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Biochemical and immunological aspects of riboflavin carrier protein*

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Abstract. Riboflavin carrier protein which is obligatorily involved in yolk deposition of the vitamin in the chicken egg, is a unique glycophosphoprotein present in both the yolk and white compartments. The yolk and egg white proteins are products of a single estrogen-inducible gene expressed in the liver and the oviduct respectively of egg laying birds. Despite the fact that the carbohydrate composition of the yolk and white riboflavin carrier proteins differ presumably due to differential post-translational modification, the proteins are immunologically similar and have identical amino acid sequence (including a cluster of 8 phosphoser residues towards the C-terminus) except at the carboxy terminus where the yolk riboflavin carrier protein lacks 13 amino acids as a consequence of proteolytic cleavage during uptake by oocytes. The protein is highly conserved throughout evolution all the way to humans in terms of gross molecular characteristics such as molecular weight and isoelectric point, and in immunological properties, preferential affinity for free riboflavin and estrogen inducibility at the biosynthetic locus *viz.,* liver. Obligatory involvement of the mammalian riboflavin carrier protein in transplacental flavin transport to subserve fetal vitamin nutrition during gestation is revealed by experiments using pregnant rodent or sub-human primate models wherein immunoneutralisation of endogenous maternal riboflavin carrier protein results in fetal wastage followed by pregnancy termination due to selective yet drastic curtailment of vitamin efflux into the fetoplacental unit. Using monoclonal antibodies to chicken riboflavin carrier protein, it could be shown that all the major epitopes of the avian riboflavin carrier protein are highly conserved throughout evolution although the relative affinities of some of the epitopes for different monoclonal antibodies have undergone progressive changes during evolution. Using these monoclonal antibodies, an attempt is being made to map the different epitopes on the riboflavin carrier protein molecule with a view to delineate the immunodominant regions of the vitamin carrier to understand its structure-immunogenicity relationship.

Keywords. Riboflavin carrier protein; evolutionary conservation; transplacental transport; immunoneutralisation; monoclonal antibodies; epitope analysis.

Introduction

Vitamin carrier proteins capable of high-affinity interaction with their respective vitamins are present throughout the animal kingdom and play a vital role in the life processes of the vertebrates. A great deal of information has been accumulated during the last few years regarding the biological significance of these specific proteins, whose functions include storage and transport of vitamins and prevention of rapid losses of these vital nutrients due to excretion and/or metabolic degradation. Vitamins are known to remain biologically inert as long as they are associated with their carrier proteins and can be activated only upon dissociation. The specific interaction of vitamins with their respective carriers is through non-covalent forces,

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Abbreviations used: RCP, Riboflavin carrier protein; M_r, molecular weight; RIA, radioimmunoassay; SDS, sodium dodecyl sulphate; IgG, immunoglobulin G; MAB, monoclonal antibody.

thereby permitting a reversible dissociation of the unmodified ligands from the carriers under appropriate physiological conditions. These aspects together with their ubiquitous distribution make them attractive candidates for the study of their biochemical and physiological roles as well as their evolutionary conservation.

Riboflavin carrier proteins

Specific carrier proteins have been identified in oviparous species for riboflavin (Rhodes *et al.,* 1959; Ostrowski *et al.,* 1962), thiamin (Muniyappa and Adiga, 1979, 1981), biotin (Eakin *et al.,* 1940; White *et al.,* 1976), vitamin B_{12} (Sonneborn and Hensen, 1970), retinol (Kanai *et al.,* 1968), vitamin D (Fraser and Emtage, 1976) and folic acid (Krishnamurthy, 1984). It is appropriate to mention that some of these vitamin carriers (for retinol, vitamin D and folic acid) are constitutive to the species, and their hepatic elaboration may be significantly enhanced by appropriate endocrine stimuli to meet the accelerated demand during egg laying. In contrast, others such as those specific for riboflavin, thiamin and biotin are induced *de novo* solely as a reproductive strategem to facilitate vitamin deposition in the developing oocyte (Murthy and Adiga, 1978a; Malathy and Adiga, 1985). These then become detectable both in the eggs and in the maternal circulation. All these vitamins are present at 5–10-fold higher concentrations in the egg than in the maternal circulation; the interaction with carrier proteins apparently facilitates concentration of the vitamins for deposition in the egg (Coates, 1971). These carrier proteins bind the vitamin with a higher affinity than the respective co-enzyme derivatives and this may be a mechanism by which the developing oocyte can sequester the vitamins for its own use in the most appropriate form.

Among the various vitamin carriers hitherto studied from different avian species, chicken riboflavin carrier protein (RCP) is the best characterised, apparently because relatively simple procedures are involved in its isolation in good yields from egg white. Other attractive features associated with this vitamin carrier are: (i) its presence, unlike other major egg proteins, in both egg yolk and white, which could mean dual loci of biosynthesis *viz.,* the liver and the oviduct respectively of egg laying hens, (ii) its inducibility *de novo* presumably by sex steroids and hence its potential as a model system for studies of steroid hormone induced gene expression and (iii) its reversible, high-affinity interaction with flavin which makes it an attractive flavor-protein system with which to understand the structural features involved in ligand-protein interaction. Investigations in our laboratory and elsewhere have led to an understanding of its structure and function in well-defined terms and an overview of the available information is presented below.

RCPs have been isolated from both compartments of the egg (Ostrowski *et al.,* 1962; Murthy and Adiga, 1977) as well as from the serum of laying hens (Murthy and Adiga, 1978b) and the proteins are identical immunologically and biochemically in terms of their affinity and preference for riboflavin. Egg white RCP has recently been crystallised (Zanotti *et al.,* 1984).

Biochemical aspects

Chicken RCP is a phosphoglycoprotein with molecular weight (M_r) 37,000 and exhibits preferential affinity for riboflavin in the Ka range 10^6–10^8 M^{-1} (Rhodes *et*

al., 1959). Both egg white and yolk RCP were earlier postulated to consist of two non-identical subunits (M_r 24,000 and 8,000) joined by two interchain disulphide bridges (Philips, 1969). However, this has not been confirmed either in our laboratory or elsewhere (Murthy and Adiga, 1977; Becvar and Palmer, 1982; Kozik, 1982). Analysis of carbohydrate composition of egg white RCP has revealed that the protein contains 10% hexosamine and 4% neutral sugars with a single sialic acid residue at the terminus of a highly branched oligosaccharide chain (Philips, 1969). Egg yolk RCP contains both hexose and hexosamine as well as multiple sialic acid residues (Ostrowski *et al.,* 1968). The sequences of these oligosaccharides are yet to be elucidated.

