Highly Phosphorylated Region of Chicken Riboflavin-Binding Protein: Chemical Characterization and ^{31}P NMR Studies†

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ABSTRACT: Phosphate residues on chicken riboflavin-binding protein (RBP) have been implicated in the recognition and deposition of the protein–vitamin complex in egg yolk by the ovary. We demonstrate that all of the phosphate of RBP is linked to serine and that most if not all of the phosphoserine residues are contained in a single tryptic peptide having a composition $SerP_8$, Glx_6, His_2, Leu_2, Ala, Met, Lys. Despite differences in the tissue of synthesis, RBPs isolated from egg yolk and egg white yield phosphopeptides virtually identical in their amino acid composition and 101-MHz ^{31}P NMR spectra. This implies that posttranslational phosphorylation of RBP is the same in liver and oviduct. The ^{31}P NMR spectrum of the phosphopeptide is quite different from that of the phosphoprotein. The ^{31}P NMR spectra of egg yolk and egg white RBP are quite similar but not identical and are unaffected by the binding of riboflavin. Optimal resolution of seven phosphorus resonances was obtained near pH 7.0. The titration behavior of all the phosphoserine residues is similar. We propose that this highly anionic peptide, exposed at the surface of RBP, is the principal determinant for the uptake of RBP by the vitelline membrane of the ovarian follicle.

Riboflavin in chicken egg yolk and albumen is tightly bound to a specific phosphoglycoprotein (Ostrowski et al., 1962; Rhodes et al., 1959). The riboflavin-binding protein (RBP)[1] deposited in the egg yolk is synthesized in the liver and is transported by the blood to the developing ovarian follicles as are other yolk proteins (Froelich et al., 1980; Schjeide et al., 1963; Blum, 1967). The riboflavin-binding protein in egg white is synthesized in the oviduct (Mandeles & Ducay, 1962). A mutant strain of chickens unable to synthesize RBP is also unable to deposit adequate amounts of riboflavin in its eggs even when given large amounts of the vitamin (Maw, 1954; Boucher et al., 1964; Winter et al., 1967). Embryos in these eggs die of riboflavin deficiency unless they are rescued by injecting the vitamin into the egg. Clearly, the deposition of riboflavin in chicken eggs depends upon the recognition and transport of the protein–vitamin complex and not the vitamin alone. Similarly, a circulating riboflavin-binding protein is found in the plasma of pregnant mammals and is required for the transport of riboflavin across the placenta to the fetus (Muniyappa & Adiga, 1980; Murthy & Adiga, 1982). Our objective is to characterize the structural features of RBP which are required for the deposition of riboflavin in the ovarian follicle.

Although RBPs from egg yolk and egg white are products of the same gene, there are tissue-specific differences in posttranslational glycosylation (Miller et al., 1982b). Attempts to demonstrate an ovarian receptor that recognizes the oligosaccharide moieties of riboflavin-binding protein have been inconclusive (Miller et al., 1981, 1982b). On the other hand, attempts to implicate covalent phosphate residues on RBP in this ovarian recognition–transport process have been promising.

Riboflavin-binding proteins from hen egg white and egg yolk both contain about eight residues of phosphate per molecule of protein (Miller et al., 1982a). All of these phosphates can be removed by treatment with acid phosphatase with no effect on the riboflavin-binding capacity and little effect on the conformation of the protein (Rhodes et al., 1959). However, within the laying hen, the ability of RBP to deliver riboflavin to developing oocytes is greatly diminished by dephosphorylation (Miller et al., 1982a). We have postulated that there are receptors within the ovarian follicles which recognize RBP, bind it, and deliver it into the oocyte by endocytosis. We feel that the phosphorylated portions of RBP may be recognized specifically by these receptors.

In our previous experiments (Miller et al., 1982a), 10.5% of ^{125}I-labeled yolk RBP was incorporated into oocytes following intravenous injection of tracer quantities, while only about 1.4% of the dephosphorylated yolk RBP was transported under similar conditions. Progressive removal of phosphate residues resulted in a progressive decrease in the uptake of RBP by oocytes. Furthermore, uptake could not be restored by adding back anionic groups to the dephosphorylated protein by succinylation, indicating that recognition of the protein is not simply a nonspecific charge phenomenon.

In this paper, we show that all the phosphate in RBP occurs as phosphoserine. Furthermore, most if not all of the phosphoserine residues occur in a single, highly acidic, tryptic peptide. Comparison of ^{31}P NMR spectra of egg yolk and egg white RBP and of their respective phosphopeptides indicates that the distribution of phosphate residues is similar if not identical in the two proteins.

Materials and Methods

Purification of Riboflavin-Binding Proteins. Yolk RBP was prepared from oocytes collected from Single Comb White Leghorn chickens (*Gallus domesticus*) at Dover Poultry Products, Baltimore, MD, or at United Poultry, Vineland, NJ. The oocytes were slit open and drained of their contents. The yolk was diluted with an equal volume of 50 mM sodium acetate buffer (pH 4.3) containing 10 mM NaF to inhibit endogenous phosphatases. Following this step, yolk RBP was isolated as described by Miller et al. (1981). Egg white RBP was purified from the albumen of White Leghorn eggs by the

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[1] Abbreviations: RBP, riboflavin-binding protein; TPCK, tosylphenylalanine chloromethyl ketone; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

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method of Farrell et al. (1969). RBP was maintained as the holoprotein throughout its isolation by prior saturation with riboflavin (Sigma Chemical Co., St. Louis, MO). RBP from both of these sources was homogeneous by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (Weber & Osborn, 1969). Apo-RBP was prepared from the holoproteins by chromatography on CM-cellulose (Whatman CM-52) at pH 3.14 followed by elution of the protein at pH 5.8 (Farrell et al., 1969).

