

Comparison of biotin binding protein of pregnant rat serum with rat serum albumin

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Abstract. The purified biotin binding protein of pregnant rat serum was shown to be immunologically similar to rat serum albumin as assessed by a sensitive radioimmunoassay. In radioimmunoassay for rat biotin binding protein, the binding of [125 I] rat biotin binding protein to anti-chicken egg yolk biotin binding protein antibodies was displaced by both rat serum (10–100 nI) and purified rat serum albumin (0.1–10 ng). Similarly, in radioimmunoassay for rat serum albumin the binding of [125 I] rat serum albumin to either anti-rat serum albumin antibodies or anti-chicken egg yolk biotin binding protein antibodies was displaced by unlabelled rat biotin binding protein at comparable concentration range (0.5–10 ng). Significant fractions of radioiodinated rat biotin binding protein and rat serum albumin bound to antibodies to chicken egg yolk biotin binding protein. In immature rats, the circulating half-lives of rat biotin binding protein and rat serum albumin were determined to be 12 and 17 h respectively. The rat biotin binding protein and rat serum albumin were analysed by techniques that exploit their physicochemical properties. They displayed similar electrophoretic mobilities in alkaline as well as denaturing sodium dodecyl sulphate-polyacrylamide gels. However, in non-equilibrium pH gradient polyacrylamide gel electrophoresis, they resolved clearly. In two-dimensional tryptic peptide map analysis, the two proteins showed similarities as well as significant differences in the relative distribution patterns of their iodopeptides. These results showed that the primary structure of rat biotin binding protein and rat serum albumin were different in finer details despite the fact that they shared significant immunological cross-reactivity.

Keywords. Biotin binding protein (chicken, rat); rat serum albumin; immunological-crossreactivity.

Introduction

In some mammalian species, carrier proteins for water-soluble vitamins present in maternal circulation during gestation are obligatorily required for the transplacental transport of vitamins to the developing fetus (Adiga and Murty, 1983; Adiga *et al.*, 1986). In the rat, carrier proteins for riboflavin (Murty and Adiga, 1982a) and thiamin (Malathy and Adiga, 1985) are estrogen-inducible proteins of hepatic origin and immunological interference with the functioning of these vitamin carriers during pregnancy is detrimental to embryo development, culminating in pregnancy termination (Muniyappa and Adiga, 1980; Murty and Adiga, 1981, 1982b; Malathy 1985). Such selective immunoneutralisation of vitamin carriers bring about drastic

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Abbreviations used: BBP, Biotin binding protein; rBBP, rat biotin-binding protein; RIA, radioimmunoassay; RSA, rat serum albumin; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; SA, serum albumin; M_r , molecular weight.

curtailment of transport of the particular vitamin to the developing embryo and hence early embryonic mortality (Murty and Adiga, 1981; Surolia *et al.*, 1985).

Recently, biotin binding protein (BBP) was purified from pregnant rat serum (Seshagiri and Adiga, 1987) and its functional significance during pregnancy was demonstrated (Seshagiri and Adiga, 1989). This rat BBP (rBBP) is a glycoprotein of molecular weight (M_r) 66,000, has pI of 4.1 and specifically binds [^{14}C] biotin. This protein exhibited immunological cross-reactivity with the well characterised chicken egg yolk BBP (yolk BBP). The rBBP was isolated in significant amounts from the sera of pregnant or estrogen-treated females but not from those of the males, implying thereby the involvement of the steroid hormone in the elaboration of this protein (Seshagiri and Adiga, 1987). Therefore, it was considered desirable to develop a specific radioimmunoassay (RIA) for rBBP for quantification to explore the specificity and the pattern of hormonal modulation of rBBP induction, on lines similar to induction of avian egg yolk BBP (Murty and Adiga, 1985). During such attempts, it was surprising to find that rBBP and rat serum albumin (RSA) exhibited significant immunological cross-reactivity in RIA. Subsequent experiments however revealed that rBBP can be distinguished from RSA in terms of certain physicochemical properties.