Analysis of the amino acid composition of RCP shows the presence of all the common amino acids (Norioka *et al.,* 1985); the protein is particularly rich in glutamic acid, serine and aromatic amino acids. The amino acid sequences of RCP (figure 1) from egg white and serum have been compared (Norioka *et al.,* 1985) and found to be identical; the yolk RCP also has identical sequence except at the carboxyl terminus where it lacks 13 amino acids. It is therefore reasonable to

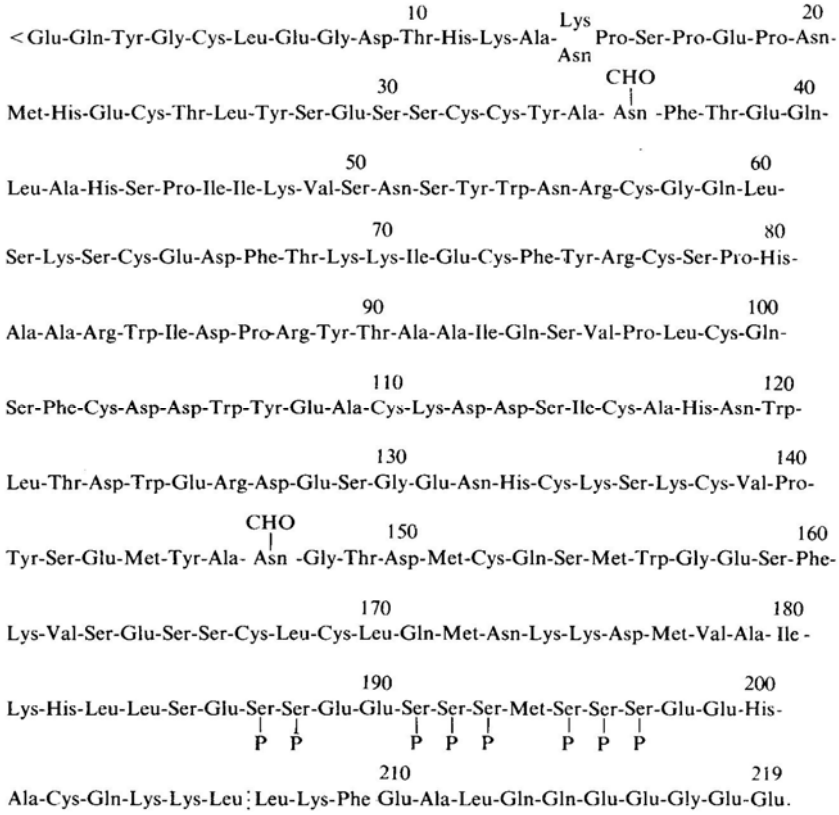

Figure 1 Amino acid sequence of chicken egg white riboflavin carrier protein (from Hamazume *et al.,* 1983).

<Glu, Pyroglutamyl residue; CHO, carbohydrate chain; Ser phosphoserine,: cleavage site for yolk RCP. P

assume that during incorporation of circulatory RCP into the oocyte or yolk fluid, the C-terminal end is cleaved in a specific manner (Deeley *et al.,* 1975), somewhat like the processing by proteolysis of vitellogenin to form lipovitellin and phosphovitin (Bergink *et al.,* 1974). Thus RCP is the second example among yolk proteins that is enzymatically modified during incorporation into the yolk. In other respects, yolk RCP has the same characteristics as egg white RCP, and these include the N-terminal pyroglutamic acid, polymorphism in the amino acid sequence (lysine/asparagine at the 14th residue from the N-terminus) and carbohydrate chains attached to Asn-36 and Asn-147 residues. Phosphate groups are also bound to the same serine residues which occur in a cluster between positions 187 and 197 in both yolk and white RCPs. All these observations confirm the earlier hypothesis based on genetic analysis that yolk, plasma and white RCPs are derived from the same structural gene (Norioka *et al.,* 1985).

An intriguing aspect of this phosphoprotein is that all the phosphate groups are localised as phosphoserine moieties in a restricted, highly anionic region of the peptide chain (Norioka *et al.,* 1985); thus within a 21 amino acid segment are found 8 phosphoserine residues and 5 glutamate residues (figure 1). This peptide can be isolated by trypsin digestion of reduced and carboxymethylated RCP (Miller *et al.,* 1984) and has lysine at its C-terminal and histidine at the N-terminal ends. Sandwiched between these two basic amino acids is a sequence of 21 amino acids among which 14 carry one or two negative charges at physiological pH. In view of the high degree of charge repulsion, it is assumed that this phosphopeptide is rigid, with little or no ordered secondary structure. A highly conspicuous feature of this phosphopeptide is the palindromic sequence around Met-194. This residue is sandwiched between 6 phosphoserine and 4 glutamic acid residues in a defined sequence. The biological significance of this rather unique amino acid sequence is currently unknown.

The carbohydrate composition of yolk RCP is identical to that of plasma RCP, but both differ from that of egg white RCP showing that the post-translational attachment of carbohydrate chains of RCP differs in the liver and oviduct. However, it is intriguing that the phosphorylation sites of egg yolk RCP are similar to those of egg white RCP, indicating that protein kinases with similar specificities participate in the phosphorylation of the protein in the liver and oviduct (Norioka *et al.,* 1985).

