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Refolding of native and recombinant chicken riboflavin carrier (or binding) protein: evidence for the formation of non-native intermediates during the generation of active protein

Priyaranjan PATTANAIK, SOORYANARAYANA, P. Radhakantha ADIGA and Sandhya S. VISWESWARIAH

Department of Molecular Reproduction, Development and Genetics, Indian Institute of Science, Bangalore, India

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Riboflavin carrier (or binding) protein (RCP) is a phosphoglycoprotein originally purified from the egg white, yolk and serum of laying hens. The 18 cysteine residues present in RCP form nine disulfide bridges, allowing the protein to form a compact structure to generate a hydrophobic pocket in which riboflavin sits. We studied the refolding of totally reduced and denatured egg white RCP and found that the protein initially folded to generate a molecule that did not possess riboflavin-binding activity, despite near-complete oxidation of the cysteine residues. Riboflavin-binding activity was then slowly regained, but the final refolded form of the protein was less compact in structure than the native molecule, due to incomplete oxidation of all the cysteine residues. Denatured and reduced dephosphorylated RCP refolded as efficiently as the native protein, with similar rates of disulfide-bond oxidation and generation of riboflavin binding, showing that the phosphoserine stretch of RCP has little role to play during refolding. In order to study the role of glycosylation in the refolding process, the cDNA for full-length RCP was expressed in *Escherichia coli* and purified. Recombinant RCP refolded only in the presence of redox buffers, demonstrating that glycosylation of RCP could allow the formation of high yields of productive intermediates in the folding pathway. Using a panel of conformation-specific monoclonal antibodies to RCP, it appeared that the folding intermediates of RCP possessed a structure distinctly different to the native protein, indicating that the correct folding pathway of RCP passed through conformation(s) generated by non-native disulfide bridges.

Keywords: riboflavin carrier protein; refolding; monoclonal antibodies.

Riboflavin carrier protein (RCP) is a phosphoglycoprotein found in the egg white, yolk and serum of laying hens and other birds [1, 2]. The egg-white protein is synthesized in the oviduct of the laying hen [3]. The liver, under the influence of estrogen, synthesizes serum RCP [3, 4] which is then deposited through receptor-mediated uptake into the yolk of the developing egg [4]. RCP delivers riboflavin to the developing embryo in the egg, and birds that harbor a mutant RCP gene fail to lay eggs that hatch, as a result of insufficient deposition of the vitamin in the egg [5, 6]. Egg-white RCP differs from the yolk and serum RCP only in the pattern of glycosylation [7] and removal of the C-terminal 11−13 amino acids in yolk RCP, presumably as a result of proteolytic cleavage during oocyte uptake [8]. A stretch of phosphoserine residues is present towards the C-terminal end of RCP [9]. We and others have shown the critical importance of these residues in mediating oocyte-receptor-binding and uptake of RCP during egg development [10, 11].

RCP is a complex protein with 18 cysteine residues all involved in disulfide-bridge formation [12]. Early studies had indicated that a single disulfide bridge appears to be critical for riboflavin binding [13], but the specific disulfide bridge involved has not been identified. The crystal structure for the egg-white protein has been recently described [14] and provides a basis for earlier biochemical observations that indicated the essentiality of the disulfide bridges for high-affinity riboflavin binding [15], as well as the role of tryptophan residues present in the riboflavin-binding site of RCP [16]. The stretch of phosphoserine residues is present between two helices that are linked to the main riboflavin-binding core of the protein, therefore allowing the phosphopeptide to function autonomously in mediating receptor interaction [14].

In view of the complex structure of RCP, it is of interest to study the refolding of the protein *in vitro,* since it provides a model system to study the formation of intermediates during the folding pathway, as well as the role of post-translational modifications, such as phosphorylation and glycosylation, in the folding pathway. Early work indicated that if the disulfide bonds in RCP were not reduced, but the protein treated with high concentration of urea or guanidinium hydrochloride, refolding of the protein to generate a protein that could bind riboflavin was rapid and occurred in quantitative yields [17]. However, on reduction of RCP and total unfolding in the presence of chaotropes, refolding of RCP was slow and occurred with low efficiency. In the presence of protein disulfide isomerase (PDI), yields were improved to 30%. Another study indicated that reduction and denaturation, followed by rapid removal of the chaotropes by gel filtration, allowed the protein to fold almost quantitatively, at relatively high protein concentrations [18]. Moreover, the conformation of the refolded protein was similar to the native pro-

Correspondence to S. S. Visweswariah, Department of Molecular Reproduction, Development and Genetics, Indian Institute of Science, Bangalore 560012, India

Fax: +91 80 3345999.

E-mail: sandhya@serc.iisc.ernet.in

Abbreviations. PDI, protein disulphide isomerase; RCP, riboflavin carrier (or binding) protein.

tein as judged by the reactivity of a panel of monoclonal antibodies to native RCP [18]. A recent report using stopped-flow analysis has examined the early and rapid phases of the refolding process of denatured, but disulfide-bond intact, native and dephosphorylated RCP [19]. Native secondary structure was regained rapidly and there did not appear to be a dramatic difference between the native and dephosphorylated forms of RCP.

The presence of 18 cysteine residues in RCP, all involved in disulfide bridge formation [12] allows both the monitoring of the oxidation of the cysteine residues and the generation of riboflavin-binding activity simultaneously during the refolding. A sensitive and non-destructive method of monitoring riboflavin binding is to measure the quenching of riboflavin fluorescence on binding to the Apo protein [16]. The state of the free thiol content in RCP during refolding can also be measured [20] at the concentration of RCP used during refolding. Since refolding of RCP appears to be a slow process [17, 18], it is possible that a number of non-native disulfide bonds may form during the refolding process [21], leading to the formation of molecules with structures dissimilar to the native protein.

In order to study, in greater detail, the kinetics for refolding of native RCP in terms of the formation of disulfide bonds and generation of riboflavin-binding activity, as well as the role of post-translational modifications during the folding process, we have studied the refolding of native, dephosphorylated and recombinant RCP. We show here that refolding of both native and dephosphorylated RCP occurred under conditions of normal-air oxidation, passing through conformationally distinct states. Recombinant RCP refolded in low yields in the presence of oxidized and reduced glutathione, indicating that glycosylation of RCP, in contrast to the phosphorylation, appeared to play a role in efficient *in vitro* refolding of the protein.

