis (F. Gauthier, unpublished work). Pure α_1 macroglobulin is then eluted before the α_2 acute-phase macroglobulin.

The amino acid and carbohydrate contents of the two macroglobulins were shown to be very similar and are in close agreement with the results reported for α macroglobulin obtained from other species. However, Jamieson *et al.* (1972) obtained a higher hexose content (9.3%) during α_2 acute-phase-macroglobulin carbohydrate analysis. They also found a slightly higher sialic acid content (2.6%). No fucose was found.

The present study indicates that rat α_2 acute-phase macroglobulin possesses all the physicochemical properties characteristic of α macroglobulins; no significant differences from rat α_1 macroglobulin can be demonstrated, which would explain the role of α_2 acute-phase macroglobulin in response to inflammation or other acute processes. However, α_1 macroglobulin, which normally binds and regulates the activity of proteolytic enzymes, does not increase during inflammation, despite the liberation of proteolytic enzymes, particularly from injured cells. α_2 acute-phase macroglobulin could act in the same way as α_1 macroglobulin; experiments done *in vitro* have demonstrated the same binding capacities for trypsin and chymotrypsin (Gauthier & Mouray, 1975b). A

factor related to the liberation of enzymes could intervene as a 'de-repressor' of the α_2 acute-phase-macroglobulin synthesis (Sarcione, 1970) since it is known that α_2 acute-phase macroglobulin is normally present in the blood of the foetal and young rat, and diminishes after parturition.

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