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Review Article

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A critical evaluation of structure, mechanism and functions of riboflavin carrier protein

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INTRODUCTION

1.1 INTRODUCTION OF RIBOFLAVIN CARRIER PROTEIN (RCP)/ RfBP:

Vitamin binding/carrier proteins are soluble proteins present in the blood and other body fluids, which facilitate the dietary absorption, selective transport and protection against loss by metabolic degradation/glomerular filtration by the kidneys of these micronutrients. These proteins bind stoichimetrically and reversibly to vitamins with high affinity and receptor like specificity. Some of these vitamin carriers, those specific to fat-soluble vitamins, folic acid and cobalamine are constitutive while others specific to riboflavin and thiamine are apparently induced *de novo*, as a reproductive stratagem to facilitate vitamin deposition in the developing oocytes in oviparous species and to transport the micronutrients through the physiological and chemical barriers in the viviparous species. Upon physiological demand these binding proteins controll the supply of active metabolite / coenzyme. As they are not saturated with respect to their ligand, these binding proteins are able to scavenge nutrients and thereby are thought to protect the embryo from infection by microbes that require the ligands.

The specific binding/carrier proteins for fat soluble vitamins such as vitamin A and vitamin D have been identified in normal serum in all vertebrates (Kanai et.al., 1968, Thomas Jr.et.al., 1959, Edelstein et.al., 1973; Abe et.al., 1975). Proteins binding to water soluble vitamins such as Riboflavin binding/carrier proteins (Rhodes et.al., 1959; ostrowskiet.al., 1962, Karunakar et.al., 2012 and 2013; Madhukar et.al., 2011 & 2012a,b; Bindu et al., 2012), vitamin B_{12} binding protein (Grasbeek, 1969, Sonneborn et.al.,1970) and thiamin binding protein (Naber et.al., 1954, Coates, 1971) have been demonstrated in the sera, egg white and yolk of the egg laying hens.

1.2 RIBOFLAVIN CARRIER PROTEIN (RCP)

Riboflavin binding protein (RfBP) is a phospho-glycoprotein, whose primary physiological function is to store riboflavin (Rhodes et.al., 1959), wasfirst identified in the chicken egg white. Later it was isolated in egg yolk (Ostrowski et.al., 1978) and in the plasma of egg-laying hen (Murthy and Adiga, 1968). This carrier protein is essential for embryonic vitamin nutrition (Maw, 1954, Cowan et.al., 1964; 1966 and Winter et.al., 1967). Coot (*Fulica atra*) egg Riboflavin-binding protein (RfBP) was purified using DEAE-Sepharose ion exchange chromatography followed by gel filtration on Sephadex G-100 and was found to have a molecular weight corresponding to nearly 29.2 kDa. Sepharose column chromatography was used to isolate, purify and characterize Riboflavin binding protein (RfBP) from Hen (*Gallus gallus*) egg white and yolk (Rao et al.,2011). Riboflavin binding protein (RfBP) was purified from the egg yolk of *Aquila hastate* (Eagle) (Kudle et al.,2011). RfBP was isolated and purified for the first time from the yolk of 73 parrot eggs (Nikhath et.al., 2013). The purification was achieved using DEAE-Sephadex ion exchange chromatography followed by gel filtration on Sephadex G-100 suggesting that the RfBP from egg yolk of *Psittacula eupatria* had a molecular weight close to 29.2kDa. Riboflavin binding protein (RfBP) was isolated first time in India from peacock eggs (*Pavo cristatus*) (Rajendar et al., 2010).

The Rfbp was purified in two steps using DEAE-Sephadex A-50 ion exchange chromatography and sephadex G-100. These proteins showed a single band on SDS gels and the molecular weight was 30 Kilodaltons. Emu (*Dromaius novaehollandiae*) Riboflavin-binding protein (RfBP) was isolated and purified from egg white which had a molecular weight close to 43 kDa and it was approximately 10 kDa larger than the hen egg white RfBP (Bindu et al., 2012)

It was discovered that fertilized eggs of a mutant strain of homozygous recessive mutant (rd rd) chicken with an inherited disease viz., avian riboflavinuria were found to lack a functional RfBP due to splice mutation in its gene (MacLachlan et.al., 1993). The fertilized eggs failed to hatch because of the acute flavin deficiency resulting from the failure of the maternal system to deposit adequate vitamin in the egg, which could be due to impermeability of oocyte plasma membrane to free vitamin leading to embryonic mortality in between 10 and 14 days after incubation. Directly injecting riboflavin into such eggs resulted in a normal hatch.

Deficiency of riboflavin first appear after the 10th day of incubation when embryos become severely hypoglycemic and begin to accumulate intermediates of fatty acid oxidation. The major metabolic consequence of riboflavin deficiency is a severe impairment of fatty acid oxidation as there is 80% reduction in the activity of medium-chain acyl-CoA dehydrogenase. White IIIet.al.,(1996) worked on the riboflavin-deficient strain which provided numerous insights into the metabolism of normal hens and chicken embryos. In the liver, RfBP is synthesized and is secreted into the blood stream, where it complexes with riboflavin.

The vitamin-protein complex is then deposited as part of the yolk in a developing oocyte. After ovulation, this mature oocyte passes down the oviduct whereafter it is secreted by the magnum region of the oviduct. The albumin of egg comes from the diet, during restricted riboflavin intake it can come from tissues provided liver flavin is above 50% of the normal, otherwise, egg laying stops.