Materials and methods

Albino rats (Wistar) of the Institute Colony were fed a pelleted diet (Hindustan Lever, Bombay) and water *ad libitum* and exposed to a 14:10 h light-dark schedule. Purification of BBP from the sera of pregnant rats was accomplished according to the published procedure (Seshagiri and Adiga, 1987). Antisera against yolk BBP and RSA (raised in rabbits) were obtained by Standard procedures described earlier (Murty and Adiga, 1984). Carrier-free Na [^{125}I] were obtained from Amersham, UK. Iodogen (1, 3, 4, 6-tetrachloro-3 α , 6 α -diphenyl glycouril) was obtained from Pierce Chemicals, Rockford, USA. The sources of other chemicals- and reagents were the same as described earlier (Seshagiri and Adiga, 1987).

Heterologous RIA for rBBP

In view of the poor yield coupled with the batch to batch variation in purity and biological activity, monospecific antiserum to rBBP could not be obtained and therefore, heterologous RIA was developed for rBBP quantification employing rabbit anti-yolk BBP antibodies. Iodination of rBBP was performed at room temperature in the presence of iodogen (Fraker and Speck, 1978). The iodinated protein was resolved from free iodine by gel-filtration chromatography on a Sephadex G-75 column pre-equilibrated with phosphate buffered saline containing 0.3% gelatin. The immunoassay procedure employed is as described earlier (Malathy and Adiga, 1985) and used an antiserum dilution capable of binding 25–40% of the radio-labelled protein and the double antibody precipitation to separate bound from free antigen.

Determination of half-life of rat BBP

Freshly prepared [^{125}I] rBBP or [^{125}I] RSA (approx. 5×10^5 cpm) was injected to

immature male rats *via* intra-cardiac route. Blood samples withdrawn at suitable intervals were counted for radioactivity. A least square fit of the data was computed.

Gel electrophoresis

Electrophoresis was carried out on 7.5% polyacrylamide gels (PAGE) at pH 8.3, (Davis, 1964). Sodium dodecyl sulphate (SDS)-PAGE was carried out on 7.5% gels according to the standard procedure (Weber and Osborn, 1969). The proteins on the gels were stained with Coomassie brilliant blue R-250 and destained with methanol/glacial acetic acid/water (40:10:50, v/v). Non-equilibrium pH gradient two-dimensional PAGE was performed as follows (O'Farrell *et al.*, 1977). Briefly, [125 I]rBBP and/or [125 I] RSA was electrophoresed on isoelectric focussing gels for 4–6 h in capillary tubes (200 μ l) followed by SDS-PAGE (7.5%). After this, the gel was treated with 12% (w/v) trichloroacetic acid for 1 h and dried using a gel-drier (Pharmacia, Sweden, Model GSD-4) prior to exposing the gel to an X-ray film for autoradiography.

Two-dimensional tryptic peptide mapping of [125 I]-labelled proteins

This analysis was performed according to the published procedure (Elder *et al.*, 1977). Briefly, the stained protein bands resolved by SDS-PAGE were excised and the protein trapped in the gel was subjected to *in situ* radioiodination by chloramine T method (Greenwood *et al.*, 1963). The radioiodinated protein was then subjected to protease digestion for 12 h at 37°C with TPCK-trypsin (50 μ g in 0.5 ml of 0.1 M ammonium bicarbonate). The released peptides were quantitatively recovered, concentrated and then subjected to electrophoresis (100 V for 90 min) on cellulose-coated plastic sheets using the buffer, glacial acetic acid/formic acid/water (150:50:800, v/v). This was followed by chromatography in the second dimension using the solvent system butanol/pyridine/acetic acid/water (32.5:25:5:2, v/v). The plates were air-dried and then exposed to X-ray films for autoradiography.