Preparation of Two-Dimensional Maps of Tryptic Peptides. Egg white and egg yolk RBPs were separately reduced with dithiothreitol and alkylated with iodoacetic acid (Konigsberg, 1972). Following dialysis against distilled H_2O, the reduced carboxymethylated proteins were digested with trypsin (EC 3.4.21.4, TPCK treated, Worthington Biochemical Co., Freehold, NJ) for 24 h at 30 °C. The concentration of RBP was 5 mg/mL, and the reaction mixture was buffered with 1% NH_4HCO_3 at pH 8.1. Trypsin was added at the start of the incubation and after 8 h to give a final concentration of 100 μg/mL. The reaction mixtures were lyophilized. Peptide maps of the tryptic digests were developed according to the method used by Hunter & Sefton (1980) for phosphopeptide analysis. Between 0.5 and 1.0 mg of each tryptic digest was spotted near the lower left corner of a cellulose thin-layer plate (Eastman Chemical Co., Rochester, NY). Electrophoresis was carried out in the first dimension in 1% $(NH_4)_2CO_3$ at pH 8.9 for 100 min at 400 V. After drying overnight, the plates were chromatographed in the second dimension in 1-butanol/pyridine/acetic acid/H_2O (75:50:15:60 v/v). The peptides were visualized by spraying with ninhydrin (Bennett, 1967) or fluorescamine (Felix & Jiminez, 1974). Phosphopeptides were detected on the same plates (after the peptide spots were recorded) with the acid/molybdate spray reagent described by Bochner et al. (1981).

Phosphopeptide Purification. The phosphopeptides from egg white and egg yolk RBPs were prepared by ion-exchange chromatography of tryptic digests of the reduced carboxymethylated proteins prepared as described for peptide mapping. The tryptic peptides from 500 mg of yolk holo-RBP or 50 mg of egg white holo-RBP were applied to a 1.5 × 20 cm column of DEAE-cellulose (Whatman DE-52) in 0.1 M ammonium formate at room temperature. The column was eluted with a gradient of 0.1 M ammonium formate to 0.1 M HCOOH followed by 0.25 M HCOOH followed by a gradient of 1 M HCOOH to 1 M HCl (100 mL of each). The column was monitored at 280 nm, and phosphopeptides were identified by assaying 25-μL aliquots for phosphate (see Analytical Methods). The phosphopeptide fractions were pooled and evaporated to dryness under reduced pressure. These fractions were then rechromatographed on DEAE-cellulose (1 × 60 cm column) in 0.1 M sodium acetate buffer (pH 3.5) and eluted with a linear 0.1–0.4 M NaCl gradient in the same buffer. The phosphate-containing peaks were collected individually, concentrated by lyophilization, and desalted on a Sephadex G-15 (Pharmacia) column (1.5 × 25 cm) in H_2O. Homogeneity of the isolated phosphopeptides was determined by electrophoresis on cellulose thin-layer plates (Eastman Chemical Co.) at pH 3.5 in pyridine/acetic acid/H_2O (5:50:945) for 2 h at 600 V.

Analytical Methods. Phosphate was determined by the method of Chen et al. (1956) following ashing of the protein as described by Ames & Dubin (1960). Protein concentration was determined by the method of Lowry et al. (1951) or, on pure RBP samples, spectrophotometrically at 280 nm after subtracting the contribution of protein-bound riboflavin at this wavelength (absorbance at 280 nm minus 1.55 times the absorbance at 455 nm) as described by Kozik (1982). RBP samples were deglycosylated with trifluoromethanesulfonic acid (Aldrich Chemical Co.) by the method of Edge et al. (1981). Hexoses were determined colorimetrically with orcinol/H_2SO_4 (Francois et al., 1962) and hexosamines by the method of Rondle & Morgan (1955) after hydrolysis for 4 h at 100 °C in 4 N HCl under N_2. Qualitative analysis of carbohydrates was by gas chromatography as described previously (Miller et al., 1982b).

Amino acid analyses were performed on a Beckman amino acid analyzer according to the manufacturer's directions after hydrolysis of samples for 20 h at 110 °C in 6 N HCl under N_2. Phosphoamino acids were identified after partial acid hydrolysis of RBP samples in 6 N HCl for 1 h at 110 °C under N_2. Following removal of acid by rotary evaporation, the samples were applied to Eastman cellulose thin-layer plates and electrophoresed at pH 3.5 in pyridine/acetic acid/H_2O (5:50:945 v/v) for 1 h at 600 V. Phosphoamino acids were visualized with acid/molybdate spray reagent (Bochner et al., 1981) and identified by comparison of their mobilities with those of standard phosphoamino acids (phosphoserine and phosphothreonine, Sigma Chemical Co.) relative to the mobility of inorganic orthophosphate. Derivatization of the N-terminal amino acid residue was by the dansylation procedure of Gray (1967). Following hydrolysis of the dansylated peptides for 16 h at 105 °C in 6 N HCl under N_2, the derivatized amino acids were separated by two-dimensional thin-layer chromatography on polyamide sheets (Pierce Chemical Co.) by using the solvent systems described by Gray (1967).

^{31}P NMR Spectroscopy. Samples of lyophilized egg yolk and egg white RBPs, and their respective phosphopeptides, were dissolved in 2 mL of 0.25 mM Hepes (pH 7.0) and placed in 10-mm precision tubes (Wilmad) equipped with Teflon vortex plugs. The samples contained 20% D_2O as an internal field frequency lock and 10 mM EDTA to eliminate line broadening due to paramagnetic impurities. ^{31}P NMR spectra were measured at 27 °C with a Bruker 250-MHz spectrometer at 101.27 MHz with quadrature phase detection. All spectra were recorded with broad-band proton decoupling. Typical conditions of the NMR experiment were 16K memory size, 2000-Hz sweep width, 10-μs pulse width, and 4.096-s acquisition time. Exponential multiplication of the free induction decay signal was applied to reduce noise, with the resulting line broadening of 0.5 Hz. All chemical shifts were determined relative to an external standard of 85% H_3PO_4. Downfield shifts are given a positive sign. Conditions for individual NMR experiments are given in the legends to the figures.