Riboflavin binding characteristics of chicken RCP

Every region of RCP has been investigated for flavin binding, receptor recognition as well as antigenicity. Extensive work has been carried out on the flavin binding characteristics of the protein in an attempt to understand the sites of flavin-protein interactions apparently as a model for flavin co-enzyme-enzyme interaction. The apoprotein binds to a variety of flavin analogues in a 1:1 stoichiometry and shows a preferential affinity for riboflavin (Nishikimi and Kyogoku, 1973). The fluorescence of flavin is completely quenched on binding to egg white apo-RCP while 80% of the protein fluorescence is quenched on binding to riboflavin and 3-methyl riboflavin and 77% on binding to lumiflavin (Nishikimi and Kyogoku, 1973). Nuclear magnetic resonance data indicate that the chemical shifts of the carbon and nitrogen atoms of riboflavin hardly differ on binding to either egg white or yolk RCP indicating that the binding site for the oxidised isoalloxazine ring are similar in the two isoproteins

(Nishikimi and Kyogoku, 1973). However, thermodynamic analysis indicates that the cavity in which riboflavin sits in the protein is relatively smaller in yolk RCP than in egg white RCP (Nishikimi and Kyogoku, 1973; Matsui *et al.,* 1982a). Moreover, N-3 of riboflavin is exposed to the solvent while N-10 and ribityl side chain are strongly involved in the interaction with the protein by formation of hydrogen bonds (Matsui *et al.,* 1982b; Moonen *et al.,* 1984). Studies using model compounds with different modifications of the flavin molecule reveal that the dimethyl benzenoid part of the ring is the predominant portion involved in interaction with the apoprotein and gets buried to a large extent in the protein (Choi and McCormick, 1980).

The flavin binding site in the protein has been studied by chemical modification of the protein. From the data obtained hitherto, it appears that tryptophan residues are essential for the binding of riboflavin (Murthy *et al.,* 1976; Blankenhorn, 1978). Modification of 5 tryptophans completely abolished the flavin binding ability of yolk RCP (Miller *et al.,* 1981a) and it has been proposed earlier that 1–2 tryptophans are essential for riboflavin binding in egg white RCP (Murthy *et al.,* 1976). More recent studies have however revealed that one of these tryptophan residues is critically involved in the binding of flavin and this tryptophan is not protected by bound flavin against chemical modification. Tyrosine is not critically involved in flavin binding because extensive iodination or nitration does not alter the flavin binding capacity (Farrell *et al.,* 1969). However, a further study has established that one tyrosine is apparently located at the binding site since it is protected against chemical modification by bound flavin (Blankenhorn, 1978).

Earlier work (Murthy, 1977) has indicated that at a low pH, the protein undergoes self-aggregation leading to a reduced affinity for the vitamin. In fact, there is nearly 100-fold reduction in riboflavin binding capacity on lowering the pH from 7·4–4. The presence of sodium dodecyl sulphate (SDS) also reduces the binding. However, interaction of the protein with polyclonal antibody or concanavalin-A does not seem to change its affinity for riboflavin, suggesting that the riboflavin binding site is distinct from antigenic sites (Murthy, 1977).

Receptor recognition sites on RCP

The function of RCP, as mentioned earlier, is confined to the deposition of the vitamin in the developing oocyte. It is also believed that RCP, mostly present in apoprotein form in egg white, may have a bacteriostatic role in sequestering the free vitamin released into the albumen during embryonic development (Board and Fuller, 1974). On the other hand, RCP present in the yolk is involved essentially in meeting the nutritional requirements of the growing embryo. Yolk RCP is deposited into the yolk from the blood, with the vitamin firmly bound to it; direct evidence for this stems from experiments using mutant hens afflicted with the hereditary syndrome avian riboflavinuria (Maw, 1954). When RCP isolated from the eggs of normal hens was injected into laying hens homozygous for the avian riboflavinuria trait, and the eggs examined for evidence of transfer of RCP by immunoprecipitation, the protein was detected at low levels in the egg yolk at 2 days following injection, but none was found in the egg white (Hammer *et al.,* 1971). This shows clearly that the blood protein is directly incorporated into the yolk. Furthermore, removal of sialic acid reduced the transport of RCP to yolk by 88% (Miller *et al.,* 1981a), while oxidation

of galactose and removal of N-acetylglucosamine and galactose also led to diminished transport of the protein into the yolk (Miller *et al.,* 1981b) despite the fact that the protein still retained riboflavin binding activity. A comparison of the carbohydrate composition of yolk and circulatory RCPs indicates removal, during ovarian uptake, of one sialic acid, one fucose, two galactose, and 3 N-acetylglucosamine residues from the precursor serum RCP. However, it may be pointed out, despite the obvious implication that the carbohydrate (especially the sialic acid) residues are involved in uptake by the oocyte, that no direct evidence for binding of RCP to the ovarian follicular membrane has been demonstrated so far (Miller *et al.,* 1982a).

Another region of RCP which has been implicated in oocyte membrane recognition is the phosphopeptide moiety; dephosphorylation of egg white RCP or yolk RCP has no effect on the binding of riboflavin by the protein, but oocyte uptake of the dephosphorylated protein is greatly reduced (Miller *et al.,* 1982b). Removal of a single phosphate residue from yolk RCP decreases follicular uptake by 60% and this cannot be restored by the addition of anionic groups such as by succinylation (Miller *et al.,* 1982b). The phosphopeptide portion appears to function autonomously of the rest of the protein and could be involved in recognition of the putative receptor on the oocyte membrane, either through direct interaction or by directing the protein in such a way as to facilitate subsequent interaction with the receptor in a potential gradient (Miller *et al.,* 1984).

Interestingly, succinylation of the native protein also decreases its uptake by the oocytes (Miller *et al.,* 1981b) indicating that uptake also involves other segments of the peptide chain. Hence, uptake of RCP by oocytes may be a complex sequence of protein-receptor interaction involving phosphate, sialic acid and lysine residues and elucidation of the mechanism awaits further research.

Mammalian RCPs

In contrast to the extensive knowledge available on chicken RCP, the information on mammalian RCPs is limited to a few cases. The first demonstration of RCP from a mammalian source was reported from our laboratory (Adiga and Muniyappa, 1978; Nutrition Reviews, 1979). Using a sensitive radioimmunoassay (RIA) involving iodinated chicken RCP and antiserum to chicken RCP, a protein cross-reacting with chicken RCP could be detected in pregnant rat serum (Muniyappa, 1980). The protein has been purified by lumiflavin-affinity chromatography, though its M_r was ambiguous. More recent data from our laboratory however reveal that the rodent RCP purified by fast protein liquid chromatography has a molecular size comparable to that of chicken RCP (Karande, A. A. and Adiga, P. R. unpublished results). Evidence for the functional role of rat RCP has also been obtained. Administration of antibodies to chicken RCP to pregnant rats leads to pregnancy termination (Muniyappa and Adiga, 1980) consequent to a decrease in uptake of riboflavin by the developing embryo (Murty and Adiga, 1981). Further studies on the mechanism of fetal wastage has revealed (Krishnamurthy *et al.,* 1984) that the drastic curtailment of vitamin supply to the embryo leads to profound changes in the pattern of co-enzyme levels particularly that of FAD as a consequence of inhibition of FAD synthesis coupled with high activity of the catabolic enzyme present in the embryonic tissue (Surolia *et al.,* 1985).