Results

A heterologous RIA to rBBP was developed employing anti-yolk BBP antibodies. The Standard curve of rBBP-RIA had a linearity range of 500 pg–10 ng with a sensitivity of 0.5 ng per tube (figure 1A). Both male and estrogen-treated female rat serum could displace the binding of [125 I]rBBP from the heterologous anti-yolk BBP antibodies in a parallel fashion; nearly 80% inhibition of binding could be achieved by 100 nl of either of the sera tested (figure 1A). The cross-reacting protein of the sera, present in such an high concentration, was suspected to be RSA, since the M_r of the isolated rBBP was close to that of RSA (66,000). This premise was further strengthened by the observation (figure 1B) regarding the dose-dependent displacement of [125 I]rBBP from anti-yolk BBP antibodies by RSA (either commercial preparation or that purified by passing through an immuno-affinity column of anti-yolk BBP-IgG coupled to Sepharose 4B).

In order to determine whether rBBP also inhibits the binding of RSA to anti-

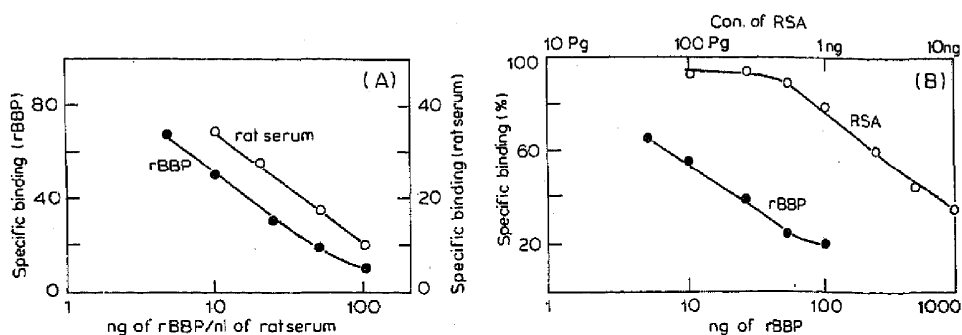


Figure 1. Displacement of $[^{125}\text{I}]$ rBBP binding to yolk BBP antibodies by rat serum (A) and rat serum albumin (B). The dilution of the yolk BBP antiserum used was 1:1000.

RSA antibodies, an RIA for RSA was developed (figure 2A). This had a linearity range of 250 pg–10 ng and a sensitivity of 0.25 ng per tube. By utilising this assay, it could be shown that purified rBBP could significantly displace the binding of $[^{125}\text{I}]$ RSA from anti-RSA antibodies (figure 2A) and the concentration of rBBP required

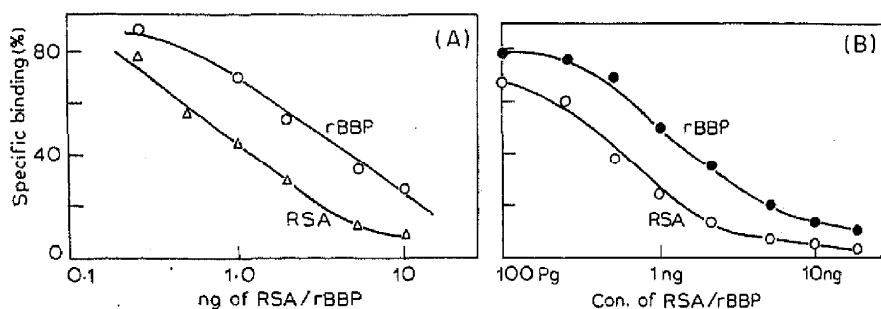


Figure 2. Displacement of $[^{125}\text{I}]$ RSA binding to either RSA antibodies (A) or yolk BBP antibodies (B) by rBBP. The dilutions of RSA antiserum and yolk BBP antiserum used were 1:100,000 and 1:1000 respectively.