EXPERIMENTAL PROCEDURES

Purification of chicken egg-white RCP. RCP was purified to homogeneity from chicken egg white by the method of Farrell et al. [22], with slight modifications [23]. The protein obtained by this method was complexed with riboflavin, and apoRCP was prepared by the method of Miller and White [24]. The RCP-containing fractions were pooled and dialyzed against water and concentrated by lyophilization.

Dephosphorylation of cRCP. ApoRCP (10 mg) was dissolved in 1 ml 10 mM sodium phosphate, pH 4.8, to which 2 U acid phosphatase (Sigma) was added. The sample was dialyzed against the same buffer at 37 °C for 10 h. Alkaline gel electrophoresis under non-denaturing conditions [25] was performed to check for dephosphorylation, and dephosphorylated RCP migrated less than the native protein.

Unfolding and refolding of native and dephosphorylated RCP. All buffers and solutions contained 1 mM EDTA and were freshly purged with N_2 gas. Solutions of native or dephosphorylated RCP (2 mg/ml) in 50 mM Tris/HCl, pH 7.4 were incubated in 8 M urea and dithiothreitol (100-fold molar excess to cysteine residues in RCP) for 2 h at 37 °C. Proteins were subjected to gel filtration on a PD10 column (Pharmacia) equilibrated with 10 mM sodium acetate, pH 4.5, to prevent air oxidation of cysteine residues during desalting, and protein-containing fractions were pooled. Refolding was initiated by adjusting the concentration of RCP to 100 μg/ml and the pH of the solution to 8.5 by the addition of 1 M Tris/HCl, pH 8.5, to a final concentration of 50 mM. Oxidation was carried out at 25 °C with gentle shaking. Samples were removed from the refolding solution at various times during refolding and subjected to free thiol estimation [20], or assayed for riboflavin binding activity by fluorescence titration (see below) and native gel electrophoresis using the method of Davis [25]. Free sulfhydryl content in the refolding solution was estimated at different time intervals using the method of Ellman described in [20].

Tryptophan fluorescence of the refolding mixture was measured at different intervals at an excitation wavelength of 290 nm using a Hitachi spectrofluorimeter. Emission spectra were recorded from 300−400 nm.

For alkylation of free thiol during folding and analysis of folding intermediates, samples (1 ml) were removed from the refolding mixture and alkylated by adding iodoacetamide to a concentration of 0.1 M, followed by incubation in the dark for 1 h. Samples were stored at −20°C and used for electrophoresis under non-denaturing conditions as per the method of Davis [25].

To monitor the free thiol content of the refolded protein, ^{14}C-iodoacetic acid (56 mCi/mmol; Amersham) was added at a tenfold molar excess to refolded protein. Incubation was continued for 30 min in the dark at 25°C. The solution was filtered through a nitrocellulose membrane and washed with chilled 50 mM Tris/HCl, pH 7.5. The filter was air dried and radioactivity bound to the filter monitored by liquid scintillation counting.

Gel-filtration analysis of the refolded RCP was performed on a Superose-12 column (2 cm×50 cm) fitted to the FPLC system (Pharmacia). The column was equilibrated with 50 mM Tris/HCl, pH 7.5, and developed with the same buffer at a flow rate of 1 ml/min.

Overexpression of RCP in *Escherichia coli* and purification of recombinant protein. All molecular biological procedures were performed using standard protocols [26]. The cDNA encoding the cRCP gene was a kind gift from Prof. H. B. White III (Department of Chemistry, University of Delaware, Newark, USA) and was originally cloned in plasmid pIBI21 [27]. Complementary 3′ and 5′ primers were synthesized for PCR amplification of the coding sequence of mature RCP without the signal sequence present in the original cDNA clone. The 5′ and 3′ oligonucleotides had the sequences 5′-GGCCATGGCGCAGCAGTATGGATGT-3′ and 5′-TTGGATCCTCACTCTTCCCCTTCCTC-3′, respectively. The 5′ primer contained a *Nco*I site and the 3′ primer contained a *Bam*HI site. The 678-bp PCR product obtained after amplification using the above primers was digested with *Nco*I and *Bam*HI and cloned into *Nco*I/*Bam*HI-digested pET 12d vector (Invitrogen) to obtain plasmid pSV5. Transformants were obtained initially from DH5α and subsequently in BL21 (DE3) to allow expression from the T7 promoter [28]. The DNA sequence of the clone used for expression studies was confirmed by automated DNA sequencing performed in the DNA sequencing facility of the Indian Institute of Science. A single change from A to T was observed at the third position in the codon for the amino acid proline (residue 15 from the N terminus) and, therefore, did not change the coded amino acid which remained proline.

BL21 (DE3) cells harboring the recombinant plasmid were grown in Luria-Bertani broth at 37 °C with 100 μg/ml ampicillin. Expression of the protein was induced with 500 μM isopropyl-thio-β-D-galactoside when cultures had reached an absorbance of 0.8 at 600 nm. Cells were cultured for a further 5 h, collected by centrifugation and washed in 10 mM sodium phosphate, pH 7.5, containing 0.9% NaCl. The pellet was suspended in 50 mM Tris/HCl, pH 8.0, containing 5 mM dithiothreitol, 0.1 M NaCl, 1 mM benzamidine, 5 μg/ml soybean trypsin inhibitor, 5 mM phenylmethylsulfonyl fluoride, 5 mM EDTA and 1% Triton X-100 (lysis buffer). Cells were sonicated in an ice bath using a Virsonic sonicator at a setting of 7. The lysate was centrifuged at 500 *g* for 15 min and the resultant supernatant was