Following active immunisation of normal female rats with chicken RCP the consequent chronic *in vivo* immunoneutralisation of maternal RCP leads to termination of pregnancy around days 8–10 of gestation. It has been proposed that rat RCP may not be involved in the fertilization/implantation process *per se* but is definitely required for providing adequate riboflavin to the developing fetus (Murty and Adiga, 1982a). Pregnancy continues to term with the delivery of normal pups when circulating antibody concentrations are allowed to wane with time in actively immunized rats (Murty and Adiga, 1982a), showing the reversibility of the immuno-neutralisation process.

Another claim for the occurrence of RCP in a mammal was made by Merrill *et al.* (1979), who purified RCPs from bovine plasma and adduced evidence for a Pregnancy-specific riboflavin binding protein. These proteins have been isolated by affinity chromatography using N^3-carboxymethyl riboflavin coupled to AH-Sepharose. At least 3 major protein bands could be observed migrating in regions ascribed to the β- and γ-globulins of plasma following cellulose acetate electro-phoresis. The M_r of one of these proteins was 150,000 as assessed by gel filtration, but the interesting observation was that a small amount of another riboflavin binding protein of M_r 37,000 was also present. All 3 proteins bound [^{14}C]-riboflavin avidly, with high affinity (kd= 10^{-6} mol/litre). The presumed pregnancy-specific, low M_r protein from bovine serum was purified to apparent homogeneity and appeared to have an even higher affinity for riboflavin. No further analysis of these proteins has been forthcoming, but it is claimed that a certain protein binding riboflavin with high affinity is associated with pregnancy in higher mammals with a function analogous to that of serum RCP in laying hens.

Earlier studies on RCPs in higher mammals were confined to their detection and partial characterisation in human sera. Merrill *et al.* (1979) have reported that besides albumin, which is known to associate with riboflavin with low affinity (Jusko and Levy, 1975), a certain fraction of immunoglobulin G (IgG) also binds riboflavin with a reasonably high affinity (4 μM) (Merrill *et al.,* 1981). This fraction (about 1% of total IgG) could be isolated by affinity chromatography and is non-specific in the sense that it is present in the sera of male and female non-pregnant and pregnant individuals. However, because of the relatively higher concentrations of albumin in the serum it has been suggested that only 5-6% of protein-bound riboflavin in normal human plasma is associated with this IgG fraction (Merrill *et al.,* 1981).

It seems unlikely that riboflavin is the antigen inducing these immunoglobulins and it is conceivable that ligand binding is accomplished on a site on these proteins which accomodates the flavin and/or ribityl side-chains. Eisen *et al.* (1970) have observed that a monoclonal immunoglobulin A produced by mouse plasmacytoma MOPC-315 binds riboflavin (Kd 36 μM) and other hydrophobic compounds moderately tightly. A much tighter binding of riboflavin has been reported by Farhangi and Osserman (1976) for a monoclonal IgG (IgG^{gar}) produced by a patient with multiple myeloma. This human monoclonal IgG 2 (λ) is separable into two similar fractions by ion-exchange chromatography; one of these fractions is nearly saturated with the ligand with an average of about 2 equivalents of riboflavin/mol, while the second, slightly more acidic, fraction has a small amount of riboflavin associated with it (about 0·2 equivalent flavin/mol) (Farhangi and Osserman, 1976). The vacant site on native IgG^{gar} reversibly binds up to a total of 2 equivalents of riboflavin per mol of protein. Sites already occupied by riboflavin bind the flavin irreversibly and the vitamin is dissociated only on denaturation by urea (Chang *et*

al., 1981a, b). Using a variety of flavin analogues, the regions on the riboflavin molecule which associate with the binding site have been identified (Pologe *et al.,* 1982). The pyrimidine edge of the isoalloxazine does not interact with the combining site, particularly around N-3. The ribityl side chain and dimethyl benzene edge of the flavin ring are critical for binding and are probably not exposed (Pologe *et al.,* 1982). A comparison with flavoproteins show that IgG^{gar} binds riboflavin in a rather novel way. The isoalloxazine ring interacts in a way similar to that in FMN binding proteins, but unlike the situation in flavoproteins, the ribityl side chain is not essential for binding. Further investigations are required to shed light on the essential features of this flavin binding site and the way in which such a site is manifest as an integral part of a human monoclonal immunoglobulin.

There has been a recent claim that another protein fraction which binds riboflavin with high affinity can be obtained from human fetal cord blood and is present in relatively smaller amounts (25μg/15ml blood) (Merrill *et al.,* 1981). Further information regarding molecular size or other characteristics is not available at present, but it has been proposed that human blood like blood of other mammals, contains proteins which may serve an ancilliary role to albumin in pregnancy, analogous to the role of RCP in avian eggs.

We have recently isolated and characterised RCPs from pregnant bonnet monkey and human sera (Visweswariah and Adiga, 1987a, b). A heterologous RIA using [^{125}I]-labelled chicken RCP and antiserum to chicken RCP was employed to show that sera from pregnant bonnet monkeys and humans (Visweswariah and Adiga, 1987a, b) are able to inhibit the binding of chicken RCP to specific antiserum. Human umbilical cord serum also contains a cross-reacting protein, in higher concentrations than in maternal pregnancy sera. Isolation of these proteins involves sophisticated protein purification techniques such as fast protein liquid chromatography involving ion-exchange and chromatofocusing. The purified proteins exhibited properties with remarkable similarities to those of the chicken vitamin carrier. Thus, the M_r of both monkey and human RCP (from either pregnancy or umbilical cord sera) are similar to that of chicken RCP (37,000). All the proteins are acidic in nature (pI < 4) and preferentially bind riboflavin *vis-a-vis* FMN and FAD. The purified proteins bind to specific antibodies against chicken RCP which is indicative of extensive sequence similarity amongst the proteins. The sequence similarity could be confirmed by comparing the tryptic peptide maps of [^{125}I]-labelled monkey RCP and chicken RCP. Thus, RCP is a protein which has been retained to near identity from aves to primates. This is strongly suggestive of a vital role for this protein in primate reproduction as well.

