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Isolation and Characterization of Riboflavin-Binding Protein from Pregnant-Rat Serum

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A high-affinity riboflavin-binding protein was isolated and characterized for the first time from pregnant-rat sera by affinity chromatography on a lumiflavin–agarose column. The purified protein was homogeneous by the criteria of analytical polyacrylamide-gel disc electrophoresis, gel-filtration chromatography on Sephadex G-100 and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. It had a molecular weight of 90000 ± 5000 and interacted with [^{14}C]riboflavin with a 1 : 1 molar ratio with a dissociation constant (K_d) of 0.42 μM.

Notwithstanding the relative impermeability of the placental membranes to riboflavin and its coenzyme forms (Dancis, 1962), the vitamin is actively concentrated by the conceptus in pregnant humans and higher animals (Dancis & Schneider, 1975; Clarke, 1977). But the molecular mechanisms underlying this phenomenon of facilitated transplacental vitamin delivery are largely unknown. Of relevance in this context is the evidence in the egg-laying chicken for the obligatory participation of a specific high-affinity riboflavin-binding protein to ensure adequate vitamin deposition in the developing oocytes (Hammer *et al.*, 1973; Murthy & Adiga, 1977*a*). During our previous studies on the molecular aspects and hormonal induction of the chicken riboflavin-binding protein (Murthy *et al.*, 1976; Murthy & Adiga, 1977*b*, 1978) we obtained immunological evidence for the existence of a similar protein in the sera of pregnant rats but not of male or immature animals. The paramount functional importance of this protein for foetal survival was demonstrated by passive immunoneutralization with the specific antiserum to the chicken riboflavin-binding protein, with the resultant abrupt termination of pregnancy (Adiga & Muniyappa, 1978). In the present communication we describe the isolation of such a riboflavin-binding protein from pregnant-rat serum and some of its physicochemical characteristics.

Materials and Methods

Materials

[2-^{14}C]Riboflavin (specific radioactivity 31 mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Unlabelled riboflavin, dextran and active charcoal (Norit) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Lumiflavin–agarose (Blankenhorn *et al.*, 1975) was kindly given by Dr. G. Blankenhorn, Universität Konstanz, Konstanz, Germany. Inbred Wistar rats (60 days old; 100–120 g body wt.) from our Institute colony were fed on a pelleted diet (Hindustan Lever Products, Bombay, India) and water *ad libitum*. They were exposed to a 14h–10h light–dark schedule. The sources of other biochemicals and reagents were detailed elsewhere (Muniyappa & Adiga, 1979).

Binding of ^{14}C-labelled riboflavin to pregnant-rat serum protein

The pooled sera (5 ml) from pregnant rats at day 12 of gestation were concentrated against Aquacide to 1 ml, incubated with 1 μCi of [^{14}C]riboflavin at 30°C for 1 h and subjected to gel-filtration chromatography on a Sephadex G-75 column (1.0 cm × 5.8 cm) to separate the free vitamin from the protein-bound form. Each fraction was monitored for absorbance at 280 nm and [^{14}C]riboflavin. The radioactivity in 0.1 ml samples was measured by liquid-scintillation spectrometry as detailed elsewhere (Muniyappa & Adiga, 1979).

Isolation of riboflavin-binding protein by affinity chromatography

The pregnant rats exhibit elevated concentrations of circulatory riboflavin-binding protein during days 10–13 of gestation, as measured by a heterologous radioimmunoassay (Muniyappa & Adiga, 1980). Adult female rats with a regular 4-day oestrous cycle were mated with adult males, and the day on which sperms were detected in the vaginal

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smear was taken as day 1 of pregnancy. Animals were killed on day 12 of gestation, and sera were prepared and stored at 4°C until used for isolation of protein.

