Biosynthesis of Serum Albumin in Rat Liver

ISOLATION AND PROBABLE STRUCTURE OF 'PROALBUMIN' FROM RAT LIVER

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1. Two methods are described for the preparation of 'proalbumin' in good yields from rat liver. 2. One of the methods does not depend on the use of specific antisera. 3. The product from both methods is identical as judged by electrophoresis on polyacrylamide gel, isoelectric focusing on pH gradients, ion-exchange chromatography and quantitative immunoelectrophoresis. The protein also appears to be radiochemically pure by these criteria. 4. The protein is free from serum albumin as judged by isoelectric focusing and co-chromatography on ion-exchange columns. It is judged to be free from other proteins by these same criteria and by specific precipitation with antibody. 5. It is converted into serum albumin by limited tryptic hydrolysis. The albumin so produced has the same *N*-terminal (glutamic acid) and *C*-terminal (alanine) amino acids as reported for rat serum albumin. 6. A hexapeptide is liberated from the *N*-terminal end of 'proalbumin' simultaneously. It contains three arginine, one phenylalanine, one valine and one glycine residues.

In our previous work we suggested that rat serum albumin was synthesized by way of 'proalbumin', which was converted into serum albumin by the proteolytic removal of a small peptide just before secretion (Judah & Nicholls, 1971; Geller *et al.*, 1972; Judah *et al.*, 1973; Russell & Geller, 1973). In the present paper we describe methods for the preparation of 'proalbumin' from rat liver.

Materials and Methods

These are all exactly as referred to already in previous papers (Geller *et al.*, 1972; Judah *et al.*, 1973), except for the following. Quantitative immunoelectrophoresis was done as described by Laurell (1966), and electrophoresis on polyacrylamide gel was by the method of Neville (1971).

Labelling of protein with ^{14}C

In all the methods described in this paper 'proalbumin' was identified by the addition of trace quantities of ^{14}C -labelled 'proalbumin' prepared as described by Judah *et al.* (1973). However, identical results were obtained if the experiments were done with livers which were labelled by injecting L-[1- ^{14}C]leucine intravenously into a proportion of the rats used for the preparation, and killing them 10 min after the injection.

Limited tryptic digestion of 'proalbumin'

This was based on the method of Judah *et al.* (1973). The system contained 'proalbumin' (labelled with [^{14}C]arginine; sp. radioactivity 10000 d.p.m./mg; 5 mg) and trypsin (1 μg) in 1 ml of 50 mM- K_2HPO_4 -HCl buffer, pH 7.5, 50 μM with respect to CaCl_2 . The temperature was 22°C and the time of incubation 60 min. Soya-bean trypsin inhibitor (2 μg) was added to the reaction mixture, which was then passed through a Sephadex G-25 column (1 cm $\times$ 40 cm) equilibrated with 10 mM- K_2HPO_4 -HCl buffer, pH 7.5. Protein emerging at the front was concentrated to 0.5 ml (by using a Minicon B15 filter, obtained from Amicon Co., High Wycombe, Bucks., U.K.), before determination of *N*- and *C*-terminal residues, and electrofocusing. The small peptide severed from 'proalbumin' was found in the fraction emerging after the passage of 17–22 ml of eluent and contained about 10% of the radioactivity originally applied. It was dried on a rotary evaporator, taken up in 6 M-HCl and used for amino acid analysis as described below.

Determination of *N*- and *C*-terminal amino acids

N-Terminal residues were determined by the methods of Hartley (1970). *C*-Terminal amino acids were established by the use of carboxypeptidase A (obtained from Sigma Chemical Co., London S.W.6, U.K.) as described by Harris & Knight (1955). Chromatography of the dansylated amino acids was

done on polyamide sheets (obtained from BDH Ltd., Poole, Dorset, U.K.) by the method of Woods & Wang (1967).

Protein assay

Protein was measured by E_{280} throughout, with rat serum albumin as standard.

Antiserum to rat albumin

This was raised in sheep by the Department of Biological Reagents, Wellcome Research Laboratories, Beckenham, Kent, U.K. The purity of the antiserum was checked by immunoelectrophoresis against rat serum.

Experimental

All the operations were done at 2°C, except the chromatographic steps, which were done at 22°C.

Method 1: chemical fractionation

Extraction of microsomal fractions. The usual method for the extraction of albumin from microsomal fractions starts with the preparation of a deoxycholate extract. This was found to be unsatisfactory for large-scale preparations because the yields were low when compared with those expected from small-scale experiments.