For experiments where the pH was varied, the pH was adjusted by dropwise addition of NaOH or HCl. The pH was measured on a Fisher Accumet standard pH meter (Model 310) equipped with a glass electrode, standarized by using normal H_2O buffers. The pH values reported have not been corrected for the deuterium isotope effect at the glass electrode. The NMR pH titration curves were analyzed by using a nonlinear least-squares curve-fitting computer program based on Marquardt's algorithm (Marquardt, 1963) for the single-site equilibrium (Henderson–Hasselbach type) model.

Results

Phosphopeptide Mapping of Egg White and Egg Yolk RBPs. The peptide maps prepared from tryptic digests of reduced and alkylated samples of RBP are shown in Figure 1. The two-dimensional tryptic maps revealed with ninhydrin are essentially identical for the egg white and egg yolk proteins.

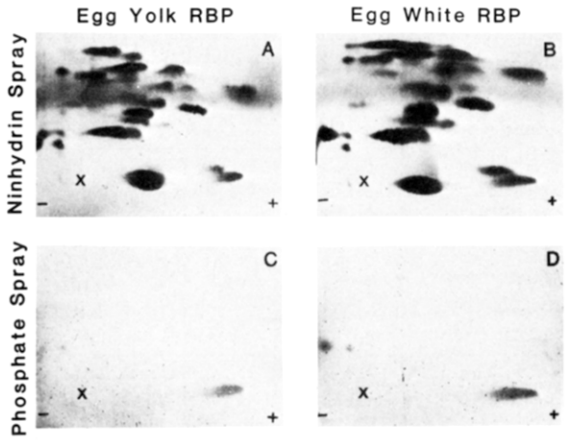

FIGURE 1: Peptide maps of RBP. Tryptic digests (~1 mg) of reduced, carboxymethylated egg white RBP (A) and egg yolk RBP (B) were spotted on cellulose thin-layer plates at the lower left (x). Electrophoresis in the horizontal direction was in 1% NH_4HCO_3, pH 8.9, at 400 V for 100 min. Chromatography in the vertical direction was in 1-butanol/pyridine/acetic acid/H_2O (75:50:15:60 v/v). Peptides were detected with ninhydrin spray. The same plates were then sprayed with acid/molybdate reagent to reveal the phosphopeptides of egg white RBP (C) and egg yolk RBP (D).

Table I: Deglycosylation of Riboflavin-Binding Protein with Trifluoromethanesulfonic Acid[a]

sample	total hexose (residues/mol)	*N*-acetylglucosamine (residues/mol)	phosphate (residues/mol)
native yolk RBP	12.1	11.8	8.10
deglycosylated yolk RBP	0.14	2.27	7.60
native white RBP	9.36	14.6	7.60
deglycosylated white RBP	0.11	2.59	7.27

[a] All values are based on 36 000 g/mol of RBP and were corrected for protein concentration by using holo white RBP as the reference protein. Each value is the average of at least two determinations as described under Materials and Methods. Deglycosylation was carried out for 5 h at room temperature in a mixture of trifluoromethanesulfonic acid and anisole (2:1 v/v) (Edge et al., 1981).

On the basis of a total of 23 lysine and arginine residues per molecule (Farrell et al., 1969), 24 peptides were expected. Although some of the spots are not as distinct as others, 24 peptides can be identified on the peptide maps from both egg white and egg yolk RBPs. The phosphopeptides were also found in identical positions on the egg white and egg yolk peptide maps. One peptide spot containing most if not all of the phosphate residues is readily apparent in the lower right corner of both maps.

Linkage of Phosphate to RBP. Since RBP contains carbohydrate as well as phosphate prosthetic groups, we first considered that the phosphate might be linked to the protein via its oligosaccharide moieties. We examined this possibility by deglycosylating RBP with trifluoromethanesulfonic acid. The results of this modification are given in Table I. Since trifluoromethanesulfonic acid cleaves only the O-glycosidic linkages (Edge et al., 1981), the only residues which remain are the core *N*-acetylglucosamine residues which are in an N-glycosidic linkage to asparagine. Although the neutral sugars were completely removed by this treatment, about two residues of *N*-acetylglucosamine remained, confirming a previous report that there are two asparagine-linked oligosaccharide chains associated with RBP (Mega & Ikenaka, 1982). An insignificant amount of the phosphate was lost during deglycosylation, demonstrating that the phosphate is not linked via carbohydrate.

Table II: Phosphoamino Acid Analysis of Riboflavin-Binding Protein

sample	relative mobility[a]	amino acid analysis[b]
yolk RBP		
spot 1	0.83	nd[c]
spot 2	0.75	nd
white RBP		
spot 1	0.80	serine (100%)
spot 2	0.73	serine (93%), glutamic acid (7%)
standards		
phosphoserine	0.81	nd
phosphothreonine	0.73	nd

[a] Mobility on thin-layer electrophoresis at pH 3.5 relative to inorganic orthophosphate of partial acid hydrolysates of holo egg yolk and egg white RBPs. [b] Analysis of phosphoamino acid spots, recovered from thin-layer plates and subjected to complete acid hydrolysis. [c] Not determined.

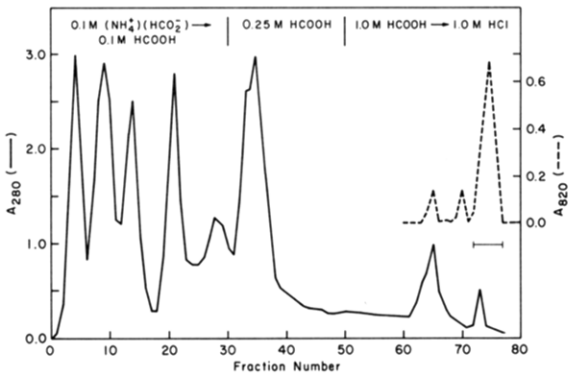

FIGURE 2: Chromatography of yolk RBP peptides on DEAE-cellulose. The tryptic digest of 500 mg of reduced, carboxymethylated yolk RBP was applied to a DEAE-cellulose column (1.5 × 20 cm) and eluted with the indicated gradient at room temperature. The column was monitored at 280 nm (—), and 25-μL aliquots were analyzed for phosphate by the acid/molybdate reaction (A_{820}, ---). The phosphopeptide was pooled as indicated by the horizontal bar.