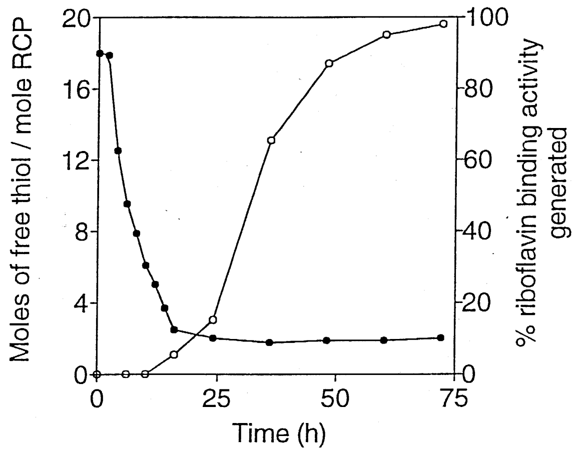

Fig. 1. Kinetics of refolding of native cRCP. ApoRCP was reduced and denatured with urea and dithiothreitol and refolding was carried out as described in the text. Data shown are representative of three experiments. Free thiol content (●) was estimated by the method of Ellman [20] and riboflavin binding activity (○) was measured by fluorescence quenching [29].

centrifuged again at 13000 *g* to sediment the inclusion bodies. The pellet was washed four times in lysis buffer followed by washing with 50 mM Tris/HCl, pH 8.0, and was analyzed by SDS gel electrophoresis. Western-blot analysis using polyclonal antibodies to RCP confirmed the expression of the specific protein and also indicated that the recombinant protein was almost exclusively localized in inclusion bodies (data not shown).

To purify RCP from inclusion bodies, the pellet was suspended in 50 mM Tris/HCl, pH 8.0, containing 8 M urea and 1 mM dithiothreitol. The suspension was centrifuged and the supernatant was subjected to desalting on a Sephadex G25 column equilibrated with 50 mM Tris/HCl, pH 8.0, containing 5 mM dithiothreitol. A high concentration of the reducing agent helped the protein remain in solution even in the absence of urea. The A_{280}-positive fractions were pooled and subjected to anion-exchange chromatography using a MonoQ column (2 cm×10 cm) connected to the FPLC system. The column was equilibrated with 50 mM Tris/HCl, pH 8.0, containing 5 mM dithiothreitol. Elution was carried out using a linear gradient of 0−1 M NaCl over 60 min at a flow rate of 2 ml/min. A_{280}-positive fractions were pooled and purity of the eluted peak was checked by SDS/PAGE. The protein was dialyzed against 50 mM Tris/HCl, pH 8,

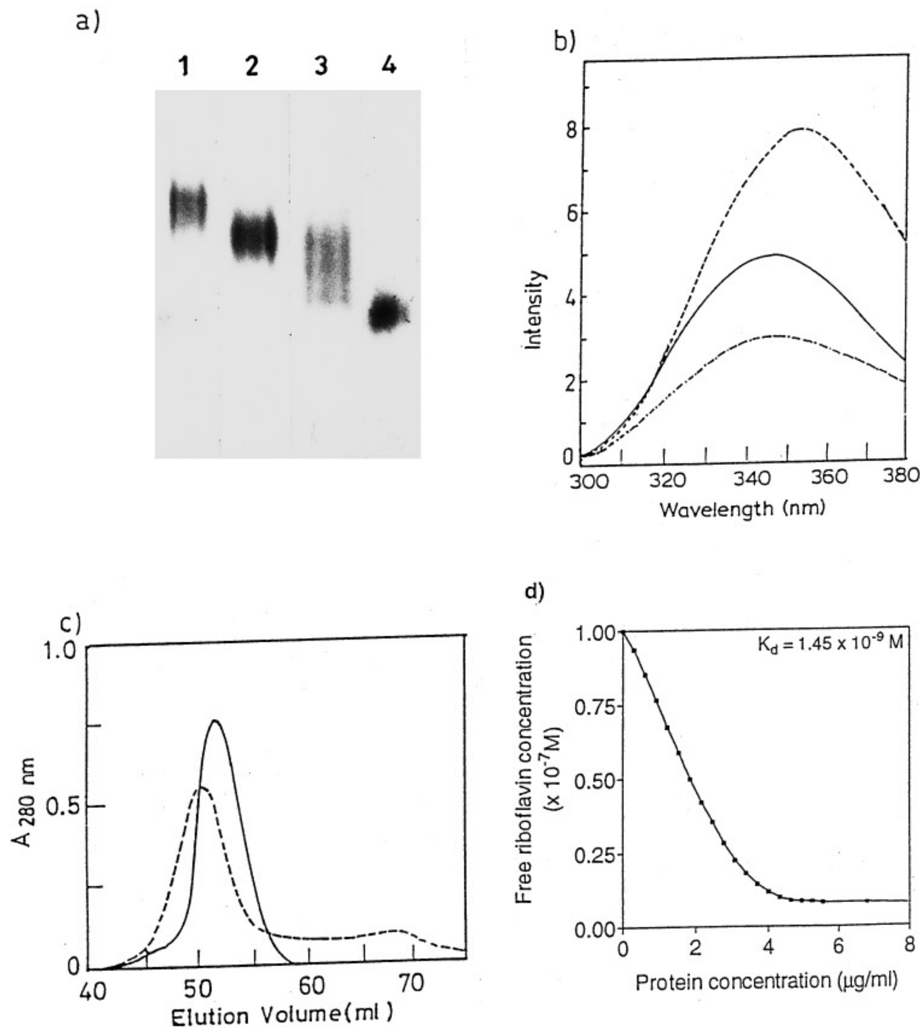

Fig. 2. Characterization of refolded native cRCP. (a) Non-denaturing gel electrophoresis of refolding samples after 2 h (lane 1), 16 h (lane 2), 72 h (lane 3) of air oxidation and native RCP (lane 4). (b) Tryptophan fluorescence of reduced and denatured RCP (– – –), native RCP (——), and refolded RCP after 72 h of refolding (– · – · –). (c) Gel filtration analysis of refolded (– – –) and native (——) RCP. (d) Riboflavin binding to renatured RCP as determined by fluorescent titration.

containing 5 mM dithiothreitol, and stored at $-20°C$ until further use.

Refolding of recombinant RCP. Purified recombinant RCP was treated with 6 M guanidine hydrochloride and a 100-fold molar excess of dithiothreitol to cysteine residues. Desalting was carried out in 50 mM Tris/HCl, pH 8.5 containing 1 mM reduced glutathione on a PD-10 column (Pharmacia). Refolding was performed at a protein concentration of 100 µg/ml following the addition of oxidized glutathione to the solution to a final concentration of 0.1 mM.