Dissociation of bound flavin

The pooled sera from 15 animals were stirred for 24h at 4°C with an equal volume of an aqueous suspension of dextran/charcoal (500mg of charcoal and 50mg of dextran in 100ml) to remove endogenously bound ligand, if any. The charcoal was sedimented by centrifugation at 25000*g* for 20min, and the clarified protein solution was applied to a column of lumiflavin–agarose and extensively washed with equilibration buffer (0.1M-sodium phosphate buffer, pH7.0) until the effluent was free of detectable 280nm-absorbing material. The protein retained on the affinity matrix was eluted specifically with 10 bed volumes of 10μM-riboflavin in the above buffer. The unbound flavin was removed from the effluent by extensive dialysis against water and the protein fraction was concentrated over Aquacide to a small volume (10ml). The flavin bound to protein was removed by treatment with dextran/charcoal as before. The protein thus obtained had no significant 445nm-absorbing material (flavin), as monitored spectrophotometrically.

Polyacrylamide-gel disc electrophoresis

Electrophoresis of the purified protein on analytical polyacrylamide gel (7.5%) was conducted (Davis, 1964) at 4mA/tube for 2h at 25°C (pH8.3). After electrophoresis the protein on the gel was stained and destained as described elsewhere (Muniyappa & Adiga, 1979). A duplicate gel containing the protein saturated with [^{14}C]riboflavin was sliced into 2.5mm-thick discs, and individual discs were digested and counted for radioactivity (Young & Fullhorst, 1965).

Molecular-weight determination

The molecular weight of the protein was estimated by gel filtration (Andrews, 1965) on Sephadex G-100 with 0.1M-sodium phosphate buffer (pH7.0)/1M-NaCl as the eluent. Electrophoresis on sodium dodecyl sulphate/polyacrylamide gels (Weber & Osborn, 1969) was also employed for this purpose. The protein sample (300μg) was treated with sodium dodecyl sulphate (1mg), dithiothreitol (1.5mg) and Tris base (1.2mg) in a total volume of 0.2ml (final pH8.6) and heated at 100°C for 2–5min before application on the gel after addition of glycerol. The following proteins of known molecular weight served as markers in both the procedures: cytochrome *c* (13700), chymotrypsinogen (25000), ovalbumin (44000), bovine serum albumin (68000) and hexokinase (102000).

[^{14}C]*Riboflavin binding*

The affinity constant of the interaction between [^{14}C]riboflavin and the apoprotein was determined by equilibrium dialysis in a Perspex dialysis cell as described previously (Muniyappa & Adiga, 1979).

Results and Discussion

Earlier evidence for the premise that, in the rat, adequate riboflavin supply from the maternal circulation to the developing embryos is carrier-protein-mediated stemmed from the observation that pregnant-rat serum contained a protein that cross-reacted with monospecific antiserum to the purified chicken riboflavin-binding protein and that, like the latter, was oestrogen-inducible. Furthermore, profound foetal wastage followed by rejection noticed as a consequence of passive immunoneutralization of the protein by the heterologous antiserum reinforced this postulate (Adiga & Muniyappa, 1978). Direct chemical evidence for such a molecular entity capable of specific and tight interaction with the flavin was sought in experiments wherein pregnant-rat serum proteins were preincubated with [^{14}C]riboflavin followed by gel filtration on Sephadex G-75. Fig. 1 depicts the profile of association of the labelled flavin with the proteins that were

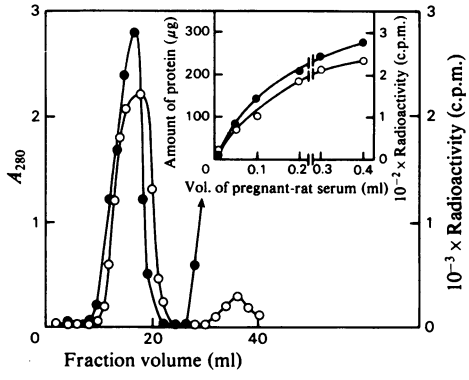

Fig. 1. *Gel filtration of pregnant-rat serum saturated with* [^{14}C]*riboflavin on Sephadex G-75*

The column (1.0cm × 58cm) was pre-equilibrated and eluted with 0.1M-sodium phosphate buffer (pH7.0)/1M-NaCl, and the effluent was collected in 1ml fractions. Inset: immunoprecipitation of pregnant-rat serum saturated with labelled riboflavin with the antiserum (0.1ml) to chicken riboflavin-binding protein. The immunoprecipitate was washed four times with 10mM-sodium phosphate buffer (pH7.0)/0.15M-NaCl and dissolved in 0.2ml of 0.1M-NaOH before determination of protein (○) and the bound radioactivity (●). For other details see the text.