1.3 Riboflavin Carrier Protein (RCP): Biochemistry and Structural Biology

A) Egg white Riboflavin Binding Protein (or) RCP

RfBP isolated from the Chicken egg white, yolk and plasma show similar physicochemical characteristics. Coates et.al.,(1971) have reported that the concentration of this protein is 9-folds higher in the egg than in the maternal circulation during egg-laying which indicates that the protein participates in vitamin sequestration from the maternal circulation against a concentration gradient for deposition in the egg . Egg white RfBP is more abundant (Rhodes et.al., 1959, Whiteet.al., 1986) than the serum or yolk RfBP in the chicken. Durgakumari et.al., (1984) and Adiga et.al., (1986) have reported that the Egg white RfBP is synthesized exclusively by the oviduct while the yolk RCP is synthesized exclusively by the liver, both of which are under the regulation by steroid hormones.

Egg white RfBP is a phospho-glycoprotein having a molecular weight of 29,200 Da and contains 219 amino acids with several post–translational modifications (Hamazume et.al., 1984) including glycosylation and phosphorylation. An open reading frame (ORF) of the corresponding cDNA clone (Zheng et.al.,1988) was similar to the aminoacid sequence except that the ORF contains an additional 17 amino acid signal peptide at the N- terminus and an Arg-Arg dipeptide at the C- terminus both of which are absent in the mature secreted protein.

Amino acids especially rich in glutamic acid, serine and aromatic amino acids were found in RfBP (Norioka et.al., 1985). Egg white and yolk RfBPs had almost same amino acid composition except for glutamic acid, white RfBP apparently contain more glutamic acids than yolk RfBP although their carbohydrate compositions were different from each other (Hamazume et.al., 1984). Higher levels of mannose and GlcNAc and lower levels of galactose and sialic acid in white RfBP suggest that the carbohydrate moieties have the hen egg white ovomucoid type structure. The yolk RfBP has a complex type structure. Analysis of N-glycosidic carbohydrate chains indicate that white and yolk RfBPs contain two N-glycosidic carbohydrate chains per molecule, respectively.

At the amino terminus, Lysine and Asparagine were found at the fourteenth residue. The carbohydrate chains were linked to asparagine residue at position 36 and 147. The amino acid sequence followed by these asparagine residues satisfies the general rule for the N-glycosidic carbohydrate chain attaching site, Asn (CHO) – X ser / Thr. Gln-Gln-Glu-Glu-Gly-Glu-Glu is the carboxy-terminal sequence which is seen in white RfBP. Except for the contents of glutamic acid, the amino acid composition of white and yolk RfBP were almost the same. The presence of low amounts of glutamic acid in yolk RfBP suggests that yolk-RfBP lacks a glutamic acid group in the carboxyl terminus.

The phosphates (Rhodes et.al., 1959) in white and yolk RfBPs were found to be attached to 6 to 7 serine residues located at the carboxyl terminal region. Phosphorylation of three serine residues Ser (195), Ser (196) and Ser (197) is found. Hamazume et.al., (1984) reported that the other 6 serines, serine (185) to serine (193), are partially phosphorylated. The phosphorylation might occur at a serine residue in one of the following sequence Ser-X-Glu or Ser-X-Ser (P). Crystallized form of egg white RfBP was achieved by using iso-electric focusing by Zanette et.al. (1984).

Hamazume et.al.,(1987) found that the protein contains 18 cysteine residues, all of which are involved in disulfide bond formation, which explains the resistance of the protein to denaturation conditions. Heating the apoprotein at 90–100^{0}C in 0.05 M Tris-HCl buffer, pH 7.4 for 15 minutes does not lead to detectable loss of binding. Denaturation of the protein occurs between 120–130^{0}C. All the disulfide bonds are essential for flavin binding (Murthy et.al., 1976 and Kozik, 1982). The iso-electric pH of RfBP is about 4.0. Rhodes et.al.,(1959) using different derivatives of the ligand, has shown that changes in the ribitol residue on the position 9 of the isoalloxazine ring of riboflavin had a major effect on binding. Dissociation constant of riboflavin at 25^{0}C was determined to be 1.3 nm and was nearly same between pH 6.0 and pH 9.0 (Becvar and Palmer et.al., 1982). Based on spectral studies and chemical modifications it is seen that Riboflavin is released from the RfBP between the pH 3.0 and 4.0. Blankenhornet.al.,(1978) concluded that one of the nine tyrosines and one of the six tryptophan residues in RfBP were essential for binding the riboflavin. Sialic acid, galactose, mannose and large amounts of N-acetyl glucose amineare present in sugars which, is distinct from other egg white proteins (Miller et.al., 1981; Milleret.al., 1984; White et.al.,1985 and Hamazume et.al.,1984) have reported the presence of a highly anionic region between residues 186 and 199 that contain 5 glutamate, 8 phosphoserine and 10 methionine residues.