for a 50% displacement was approximately 3-fold higher than that required by RSA (figure 2A), thereby implying that both rBBP and RSA appear to recognise similar population of antibodies. In order to further substantiate this observation, binding of iodinated rBBP and RSA to both the anti-RSA antibodies and the anti-yolk BBP antibodies was determined. From figure 3A, it was evident that at 1:100 dilution, the extents of binding of $[^{125}\text{I}]$ rBBP to anti-yolk BBP antibodies and to anti-RSA antibodies were 35 and 25% respectively. On the other hand, at 1:100 dilution, the extents of binding of $[^{125}\text{I}]$ RSA to anti-RSA antibodies and to anti-yolk BBP antibodies were 90 and 80% respectively (figure 3B). This consistent finding of $[^{125}\text{I}]$ RSA binding to anti-yolk BBP antibodies enabled construction of a Standard curve for RSA using anti-yolk BBP antibodies. The linearity range of the standard curve thus obtained was 200 pg–3 ng with a sensitivity of 0.2 ng per tube (figure 2B). In this experiment also, $[^{125}\text{I}]$ RSA binding to anti-yolk BBP antibodies could be

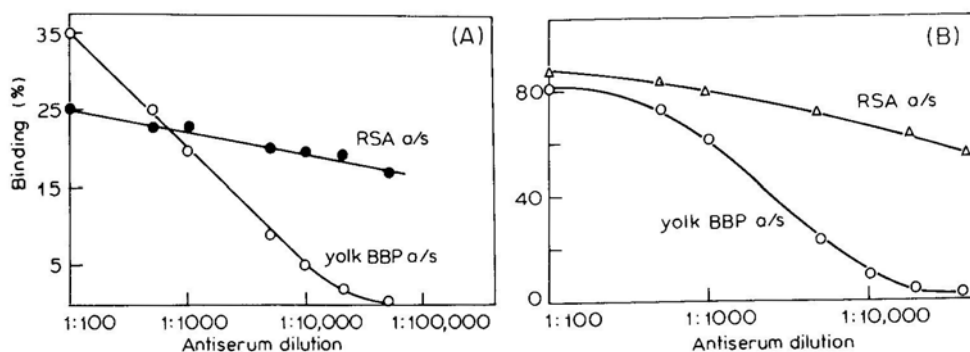


Figure 3. Binding of $[^{125}\text{I}]$ rBBP (A) or $[^{125}\text{I}]$ RSA (B) to either yolk antibodies or RSA antibodies. The input radioactivity for both the radioiodinated proteins was approx. 30,000 cpm.

displaced by unlabelled rBBP; an approximately 3-fold higher concentration of rBBP *vis-a-vis* RSA was required for 50% displacement (figure 2B).

Rate of disappearance of rBBP and RSA

The data of figure 4, show the kinetics of disappearance of the injected $[^{125}\text{I}]$ rBBP and $[^{125}\text{I}]$ RSA from the circulation of immature rats. The rates of disappearance

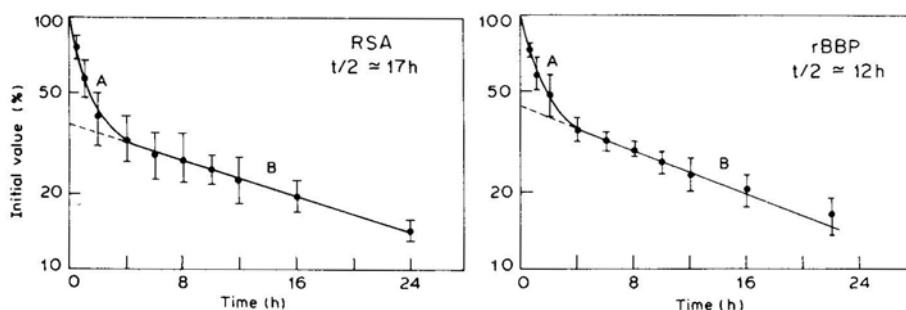


Figure 4. Rate of disappearance of $[^{125}\text{I}]$ labelled RSA and rBBP from the plasma of immature rats. Freshly prepared radio-labelled RSA or rBBP (approx. 5 lakhs cpm) was injected into immature male rats and at the indicated time intervals blood samples were collected and plasma prepared were monitored for radioactivity. The values represent mean and the vertical bars represent SD ($n=5$). (A) Phase of rapid disappearance; (B), phase of catabolism.

of both the proteins exhibited a biphasic pattern, the rapid initial phase (A) presumably representing the dilution of the label with the extravascular space, which was then followed by a slow phase (B) of disappearance. The latter phase is considered to correspond to true catabolism. The slower phase followed a first order kinetics and the least square fit of the data yielded a half life of 12 and 17 h for rBBP and RSA, respectively.