well resolved from the free vitamin. The finding that a sizeable portion of the bound vitamin readily dissociated from the protein on extensive dialysis or dilution lends credence to the reported low-affinity flavin binding to albumin and other serum constituents (Jusko & Levy, 1969; Frank *et al.*, 1970). However, significant amounts of [^{14}C]riboflavin were still tightly associated with the proteins under these conditions; this could, however, be severely curtailed only either by preincubation with 100-fold excess of unlabelled flavin or by prolonged treatment with dextran/charcoal. That this phenomenon is attributable to a high-affinity flavin-binding component in the serum is suggested by the results obtained by immunoprecipitation with the specific antiserum to the chicken riboflavin-binding protein (Fig. 1 inset). Excellent correlation between the contents of precipitated protein and its associated [^{14}C]riboflavin strongly supports the above premise, besides substantiating the immunochemical evidence for its occurrence alluded to above.

In attempts to purify this minor protein component from the serum, bio-affinity chromatography on an immobilized flavin was the method of choice. The pooled dialysed sera were treated with dextran/charcoal to remove endogenously bound flavin, and the clarified protein was applied on a lumiflavin–agarose column and extensively washed with the equilibration buffer until the effluent was free from 280nm-absorbing material. The protein still retained was eluted with 10μM-riboflavin in 0.1M-sodium phosphate buffer, pH 7.0. The protein concentration in the yellow effluent could not be accurately monitored because of flavin interference with quantitative determination either by the method of Lowry *et al.* (1951) or by measurement of absorbance at 280nm. However, an alternative elution procedure that employed 0.1M-ammonium acetate buffer/1.0M-NaCl (pH 3.2) (Blankenhorn *et al.*, 1975) did yield a symmetrical protein peak that bound [^{14}C]riboflavin but with an affinity much lower than that expected of high-affinity vitamin-binding proteins ($K_a \simeq 10^7$–10^9 M^{-1}), presumably as a consequence of prolonged exposure to low pH. Hence specific elution with riboflavin was preferred. The yellow protein eluted from the affinity matrix was dialysed, concentrated to a small volume and treated with dextran/charcoal to remove firmly bound flavin. The resultant apo-(riboflavin-binding protein) exhibited a single stainable band on analytical disc gel electrophoresis, and if saturated with [^{14}C]riboflavin before electrophoresis firmly retained almost all the bound radioactivity that banded at a position coincident with the protein stain (Fig. 2). These findings thus clearly show that the isolated protein was homogeneous and interacts with the flavin with an affinity strong enough to withstand prolonged gel electrophoresis.

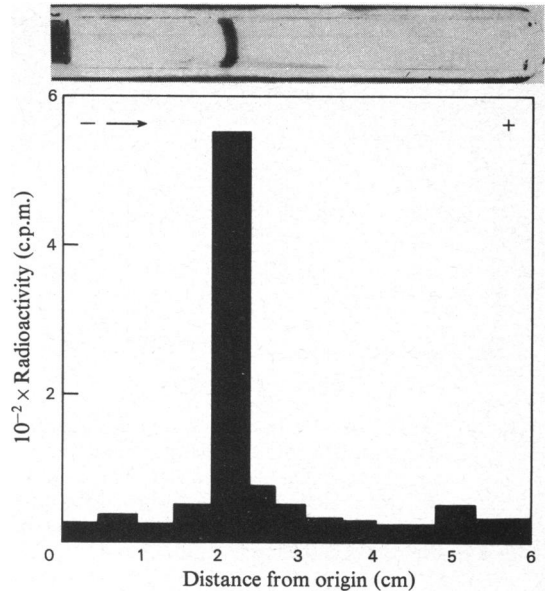

Fig. 2. *Distribution of radioactivity and protein stain after electrophoresis of purified riboflavin-binding protein on polyacrylamide gels*

The purified protein (approx. 300μg) was loaded on duplicate gels and electrophoresed in 0.2M-Tris/glycine buffer, pH 8.3. For further details see the text.