Riboflavin binding monitored by fluorescence quenching. Monitoring of riboflavin binding to RCP was performed by detecting the quenching of riboflavin fluorescence on binding to the apo protein. Excitation was at 370 nm and emission spectra were recorded from 500 nm to 550 nm on a Hitachi spectrofluorimeter. A standard titration curve was plotted using different concentrations of apoRCP with constant riboflavin concentration of 1 µM. This was used to estimate the fraction of protein in the refolding mixture capable of binding riboflavin. The dissociation constant (K_d) was calculated from the titration curves as described by Hu et al. [29].

Radioimmunoassay of RCP. RCP was radiolabeled by means of the lactoperoxidase method as described previously [30]. Varying concentrations of native or refolded RCP were incubated at 4°C for 12 h with a fixed dilution of each monoclonal antibody in the presence of radioiodinated apoRCP, and radioactivity bound to the antibody was monitored by means of precipitation of the antibody−RCP complex using anti-mouse IgG [30].

RESULTS

Refolding of native RCP. Purified egg-white apoRCP was used for these studies, since information on folding conditions and yields obtained by earlier workers suggested that a high yield of refolded protein could be obtained [17, 18]. The complete denaturation of RCP after urea and dithiothreitol treatment was confirmed by intrinsic protein fluorescence and the reduced and denatured protein was subjected to gel filtration at pH 4.5 to remove these subsances. Immediately after desalting, the free thiol present in the protein corresponded to 18 mol cysteine/mol RCP (data not shown).

The data shown in Fig. 1 indicate that oxidation of the cysteines in RCP was slow and required more than 12 h for near-complete oxidation. Interestingly, when the protein in the refolding mixture was checked for riboflavin-binding activity, the ability to bind riboflavin was barely detected after the near complete oxidation of the protein (at 14 h), and recovery of full riboflavin binding was observed only at the end of 72 h, more than 48 h after apparent near-complete oxidation of the sulfhydryl groups. This implies that the initial oxidation of the sulfhydryl groups resulted in the formation of a protein that possessed a conformation that did not allow riboflavin binding, therefore, representing a non-native intermediate(s).

To investigate this further, samples from the folding mixture were removed at various times, iodoalkylated and then subjected to native gel electrophoresis. The data shown in Fig. 2a indicate that the mobility of the protein increased during the folding process, suggesting more compaction of the structure. However, the completely refolded and active protein did not migrate to a position identical to that of native RCP. The tryptophan fluorescence spectrum (Fig. 2b) of reduced and denatured cRCP showed a red shift of about 8 nm and greater intensity than the native protein. Interestingly, the spectrum of the protein refolded for 72 h had emission maxima comparable with the native protein, but almost half the intensity.

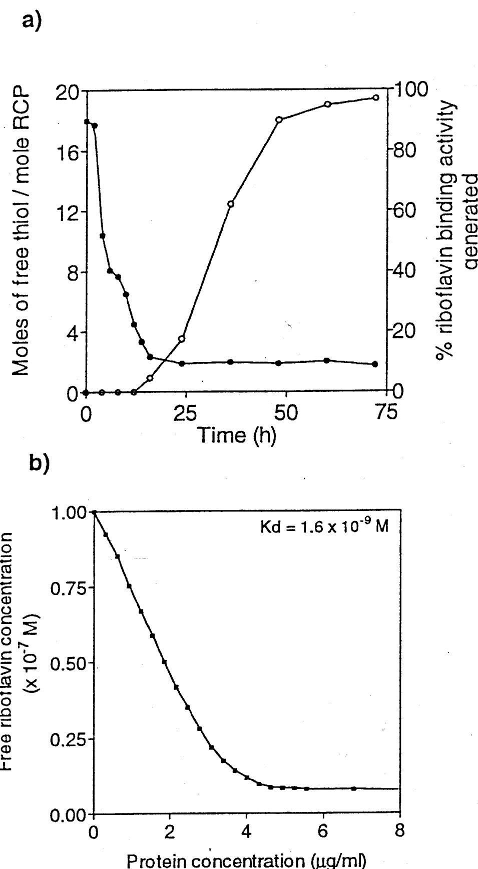

Fig. 3. Refolding of dephosphorylated RCP. Data shown are representatives of three experiments. Reduction, denaturation and refolding were carried out under similar conditions as for native RCP. (a) Kinetics of refolding. Free thiol content (●) and generation of riboflavin binding activity (○) were measured at different intervals. (b) Fluorescence titration of refolded protein.

Gel-filtration analysis (Fig. 2c) of the refolded protein also indicated that the Stokes radius of the protein was larger than that of the native protein, in agreement with the gel-electrophoresis pattern. Gel electrophoresis under denaturing conditions in the absence of reducing agent indicated that the increase in apparent molecular size was not due to inter-molecular disulfide bridging (data not shown). The data in Fig. 2d indicate that the refolded protein had an affinity comparable with native RCP, demonstrating that the more 'open' structure of the refolded protein did not dramatically alter the confirmation of the riboflavin-binding domain of RCP.

Since the structure of RCP is largely determined by the nine disulfide bridges present in the protein [12, 14], it is conceivable that the larger Stokes radius of the refolded protein could be due to one or more non-oxidized cysteine residues in the protein. The refolded protein was treated with ^{14}C-iodoacetic acid and, in a number of experiments, nearly 2 mol free thiol/mol protein was present as judged by the incorporation of ^{14}C into the refolded protein. Negligible amounts of radioactivity could be detected in the native protein (data not shown) suggesting that, in the final folding mixture, a single disulfide bridge not critical for the generation of the riboflavin-binding pocket remained reduced in the refolded protein.