The use of ethanolic solutions of trichloroacetic acid (which is common in the preparation of albumin from serum) for the extraction of microsomal fractions gives a very poor yield. We noticed, however, that the residue left after ethanolic trichloroacetic acid treatment could be extracted with aqueous buffers to provide significant amounts of albumin and 'proalbumin'. We therefore tried acetone-dried powders of microsomal fractions and found them to be convenient sources of liver albumin fractions. The stability of the acetone-dried powders has not been investigated. We store them at room temperature *in vacuo*, for up to 2 weeks without loss. Perhaps they could be kept longer, and no doubt refrigeration would help.

Preparation of acetone-dried powder of microsomal fractions. Microsomal fractions were prepared from approx. 150g of rat liver at a time. The livers were homogenized in a blender in approx. 750ml of 0.25M-sucrose. The homogenate was centrifuged at 2000g for 10min. The supernatant was adjusted to approx. 10mM with respect to CaCl_2 and centrifuged at 20000g for 20min in the GSA rotor of a Sorvall RC2B centrifuge. The precipitate was washed once with 600ml of 150mM-NaCl and the pellet was then suspended in 100ml of ice-cold water. Ice-cold acetone (1.2 litre) was poured slowly into this

suspension with constant stirring. The protein precipitate was filtered off (Whatman 54 filter paper) and then washed repeatedly with acetone (total volume approx. 250ml). Care must be taken not to let the surface of the protein precipitate crack during this procedure. The precipitate was then washed with about 250ml of diethyl ether, which was allowed to drain through. The protein was then dried over CaCl_2 and liquid paraffin *in vacuo* at room temperature (22°C). The yield is about 8g of dry powder from the microsomal fraction of 150g of liver.

The method described is convenient because relatively large-scale operations can be undertaken without the use of high-speed centrifuges. It is also a quick method, but of course no claim is made about the purity of the microsomal fractions so prepared.

Extraction of the acetone-dried powder. The acetone-dried powder of microsomal fraction was extracted with constant stirring for 60min in 200ml of 100mM-Tris-HCl, pH 7.8, at 2°C. The suspension was centrifuged at 20000g for 20min in the GSA rotor of a Sorvall RC2B centrifuge. ^{14}C -labelled marker 'proalbumin' was added to the clear supernatant.

Trichloroacetic acid precipitation. The extract was made to 5% (w/v) trichloroacetic acid and the precipitate was collected by centrifugation (3000g for 10min). It was then suspended in 150ml of water and the pH adjusted to 6.0 with 1M-Tris base. The insoluble residue was removed by centrifugation (20000g for 20min) and the clear supernatant retained.

Ammonium sulphate precipitation. To the above extract (170ml) enough 1M-acetic acid was added to bring the pH to 5.0. The volume was measured and 50g of solid $(\text{NH}_4)_2\text{SO}_4$ added to each 100ml. It was checked that the pH was still 5.0 and the suspension was centrifuged (12000g for 10min). The precipitate was collected and dissolved in about 40ml of 100mM-Tris-HCl, pH 7.8. The solution was concentrated on an Amicon filter (PM10 membrane; obtained from Amicon Co., High Wycombe, Bucks., U.K.) to about 10ml.

Preparative chromatography on Sephadex G-100. This was done on a column (2.5cm × 90cm) equilibrated with 100mM-Tris-HCl, pH 7.8, at 22°C. The flow rate was 40ml/h. The protein fraction eluted between 180 and 220ml was collected and concentrated to about 10ml on an Amicon filter with PM10 membrane.

Preparative chromatography on DEAE-cellulose. This was done on columns (1.5cm × 10cm) equilibrated with 100mM-Tris-HCl, pH 7.8, at 22°C. A flow rate of 40ml/h was used. The sample in 100mM-Tris-HCl, pH 7.8, was put on the column usually in a volume of 10ml and the column was then washed with a further 60ml of the same buffer. Elution was done with 125mM-Tris-HCl, pH 7.8. 'Proalbumin' began to appear after the passage of 20ml of this

Table 1. *Purification of 'proalbumin' from acetone-dried powder of microsomal fractions*

Marker 'proalbumin' (100 µg; sp. radioactivity 750000 d.p.m./mg) was added after the extraction of the acetone-dried powder. All operations were done at about 2°C except for the chromatography, which was done at room temperature (22°C).