Phosphorylation pattern is similar to that found in casein (West, 1986). The N-terminus in RfBP is blocked due to cyclization to pyroglutamic acid. Miller et.al.,(1982) and Hamazume et.al.,(1984) have observed that the complex oligosaccharides are attached to two sites, Asn 36 and 147. The composition of the carbohydrates shows 10% hexosamine, 4% neutral sugars and a single sialic acid residue at the terminus of highly branched oligosaccharide chains

Oligosaccharides were cleaved off the hen egg white riboflavin binding glycoprotein by using $LiBH_4$/ButOH treatment. HPLC analysis led to the isolation of four fucose-containing oligosaccharide alditols (Valadimire, et.al., 1990), whose structures were elucidated by means of 1H NMR 500 mHz spectroscopy. Pisakarev et.al.,(1990) found that the main fucosylated oligosaccharide also present in hen ovomucoid, was a biantennary carbohydrate chain of N-acetyl lactosamine type. Amorosamo et.al., (1999) have structurally characterized the carbohydrate chains of 9 isoforms of chicken egg white RfBP and six isoforms of Quail egg white and yolk RfBPs. The most abundant isoform of each of the three proteins containing the two N-glycosylation sites, Asn 36 and Asn 147 were studied in further detail leading to the identification of different glycosylation patterns. In both chicken and quail egg RfBP, the carbohydrates attached to position 36 had a lower degree of branching while in quail egg white RfBP, this site was only partially glycosylated. Other glycosylation sites analysis showed a characteristic very heterogeneous mixture of complex structures. Analysis of the above N-glycosylation sites, Asn 36 and Asn 147 in quail yolk RfBP showed that it was the same as in the hen yolk RfBP.Copper has been detected at very low levels in the protein as purified (Shaw et al., 2006). It was shown that RBP binds copper in a 1:1 molar ratiowhich suggests a possible additional role for RBP in the transport and storage of copper in avian embryo.

RfBP-riboflavin complex lacks the characteristic fluorescence of free riboflavin as upto 80% of the protein fluorescence is quenched due to ligand binding (Nishikimi and Kyoguku 1973 and Murthy et.al., 1976). Between pH 6 and pH 9. The dissociation constant for riboflavin at 25^{0}C is 1.3nm which is unaltered. Becvar and Palmer et.al.,(1982) have reported the binding of Flavin nucleotides, FMN and FAD to RfBP but with much lower affinities. Decrease in environmental pH to 3 and methanol extraction can release the bound riboflavin. Choi & McCormick et.al., (1980) found that the charged flavin species do not bind well, and the isoalloxazine ring and ribityl side chain modifications also decreased ligand binding. Blankenhorn et.al., (1978) have found that the modifications of C-2 and N-3 of the iso-alloxazine ring have the least effect on flavin binding upon binding to the apo-RfBP. The spectral characteristics of riboflavin and flavin analogs viz, 3-methyl riboflavin and lumiflavin are altered. Nishikimi and Kyoguku et.al., (1973), have observed that the spectral changes are characterized by a red shift of the 450nm band which accompanies the appearance of shoulders and a remarkable hypochromism of the 370 nm band. The egg white RfBP was crystallized by Monaco (1997) and elucidated its 3-D structure at 2.5A^o resolution.

The covalent and ribbon structure diagrams of RfBP. Chicken RfBP which is a globular protein with about 30% of the residues lying in six –helices namely, A-F and less than 15% of the residues distributed among four discontinuous areas a, b, c and d of β-structures. These β-structures were further analyzed and were found to contain gaps and thus are not really of canonical type. The longest uninterrupted stretch of β-structure found in the entire protein sequence is the β-strand (residues 158-162) made up of five amino acids. A search for homology with other sequenced proteins revealed a similarity with bovine folate binding protein, which is also a vitamin binding protein.

Comparison with the completed FBP sequence by Svendsenet.al.,(1984) has extended the region of apparent homology. Alignment of the two sequences showed that RfBP has a carboxyl-terminal extension relative to FBP, whereas FBP is extended at the amino terminus. In the region from residues 5 to 172 of sequence clearly corresponds to that of FBP, which contains many small insertions or deletions. Sequence identity is more than 30%, a value that greatly exceeds the null hypothesis that the identities are due to chance. Further, this presumption of homology is supported by structural and functional considerations. nine disulfide bonds are present in RfBP. Hamazume et.al., (1987) have determined the specific cysteines involved in each disulfide. All of them except for one of these pairs of cysteine residues are conserved in FBP, suggesting that the disulfide bonds in the two proteins are probably the same. Blankenhorn et.al.,(1978) have reported that tryptophan residues are involved in riboflavin binding to RfBP. Six of the tryptophan residues in RfBP were also found to be conserved in FBP and one of the two N linked oligosaccharides in each protein occur at a conserved asparagine residue. The conserved residues account for 23 of the 59 sequence identities between the two vitamin-binding proteins. The highly anionic region at the carboxyl terminus of RfBP which includes a 14-residue sequence that contains 8 phosphorylserine and 5 glutamic acid residues (Fenselac, et.al., 1985 and Mega et.al., 1986) is absent in the FBP sequence. Cooke, (1986) has shown that the vitamin D-binding protein is homologous with albumin and α-fetoprotein while retinol-binding is homologous with bilin-binding protein and milk β-lactoglobin (Sawyer, 1987). Eventhough folates and flavins are functionally distinct in their catalytic function in cells, they have structural resemblance in the pterin ring systems. Thus, it is logical to conclude that an ancestral protein had the capacity to bind both vitamins and a few amino acid replacements could interconvert the binding specificities.