Comparison of electrophoretic mobilities of rBBP and RSA

Resolution by alkaline PAGE (pH 8.3) as well as SDS-PAGE revealed that rBBP and RSA with similar mobilities migrated in both the types of gels with no discernible separation when applied either singly or as a mixture of these two proteins (figure 5). On the other hand, when a mixture of radioiodinated rBBP and

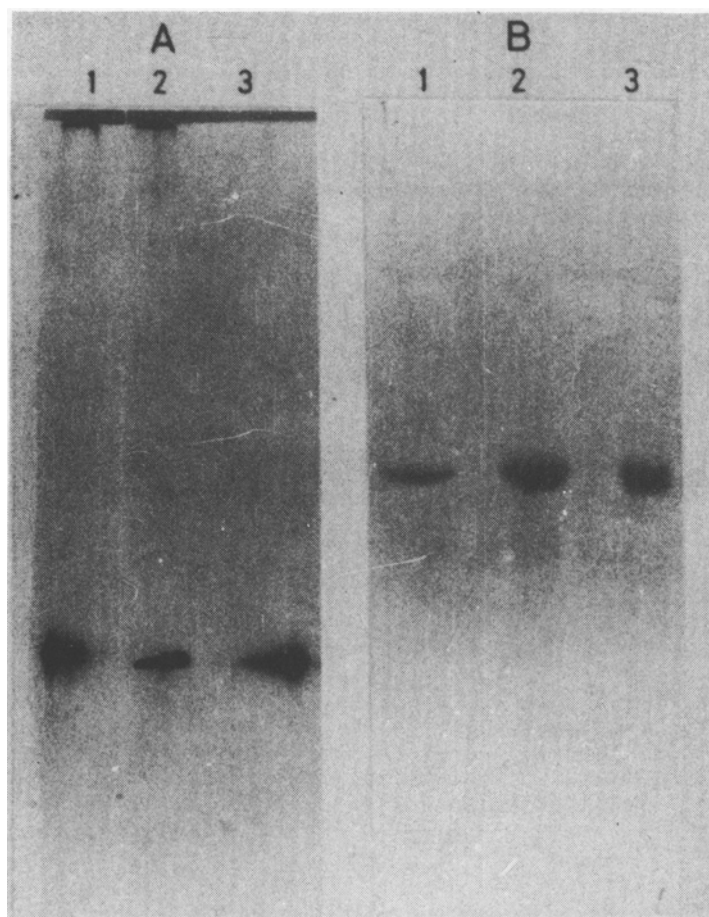


Figure 5. Comparison of electrophoretic mobilities of rBBP and RSA. Alkaline (A) and SDS-denatured (B)—PAGE on 7.5% slab gels were performed as detailed in the text. Lanes 1-3 contained 20 μ g of rBBP (1), a mixture of 15 μ g each of rBBP and RSA (2) and 20 μ g of RSA (3).

RSA was subjected to resolution by two-dimensional non-equilibrium pH gradient PAGE, the autoradiograms revealed two distinct well separated spots (figure 6), the more acidic one corresponding to rBBP. These results clearly reveal a significant pI difference between rBBP and RSA.