Step no.	Purification procedure	Protein recovered (mg)	Sp. radioactivity (d.p.m./mg of protein)	Yield of ¹⁴ C added (%)
1	pH 7.8 extract of acetone-dried powder of microsomal fraction from 150g of liver	9150	8	100
2	Trichloroacetic acid precipitate	810	73	79
3	(NH ₄) ₂ SO ₄ precipitate	396	123	65
4	Chromatography on Sephadex G-100	97	410	53
5	First chromatography on DEAE-cellulose	10	2750	36
6	Second chromatography on DEAE-cellulose	5	3500	24

buffer, and elution was complete after a further 50ml had been collected. The inclusion of labelled serum albumin in the sample in several trials showed that the 'proalbumin' sample was not contaminated by serum albumin. However, the 'proalbumin' fraction obtained was contaminated as judged by an electrofocusing experiment and was further purified.

Second chromatography on DEAE-cellulose. This was done exactly as the first. Table 1 summarizes the procedure.

Method 2: use of antiserum

Despite the success of chemical fractionation for the isolation of 'proalbumin', we decided to use an antibody method because of the ease of the operation, because it made use of the only biological 'marker' of the protein, and because it was desired to establish the identity of the products obtained by the different methods.

Extraction of acetone-dried powder. This was done exactly as for method 1. ¹⁴C-labelled marker 'proalbumin' was added at the same stage.

Antibody precipitation. Enough antiserum was added to the extract to precipitate the albumin and 'proalbumin' therein. The amount required was established by a preliminary small-scale experiment. The mixture was left for 30min at 38°C, during which a heavy curd-like precipitate appeared. The vessel was then kept at 2°C overnight (approx. 16h) and the precipitate collected by centrifugation (10000g, 5min). The precipitate was then washed three times in 150mM-NaCl, about 100ml being used each time. The centrifuge tubes were carefully drained and wiped.

Separation of antigens from antibody precipitate. The precipitate was suspended carefully in 6ml of 1% (w/v) trichloroacetic acid. Then 24ml of 1% (w/v) trichloroacetic acid in ethanol was added to the uniform suspension, mixed well and the mixture kept at 2°C for a minimum period of 4h. It is often convenient to leave it overnight and no harm is done.

A further 40ml of 1% (w/v) trichloroacetic acid in ethanol was then added, the suspension well mixed and the precipitate removed by centrifugation (10000g for 20min). The water-clear supernatant was collected and 2 vol. of diethyl ether added. The mixture was well stirred. After a few minutes a flocculent white precipitate appeared. It was centrifuged off and dissolved in approx. 10ml of 100mM-Tris-HCl buffer, pH 7.8, ready for the next step. The above volumes of aq. 1% (w/v) trichloroacetic acid and of ethanolic trichloroacetic acid are designed for use with the antibody precipitation from the microsomal fractions of 150g of rat liver. They can be scaled up or down, but it is important that the proportions remain constant.

Chromatography on DEAE-cellulose. This was done exactly as described for method 1, except that rechromatography was not required. The yield of 'proalbumin' from 150g of liver was 6.8mg, and the recovery of ¹⁴C-labelled marker was 60%.

Proof of purity

¹⁴C-labelled 'proalbumin' was used to test the purity of these products. Freedom from contamination by serum albumin was easily shown by addition of ³H-labelled albumin to the sample of 'proalbumin' before chromatography on DEAE-cellulose. No ³H appeared in the 'proalbumin' fraction.

Electrofocusing of the material prepared by method 1 showed the typical pattern of 'proalbumin', with the major peak at pI 6.1 (Geller *et al.*, 1972; Russell & Geller, 1973; Judah *et al.*, 1973). At the end of the electrofocus run, it was found that the ¹⁴C and E₂₈₀ traces were identical with those obtained with 'proalbumin' prepared by the antibody procedure. The apparent variation in specific radioactivity in the two minor peaks is due to the disproportionate effect of the optical absorption of the Ampholine. This is exaggerated in the peak at the lowest pI by the presence of the 'end absorption' always seen with this system.

Fig. 1. Electrofocus of purified 'proalbumin' before (a) and after (b) limited tryptic hydrolysis

The 'proalbumin' was prepared by method 1. In (a), 3.6mg of 'proalbumin' (sp. radioactivity 18000 c.p.m./mg) was placed on the column. In (b) 2.7 mg of 'proalbumin' (sp. radioactivity 5800 c.p.m./mg) was subjected to trypsin treatment in the presence of 15 mg of unlabelled rat serum albumin as described in the Materials and Methods section, and then placed on the column. Electrofocusing was done at 600V for 48h at 2°C, in the presence of 6M-urea between pH5 and 7; 0.5ml of each fraction (1.0ml) was taken for determination of radioactivity. ○, E_{280} ; ●, ^{14}C radioactivity; ▲, pH.