Hormonal modulation of RCP

It is now well established that the *de novo* synthesis of yolk proteins in the liver of oviparous vertebrates is clearly in response to augmented circulatory levels of estrogen during egg-laying; synthesis can also be induced by administration of the steroid (Tata and Smith, 1979). Egg white protein synthesis in the oviduct is also hormone-dependent (Kohler *et al.,* 1968; Oka and Schimke, 1969; Palmiter and Gutman, 1972). Since RCP is present in both yolk and white of chicken eggs, we have investigated the details of hormonal modulation of chicken RCP synthesis in the liver and oviduct by administration of hormones to immature male and female

chicks. The induced proteins are secreted by the respective biosynthetic organs even in the absence of a developing oocyte in which they are normally sequestered (Cecchini *et al.,* 1979) and hence plasma or oviduct tissue concentrations reflect the synthetic capacities of liver and oviduct, respectively.

We developed (Murthy and Adiga 1978a) a RIA for chicken RCP to monitor the circulating levels of the protein following estradiol-17β administration to immature male chickens. After a single injection of the hormone, the plasma RCP level is enhanced several-fold at 6 h, reaching peak levels around 48 h and declining thereafter to the basal level. A 2-fold amplification of the response is observed on secondary stimulation with the hormone. A 4h lag phase prior to the onset of induction is noticed during both primary and secondary stimulations with the hormone. The synthesis of the protein is dependent on the dose of hormone administered, with the maximum effect observed with 10 mg/kg body weight. There is no appreciable change in the half-life of the protein on estradiol-17 β administration but the half-life is modulated by the thyroid status of the animal (Murthy and Adiga, 1978a). Progesterone is unable to affect the kinetics of estradiol-17β induced RCP production, but antiestrogens are potently capable of blocking the response.

A detailed comparison of the induction of RCP in the liver and oviduct has been carried out in our laboratory (Durga Kumari, 1984). On primary stimulation of immature female birds with estradiol-17β (10 daily injections), there is a rapid increase in oviduct weight and total RCP after an initial lag period of 2–3 days. Secondary stimulation with estradiol-17β results in a rapid increase in RCP levels without the lag period. Progesterone treatment results only in a slight increase in oviduct weight; it can also activate the differentiated oviduct cell function in terms of RCP synthesis, but only during secondary stimulation, *i. e.,* after primary stimulation with estradiol- 17β. The plasma levels of RCP in these birds reflect progressive increase in synthesis of RCP on primary stimulation with estradiol- 17 β and the characteristic memory effect with attendant amplification of the inductive response during secondary stimulation. However, progesterone is unable to stimulate the synthesis of RCP by the liver when administered as secondary inducer, unlike the phenomenon observed in the oviduct (Durga Kumari and Adiga, 1986). These observations bring into focus subtle qualitative differences in the hormonal regulation of the RCP gene in the two estradiol-17 β dependent avian tissues. The differences may be a reflection of differential modulation of tissue-dependent regulatory elements governing RCP gene expression in the two biosynthetic loci.

Cell-free translation of (polyA$^+$) RNA from both liver and oviduct has revealed that enhanced RCP mRNA levels account for the increased synthesis of RCP in these two tissues (Durga Kumari, 1984). In a heterologous cell-free translation system, *viz.,* rabbit reticulocyte lysate, a precursor RCP of M_r 38,000 is identified which is processed to native RCP in the presence of stripped microsomes from avian liver. The increased mRNA activity associated with chicken RCP production on secondary stimulation could be correlated with greater number of mRNA molecules due to enhanced transcription and/or due to stabilization of cytoplasmic RCP mRNA during secondary stimulation, as shown for vitellogenin, ovalbumin and conalbumm (Palmiter, 1972).

Recent investigations have established that rodent RCP synthesis is also modulated by estrogen. Immunological studies have shown that a protein cross-reacting with chicken RCP is detectable in the sera of ovariectomised female rats on

administration of estradiol- 17*β* (Muniyappa and Adiga, 1980). Moreover, the levels of the protein is clearly modulated by circulatory concentrations of estrogen during the 4-day estrous cycle: the concentration of the protein is highest during pro-estrous when estrogen concentration is the highest. The concentration of the protein appears to change during pregnancy with a gradual increase to peak around day 10 of gestation and is maintained more or less at high levels till term. These studies employed a heterologous RIA utilising [^{125}I]-labelled chicken RCP and antiserum to chicken RCP. Using a homologous RIA for rat RCP, the hormonal induction of the protein has been investigated (Murty and Adiga, 1982b) and the data are essentially in agreement with the results reported earlier. It is noteworthy that the rodent protein is also induced specifically by estradiol-17*β* in a dose-dependent manner and its synthesis is blocked by cycloheximide (Murty and Adiga, 1982b).

The circulatory concentrations of monkey and human RCPs are also modulated by physiological changes in estrogen concentration that occur during the menstrual cycle and pregnancy. Administration of estradiol- 17 *β* to immature male or female monkeys is able to enhance circulatory concentrations of monkey RCP (Adiga *et al.,* 1986; Visweswariah, 1986). These results clearly reveal that the evolutionary conservation of RCPs extends not only to structural and physicochemical properties but also to their estrogen- denpendent elaboration by the liver. This implies an important role for these proteins during gestation in primates, presumably analogous to that established in the avian and rodent models. Confirmatory evidence for this premise stems from recent observations in our laboratory that active immunisation of adult female bonnet monkeys with chicken RCP leads to early termination of pregnancy, provided that the antibody titres in circulation are high enough to neutralise endogenous protein (Adiga *et al.,* 1986). These results lend credence to our working hypothesis that the immunological similarities between the RCPs is a reflection of the vitamin carrier performing a definite and important function in primate reproduction in terms of facilitating transplacental flavin transport from the maternal supply line to the developing fetus (figure 2).