Two-dimensional tryptic peptide map analysis of radioiodinated rBBP and RSA

The autoradiograms (figure 7) of the tryptic peptide maps of RSA and rBBP

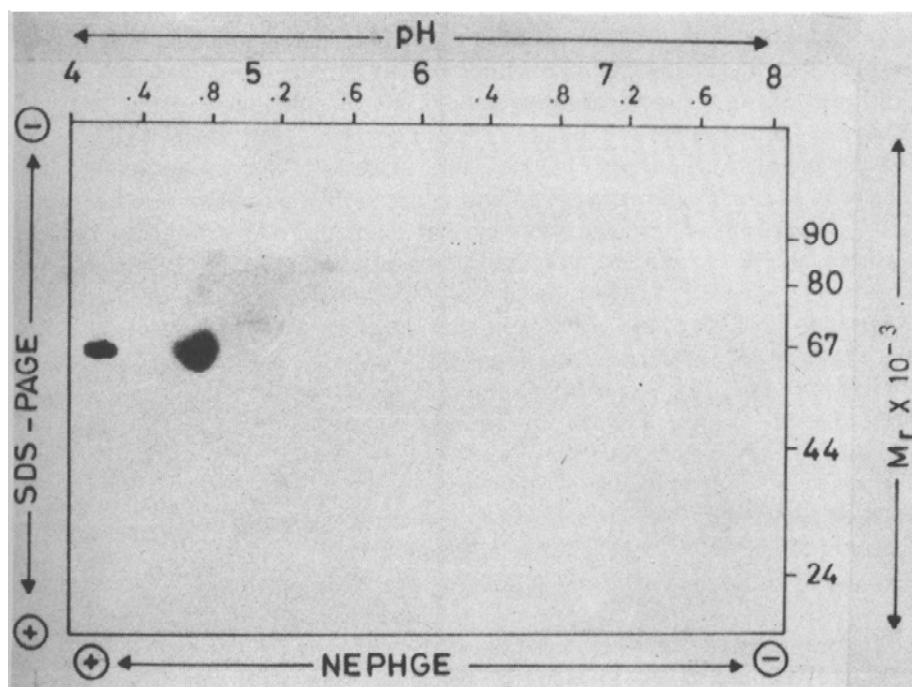


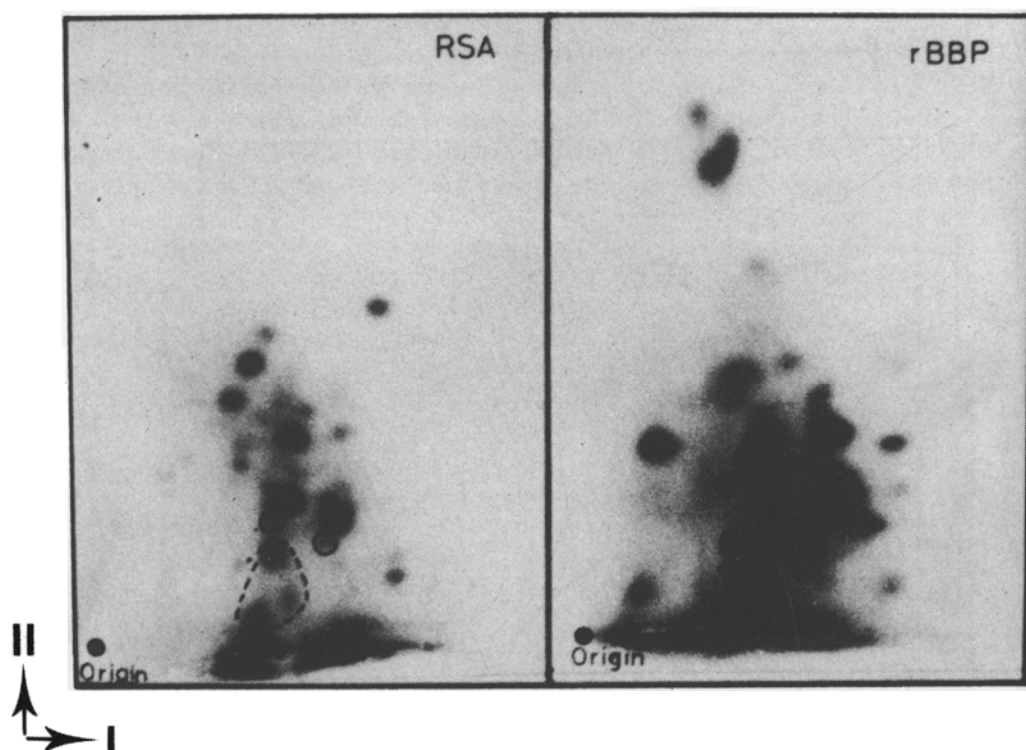
Figure 6. Two-dimensional non-equilibrium pH gradient PAGE of a mixture of radioiodinated rBBP and RSA. A mixture of radioiodinated rBBP and RSA (each approx. one million cpm) was subjected to isoelectric focusing (first dimension) followed by SDS-PAGE (second dimension) on 7.5% gels. An autoradiogram was prepared from this gel, as described in the text.