Following partial acid hydrolysis, phosphoamino acids were identified by thin-layer electrophoresis (Table II). Two spots were visible in both the egg white and egg yolk samples upon spraying with acid/molybdate reagent. These components had relative mobilities which were similar to the standards, phosphoserine and phosphothreonine. However, additional evidence was needed to confirm the identity of these phosphoamino acids. A duplicate thin-layer plate was prepared which was electrophoresed identically but was sprayed lightly with fluorescamine instead of with acid/molybdate. The fluorescamine-positive spots corresponding to the phosphoamino acids were scraped from the plates, recovered from the cellulose by elution with H_2O, and subjected to conventional amino acid analysis after complete acid hydrolysis. The phosphoserine spot contained only serine. The spot corresponding to the phosphothreonine standard contained no threonine but mostly serine and some glutamic acid. We conclude that the apparent phosphothreonine was probably a dipeptide or a mixture of dipeptides (SerP-SerP, SerP-Glu, or Glu-SerP) which resulted from incomplete hydrolysis and which comigrated with phosphothreonine. All of the phosphate in egg white and egg yolk RBPs in linked via serine.

Isolation of Yolk RBP Phosphopeptide. Because of its highly anionic nature, the phosphopeptide of yolk RBP was easily separated from the rest of the tryptic peptides on

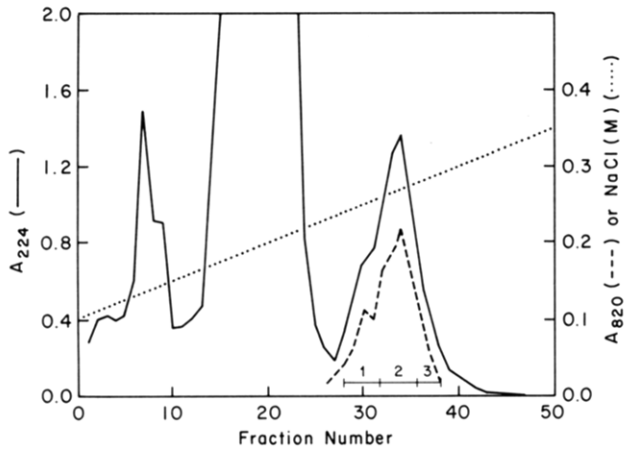

FIGURE 3: Chromatography of yolk RBP phosphopeptide on DEAE-cellulose. The pooled phosphopeptide fractions (Figure 2) were applied to a DEAE-cellulose column in 0.1 M sodium acetate (pH 3.5) and eluted with a linear NaCl gradient as shown. The column was monitored at 224 nm (—), and 25-μL aliquots were analyzed for phosphate (A_{280}, - - -). The phosphopeptides were divided into three pools as indicated.

FIGURE 4: Thin-layer electrophoresis of yolk RBP phosphopeptides. Samples (~50 μg) of the phosphopeptide pools (Figure 3) were spotted on cellulose thin-layer plates and electrophoresed at pH 3.5 in pyridine/acetic acid/H_2O (5:50:945 v/v) for 2 h at 600 V. The tracking dye was bromphenol blue. The peptides were detected with ninhydrin spray.

DEAE-cellulose. The elution profile of the separation of the tryptic peptides from yolk RBP is shown in Figure 2. It is noteworthy that the phosphopeptide could not be eluted until a gradient of 1 M formic acid to 1 M HCl was applied to the column. Most and perhaps all of the phosphate was associated with this one peak. When subjected to a two-dimensional thin-layer peptide map under the same conditions as the tryptic peptide maps in Figure 1, only one spot could be seen with either fluorescamine or acid/molybdate spray. This spot corresponded to the phosphopeptide spot in Figure 1. However, upon thin-layer electrophoresis at pH 3.5, the apparently homogeneous preparation of phosphopeptide was found to contain at least five components. For this reason, the phosphopeptide was rechromatographed on DEAE-cellulose at pH 3.5 with a shallow NaCl gradient (Figure 3). The phosphopeptide material was separated from some nonphosphorylated peptides and was resolved into two components. When pooled as indicated in Figure 3 and then subjected to thin-layer electrophoresis at pH 3.5, pool 2 was found to be homogeneous while pool 1 was enriched in the less anionic phosphopeptides (Figure 4). Although it is not apparent in Figure 4, pool 3 contained a small amount of some more anionic species. Starting with 500 mg of yolk RBP, approximately 17 mg of pure pool 2 phosphopeptide was recovered. By the same procedure, 2 mg of pure phosphopeptide was prepared from 50 mg of egg white RBP.

Amino Acid Analysis of RBP Phosphopeptide. The amino acid analyses of purified phosphopeptide from egg white and egg yolk RBPs are presented in Table III. The analyses show that the phosphopeptides from both proteins are identical in their amino acid compositions. Both contain 21 amino acids, of which 14 residues are serine and glutamic acid (or glutamine). The two pools of yolk RBP phosphopeptide, which had been separated by DEAE-cellulose chromatography, are identical in their amino acid compositions but differ in the number of phosphate residues they contain. It is not known whether the difference in phosphate content is a characteristic of the native protein or an artifact of the isolation procedure. Yolk RBP pool 2 phosphopeptide was analyzed for eight phosphate residues. If one assumes a 20% destruction by acid hydrolysis, eight serine residues are accounted for. The phosphate content of the egg white phosphopeptide was not determined due to insufficient amounts of pure material. However, since it has the same amino acid composition as the yolk phosphopeptide and since it migrated identically during analytical thin-layer electrophoresis, it is assumed that the pure egg white RBP phosphopeptide has the same phosphate composition as the pool 2 yolk RBP phosphopeptide. This is confirmed by the ^{31}P NMR experiments (see below).