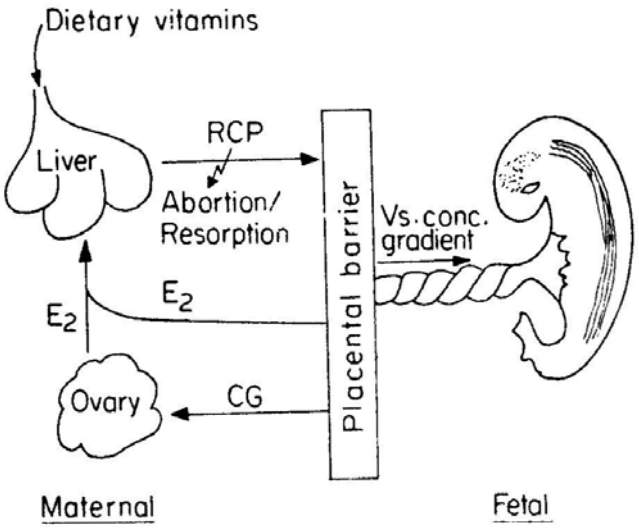

Figure 2. Schematic representation of mechanisms of induction and transplacental transport of RCP in the pregnant rat.

From the foregoing, it is clear that RCP is highly conserved throughout evolution from the aves to humans, not only in terms of similarities in gross structure, but also with regard to the hormonal modulation of its induction during the reproductive phase and its obligatory role as vitamin carrier from the maternal system to the developing oocyte/embryo. It is now well recognised that fixation of changes in protein sequence or structure depend on whether the changes will be compatible with the biochemical function of the protein and the degree of dispensability of the protein for the survival of the organism. The gross similarities among the RCPs throughout evolution therefore emphasise the vital role this protein has to play during reproduction.

Immunological studies on RCP

The similarities in the physicochemical properties of various RCPs extend to the extensive immunological cross-reactivity observed with polyclonal antisera to chicken RCP. A strong, although not perfect, quantitative correlation exists between amino acid sequence similarity and immunological resemblance. Thus, proteins which have a greater than 40% divergence in amino acid sequence, show no immunological cross-reactivity (Arnheim, 1973; Wilson *et al.,* 1977), and in general, the degree of cross-reactivity observed between two homologous globular proteins is directly related to the degree of resemblance between their amino acid sequences (Arnheim, 1973; Wilson *et al.,* 1977). Therefore, by implication, the immunological cross-reactivity observed amongst RCPs is highly suggestive of similarities in amino acid sequences. However, the cross-reactivities observed with whole polyclonal antisera are influenced by a number of variables, such as the relative and absolute concentration and affinities of the different determinant-specific antibodies which comprise the antisera as well as the inevitable variations in response to antigen by individual animals. Monoclonal antibodies (MAbs), however, can provide an immunological comparison of proteins on a determinant-by-determinant basis, since small changes in protein structure may produce large changes in immunological cross-reactivity. Certain MAbs have been known to discern even single amino acid changes in the sequences of two proteins (Harris, 1983) and hence, by virtue of their property of each reacting only with a single determinant, may provide exquisitely sensitive probes for discriminating between structurally related proteins. With this view in mind, we have raised MAbs to chicken RCP in an attempt to study more closely the homology in various determinants between RCPs of different species and in order to gain further insight into the antigenic map of chicken RCP and sequence divergence, if any, in the RCPs present in mammalian sera.

Studies hitherto on the antigenicity and antigenic domains of chicken RCP are few and more detailed analysis is needed. The protein is highly antigenic and antibodies can be raised in a variety of mammalian species, *viz.,* rabbit, rat and monkey (Cotner, 1972; Ramanathan *et al.,* 1979). Chemical modifications of RCP reveal that the protein moiety largely contributes to the antigenicity of the protein (Ramanathan *et al.,* 1980). Total reduction of the disulphides, NBS-oxidation of the tryptophans and succinylation or dinitrophenylation of the lysine residues results in a loss of flavin binding capacity as well as antigenicity (Ramanathan *et al.,* 1980). Deglycosylation apparently does not alter the antigenicity of the protein in a significant way (Ramanathan *et al.,* 1980). Further investigation clearly shows that modification of

lysine residues affects the antigenicity drastically . Thus, a dinitrophenyl derivative of the holoprotein shows some antigenic similarity with the native protein, while DNP-apoprotein fails to give a similar reaction. Amidation of 88% of the lysines in the apoprotein is accompanied by 82% decrease in potential antigenicity with complete retention of flavin binding activity, but the slope of the inhibition curve of the amidiated derivative in RIA is different from that of the unmodified RCP indicating a weakening of almost all antigenic determinants (Cotner, 1972; Ramanathan *et al.,* 1980). Modifications of tryptophan and tyrosine residus in the protein do not alter its antigenic properties, but leads to a complete loss of flavin binding properties (Ramanathan *et al.,* 1980). These observations lead to the inevitable conclusion that antigenic sites on the molecule are mostly localized in areas different from the riboflavin binding site. Moreover, holoflavoprotein and apoflavoprotein react similarly in RIA and Ouchterlony immunodiffusion analysis; the apoprotein bound to its antibody on an affinity column still interacts with flavin at 97% of the theoretical amount. The absence of any cross- reacting peptides in the trypsin hydrolysate of the citraconylated, totally reduced and alkylated apoprotein suggests that the antigenic determinants depend on secondary and/or tertiary structure (Murthy and Adiga, 1978a). Lysine residues may be involved either at the actual antigeneic sites and/or their modification leads to drastic changes in the conformation of the protein.

Lysine residues of globular proteins are mainly found localized on the surface of the molecule and protrude into the solvent rather than react with other residues (Arnheim, 1973; Wilson et al., 1977). There are several cases reported where the biologically active site is independent of the antigenic site and this is in agreement with the much studied phenomenon of the conservation of the active site of many enzymes through various stages of polygenetic development (Arnheim, 1973; Wilson et al; 1977). It is attractive to raise the question at this stage wheather various RCPs have retained an identical amino acid sequence/tertiary structure at the riboflavin binding region during evolution. With a view to study in greater detail the immunological cross-reactivity amongst avian and mammalian RCPs by a sensitive determinant-by-determinant approach, we have generated MAbs to chicken RCP by employing the hybridoma technique developed by Kohler and Milstein (1975). We have used the myeloma SP2/0-Ag as the fusing partner of mouse splenocytes and optimised immunisition protocols. Mice were immunised with chicken egg white RCP (Visweswariah, 1986). Fusion was performed with 50% polythylene glycol (PEG 4,500) and 10% dimethyl sulphoxide and the hybrid clones were screened by enzyme linked immunosorbent assay and a solid phase protein. A binding assay (Visweswariah et al., 1987). Three MAbs have been extensively characterised and their properties have been described recently (Visweswariah et al., 1987). The affinity calculated by Scatchard analysis for the parent antigen varies as expected with each antibody. These antibodies do not appear to differentiate between holo-and apo-RCP, in agreement with observations with polyclonal antisera. Denaturation of RCP with SDS also does not modify the interaction of the protein with these MAbs, but earlier results from this laboratory have shown that chicken RCP treated with SDS has significantly reduced affinity for riboflavin (Murthy, 1977). This is consistent with the premise that the flavin binding site is distinct from the major antigeneic determinants recognised by the 3 MAbs. However, as shown recently(Visweswariah *et al.*, 1987), total denaturation of RCP by reduction and carboxymethylation eliminates recognition of the modified protein by the MAbs. This shows that none