revealed that the overall pattern of distribution, especially the orientation of closely spaced peptides is not identical thereby indicating distinct differences in the primary structure between these two proteins. The only significant commonality between their peptide maps (figure 7) was with regard to the 3 peptides arranged in a triangle with comparable migrational properties; this might be indicative of similarities in limited amino acid sequences between rBBP and RSA.

Discussion

The primary aim contemplated in the present study was to develop a heterologous RIA for rBBP in order to examine the kinetics of hormonal induction of this protein. However, such an investigation was rendered difficult in view of the rather unexpected immunological cross-reactivity in RIA between rBBP and the abundant serum protein *viz.*, RSA. It was however noticed that other gross immunological methods such as immunodiffusion, immunoprecipitation and Immuno-electrophoresis failed to show this cross-reactivity to any discernible extent (Seshagiri, P. B. and Adiga, P. R., unpublished results).

In displacement studies involving RIA of rBBP and RSA, the puzzling observation was the requirement of 3–5-fold lower concentration of unlabelled RSA



rBBP. Radioiodinated tryptic peptides from RSA and rBBP were obtained as described in the text. These were dissolved in 10 μ l of electrophoresis buffer, applied on one corner of the cellulose-coated plastic sheets and subjected to electrophoresis (first dimension: I), followed by chromatography (second dimension: II). The plates were air-dried and the autoradiograms were prepared. Peptides circled have comparable migrational properties in the maps of RSA and rBBP.

than that of rBBP for equivalent displacement (figures 1B and 2B). Although the precise reason for this finding is not clearly understood at present, it is conceivable that the extent of immunological inactivation of rBBP, absolute concentrations of the unlabelled proteins and their specific activities may be some of the major determining factors for such unusual behaviour. A similar situation was encountered in the case of displacement studies with different serum albumins (SAs). For example, complete inhibition of [125 I] rabbit SA binding to rabbit antibodies to human SA or bovine SA was obtained at 5-fold lesser concentration of unlabelled rabbit SA than the unlabelled human SA or bovine SA (Sakata and Atassi, 1981).

Another significant observation made in the present study was with regard to the recognition of antibodies specific to the protein of an avian origin (yolk BBP) by an entirely different protein of mammalian origin (RSA). A similar observation was recorded with regard to immunological cross-reactivity between the lysozyme of the hen's egg white and bovine α -lactalbumin (Brew *et al.*, 1967). Such seemingly paradoxical cross-reactivity between two unrelated proteins from different species that are distant in the evolutionary scale could be explained to a certain extent as

being due either to close higher order structural similarities or to a limited amino acid sequence relatedness that might exist between proteins. It would therefore seem that such unusual cross-reactivity is attributable to sequence/conformation-specific antibodies which could bind to the related sequence/domain in the heterologous protein (Todd *et al.*, 1982). Among other examples belonging to this category are (i) SA and α -feto protein (Rouslahti and Terry, 1976), (ii) RSA and a M_r 64,000 protein induced under compensatory muscular hypertrophy (Yamada *et al.*, 1984), (iii) BSA and inhibin-like substance (Duraiswamy, S., unpublished results), (iv) rat testis fluid protein and SA (Skinner *et al.*, 1987) and (v) different SAs of species that are in close evolutionary proximity (Wilson *et al.*, 1977).