All the 'proalbumin' recovered from the column was pooled and its specific radioactivity (when corrected for absorption by the Ampholine) was found to be unchanged from that of the starting material. The point of this experiment was to show

Table 2. *N*- and *C*-Terminal amino acids of albumin and 'proalbumin'

N- and *C*-Terminal amino acids were identified as described in the Materials and Methods section. The *N*-terminal residue of 'proalbumin' was not detectable by the methods we used. The identification comes from the data of Russell & Geller (1975).

	Albumin	'Proalbumin'	Albumin derived from 'proalbumin'
<i>C</i> -Terminal	Ala	Ala	Ala
<i>N</i> -Terminal	Glx	Arg	Glx

that the 'proalbumin' preparation contained little or no serum albumin, since 'proalbumin' and albumin are easily separated by the electrofocus procedure, albumin having a pI for the major peak of pH5.7 under these conditions.

The pooled material from the electrofocus run was then treated with anti-(rat albumin). The protein precipitated quantitatively without alteration in specific radioactivity. This proved that the protein obtained from the column was not contaminated with proteins other than albumin.

The 'proalbumin' prepared by chemical fractionation could not be separated from that prepared by antibody precipitation when subjected to electrophoresis on polyacrylamide gel by the method of Neville (1971). When the chemically fractionated product was subjected to quantitative immunoelectrophoresis (Laurell, 1966), there was no change in specific radioactivity.

Effect of trypsin

When the 'proalbumin' preparations were treated with trypsin they yielded albumin which migrated with marker serum albumin on electrofocusing on a pH gradient of 5-7 (Fig. 1b). This experiment was done in the presence of 15 mg of unlabelled rat serum albumin, which accounts for the much higher E_{280} in Fig. 1(b), and explains why there is a much smaller apparent discrepancy in the specific radioactivity of the minor peaks, since the background absorption due to the Ampholine has a smaller effect. The same result was obtained on ion-exchange chromatography on DEAE-cellulose, with a linear salt gradient, as described previously (Judah *et al.*, 1973).

Identification on *N*- and *C*-terminal amino acids

Table 2 shows the *N*- and *C*-terminal residues for serum albumin, 'proalbumin' and albumin derived from 'proalbumin' by tryptic hydrolysis. The data indicate that trypsin attacks 'proalbumin' at the *N*-terminal end, and provide further evidence that

the protein product of this attack is indeed serum albumin, since its *N*- and *C*-terminal amino acids are identical with those of rat albumin.

Amino acid composition of peptide

The small peptide liberated from the *N*-terminal end of 10mg of 'proalbumin' was hydrolysed in 6M-HCl for 16h at 105°C. The hydrolysate was run on an automatic amino acid analyser. This showed the presence of arginine, glycine, phenylalanine and valine, and traces of other amino acids. The ratios were arginine, 2.6, phenylalanine, 1.07, glycine, 1.17 and valine, 1.0. The ratio of the next most abundant amino acid (leucine) was 0.6. This was eliminated from our calculations because the peptide would then have to be a dodecapeptide, which would disagree with its behaviour on Sephadex columns (Judah *et al.*, 1973). Further, we have never been able to label the peptide with [¹⁴C]leucine. Therefore we rounded off the values to arginine 3, phenylalanine 1, glycine 1 and valine, 1. This agrees with the findings of J. H. Russell & D. Geller (personal communication) and also with the amino acid sequence at the *N*-terminal end of 'proalbumin' reported to us by Dr. D. M. Geller (personal communication; Russell & Geller, 1975); this gives the amino acid sequence as Arg-Gly-Val-Phe-Arg-Arg and then continues with the known sequence for rat albumin (Bradshaw & Peters, 1969).

Heterogeneity of 'proalbumin'

The protein as isolated by either of the methods described in this paper is heterogeneous. This is clearly shown by the electrofocus pattern (Fig. 1a), which is in good agreement with those previously published. There is a major peak with pI 6.1 and a minor species on either side (Geller *et al.*, 1972; Judah *et al.*, 1973; Russell & Geller, 1973). The interesting point is that after limited tryptic hydrolysis,

the three species appeared to shift together to lower isoelectric points corresponding to the serum albumin marker (Fig. 1b), the major peak having a pI of 5.85. We draw attention to this point because it might reflect the presence of several species of 'proalbumin' and the albumin derived therefrom.

Conclusions

This work provides further strong support for our suggestion that albumin synthesis in rat liver proceeds by way of a protein precursor. The forthcoming paper of Russell & Geller (1975) provides independent support by a somewhat different route.

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