The binding of riboflavin takes place at the hydrophobic cleft in the ligand binding domain where the vitamin's isoalloxazine ring is stacked between the parallel planes of Tyr 75 and Trp 156. The crystal structure revealed that unlike the rest of the protein molecule, the anionic region (which includes 8 phosphoserines) is not ordered in the electron density map. The phosphorylated motif is made up of a flexible anionic region inserted between 2 antiparallel helices. The two helices are found on the surface of the molecule while the anionic region protrudes into the solvent facilitating the interaction between the phosphorylated region and the plasma membrane receptors on the oocyte. The structural integrity of secondary and tertiary structures of the protein and the vitamin binding capacity of RBP have been investigated using UV-Vis, Circular dichroism (CD) and Dynamic light scattering (DLS) studies in the crowding environments. The effect of molecular/macromolecular crowding could have major implication in the intra-protein ET (electron transfer) dynamics in cellular environments (Rakshit et.al., 2012). Electrochemical studies of the riboflavin–RBP interactions are limited as there is a lack of methods capable to detect electrochemical changes in the RBP responses. Martin et.al., (2009) used Constant current chronopotentiometric stripping analysis (CPSA) with the hanging mercury drop electrode (HMDE) and square wave voltammetry (SWV) with carbon paste electrode (CPE) to investigate RBP. Constant current chronopotentiometric stripping analysis (CPSA) of RBP produces electro catalytic peak H, capable to discriminate between apoprotein and holoprotein forms of RBP which is suitable for studies of RBP–riboflavin interaction at nanomolar concentrations. No sign of a release of riboflavin from holoprotein adsorbed at the HMDE surface was seen. SWV with CPE required higher concentrations of RBP which displayed almost similar oxidation peaks of apoprotein and holoprotein.

B) Egg Yolk RfBP

Presence of large amounts of lipids and other proteins make yolk RfBP purification difficult. Isolation of RfBP from egg yolk was first reported by Ostrowiski et.al.,(1962) and improved methods were subsequently developed by Miller, (1976), Miller et.al.,(1981) and Murthy et.al.,(1979). The synthesis of yolk RfBP takes place in liver transported via the blood to the ovary and deposited in the yolk. White et.al.,(1986) found that the concentration of RfBP in yolk is slightly lesser than egg white. Egg yolk RfBP has a molecular weight of 29,000 to 30,000Da and contains 219 amino acids based on composition (Hamazume et.al., 1984). For elucidation of the mechanism of the uptake of yolk proteins from the blood into the developing oocyte, structural investigations of yolk RfBP are important.Yolk RfBP also is glycosylated (Winter et.al., 1967) like several other nutrient-binding proteins found in egg-yolk, such as Biotin-binding protein (White et.al., 1976), transferrin (Williams, 1962 & 1968) and phosvitin

(Shainkin et.al., 1971 and Christmann et.al., 1977). Occurrence of these glycoproteins suggested that their oligosaccharide moieties might contain important recognition features for their specific uptake into the yolk. This possible function by modifying the carbohydrate moieties of native and modified proteins which were transferred to yolk following injection into the blood stream of laying hens was examined by Neufeld et.al., (1980) & Miller et.. al., (1981). They have also found that sialic acid may be important for ovarian recognition and uptake of RfBP. Two chains of carbohydrate are present in white and yolk RfBP (Mega et.al., 1982). Except for the contents of glutamic acid the amino acid composition of white and yolk RfBPs were almost the same. In the ligand-protein interaction, Tryptophan residues were involved. On selective oxidation of 5 out of the 8 tryptophan residues with N-Bromo succinimide (NBS) a complete loss in binding was noticed.Yolk RfBP Sequence analysis has revealed the absence of 11-13 amino acids at the C-terminus. Limited proteolytic cleavage of proteins deposited in yolk has also been observed in the conversation of vitellogenin to lipovitellin and phosvitin by Christmannet.al., (1977) and for yolk very-low-density lipoprotein. Evanset.al.,(1987) and Matsui et.al.,(1982) observed that yolk RfBP binds 8-substituted riboflavin slightly less tightly than does egg white RfBP which may explain the difference in C-terminal amino acid sequence. The difference in C-terminal amino acid sequence could be due to specific but limited proteolytic cleavage during oocyte uptake/yolk deposition in a manner which has been observed with vitellogenin and apo-lipoprotein B. One of the interesting structural feature of RfBP is the presence of a highly anionic phosphorylated region between residues186 and 199 which contains 5 Glu, 1 Met and 8 phosphoserine residues, the pattern of phosphorylation being very similar to casein. The binding of RfBP to an oocyte membrane receptor takes place through this phosphopeptidyl fragment (Sooryanarayana et.al.,1998) and also harbors a palindromic sequence around Met 194. Miller et.al., (1982b) has found that the removal of even a single phosphate residue reduces the concentrative uptake of the protein by the oocyte plasma membrane by more than 60%.

The structure of the sugar chains of hen yolk riboflavin-binding protein were established by Tarutani et.al., (1973) in the following way. Asparagine-linked sugar chains of yolk–RfBP were liberated by hydrazinolysis. Free amino groups of the sugar chains were acetylated and the reducing-end sugar residues were tagged with 2-aminopyridine. Fluorescent pyridylamino (PA-) derivatives of the sugar chains were purified by gel-filtration and Reverse-phase HPLC. Seven PA-sugar chains were isolated and the structure of each was determined by composition analysis, sequential exoglycosidase digestion, methylation analysis and 500-mHz 1H-NMR spectroscopy. These analyses showed that the main sugar had sialylbiantenna and sialyltriantenna structures. The plasma RfBP had almost the same sugar chains as the yolk RfBP did, indicating that sugar chains are not modified during incorporation into the oocyte.