Table III: Amino Acid Analysis of RBP Phosphopeptides[a]

amino acid	egg white RBP (ratio, Lys = 1)	yolk RBP, pool 2 (ratio, Lys = 1)	yolk RBP, pool 2 (ratio, His = 2)
serine	6.40 (8)[c]	6.43 (8)	6.46 (8)
glutamic acid	5.77 (6)	5.58 (6)	5.58 (6)
alanine	1.13 (1)	1.15 (1)	1.15 (1)
methionine	1.14 (1)	1.15 (1)	1.04 (1)
leucine	2.03 (2)	2.04 (2)	1.97 (2)
histidine	1.83 (2)	1.98 (2)	2.00 (2)
lysine	1.00 (1)	1.00 (1)	1.16 (1)
phosphate[b]	nd[d]	7.81 (8)	6.92 (7)

[a] The data are the average of two determinations on each of the peptides. The value of methionine represents the sum of methionine and its oxidation products. [b] Phosphate was determined colorimetrically on samples of yolk RBP pools 1 and 2 only. [c] The numbers in parentheses are the nearest integer values for each of the components. Serine was corrected for 20% loss during hydrolysis before assigning its integer value. [d] Not determined.

Several attempts were made to determine the N-terminal residue of the yolk RBP phosphopeptide by dansylation. The N-terminal remained refractory to derivatization, although the ϵ-amino group of the C-terminal lysine was dansylated. It now appears likely that the anionic nature of the peptide hindered the dansylation reaction (V. Chowdhry, R. Kutny, M. S. Miller, and H. B. White, unpublished results).

Comparison of the ^{31}P NMR Spectra of Holo- and Apo-RBPs. The spectra at pH 7.9 of apo- and holo-RBPs are presented in Figure 5. Addition of riboflavin to either apo egg yolk RBP or apo egg white RBP does not produce noticeable changes in the ^{31}P NMR spectra. Despite slight displacement in chemical shifts, a striking similarity of all four spectra is apparent. Five major phosphate resonances are observed in each spectrum within a 0.29 ppm range (4.63–4.35 ppm) for egg white RBP and a 0.45 ppm range (4.43–3.98 ppm) for egg yolk RBP with the intensity ratios as given in Table IV. The chemical shifts of two phosphoserine resonances of ovalbumin (4.93 and 4.66 ppm) and of free phosphoserine (4.53 ppm) are given for comparison. Better resolution of phosphoserine resonances in RBP spectra can be obtained at a pH close to neutrality (see below).

^{31}P NMR of Egg Yolk and Egg White RBPs as a Function of pH. Titration of egg yolk and egg white holoproteins was

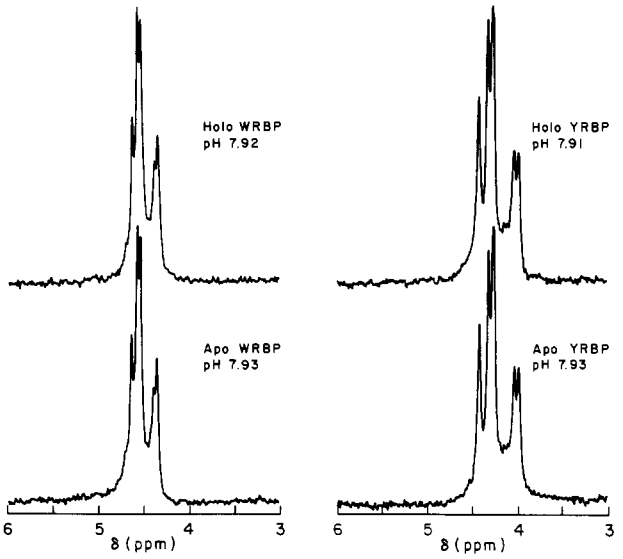

FIGURE 5: ^{31}P NMR spectra of holo and apo egg white and egg yolk RBPs at pH 7.9. The concentration of apoproteins was 100 mg/mL in 0.5 M Hepes (pH 7.9), 10 mM EDTA, and 20% D_2O. For holoprotein spectra, 10 μg of solid riboflavin was added per mg of RBP. Following a 15-min equilibration, samples were centrifuged, and the pH was readjusted; 500 scans.

Table IV: Chemical Shifts of ^{31}P Resonances of Riboflavin-Binding Proteins, Ovalbumin, and Phosphoserine[a]

protein[c]	chemical shift range (ppm)	no. of major peaks	relative intensity[b]
holo-WRBP	4.63–4.35	5	1.5:2.2:2.3:1.0:1.2
apo-WRBP	4.64–4.36	5	1.5:2.0:2.1:1.0:1.2
holo-YRBP	4.43–3.99	5	2.2:2.2:2.6:1.3:1.0
apo-YRBP	4.43–3.98	5	1.5:1.9:2.6:1.2:1.0
ovalbumin	4.93–4.66	2	1.0:1.1
phosphoserine	4.53	1	

[a] Experimental conditions are the same as those in Figure 5.
[b] These ratios are calculated from the areas of each peak.
[c] Abbreviations: WRBP, egg white RBP; YRBP, egg yolk RBP.

performed over the pH range of 4–10 in order to achieve maximum resolution of phosphate resonances as well as to estimate the pK values for phosphoserine residues. The pH profiles shown in Figure 6 demonstrate a strong pH dependence of the chemical shifts of phosphoserine resonances for both proteins. Maximum resolution of phosphoserine resonances is observed at pH 7–8 (seven resonances). Chemical shifts of the peaks in the egg yolk RBP spectrum at pH 7.12 are in the range of 3.97–3.26 ppm and in the egg white RBP spectrum at pH 7.06 occur in the 3.67–2.97 ppm range. Nonstoichiometric intensity ratios of the peaks may result from a heterogeneity of the samples (with respect to the phosphate content) and/or the nuclear Overhauser effect due to broadband decoupling of the spectra. Close similarity of the spectra of egg yolk and egg white RBP is evident at any given pH studied. The titration behavior of all seven individual resonances resolved at a pH close to neutrality could not be traced throughout the entire pH range, due to the overlap with other resonances. Therefore, determination of pK values for individual phosphoserine residues was not possible. However, the range of pK values could be estimated by following the pH dependence of the chemical shifts of the left, right, and major resonances. The pH titration curves for these resonances are presented for egg yolk RBP and egg white RBP in panels A and B, respectively, of Figure 7. The experimental points were obtained from the data in Figure 6. The solid lines in Figure