of these MAbs recognize a linear sequence of amino acids *per se* and that at least a partially native conformation of chicken RCP is essential for interaction with these antibodies. Interestingly, succinylated chicken RCP is also not able to inhibit the bindingof [^{125}I]-labelled native chicken RCP to these MAbs even at a 100–fold excess concentration, indicating that lysine residues are involved in the recognition of the protein by these antibodies, in agreement with the observations made with polyclonal antisera.

Using a novel method of epitope analysis using Superose 12 gel filtration in conjunction with fast protein liquid chromatography, it could be shown that the 3 MAbs are directed to 3 different and distinct epitopes on the chicken RCP molecule (Karande *et al.,* 1987). Employing these MAbs, studies were initiated to ascertain whether the epitopes on chicken RCP to which these MAbs are directed are conserved in mammalian RCPs. As expected we could indeed observe an inhibition of binding of [^{125}I]-labelled chicken RCP to each of these MAbs by different concentrations of rat, monkey and human RCPs (Visweswariah *et al.,* 1987). This indicates that the epitopes defined by these MAbs are clearly present in mammalian RCPs as well. By employing RCP isolated from human pregnancy serum and umbilical cord serum, we could show that at least in the regions defined by the 3 MAbs, the two proteins were nearly identical, as gauged by very similar affinities of the MAbs for them (Visweswariah, 1986). If one assumes that the protein from cord serum is largely of fetal liver origin, then there is apparently no significant difference between the embryonic and the adult RCP gene products at least in terms of these epitopes. The results obtained using MAbs are in close agreement with our earlier observations employing polyclonal antisera and provide a means of mapping the various epitopes on the chicken RCP molecule.

Towards this end, a number of other MAbs to chicken RCP have been produced and are being characterised at present (Kuzhandhaivelu, N., Karande, A. A. and Adiga P. R., unpublished results). Preliminary results indicate that one of the MAbs is able to recognise egg white RCP but not egg yolk RCP. As stated earlier, the difference between these isoproteins is the two carbohydrate chains and the 13 amino acid chain at the C-terminus which is present in egg white and serum RCP but is cleaved off during uptake by oocytes to produce mature yolk RCP (Norioka *et al.,* 1985). It is therefore likely that one of the MAbs recognises the conformation associated with these 13 amino acids, and this region could represent a continuous epitope on the chicken RCP molecule (Sela, 1969). Alternatively, this MAb could recognise one of the carbohydrate chains exclusive to the egg white. We also have preliminary evidence to show that one of the other MAbs recognizes in solid-phase RIA the phosphopeptide isolated from egg white RCP representing the sequence of amino acids from 181-204 (figure 1).

An attempt has been made to theoretically predict the possible antigenic domains on the chicken RCP molecule by performing a hydrophilic analysis (Hopp and Woods, 1981; Visweswariah, 1986) using the known amino acid sequence of chicken RCP (Norioka *et al.,* 1985). This exercise was prompted by the recent evidence that antigenic domains on globular proteins are localised on the surface of the protein molecule in regions of high atomic mobility (Tainer *et al.,* 1985) and as a consequence, hydrophilic regions of the protein are most likely to be antigenic as they are found predominantly on the surface of the molecule (Benjamin *et al.,* 1984). The results of the hydrophilic analysis are shown in figure 3. The region of highest

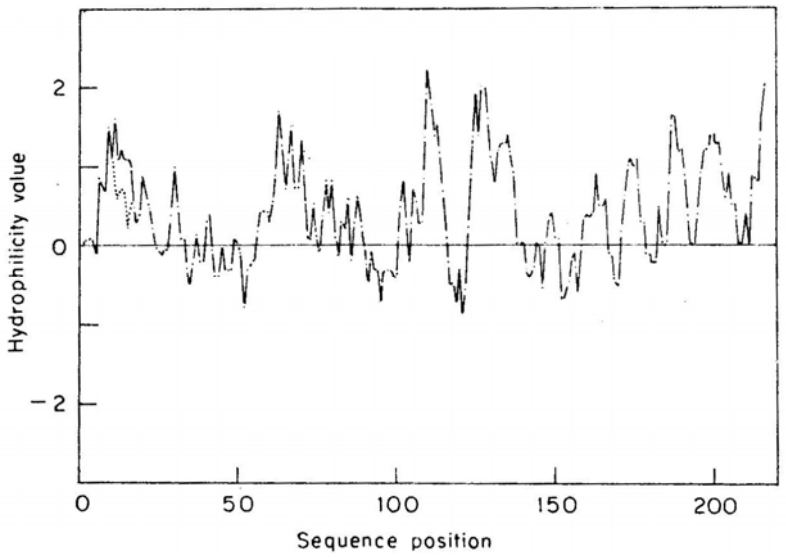

Figure 3. Hydrophilicity profile of chicken RCP. The hatched line indicates the profile obtained when asparagine is present in the sequence at position 14 instead of lysine. The averaged hydrophilicity values are plotted versus position along the amino acid sequence. The x-axis contains 214 increments, each representing an amino acid in the sequence of chicken RCP. The y-axis represents the range of hydrophilicity values from – 3 to + 3. The data points are plotted at the centre of the averaging group from which they were derived.

hydrophilicity is in sequence (108–118) and this is likely to be an antigenic domain. It appears that there are 3 major hydrophilic regions in the molecule (60–70, 105–115, 120–140) and it is attractive to propose that the MAbs described here are directed to these 3 regions.