C) Serum RfBP / Riboflavin carrier Protein

The first serum RfBP/RCP was identified in the serum of laying hens Blum (1967 & Winter et.al., (1967) showed it to be the product of the same gene as the egg white and yolk RfBP, and it was shown by Farrel et.al.,(1970) to be serologically indistinguishable from the egg white and yolk proteins. Murthy and Adiga (1978) first purified serum RfBP from estrogen stimulated male chicks. Many properties of the egg white RfBP and serum RfBP are same because they are products of a single gene. The primary sequence of both RfBPs is the same (Hamazume et.al., 1984). Serum RfBP is normally saturated with riboflavin in hens fed adequate amounts of riboflavin (Blum et.al., 1967 and White et.al., 1986) unlike egg white RfBP.

The carbohydrate composition is more complex which is the major difference (Hamazume et.al., 1984). There is significantly more sialic acid and galactoses along with several residues of fucose in serum RfBP than in egg white RfBP, the presence of which increases the molecular weight of serum RfBP by few percent. Composition of oligosaccharides attached to the asparagines 36 and 147 are similar, suggesting structural similarity (Winter et.al., 1967 and Rohrer et.al., 1987). Miller et.al.,(1982) reported that the extent of phosphorylation (7-8 residues/mol) is also similar in egg white, egg yolk and serum RfBPs. Amino acid sequence around two attachment sites, as well as other post translational modifications such as phosphorylation, presence of N-terminal pyroglutamate are similar to that present in egg white RfBP.

D) Transport of RfBP

Serum RfBP which is synthesized in the liver, later complexes with riboflavin in the plasma to form the holoprotein. If riboflavin absorbed from the lumen of the small intestine, is not trapped by complexing with serum RfBP, it is excreted by the kidney (Cowan et.al., 1964 & 1966). In the rapid deposition phase the holoserum RfBP is removed from circulation by ovarian follicles and transported into the developing oocytes. To see that no riboflavin is lost by the laying hens, serum RfBP plays a protective role which is importantin a riboflavin-poor diet, so that each egg

receives a sufficient quantity of this essential nutrient to assure viability. Holo-serum RfBP is transformed into holo-yolk RfBP upon modification of its oligosaccharide moieties. The magnum of the oviduct removes another portion of the holo-serum RfBP. This tissue synthesizes all egg white proteins and removes many proteins from the plasma as a source of its amino acid pool (Miller et.al., 1982). In the magnum, holo-serum RfBP is catabolised with the subsequent release of riboflavin. This riboflavin is then captured by egg white RfBP, synthesized de novo by secretory cells of the magnum. Egg white RfBP is never fully saturated with its ligand and the level of saturation of egg white RfBP reflects the availability of holo serum RfBP unlike yolk RfBP (Feeny et.al., 1969).

Additionally in liver, kidney and intestine, removal of serum RfBP from circulation takes place. Although the liver site is more specific for egg white RfBP significant quantities of serum RfBP are not removed by the liver. After degradation of the protein serum RfBP would release its bound riboflavin making it available for use by liver flavoenzymes. Even though serum RfBP does not have any role in intestinal absorption of riboflavin (Cowan, 1964), its specific uptake by intestinal tissue indicates a role in preferential binding of riboflavin immediately after its absorption. Miller et.al., (1982) has observed similar role for serum RfBP in kidney although here it would function to rescue any free riboflavin before it is excreted. RfBP in different tissues was investigated by Murthy et al(1978), Frolich et al (1980) and Winter et al (1967).

Circular Dicroism (C.D) and fluorescence spectroscopy were used for stufying unfolding and refolding of RfBP from hen egg-white induced by addition of guanidinium chloride. Reduction of its nine-disulfide bonds caused a reduction in the secondary structure (alpha-helix plus beta-sheet) from 63% to 33% of the amino acid residues. The unfolding of the native protein occurred in two phases; the first involving a substantial loss of tertiary structure, followed by a second phase involving loss of secondary structure at higher guanidinium chloride concentrations. By contrast, this biphasic behavior was not discernible in the reduced protein. After the first phase of unfolding, the loss of ability to bind riboflavin occurred. Comparing the unfolding of the holoprotein and apoprotein the riboflavin has only a small stabilizing effect on the unfolding process. Allen et.al., (1992) reported that the holoprotein, apoprotein and reduced protein assumed their original conformation. After removal of guanidinium chloride, Hen egg white riboflavin-binding protein contains nine disulfide bonds. The refolding of RfBP after incubation in 6M guanidinium chloride is highly efficient with at least 95% binding activity regained in 3mins. Kinetic studies on regaining shows the process consists of at least two phases. When disulfide bonds of RfBP are reduced, reoxidation using a mixture of oxidized and reduced glutathione leads to less than 5% recovery of activity. When protein disulfide isomerase (PDI; EC 5.3.4.1) is present during the reoxidation, nearly 50% activity can be regained, suggesting that PDI may play an important role in the maturation of RfBP *in vivo* (McClelland et.al., 1995). Disulfide reduced and carboxymethylated riboflavin binding protein (RCM-RfBP) a derivative of chicken RfBP, does not bind riboflavin and also there is a drastic reduction in its ability to interact with antiserum to cRfBP (Pereira et.al., 1993).