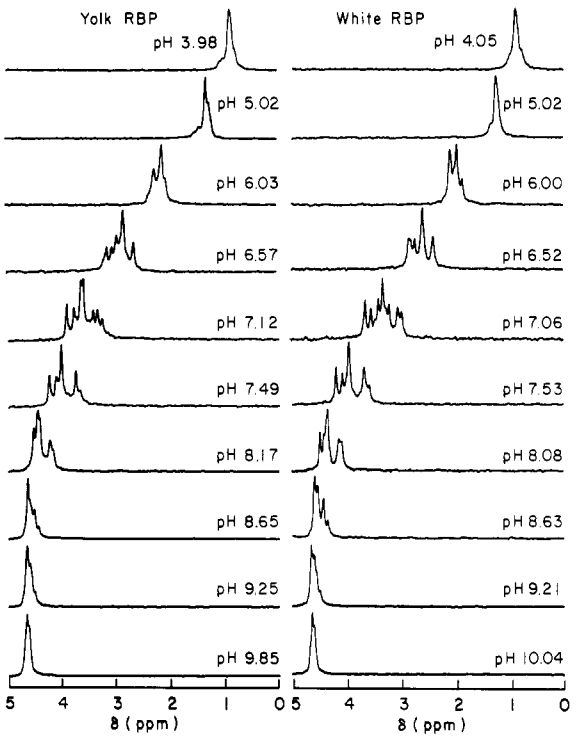

FIGURE 6: ^{31}P NMR pH titration of egg white and egg yolk RBP holoproteins. Egg white and egg yolk RBPs which had been purified as apoproteins were saturated with riboflavin, dialyzed, and lyophilized in the form of holoproteins. The holoproteins were dissolved in H_2O (100 mg/mL) containing 10 mM EDTA and 20% D_2O and titrated with 1 M NaOH or 1 M HCl to the desired pH as indicated. The pH was measured immediately after each spectrum was recorded; 200 scans were collected for each spectrum.

7 are theoretical curves obtained by computer curve fitting. The pH titration data for the three resonances were fitted to the simple proton association equilibrium model by using a nonlinear least-squares program based on Marquardt's algorithm (Marquardt, 1963). The best-fit pK values are 6.51, 6.70, and 6.84 for egg white RBP and 6.41, 6.59, and 6.70 for egg yolk RBP, for the left, major, and right resonances, respectively. Asymmetry of the experimental titration curves in comparison to the computer-fitted lines observed in Figure 7 indicates the inadequacy of the simple equilibrium model to describe the titration pattern observed for both proteins (experimental points in Figure 7A,B).

Mathematical models for the treatment of asymmetric NMR titration curves have been described by Shrager et al. (1972). Unfortunately, application of multisite interaction models is not feasible for our system due to the inability to resolve and assign the individual resonances at all pH values. The application of a two-site model has been recently applied for analysis of the titration behavior of phosphoserine resonances of bovine β-casein (Humphrey & Jolley, 1982). Despite the deviation from the assumed model, it can be concluded that phosphoserine residues of egg yolk RBP and egg white RBP exhibit very similar titration behavior. The estimated pK values for the left, right, and major resonances differ only by 0.1 pH unit for egg white and egg yolk proteins. However, examination of the two sets of experimental data suggests a displacement of slightly more than 0.2 pH unit. This apparent difference in pK values may be due to differences in the ionic strength of the solutions (Roberts et al., 1981).

The influence of neighboring charged groups on the pH titration curves has been discussed in detail (Sachs et al., 1971; Shrager et al., 1972; Markley, 1975). It has been demon-

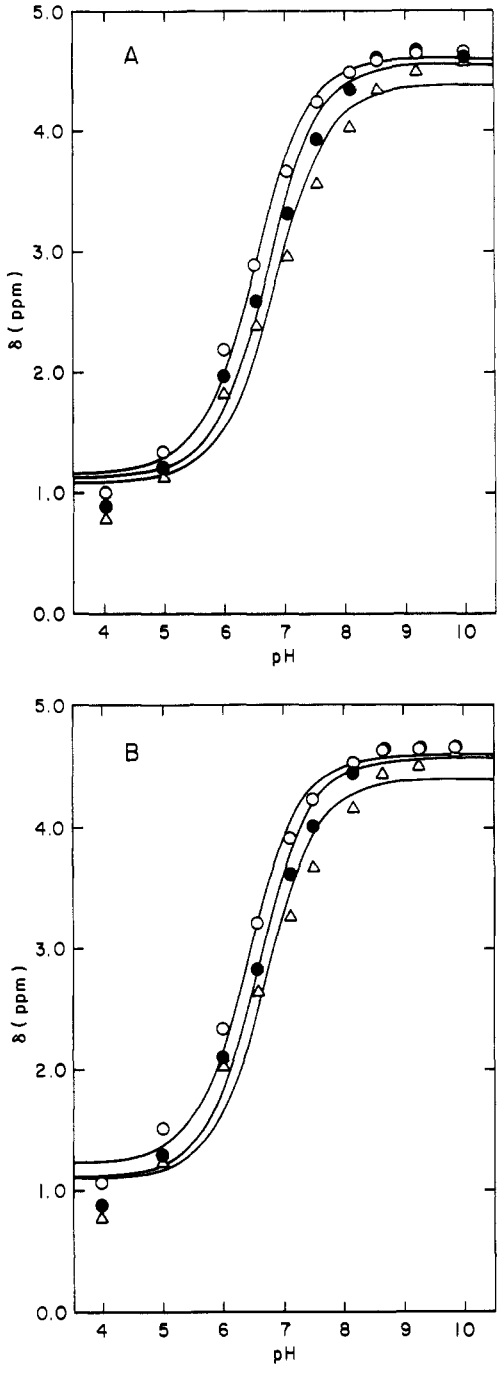

FIGURE 7: pH dependence of the ^{31}P chemical shifts of (A) egg yolk RBP and (B) egg white RBP: (○) left resonance; (●) major resonance; (△) right resonance. The solid lines are theoretical curves obtained by fitting the data to a single-site equilibrium model. Experimental points were obtained from the data presented in Figure 6.

strated that the presence of the neighboring titratable group with a pK differing by more than 2 units is reflected as an inflection in the titration curve and its pK can be determined by inspection. A neighboring charged group(s) titratable within 2 pH units lead(s) to an asymmetric titration curve, and computer-fitting procedures are necessary to calculate microscopic pK values. A neighboring charged group which retains its charge in the pH range of the titration causes a shift of the titration curve but does not lead to asymmetry. The presence of positively or negatively charged groups causes a decrease or an increase in the pK of a titrating group, respectively.