We investigated the possibility that phosphorylation of RCP could modify its folding kinetics and, therefore, performed refolding experiments using reduced and denatured dephosphorylated RCP. The data shown in Fig. 3a indicate that there are no

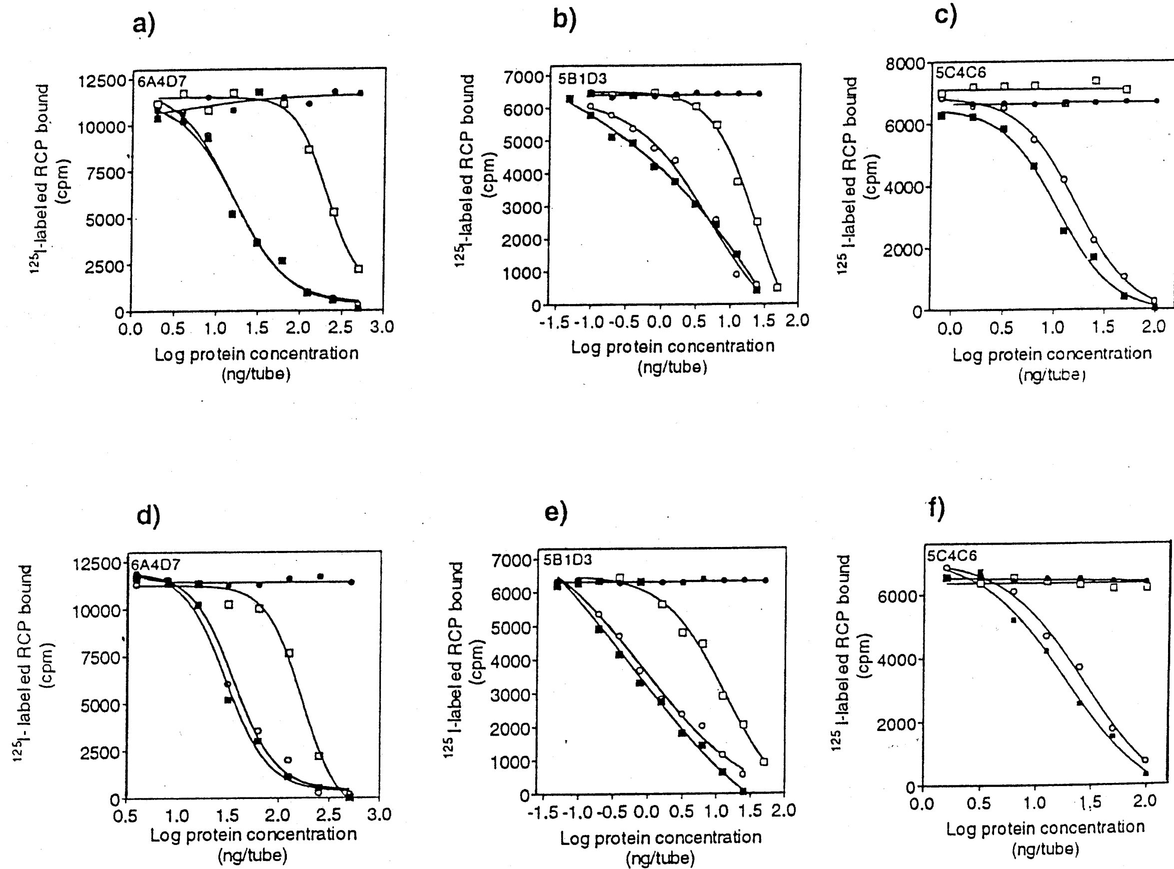

Fig. 4. Radioimmunoassay of refolded native (a–c) and dephosphorylated (d–f) RCP using conformation-specific monoclonal antibodies. Samples were taken 2 h (●), 16 h (□) and 72 h (○) after initiation of refolding and further refolding was stopped by lowering the pH to 2.0 by the addition of 1 M HCl. The inhibition of ^{125}I-labeled cRCP binding to the monoclonal antibodies by these samples was compared with that using native RCP (■).

significant differences in the folding kinetics of dephosphorylated RCP when compared with native RCP, both in terms of the rate of oxidation of the sulfhydryl residues and the regeneration of riboflavin binding in the refolding protein (Fig. 3 b). Moreover, active refolded protein was generated in high yields comparable with that obtained with the native protein. Therefore, despite the marked change in the pI of the protein on dephosphorylation, it appears that the phosphate groups do not have a profound effect on the refolding of RCP *in vitro*.

We monitored the ability of samples of both native and dephosphorylated RCP, removed during the refolding process, to interact with a number of conformational specific antibodies raised to native RCP. The data shown in Fig. 4 indicate that the conformation adopted by the native or dephosphorylated RCP, 2 h following initiation of refolding, was not recognized by any of the monoclonal antibodies, indicating that epitopes defined by these antibodies were not generated at that time. However, by 16 h, the conformation adopted by the refolding proteins was similar to that in native RCP, since two monoclonal antibodies, 6A4D7 and 5B1D3, showed appreciable cross-reactivity with the refolded protein. However, on a quantitative basis, the amount of protein required for bringing about an equivalent displacement was higher for the folding intermediate than the native protein. This difference could be due to the partial regeneration of the conformation specific for each antibody in the folding intermediate but, since the displacement curves were parallel, it is likely that only a small number of molecules of RCP had

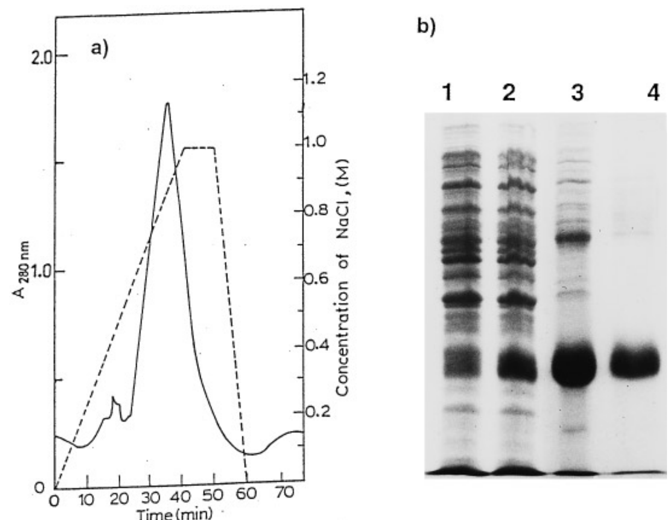

Fig. 5. Purification of recombinant RCP from inclusion bodies. BL21(DE3) cells harboring plasmid pSV5 were induced with isopropyl-thio-β-D-galactoside. Inclusion bodies were isolated as described in the text and solubilized in buffer containing 8 M urea and 1 mM dithiothreitol, desalted and purified by anion-exchange FPLC using MonoQ column. (a) SDS/PAGE of uninduced (lane 1), induced (lane 2) cultures, inclusion body fraction (lane 3) and purified recombinant RCP (lane 4). (b) Elution profile from MonoQ. A_{280} (——) and concentration of NaCl (M) (– – –) are shown.