Further evidence for the homogeneity of the isolated protein was sought by gel filtration on Sephadex G-100. A single symmetrical protein peak with a coincident profile of bound [^{14}C]-riboflavin emerged from the gel at a position corresponding to an apparent molecular weight of 90000 ± 5000, as determined by reference to standard marker proteins (see the Materials and Methods section). Our attempts to examine the possible presence of subunits in the purified protein by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Weber & Osborn, 1969) have hitherto yielded inconclusive results, presumably because of high lability of some of peptide bonds in the protein exposed to conditions described in the method. Thus, although a single protein band corresponding to a molecular weight of 90000 was obtained when the exposure to sodium dodecyl sulphate was brief (30s), highly variable bands became apparent when the treatment was prolonged (results not given). However, the presence of contaminating proteolytic activity becoming active on exposure to sodium dodecyl sulphate as a cause of this variability in the band pattern was considered unlikely, since inclusion of proteolytic inhibitors such as phenylmethanesulphonyl fluoride (10μl/ml) and leupeptin (1mg/ml) did not alter the above pattern.

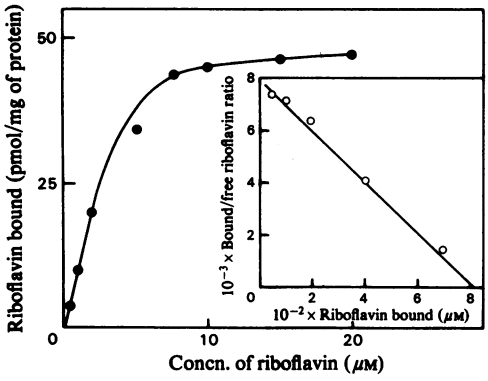

Fig. 3. *Binding of [^{14}C]riboflavin to apo-(riboflavin-binding protein) under equilibrium conditions*
For experimental details see the text. The concentration of the protein used was 10μM. The data shown are replotted according to the method of Scatchard (1949) in the inset.

When a fixed concentration of the purified apoprotein was allowed to equilibrate with [^{14}C]riboflavin in a dialysis cell, it avidly bound the labelled ligand. When the amount of the protein-bound vitamin was plotted against the concentration of the flavin, a saturation-type curve was obtained (Fig. 3). Furthermore, it could be calculated that this protein–ligand interaction had K_d 0.42μM and occurred with a 1:1 molar ratio (Fig. 3). These values were comparable with those of the purified chicken protein (Murthy *et al.*, 1976) and testify to the high-affinity vitamin–protein interaction.

Thus the above data, representing the first isolation and characterization as a distinct molecular entity of a high-affinity riboflavin-binding protein from a pregnant mammal, amply substantiate our earlier immunochemical evidence for the existence and functional importance of such a carrier protein in mammalial reproduction. The demonstration that the rodent protein, despite its larger size, still shares immunological cross-reactivity (and hence some sequence homology) with the functionally similar chicken protein assumes considerable evolutionary significance, raising the possibility of higher mammals and primates elaborating similar vitamin-carrier proteins presumably for transplacental delivery of the vital nutrient. Of relevance in this context is the communication (Merrill *et al.*, 1979) providing preliminary quantitative evidence for the existence of such a protein in pregnant cows, this being confirmatory to our earlier findings (Adiga & Muniyappa, 1978). On the assumption that this is a common mechanism of transplacental transport of water-soluble vitamins, it is conceivable that there are specific recognition sites for such carrier proteins on placental membranes to facilitate preferential foetal uptake of bound vitamins on lines suggested for transcobalamin–cobalamin complex (Seligman & Allen, 1978). Whatever be the underlying mechanisms, the unequivocal demonstration of a pregnancy-specific high-affinity riboflavin-binding protein in a higher animal has important implications, since it (*a*) shows that, besides the vitamin, the adequate availability of its specific binding protein ultimately determines the foetal vitamin status and (*b*) suggests that genetic and endocrine disturbances in the production and functionality of the carrier protein may be of grave consequence to the foetal survival and proper progression of pregnancy.

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