No additional inflections are present in the NMR titration curves within the pH range studied for egg yolk RBP and egg white RBP (Figure 7). This indicates an absence of an effect by charged groups with pK values more than 2 units different than those of the phosphoserine residues. A small difference in pK values estimated for the left and right resonances of only 0.3 pH unit has been observed for egg yolk and egg white RBP, indicating that all phosphoserine residues in these proteins titrate in a close pH region. The asymmetry observed for all titration curves suggests that there is a mutual interaction among neighboring phosphoserine residues. The effect of the proximity of the negatively charged groups is also reflected in the increased pK values for all resonances by about 1 pH unit as compared to that of free phosphoserine (pK = 5.75) (Vogel, 1983). The titration behavior of the phosphoserine residues suggests that they are exposed to the solvent and may occur as a cluster of residues.

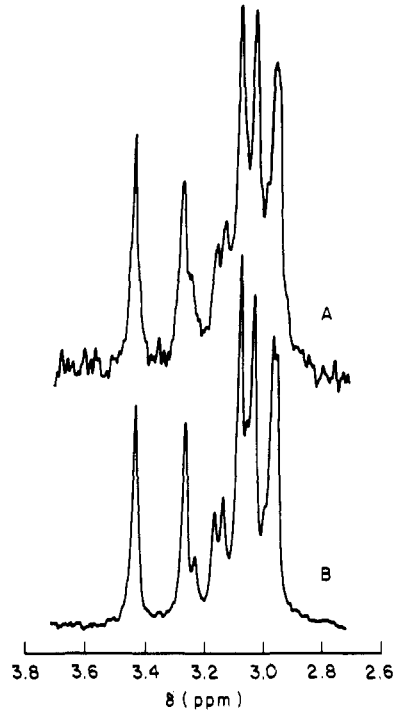

FIGURE 8: ^{31}P NMR spectra of egg white (A) and egg yolk (B) phosphopeptides. Conditions for the top spectrum were the following: 1.5 mg/mL egg white RBP phosphopeptide in 0.25 M Hepes (pH 7.04), 10 mM EDTA, and 20% D_2O; 13 217 scans. Conditions for the bottom spectrum: 15 mg/mL egg yolk RBP phosphopeptide in 0.25 M Hepes (pH 7.01), 10 mM EDTA, and 20% D_2O; 500 scans.

^{31}P NMR Spectra of Egg Yolk RBP and Egg White RBP Phosphopeptides. Any slight differences in the ^{31}P NMR spectra of egg yolk and egg white RBPs disappear when the phosphopeptide is isolated from the remainder of the protein. NMR spectra of the phosphopeptides were recorded at neutral pH, at which maximum resolution of phosphoserine resonances was achieved in the corresponding holoprotein spectra. The spectrum of egg yolk RBP phosphopeptide (Figure 8, bottom) is very similar to that of egg white RBP phosphopeptide (Figure 8, top). Six major resonances occur within a 3.43–2.95 ppm range with integral intensity ratios of 1.1:1.0:1.6:1.6:1.2:1.0 in the egg yolk RBP phosphopeptide spectrum. Five major resonances are resolved in the egg white RBP phosphopeptide spectrum within a 3.36–2.88 ppm range with intensity ratios of 1.1:1.0:2.0:1.8:2.3. Two closely located upfield resonances at 2.96 and 2.95 ppm in the former spectrum were not resolved in the latter, which exhibits one broadened peak at 2.88 ppm. This is probably the results of a 10-fold lower concentration of the egg white RBP phosphopeptide sample used for the NMR experiment. The ratios

of intensities of the resonances in both spectra are remarkably similar and suggest the presence of seven to eight phosphoserine residues in both the egg yolk and egg white RBP phosphopeptides, with agreement of the phosphate content found for both riboflavin-binding proteins (Miller et al., 1982a). It should be taken into account, however, that nuclear Overhauser effects may have influenced the absolute intensity of the individual peaks to a different extent. Four minor resonances of lower intensity which are present in the spectra of the phosphopeptides may indicate heterogeneity due to incomplete phosphorylation of all serine residues. The chemical shift range at pH 7.0 is identical (0.48 ppm) for the two phosphopeptides, suggesting a similar environment of phosphoserine residues and further indicating their close similarity. The range of the chemical shifts for the corresponding holoproteins at the same pH can be estimated by extrapolation from the pH titration data for the right and left resonances presented in Figure 8A,B. The estimated values obtained in this manner are 0.75 ppm (3.65–2.90 ppm) for egg white RBP and 0.68 ppm (3.80–3.12 ppm) for egg yolk RBP. The extended range of the chemical shifts for the holoproteins of about 0.2 ppm as compared to that for the isolated phosphopeptides may result from greater differences in the local environment of the phosphoserine residues when present in intact proteins. It can also be due to the differences in the conformation of the phosphate-containing region in the phosphopeptide and in the protein. Alternatively, the difference could be accounted for by the existence of other phosphorylation sites on the proteins, outside of the isolated major phosphopeptide.

Discussion

Since we found that covalently bound phosphate residues are importnt for the uptake of RBP into developing oocytes and possibly for recognition by specific receptors on oocyte plasma membranes, it was of interest to determine where these residues are attached to the protein. Furthermore, since removal of a single phosphate residue from yolk RBP decreases its uptake into oocytes dramatically, and since egg white and egg yolk RBPs differ in phosphate content by less than one residue, it is important to know whether there is a unique site which is phosphorylated on one protein but not the other.