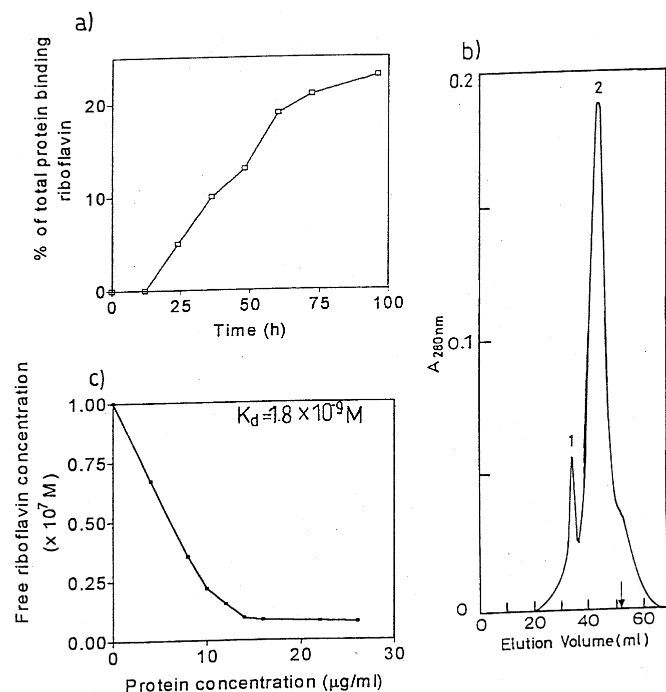

Fig. 6. Refolding of recombinant RCP. Purified recombinant RCP was reduced and denatured with guanidine HCl and dithiothreitol. Refolding was initiated by air oxidation in presence of 1 mM glutathione and 0.1 mM oxidized glutathione as described in the text. (a) Generation of riboflavin-binding activity. (b) Gel filtration of refolded recombinant RCP. Peak 2 shows riboflavin-binding activity. The arrow on the x-axis shows the position of elution of native RCP. (c) Riboflavin-binding affinity of refolded recombinant RCP. Protein in peak 2 was used for the analysis.

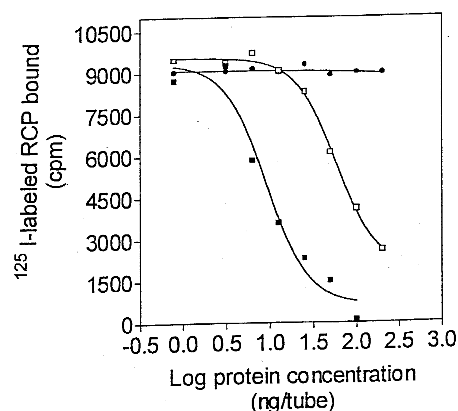

Fig. 7. Radioimmunoassay of refolded recombinant RCP using monoclonal antibody 5C4C6. Radioimmunoassay was carried out using protein in peak 1 (●), peak 2 (□) and compared with that using native RCP (■).

folded to a form present in the native protein, and these were the ones recognized by the monoclonal antibodies.

Of interest is the reactivity observed with one monoclonal antibody, 5C4C6. The data shown in Fig. 4 indicate that the epitope defined by this antibody is generated only at 72 h, when the protein had regained complete riboflavin-binding activity. This suggested that this antibody recognized a conformation generated by, and involving, the riboflavin-binding pocket of RCP. This conformation, or perhaps the riboflavin-binding pocket itself, is formed only toward the later stages of the folding process, with both the dephosphorylated and native RCP showing equivalent reactivity with the antibody.

To study the role of glycosylation in regulating the *in vitro* folding of RCP, we cloned and expressed the cDNA corresponding to the mature protein in *E. coli*. The expressed protein was localized almost exclusively in the inclusion body fraction of the cells [31]. Solubilization of the inclusion body was performed in urea and dithiothreitol and the protein purified to homogeneity by ion-exchange chromatography (Fig. 5).

Refolding of the recombinant RCP under conditions identical to those of the native protein were unsuccessful (data not shown). Desalting of the purified recombinant RCP in the absence of reducing agents prior to initiating refolding, in a manner similar to that adopted for native RCP, resulted in the rapid oxidation of the cysteine residues and the generation of aggregates, as determined by gel filtration (data not shown). We therefore performed the desalting step in the presence of reduced glutathione and initiated oxidation by the addition of oxidized glutathione. Under these conditions, however, we were unable to monitor the thiol content in recombinant RCP, but could measure the generation of active protein in terms of riboflavin binding. The time required to generate the folded protein was similar to that of the native protein, but yields finally achieved after complete refolding were 23% (Fig. 6a). Analysis of the refolded mixture by gel filtration indicated that a significant amount of refolded protein eluted in a position predicted from its molecular mass and bound riboflavin with an affinity similar to that of the native protein (Fig. 6b). However, only 25% of the total protein in that fraction could bind riboflavin. The data shown in Fig. 7 also indicate that the active protein could cross-react with 5C4C6 Mab, to an extent expected from the yield of the finally refolded protein. We can therefore conclude that, while glycosylation of RCP was not essential for the generation of active RCP which could bind riboflavin, glycosylation could have played a role in regulating and optimizing the formation of certain disulfide bonds during the folding pathway, which led to the generation of productive intermediates and, as a consequence, a higher yield of active protein. However, it can also be argued that the lack of glycosylation influenced the formation of intermolecular disulfides, leading to aggregation, rather than direct influences on the intramolecular disulfides.