E) Immunological Characteristics

Antibodies against this protein can be raised in a variety of mammalian species such as mice, rats, rabbits (Ramanathan et.al., 1980b) and subhuman primates (Seshgiri and Adiga 1987, Adiga and Karande 1991 and Nataraj, 1991). Ramanathan et. al., (1980) showed that the major determinants of immunogenicity are due to its conformational. Ramanathan et.al.,(1980b) observed that the amidation of 88% of the lysines in the apo-RfBP is accompanied by an 80% decrease in its antigenicity while retaining its flavin binding activity. Modifying tryptophan and tyrosine residues leads to a total loss of flavin binding and alter the antigenic properties. Murthy and Adiga (1977) have found that the holo and apo-forms of RfBP react similarly in radioimmunoassay and immunodiffusion analysis which suggest that there is no overlap between the antigenic sites and the ligand binding sites. Solmezynska and Zak (1984), Tarhay et.al., (1975) have found that the degradation of egg-white RfBP does not become evident before day 13 of development. Flavokinase, acts on riboflavin to convert it to a coenzymatic form and can be purified using immobilized egg white RfBP. Hence, a complex may exist in *vivo* to facilitate utilization of riboflavin when it is released (Solmezynska et.al., 1987).

F) Insect RfBP

A riboflavin-binding hexamerin from pupal hemolymph of *Hyalophora cecropiea* was purified by Magee et al., (1994). Heat denaturation released the ligand which showed the absorbancy, fluorescence spectra, and chromatographic behavior of riboflavin. During adult development all of the apoprotein and 75% of riboflavin disappear from the hemolymph; an amount of flavin, at least, equal to that stored in pupal hemolymph is transferred to the eggs formed during this period. Hemolymph proteins in insects have two proteins, a lipoprotein and a

member of hexamerin gene family that bind riboflavin (Miller and Silhacek, et.al., 1995). Silhacek et.al., (1994) have purified a flavin-binding storage protein from the hemolymph of *Galleria mellonella*. This storage protein that accumulated in the hemolymph of *Galleria mellonella* during the final larval instar was isolated and purified from newly moulted pupae.

G) Fish RfBP

In carp (*Cyprinus carpio*) a protein exhibiting immunological cross-reactivity with the chicken egg white riboflavin carrier protein was detected by radioimmunoassay in the eggs and serum. It was purified by the use of affinity chromatography. Riboflavin binding protein of fish was similar to chicken riboflavin carrier protein with respect to most of its physiochemical characteristics where the major epitopes of chicken riboflavin binding protein were shown to be conserved in the fish protein as probed with monoclonal antibodies to the avian vitamin binding protein (Malhotra et.al., 1991).

H) Amphibian RfBP

White and Merrill, (1988) have characterized the vitamin carrier from the eggs of the American alligator. RfBP in spade foot toads (*Scaphiopus couchil*) was reported by Storey et.al., (1995). Toad liver RfBP showed 50% of residues similar to the chicken and turtle liver proteins and many essential structural features were conserved in the toad protein which included 18 cysteine residues, two asparagine glycosylation sites and six tryptophan residues. But a region with eight phosphoserines in the chicken or turtle proteins that functions in RfBP binding to the oocyte membrane contained only three serine residues in toad RfBP suggesting that recognition and binding to Oocyte receptors must be different in toads.

I) Reptilian RfBP

White et.al.,(1988) have purified and characterized RfBP from the yolk of painted turtle oocytes and python eggs. The RfBPs were found to be phospho-glycoproteins with riboflavin affinities similar to that of chicken RfBP with higher molecular weights (approximately 40,000) due to greater carbohydrate content.

Hamajima and Ono et.al.,(1995) have screened total RNA from estrogen treated oviparous animals by Northern hybridization using chicken RfBP cDNA as probe to search for the existence and distribution of an RfBP. A cDNA library from estrogen-injected turtle liver, and a full-length turtle RfBP-encoding cDNA was cloned and sequenced to elucidate the structure of RfBP. Deduced amino acid sequences of turtle and chicken showed an overall 71.3% amino acid identity between them. The presence of an additional potential N-glycosylation site in the turtle sequence may provide a better explanation for the greater molecular weight of the turtle protein than chicken RfBP.

J) Mammalian RfBP: Evolutionary Conservation

Muniyappa and Adiga (1980) in an attempt to understand facilitated transplacental transport and fetal accumulation of riboflavin in pregnant higher animals and humans, found biochemical and immunological evidence for the occurrence of a reproductive specific, high-affinity RfBP in pregnant rat serum. Rodent RfBP showed immunological cross-reactivity with purified chicken RfBP. Further, the functional importance of the maternal RfBP in fetal development and proper progression of pregnancy in the rat was demonstrated by acute fetal wastage and abrupt pregnancy termination resulting from immuno-neutralization of the endogenous RfBP with specific and potent antiserum to mice (Natraj et.al., 1987) and bonnet monkey (Seshagiri and Adiga 1987). Fetal degradation is accompanied by depletion of FAD levels in the fetus and in fetal liver (Krishna Murthy et.al., 1984 and Surolia et.al., 1985). Purification of RfBPs from pregnant bonnet monkey (Visweswaraiah et.al., 1987a), human sera (Visweswaraiah et.al., 1987b) (maternal and umbilical cord) and human amniotic fluid (Subramanian and Adiga 1999) have been reported. Monoclonal antibodies and epitope specific polyclonal antibodies raised against synthetic peptides of chicken RfBP reacted with mammalian protein in immunoassays showing binding curves which are parallel to that elicited with the chicken protein which indicate that the surface topography of RfBP as represented by various epitopic conformations is similar in the avian and mammalian proteins. Polyclonal antibodies generated against the purified human RfBP, reacted significantly with the chicken RfBP. In chicken RfBP primary structure these antibodies recognized five linear b-cell epitopes corresponding to residues 37-42, 73-76,133-140,174-176 and 200-207 as assessed by Geysen's PEPSCAN ELISA. Subramanian and Adiga et.al.,(1999) suggested that the high level of immunological similarities among linear epitopes observed reflects the degree of conservation of local conformations dictated by the antigenic sequences shared by the two evolutionary distant proteins. The presence of RfBP in Marmoset (*Callithrix jachhus*), a non-human primate immunologically and functionally similar to well characterized chicken RfBP has been demonstrated in circulation in pregnant marmosets. Marmoset RfBP