This theoretical excercise may be able to predict with a certain degree of confidence some sequences which comprise antigenic domains of the protein. However, the smaller hydrophilic peaks are not always associated with immunogenic sites (Hopp and Woods, 1981). An improved method which eliminates to a great extent the redundancy of prediction makes use of the recognition factors of various amino acids (Fraga, 1982) and this correction has been applied to the analysis of chicken RCP (figure 4). Each amino acid in the chicken RCP primary sequence is assigned a recognition value and these values are repeatedly averaged over 6 residues. Figure 4 indicates the recognition value of the residue at the mid-point of each hexa-peptide. The assumption that regions of high hydrophilicity and low recognition are antigenic may lead to accurate prediction of antigenic domains with a high success rate. It can be seen that some regions of highest hydrophilicity in chicken RCP have a very low recognition value and therefore are most likely to be contained within antigenic determinants. Based on a similar consideration, the region (120–140) is also likely to comprise a determinant. However, other minor peaks of hydrophilicity appear to be non-immunogenic in that they coincide with peaks in the recognition profile. The region of the phosphopeptide (182–204) perhaps is of greatest interest, since a definite biological activity has been assigned to it recently (Miller *et al.,* 1982b). This sequence is contained within a region of high

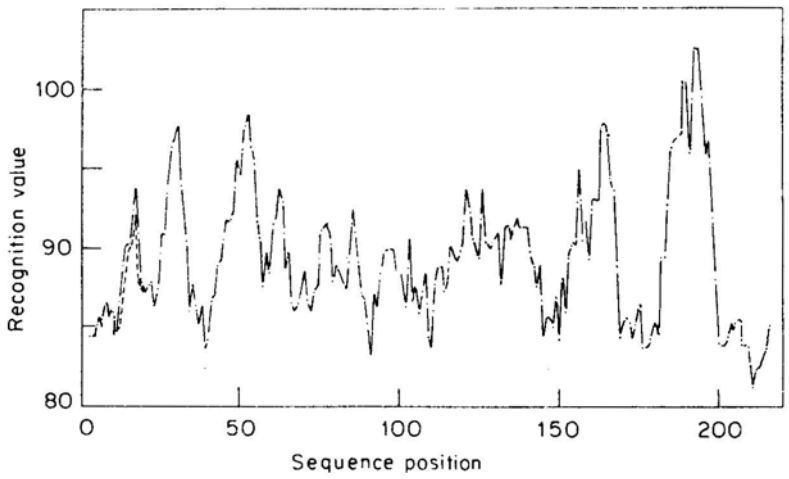

Figure 4. Recognition profile of chicken RCP. Recognition values were assigned to each residue in chicken RCP and repeatedly averaged over each hexapeptide. These averaged values are plotted versus position along the amino acid sequence. The y-axis represents the range of recognition values, and the data points are plotted at the centre of the averaging groups from which they were derived. The hatched line indicates the values obtained when asparagine is present in the sequence at position 14 instead of lysine.

recognition and is normally unlikely to be within an antigenic domain. However, in all these analysis, no correction is made for amino acid residues modified by phosphorylation or glycosylation and it is quite likely that these may cause shifts in both hydrophilicity and recognition value. The high charge of the phosphate in chicken RCP could induce certain changes in the structure of the protein such that the phosphopeptide is exposed to the surface. Isolation of the phosphopeptide and a study of its immunogenicity will provide information on whether this region is contained within an immunodominant site or not.

The hydrophilic analysis in conjuction with the recognition profile of chicken RCP could thus provide information on the possible peptide sequences to which the MAbs to chicken RCP are directed. The analysis also explains certain conclusions reached from experiments conducted with polyclonal antisera. Firstly, there are reports that there is no difference in the polyclonal response to chicken apo-RCP and vitamin bound-RCP (Cotner, 1972; Ramanathan *et al.,* 1979, 1980), despite the significant conformational changes that occur on binding of the vitamin at the active site. If tryptophan is critically involved in the binding of riboflavin to chicken RCP (Blankenhorn, 1978), and since it appears that 5 out of 6 of the tryptophan residues in chicken RCP are contained in hydrophobic pockets of the molecule (residues 54, 84, 106, 120, 156) (figure 3), these residues may not be exposed to the surface of the molecule and could thus account for the non-immunogenicity of the riboflavin binding site. Another observation made using polyclonal antisera, is that there is no difference in antigenicity of egg yolk RCP and egg white RCP (Cotner, 1972). Egg yolk RCP differs from egg white RCP only in lacking 13 residues from residue 209 onwards, and it is therefore possible that these residues are not dominant antigenic sites in the protein, despite their relative hydrophilicity and low recognition values.

Future prospects

We are currently characterising a number of other MAbs and attempting to delineate the regions of their interaction with the chicken RCP molecule. By treating the native protein with trypsin or cyanogen bromide (CNBr), a number of peptides are produced some of which are recognized by a few MAbs (Kuzhandhaivelu, N., Karande, A. A. and Adiga, P. R., unpublished results). Sequencing of these peptides should indicate the exact regions on the chicken RCP molecule which interact with the antibodies. Hopefully, a few of the peptides generated from the chicken RCP molecule could be used as immunogens to generate antibodies which might cross-react with the native protein. A particularly attractive candidate for this could be the phosphopeptide which has been shown to be involved in uptake of chicken RCP by the oocyte. It is attractive to speculate that antibodies to this peptide could inhibit the binding of the native protein to the putative placental receptor thereby resulting in reduced uptake of the vitamin by the fetoplacental unit in pregnant mammals.

Consequent to a complete understanding of the antigenic structure of chicken RCP, the MAbs could be used to probe further into the regions of homology in the mammalian proteins. The ability of MAbs to detect a single amino acid change in protein sequence should enable detection of the evolutionary conservation and divergence in the sequences of mammalian RCPs. Preliminary observations do indeed indicate that certain epitopes on mammalian RCPs are less conserved than others (Visweswariah, 1986). To confirm these observations, the cloning of chicken RCP cDNA is in progress. Cloning of the chicken RCP gene from a chicken liver cDNA library, followed by sequencing of the gene and hybridisation studies with rat and human genomic libraries should again substantiate the prediction regarding the extensive evolutionary conservation of the carrier protein. A few examples of proteins that have been conserved to a high degree are known. RCP joins this list because it is a protein whose physicochemical, immunological, functional and biosynthetic characteristics appear to remain grossly unchanged during the transition from oviparity to viviparity.

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