DISCUSSION

In vitro refolding of proteins containing disulfide bridges has always been considered a formidable task, with the complexity increasing as the number of disulfide bridges present in the protein increases [32]. A large body of information exists regarding the folding of bovine pancreatic trypsin inhibitor, and it is clear from these studies that the folding pathway proceedsvia the formation of a number of intermediates generated by disulfide bridges which are not present in the native protein [21]. While a large number of intermediates have been detected in the folding pathway, only a few of them appear to allow the folding to proceed towards the generation of active protein [21]. In this study, we have described the *in vitro* folding of native, dephosphorylated and recombinant RCP, and our results appear to indicate that non-native intermediates are formed during the folding pathway of RCP. Since RCP is abundant in sulfhydryl residues, all of which are oxidized in the native purified protein [12, 14], we could monitor the folding process as a function of formation of the disulfide bridges, generation of riboflavin binding, and the topology adopted by the intermediates using conformation-specific monoclonal antibodies. Our results clearly show that while the formation of disulfide bridges leading to almost complete oxidation of native RCP is reasonably rapid, the regeneration of

the conformation required for riboflavin binding is far slower and requires 72 h to obtain fully active protein. It is thus apparent that the final productive conformation could result from the breaking of disulfide bonds and their subsequent reoxidation to generate the bonds present in the native protein required for the formation of the riboflavin binding pocket.

Earlier reports are available on the refolding of RCP and have suggested that PDI serves to optimize the yields of native protein [17]. However, our procedure of refolding does not appear to require the presence of any chaperone, and nearly quantitative yields of refolded native and dephosphorylated protein have been obtained in our studies. It is possible that the rapid removal of urea and dithiothreitol in our studies allowed the formation of secondary structures, which, in turn, generated intermediates along the productive folding pathway. However, it is to be noted that, in our studies as well as those described earlier [17], the time required for the complete folding of the reduced protein was similar, indicating that different conditions employed during the folding may not have drastically altered the folding intermediates that are formed. PDI is known to increase the rate of the disulfide-bond formation [32]; it is, therefore, conceivable that the lower yields described in the earlier studies could have arisen as a result of the increased formation of non-productive intermediates in the folding pathway, the generation of which is enhanced by the presence of PDI. High molar ratios of PDI to RCP were required for the optimal generation of folded protein in the earlier study [17], indicating that there could have been a direct interaction of PDI with possibly multiple cysteine residues in RCP during the folding process.

While optimizing conditions for the refolding of native RCP, we observed that consistently higher yields were obtained when air oxidation was performed at a pH above 8. We observed that desalting of denatured and reduced RCP performed at neutral pH led to the rapid oxidation of sulfhydryl groups, since the freshly desalted protein possessed only 60% of its available cysteine residues in the thiol form. Consequently, a reduced yield of active protein was obtained even if refolding was performed at pH 8.5. When refolding was performed at a pH below 8.5, the maximum amount of refolded protein obtained was of the order of 40% (data not shown). At pH 8.5, the effect of the thiol pK_a in regulating the rate of its oxidation was negligible [21]. At a lower pH, the oxidation of certain thiols in the protein may not have been favored, leading to the formation of oxidized intermediates which are non-productive intermediates in the folding pathway. Our data indicate that significant disulfide-bond reshuffling could occur during the refolding process; therefore, allowing an optimum extent of disulfide-bond formation, reduction and reformation, could allow the generation of higher amounts of folded protein.

We also report in this study the generation and refolding characteristics of recombinant RCP. While our results with native and dephosphorylated RCP do not indicate an important role played by the phosphoserine residues in the folding process, our studies with recombinant RCP expressed in *E. coli* suggest that the glycosylation of RCP assists in the formation of intermediates on the productive pathway of folding. The structure of the sugar chains of yolk RCP has been described and indicates sialylbiantenna and sialylytriantenna structures [33]. However, glycosylation of egg-white RCP is slightly different in view of its alternate site of synthesis [34]. Nevertheless, it is possible that the sugar residues, while having little effect on the kinetics of the folding pathway, may reduce aggregation of the protein during the folding pathway by preventing the formation of intermolecular disulfide bridges, as well as allowing only certain disulfide bonds to form, resulting in productive intermediates. In fact, such an effect of glycosylation is suggested for yeast invertase [34]. Non-glycosylated RCP, therefore, could fold with a lower efficiency as a result of the formation of intermediates that do not allow for disulfide-bond reshuffling needed to generate the active protein.

Monoclonal antibodies have proven to be useful for the determination of structural features in proteins. We have generated monoclonal antibodies to RCP, and shown that some of these antibodies interact only with the native protein; therefore, they would be useful for probing the structure of the intermediates generated during the folding process [18]. Our data indicate that certain conformational epitopes are formed during the refolding of RCP, but a critical epitope appears to form only when the protein has achieved a structure capable of binding riboflavin. This epitope is also present in refolded recombinant RCP, and is therefore not generated by post-translational modifications. In fact, our studies are interesting in that this monoclonal antibody could provide a tool for the identification of a core structure adopted by RCP and perhaps proteins related in structure. The crystal structure of RCP indicates a new protein fold [14], and it is suggested that folate-binding proteins with a certain degree of sequence similarity to RCP could possess structural motifs similar to RCP [27].

In summary, we have monitored the refolding of a complex, multiple disulfide-bond-containing protein, with regard to the rate of oxidation of the disulfide bridges and the generation of biological activity. We suggest that non-native intermediates are formed during the refolding of RCP and extensive disulfide-bond rearrangement could occur prior to the formation of the active protein. We also report for the first time the expression of full-length RCP in *E. coli* and the refolding of the protein present in inclusion bodies to an active moiety capable of binding riboflavin with an affinity comparable to that of the native protein. Our studies suggest that RCP could serve as a good model system to study the folding of proteins containing a number of disulfide bridges. The ability of RCP, produced in *E. coli*, to refold to an active protein should allow one, in future, to critically study, through site-directed mutagenesis, the role of specific residues in the binding of riboflavin, as well as in the folding pathway.