demonstrated in circulation in pregnant marmosets which has been partially purified and the immuno-reactive protein is similar to chicken RfBP. The source of RfBP in rodents appear to be maternal liver, suggesting thereby that RfBP levels could be modulated by changing hormonal pattern that occurs during the menstrual cycle. Very low amounts of marmoset RfBP was measured during the luteal phase and a single sharp peak was observed during the follicular phase of the menstrual cycle indicating that marmoset RfBP levels are regulated by estrogen (Nataraj et.al.,1991).

K) RfBP in the Mammary Gland

The major function of estrogen inducible RfBP in mammals, is to transport the vitamin across a physiological barrier offered by the placental membrane to the growing embryo. The role of RfBP in accessory female reproductive and the male reproductive tract has been investigated keeping this in view. The mammary gland is dependent on estrogen for growth and differentiation (Lippman and Dickson 1989). This is endowed with a physiological barrier constituted by circumferential tight junctions between epithelial cells. This prevents the passage of most blood constituents other than small ions into milk (Pitilka et.al., 1973 and Linzell & Peaker 1971). It is possible that a carrier-mediated trans-epithelial delivery mechanism for the vitamin may be operative in the mammary gland since the concentration of riboflavin in colostrums and milk is several folds higher than in maternal serum (Wolfrit et.al., 1987), immunologically homologous RfBP was detected in the milk of rodent, bovine and primate species by employing a sensitive heterologous radioimmunoassay. Devi Prasad et.al., (1992) purified RfBP from bovine milk by employing riboflavin-epoxysepharose affinity chromatography which had an approx Mr of 37,000. Early evidence for the synthesis of RfBP in the mammary gland was provided by immunohistochemical localization of the vitamin carrier in the lactating rat mammary gland.

L) RFBP and Breast Cancer:

Recently vitamin carrier proteins have been shown to be over expressed in patients with malignant disease. Serum RfBP levels quantitated by a specific and sensitive radioimmunoassay validated for human samples (Visweswaraiah & Adiga 1987b) were significantly enhanced in breast cancer patients in a stage dependent manner particularly in post-menopausal patients.Rao et. al.,(1999) have observed elevated serum RfBP levels in patients with adenocarcinoma of the breast. In menstruating breast cancer patients, Serum RfBP levels are 3 to 4 fold higher ($p < 0.01$) than those in their normal counterparts. Difference in circulatory RfBP levels between cancer patients and their age matched normal counterparts is further magnified to 9 to 11 fold ($P < 0.005$) at the post menopausal stage. In immunohistochemical studies, RfBP was found in cytoplasm of malignant epithelial cells from ductal as well as lobular carcinoma of the breast. Karandeet.al., (2001) reported significantly higher RfBP concentrations ($p < 0.005$) in patients with advanced metastasizing breast cancer versus those with early disease.

Based on the above data, enhanced circulatory RfBP levels as well as the altered pattern of immunohistochemical localization of RfBP in malignant breast tissue samples could have the potential as a diagnostic tumor marker in breast neoplasia and other malignancies of various hormone dependent cancerous tissues. Riboflavin carrier protein (RCP) has been shown to be over-expressed by metabolically active cancer cells. Therefore, for visualizing tumor metabolism (Jabaduraiet al.,2012), FAD-decorated ultra small super paramagnetic iron oxide nanoparticles (FAD USPIO) were developed as the first carrier-protein-targeted molecular MR agents. *In vitro* studies showed the biocompatibility of FAD USPIO, specifically taken up by cancer cells. *In vivo* molecular MRI together with histological validation showed that FAD USPIO efficiently accumulated in tumors and tumor blood vessels, suggesting that RCP-targeted diagnostic nanoparticles can be used for the assessment of vascular metabolism in tumors.

M) Riboflavin carrier protein /RfBP as a Potential Male Contraceptive Antigen in Mammals

The flow cytometric analysis of paraformaldehyde-fixed spermatozoa using FITC-conjugated secondary antibody revealed (Sridhar et.al., 1996) that the immunostainable RfBP on the acrosomal cap of washed rat epididymal spermatozoa and electro ejaculated monkey spermatozoa and hence accessible to antibody binding. The exogenously added RfBP antibody had a significant effect on sperm motility *in vitro* and failed to acquire hyper activation due to *in vivo* capacitation and were progressively immobilized which was further aggravated by the addition of guinea pig serum as a compliment source. Yanagimachi,et.al.,(1976) have found that In zona denuded hamster egg penetration test, antibody debilitated sperms exhibited significantly impeded (>70%) capacity to penetrate the ovum investments.