The fact that there is significant cross-reactivity between rBBP and RSA as revealed by RIA using polyclonal antibodies suggests that they share major antigenic determinants. This strong immunological relatedness between rBBP and RSA raises the discrete possibility that rBBP may represent a very minute fraction of specially modified form of RSA. It is not uncommon that evolutionary mechanisms can convert a single protein with only one defined function in one species into a family of complex proteins with many different functions in another species. This is exemplified by the report that a small sub-population of human IgG specifically binds riboflavin with high-affinity, presumably acting as a carrier protein for riboflavin (Chang *et al.*, 1981).

In order to unravel other aspects of similarities and differences between the two proteins, rBBP and RSA were subjected to some of the techniques that exploit differences in physicochemical properties of these proteins. Notable among the outcome of these experiments are (i) the clear-cut pI difference i.e., 4.1 for rBBP and 4.9 for RSA (figure 6), also sustained by published reports (Seshagiri and Adiga, 1987; Calm, 1975) and (ii) significant changes in the overall distribution patterns of tryptic peptide maps of rBBP and RSA (figure 7). Other differences that differentiate (table 1) between the two proteins are (i) detection of rBBP in circulation only under experimental oestrogenisation (Seshagiri and Adiga, 1987) under such experimental conditions RSA in fact experience deinduction by estrogen (Padmanaban, G., unpublished results), (ii) glycosylated nature of rBBP unlike RSA (Calm, 1975), (iii) different kinetics of disappearance of the injected [125 I] rBBP and [125 I]RSA ($t_{1/2}$: 12 and 17 h respectively, figure 4) and (iv) the recent evidence from this laboratory (Seshagiri and Adiga, 1989) that functionally, rBBP is different from RSA. The last mentioned finding stems from the observation that active immunoneutralisation of BBP in pregnant female rats resulted in early fetal resorption without affecting the maternal health. This finding clearly discriminated BBP from SA in terms of the functional role during pregnancy in the rat. The sum total of these observations is that while rBBP and RSA have several immunological properties in common, they do exhibit many differences in physicochemical properties and more importantly in the functional property (table 1).

It is tempting to speculate that the two proteins might have had a common origin and their ancestral gene might have undergone modification by gene duplication during evolution; during this process, the rBBP gene might have acquired some additional special features like responsiveness to the steroid hormone and the ability to specifically bind biotin with high-affinity, as a consequence of restricted changes in nucleotide sequences, accounting for differences in primary amino acid sequence to acquire the exclusive function of

Table 1. Comparison of rBBP and RSA.

Property	rBBP ^a	RSA ^a
Estrogen dependence	Induced	Deinduced
Binding to biotin AH-Sepharose?	Yes	No
Yield	Low ^b	—
Glycosylated?	Yes	No
pI	4.1	4.9
Specific [¹⁴ C]biotin binding	Yes	No
Tryptic peptide maps	Different	Different
Gross immunological cross-reactivity		
with anti-yolk BBP antiserum	Yes	No
Half-life (t/2) in circulation (h)	12	17
Role in embryonic development	Yes	No

^aInformation on rBBP and RSA, listed in these two columns, is obtained from this paper and from few other publications (Calm, 1975; Seshagiri and Adiga, 1989).

^bThe yield of rBBP is approximately 800 µg/100 ml serum (Seshagiri and Adiga, 1987).

biotin transport during pregnancy. It is pertinent to recall at this stage that vitamin D binding protein of human serum has been recognised as one of the members of the albumin gene family (Cook and Davis, 1985; Yang *et al.*, 1985).

Although the above hypothesis on the mode of evolution of rBBP gene remains to be purely speculative at this juncture, with the recruitment of more sophisticated tools of recombinant DNA technology, a better understanding of rBBP/RSA gene organisation and their mode of evolution from their ancestral gene may become a reality.

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