It is apparent from our results that all of the phosphate on RBP occurs as phosphoserine residues in a localized highly anionic region of the peptide chain. Within a 21 amino acid segment[2] are found 8 phosphoserine residues as well as 6 glutamate residues (although some of these may occur as glutamine). One lysine and two histidine residues are the only basic residues in the isolated phosphopeptide. Since this is a tryptic peptide, lysine must occur at the C-terminal position. In addition, it is known that one of the histidine residues is at the N-terminal position (V. Chowdhry, R. Kutny, M. S. Miller, and H. B. White, unpublished results). Between these basic amino acids is a sequence of about 19 amino acids, of which up to 14 carry one or two negative charges at physiological pH.

Although such a highly anionic peptide is unusual, runs of phosphoserine residues have been found in other proteins. Bovine β-casein contains a segment of eight amino acids, four of which are phosphoserine and three of which are glutamate (Mercier et al., 1971). Phosvitin, the major egg yolk phosphoprotein, contains many blocks of six to eight phosphoserine residues (Shainkin & Perlmann, 1971). Because of charge repulsion, the conformation of the RBP phosphopeptide would be expected to be a rigid loop or rod, with little or no ordered secondary structure. This type of structure has been proposed for phosvitin on the basis of both chiroptical and hydrodynamic measurements [for a review, see Taborsky (1974)]. The circular dichroism spectrum of yolk RBP phosphopeptide has a very strong negative Cotton effect at 197 nm which is typical of an aperiodic peptide structure (M. S. Miller, unpublished results).

We found no evidence to support the possibility of a unique phosphorylation site on yolk RBP. Both egg white and egg yolk RBPs contain a single phosphopeptide with identical amino acid composition. The ^{31}P NMR spectra of the phosphopeptide isolated from both of these sources confirm the identity of the two peptides. A probably explanation for the difference in phosphate content of egg white and egg yolk RBP is phosphate heterogeneity within the single phosphopeptide. Yolk RBP phosphopeptide was isolated in two forms containing either seven or eight phosphate residues per peptide molecule. A small difference in the ratio of these forms could result in the observed difference in phosphate contents between the two proteins. What is more remarkable than any small differences in the degree of phosphorylation is the striking similarity between the two proteins. Although both proteins are coded by the same gene (Winter et al., 1967), they are made in different tissues and serve different functions (Froehlich et al., 1980; Blum, 1967; Mandeles & Ducay, 1962; Board & Fuller, 1974). There is no reason to assume that their posttranslational modifications should be identical. Indeed, it has been well established that the carbohydrate moieties of egg white and egg yolk RBPs differ extensively (Miller et al., 1982b). However, although the RBP-synthesizing cells of liver and oviduct may contain different glycosyl transferases, the protein kinases within these cells which phosphorylate RBP apparently have identical specificities.

Similarities in the distribution of phosphate residues in egg yolk and egg white RBP combined with the importance of phosphate residues in the deposition of yolk RBP are puzzling. Egg white RBP never enters the circulatory system and thus would seem to have no need for a receptor recognition marker. It is possible that receptors for this marker would be present in the embryo.

Some information about the microenvironment in which the phosphate residues occur in RBP is provided by the ^{31}P NMR studies of the native proteins. These studies show that there is no change in the immediate environment of the phosphate residues of either egg white or egg yolk RBP when these proteins bind riboflavin. It has been known for some time that dephosphorylation of egg white RBP (Rhodes et al., 1959) of egg yolk RBP (Miller et al., 1982a) has no effect on the binding of riboflavin to the protein. In addition, circular dichroism studies have shown that there is no significant change in the secondary structure of the protein upon dephosphorylation (Miller et al., 1982a). However, the circular dichroism spectra of the apo- and holoproteins contain differences which indicate a conformational change upon binding ligand (Zak et al., 1972). Thus, the phosphates are clearly not only uninvolved in riboflavin binding but also remain unperturbed by the conformational changes accompanying binding. The phosphopeptide seems to function autonomously

[2] The phosphopeptide has been assigned a monoisotopic mass of 3206.0 ± 0.3 amu by positive- and negative-ion FAB mass spectrometry. The same peptide with phosphate removed enzymatically was assigned a monoisotopic mass of 2566.0 ± 0.3 amu by positive-ion FAB mass spectrometry (NSF regional mass spectrometry facilities at The Johns Hopkins University, unpublished results). These masses confirm the presence of eight phosphates but exceed the compositional mass by about 247 amu, indicating that there may be additional amino acids or that there are additional modifications of the peptide.

from the rest of the protein. The biological significance of the functional independence of the phosphopeptide is that there should be little difference in the receptor recognition of apo- and holo-RBP. Although under normal nutritional conditions egg yolk RBP is almost entirely saturated with its ligand, during riboflavin deprivation a large proportion of yolk RBP might be found as the apoprotein due to lack of receptor discrimination.

The titration behavior of the phosphates in both egg white and egg yolk RBP is similar to the titration of the phosphates in β-casein (Humphrey & Jolley, 1982). The pK values for the major peaks in egg white and egg yolk RBPs (6.70 and 6.59, respectively) compare favorably with the pK values determined for β-casein which ranged from 6.4 to 6.9. The pK values of the RBP phosphates are considerably higher than that of phosphoserine, which occurs at 5.75 (Vogel, 1983). This increase in pK can be attributed to the presence of a nearby glutamic acid and other phosphoserine residues, in analogy to the phosphopeptide of β-casein (Humphrey & Jolley, 1982). Since the ^{31}P NMR spectra of the isolated egg white and egg yolk RBP phosphopeptides are identical, the origin of the slight differences in the native protein spectra is obscure.

The fact that phosphate residues on RBP are localized rather than dispersed implies some special function. Earlier experiments (Miller et al., 1982a) have shown that removal of these phosphates drastically reduced uptake of RBP by oocytes. This suggests that the phosphate residues are involved in receptor recognition either directly or indirectly, directly by being recognized by a receptor or indirectly by orienting the molecule toward a receptor in a potential gradient.

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Registry No. Serine, 56-45-1.

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