Effect of active immunization of fertile male rats with linearized denatured RfBP (RCM-RfBP) on their *in vitro* sperm characteristics and *in vivo* fertilizing ability on mating with non-immunized fertile females was studied. The mating experiments with normal fertile females, immunized male rats showed significant reduction (>85%) in fertility after 5th immune boosting. Examination by laparotomy of the uterine horns on day 8 post-coitus showed that a majority of these mated fertile female rodents lacked implantation / resorption sites while few others harbored degenerative embryonic inclusions within the pycnotic decidual chambers. The voided spermatozoa exhibited impaired mobility index *in vitro* particularly in presence of added complement when experiments were done with RCM-RfBP immunized male bonnet monkeys. Mating of these immunized males (n=4) repeatedly with fertile females (n=10) during days 10-16 of ovulated cycles, the females appear to be protected from pregnancy during a total of 29 fertile cycles (Adiga et.al., 1997).

N) RfBP as a Potential Immunocontraceptive in the Female Mammals

In mammals RfBP is synthesized primarily in response to estrogen during the reproductive phase. While in the rodents, RfBP concentration in circulation is highest during proestrous, coincident with high plasma levels of estrogen (Muniyappa & Adiga 1980). Even, higher up in the evolutionary scale i.e. in subhuman primates too the concentration of RfBP in the plasma is modulated in concert with increasing estrogen levels during days 16-19 of the menstrual cycle which is 3-4 days after the pre-ovulatory surge of the steroid (Adiga et.al., 1986 and Visweswariah and Adiga, 1988). High levels of the protein are encountered as early as day 4 of gestation with peak levels being attained between days 10-18. Through pregnancy in rodents, coincident with intense embryonic growth which indicate that RfBP is an estrogen-inducible, vitamin carrier protein that may participate in flavin transport from the maternal supply line to the developing fetus. Immunoneutralizaton of riboflavin binding protein results in the abrupt termination of pregnancy in rats, establishing the functional significance of the vitamin binding proteins for fetal growth and development. The use of RfBP as an immunocontraceptive stems from extensive studies conducted with the rodent and sub-human primate model systems. Potent polyclonal (Adiga & Murthy 1983), monoclonal (Karande et.al., 1991), peptide specific polyclonal (Koshy, et al. 1996) antibodies against chicken RfBP have shown to passively immunoneutralize RfBP in pregnant rats. Krishnamurthy et.al., (1984) traced this early embryonic loss to a drastic curtailment (>90%) of ^{14}C-riboflavin influx from the maternal to the fetoplacental unit. Severe embryonic flavin deficiency is responsible for the disturbances in relative contents and concentrations of flavin coenzymes. Adiga et.al.,(1988) have examined histology of the affected feto-placental unit which showed detachment of the placental membrane from the deciduas, drastic mitotic arrest in the neural tube, leukocyte infiltration into both the maternal blood vessels and fetal liver, characteristics of degenerative tissues and significant trophoblast degeneration. Active immunization with chicken RfBP resulted in protection from pregnancy establishment through several consecutive ovulatory cycles in fertile female rats (Murty and Adiga et.al., 1982) and bonnet monkeys (Seshagiri and Adigaet.al., 1987).

O) Immunocontraceptive Efficacy of Synthetic Peptides of Chicken RfBP

The surface topology of the protein is conserved through evolution (Adiga et.al., 1988) when a panel of 7 monoclonal antibodies (mAbs) to chicken RfBP recognized purified rat, monkey and human RfBPs. Among these, one of these mAbs, viz., 6B2C12 recognizing a sequential epitope was shown to bind to a synthetic peptide corresponding to the C-terminus (residues 200-219) of chicken RfBP with the core sequence of ^{203}QKKLLKFEAL^{212} ·Ascitic fluid administration of this mAb to pregnant mice leads to embryonic resorption. A synthetic 21 amino acid peptide (CTP) corresponding to the C-terminal fragment of RfBP, ^{2oo}HACQKKLLKFEALQQEEGEE^{219}, was immunoreactive with polyclonal antibodies to RfBP, RCM-RfBP as well as with the mAb 6B2C12 which functions as minivaccine in terms of eliciting peptide-specific, protein cross-reactive antibodies. Among these four peptides, residues 4-24, 64-83, 130-147 and 200-219, elicit neutralizing antibodies capable of curtailing pregnancy in rodents. Based on the above observations (Karande,et.al.,1991) suggested that RfBP (or its defined fragments) could have the potential to be a novel, first generation vaccine for regulating fertility in both male and female mammals. Maehashiet.al.,(2008) have shown that Riboflavin-binding protein from chicken egg was found to be a bitter inhibitor. It has been suggested that the bitter inhibitory effect of RfBP is the consequence of its ability to interact with taste. RfBP can be used for reducing bitterness of foods and pharmaceuticals.

The discovery of the vitamin carrier proteins which ensure deposition of adequate amount of the vitamins in the avian eggs and the demonstration of the existence of similar vitamin-transport proteins in the sera of higher animals prompted us to isolate these proteins in the pure form and initiate studies on the structural, functional, immunological aspects and their cytotoxic activities. Isolation and characterization of riboflavin transport proteins,

their mode of biosynthesis and regulation of synthesis from different tissues would be of considerable importance for understanding the factors involved in normal and healthy fetal growth and development. Additionally detection of increased serum levels of RCP in breast adenoma patients may help in using this vitamin binding protein as a new bio marker for the diagnosis of breast cancer.

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