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REFERENCES

1. White, H. B. III & Merill, A. H. Jr (1988) Riboflavin-binding proteins, *Ann. Rev. Nutr. 8*, 279−299.
2. Adiga, P. R. (1994) Riboflavin carrier proteins in reproduction, in *Vitamin receptors* (Dakshinamurti, K., ed.) pp. 137−176, Cambridge University Press, Cambridge, UK
3. Clagett, C. O., Buss, E. G., Saylor, E. M. & Girsh, S. J. (1970) The nature of the biochemical lesion in avian renal riboflavinuria. 6. Hormone induction of the riboflavin-binding protein in roosters and young chicks, *Poultry Sci. 49*, 1468−1472.
4. DurgaKumari, B. & Adiga, P. R. (1986) Hormonal induction of riboflavin carrier protein in the chicken oviduct and liver: a comparison of kinetics and modulation, *Mol. Cell. Endocrinol. 44*, 285−292.
5. White H. B. (1987) Vitamin-binding proteins in the nutrition of the avian embryo, *J. Exp. Zool. Suppl. 1*, 53−63.
6. Clagett, C. O. (1971) Genetic control of the riboflavin carrier protein, *Fed. Proc. 30*, 127−129.
7. Hamazume, Y., Mega, T. & Ikenaka, T. (1984) Characterization of hen egg white- and yolk-riboflavin binding proteins and amino acid sequence of egg white-riboflavin binding protein, *J. Biochem. 6*, 1633−1644.
8. Norioka, N., Okada, T., Hamazume, Y., Mega, T. & Ikenaka, T. (1985) Comparison of the amino acid sequences of hen plasma-,

yolk-, and white-riboflavin binding proteins, *J. Biochem. 97*, 19−28.

9. Fenselau, C., Heller, D. N., Miller, M. S. & White, H. B. III (1985) Phosphorylation sites in riboflavin-binding protein characterized by fast atom bombardment mass spectrometry, *Anal. Biochem. 150*, 309−314.
10. Sooryanarayana, Sarkar, S., Adiga, P. R. & Visweswariah, S. S. (1998) Identification and characterization of receptors for riboflavin carrier protein in the chicken oocyte. Role of the phosphopeptide in mediating receptor interaction, *Biochim. Biophys. Acta 1382*, 230−242.
11. Miller, M. S., Benore-Parsons, M. & White, H. B. III (1982) Dephosphorylation of chicken riboflavin-binding protein and phosvitin decreases their uptake by oocytes, *J. Biol. Chem. 257*, 6818−6824.
12. Hamazume, Y., Mega, T. & Ikenaka, T. (1987) Positions of disulfide bonds in riboflavin-binding protein of hen egg white, *J. Biochem. 10*, 217−221.
13. Kozik, A. (1982) Disulfide bonds in egg-white riboflavin-binding protein. Chemical reduction studies, *Eur. J. Biochem. 121*, 395−400.
14. Monaco, H. L. (1997) Crystal structure of chicken riboflavin-binding protein, *EMBO J. 16*, 1475−1483.
15. Becvar, J. & Palmer, G. (1982) The binding of flavin derivatives to the riboflavin-binding protein of egg white. A kinetic and thermodynamic study, *J. Biol. Chem. 257*, 5607−5617.
16. Murthy, U. S., Podder, S. K. & Adiga, P. R. (1976) The interaction of riboflavin with a protein isolated from hen's egg white: a spectrofluorimetric study, *Biochim. Biophys. Acta 434*, 69−81.
17. McClelland, D. A., McLaughlin, S. H., Freedman, R. B. & Price, N. C. (1995) The refolding of hen egg white riboflavin-binding protein: effect of protein disulphide isomerase on the reoxidation of the reduced protein, *Biochem. J. 311*, 133−137.
18. Kuzhandhaivelu, N., Karande, A. A. & Adiga, P. R. (1996) Refolding of riboflavin carrier protein as probed by biochemical and immunological parameters, *Biochim. Biophys. Acta 1293*, 231−237.
19. McClelland, D. A. & Pine, N. C. (1998) Stopped-flow analysis of the refolding of hen's egg white riboflavin binding protein in its native and dephosphorylated forms, *Biochim. Biophys. Acta 1382,* 157−166.
20. Riddles, P. W., Blakeley, R. L. & Zerner, B. (1983) Reassessment of Ellman's reagent, *Methods Enzymol. 91,* 49−60.
21. Creighton, T. E., Darby, N. J. & Kemmink, J. (1996)The roles of partly folded intermediates in protein folding, *FASEB J. 10*, 110−118.
22. Farrell, H. M. Jr, Mallette, M. F., Buss, E. G. & Clagett, C. O. (1969) The nature of the biochemical lesion in avian renal riboflavinuria. 3. The isolation and characterization of the riboflavin-binding protein from egg albumen, *Biochim. Biophys. Acta 194,* 433−442.
23. Murthy, U. S. & Adiga, P. R. (1977)Riboflavin-binding protein of hen's egg: purification and radioimmunoassay, *Ind. J. Biochem. Biophys. 14,* 118−124.
24. Miller, M. S. & White, H. B. III (1986) Isolation of avian riboflavin-binding protein, *Methods Enzymol. 122*, 227−234.
25. Davis, B. J. (1964) Disc electrophoresis II. Method and application to human serum proteins, *Ann. NY Acad. Sci. 121,* 404−427.
26. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular cloning: a laboratory manual,* 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor NY.
27. Zheng, D. B., Lim, H. M., Pene, J. J. & White, H. B. III (1988) Chicken riboflavin-binding protein. cDNA sequence and homology with milk folate-binding protein, *J. Biol. Chem. 263*, 11 126−11 129.
28. Studier, F. W., Rosenberg, A. H., Dunn, J. J. & Dubendorff, J. W. (1990) Use of T7 RNA polymerase to direct expression of cloned genes, *Methods Enzymol. 185*, 60−89.
29. Lotter, S. E., Miller, M. S., Bruch, R. C. & White, H. B. III (1982) Competitive binding assays for riboflavin and riboflavin-binding protein, *Anal. Biochem. 125*, 110−117.
30. Schulke, N. & Schmid, F. X. (1988) Effect of glycosylation on the mechanism of renaturation of invertase from yeast, *J. Biol. Chem. 263*, 8832−8837.
31. Marston, F. A. O. (1986) The purification of eukaryotic polypeptides synthesized in *Escherichia coli*, *Biochem. J. 240*, 1−15.
32. Gilbert, H. F. (1997) Protein disulfide isomerase and assisted protein folding, *J. Biol. Chem. 272*, 29 399−29 402.
33. Tarutani, M., Norioka, N., Mega, T., Hase, S. & Ikenaka, T. (1993) Structures of sugar chains of hen egg yolk riboflavin-binding protein, *J. Biochem. 113*, 677−682.
34. Miller, M. S., Burch, R. C. & White, H. B. III (1982) Carbohydrate compositional effects on tissue distribution of chicken riboflavin-binding protein, *Biochim. Biophys. Acta 715*, 126−136.