

Characterization of Hen Egg White- and Yolk-Riboflavin Binding Proteins and Amino Acid Sequence of Egg White-Riboflavin Binding Protein

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Received for publication, December 17, 1983

White- and Yolk-riboflavin binding proteins were isolated from hen eggs, and characterized as to their chemical properties. White- and Yolk-RBPs had almost same amino acid compositions except for glutamic acid, but their carbohydrate compositions were different from each other.

The complete amino acid sequence of White-RBP was determined by conventional methods. White-RBP comprised 219 amino acid residues, and the amino-terminus was pyroglutamic acid (pyrrolidonecarboxylic acid). Two amino acids, lysine and asparagine, were found at the fourteenth residue from the amino-terminus. Carbohydrate chains were linked to asparagine residues at positions 36 and 147.

Both White- and Yolk-RBPs were phosphorylated. In White-RBP either six or seven of nine serine residues between Ser(185) and Ser(197) were phosphorylated. The amino acid sequences around phosphoserines showed that phosphorylation might occur at a serine residue in one of the following sequences; Ser-X-Glu or Ser-X-Ser(P).

Hen egg white and yolk contain a yellow glycoprophosphoprotein, riboflavin binding protein (RBP). White-RBP (RBP in egg white) was isolated at first by Rhodes *et al.* (1), and Yolk-RBP (RBP in egg yolk) by Ostrowski *et al.* (2) as the proteins carrying riboflavin. RBPs bind 1 mol of ribo-

flavin reversibly, and release the vitamin below pH 4.0. The role of RBP is not definitely known, but considered to be provision of vitamin B₂ for embryos.

Yolk-RBP is synthesized in the liver, transported *via* the blood to the ovary and deposited in the yolk fluid. On the other hand, White-RBP is synthesized in the oviduct. Structural investigation of these RBPs is important for the elucidation of the mechanism of the uptake of yolk proteins from the blood into the developing oocyte. Therefore, we purified White- and Yolk-RBPs from hen eggs and characterized them as to their chemical properties, and furthermore, determined the amino acid sequence of White-RBP.

Abbreviations: RBP, riboflavin binding protein; SDS, sodium dodecyl sulfate; R-Cm-, reduced and S-carboxymethylated; HPLC, high performance liquid chromatography; TFA, trifluoroacetic acid; TPCK, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone; TLCK, 1-chloro-3-tosylamido-7-amino-L-2-heptanone; PTH, phenylthiohydantoin; Ser(P), O-phosphoserine; Thr(P), O-phosphothreonine.

MATERIALS AND METHODS

Materials—In this study fresh white leghorn eggs were used. TPCK-trypsin was purchased from Worthington Biochem. Corp. and TLCK-chymotrypsin was from Sigma. Acid carboxypeptidase was from the Protein Research Foundation, Minoh, Osaka. *Staphylococcus aureus* V8 protease was from Miles Laboratories. Pyroglutamyl aminopeptidase [EC 3.4.11.8] (3) was kindly donated by Prof. Tsuru (Faculty of Pharmaceutical Sciences, Nagasaki University). 4 N Methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole was from Pierce. Sephacryl S-200 was from Pharmacia Fine Chemicals. Phosphoserine and phosphothreonine were from Sigma.

Purification of Riboflavin Binding Proteins from Hen Eggs—White-RBP was purified from hen egg white by the procedure of Hidaka and Matsuoka (4) as follows. Egg white (5.1 liters) was homogenized and its pH adjusted to 5.5 with 1 M HCl. The precipitates were removed by centrifugation. Preswollen DEAE-Sephadex A-25 (20 g) with water was directly added to the supernatant. Then the DEAE-Sephadex A-25 was packed into a column and washed successively with 0.05 M acetate buffer, pH 5.5, containing 0.1 M and 0.15 M NaCl. Yellow RBP adsorbed to the column was eluted with the same buffer containing 0.5 M NaCl and purified by gel-filtration on Sephadex G-100 (2.5 × 133 cm).

Isolation of water soluble proteins from egg yolk was performed according to the method of Kawabata *et al.* (5). Egg yolk (2.2 liters) was mixed with 3 volumes of 0.03 M citrate buffer, pH 5.4, containing 0.04 M NaCl and centrifuged to remove egg granules. The supernatant was dialyzed against water. Precipitates appearing during dialysis were removed by centrifugation. Yolk-RBP in the supernatant was purified with DEAE-Sephadex A-25 and by gel-filtration on Sephadex G-100 as described above.

The yield of White-RBP was 2.5 g from 130 eggs, and that of Yolk-RBP was 700 mg. White- and Yolk-RBPs thus obtained gave single bands on disc gel electrophoresis, respectively (Fig. 1).

Polyacrylamide Gel Electrophoresis—Polyacrylamide gel electrophoresis was performed as described by Williams and Reisfeld (6). Electro-

phoresis was carried out using 7% acrylamide gel at pH 8.0 at a current of 3 mA per gel. The gels were stained with 0.25% Coomassie brilliant blue R-250 and destained with 7% acetic acid.

Molecular Weight Determination—Molecular weight was estimated by SDS polyacrylamide gel electrophoresis according to the method of King and Laemmli (7), using 12.6% acrylamide gel. Bovine serum albumin (M.W. 66,000), ovalbumin (M.W. 44,000), soybean trypsin inhibitor (Kunitz) (M.W. 20,400), and hen egg white lysozyme (M.W. 14,500) were used as the reference proteins.

Amino Acid Analysis—Proteins or peptides were hydrolyzed in evacuated, sealed test tubes with twice-distilled HCl or 4 N methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole (8) for 24, 48, and 72 h at 110°C. The amino acid analysis was performed with a Hitachi amino acid analyzer (model 835). Cystine was determined as pyridylethylcysteine after reduction and S-pyridylethylation.

Sugar Analysis—Analysis was performed by the method of Mega and Ikenaka (9). Methanolysis was performed at 90°C for 4 h with 1.5 M methanolic HCl in a sealed test tube. Glucosamine content was determined with a Hitachi amino acid analyzer (model 835) after hydrolysis in an evacuated, sealed test tube with 4 M HCl at 100°C for 6 h.

Determination of the Number of N-Glycosidic Carbohydrate Chains—The number of N-glycosidic carbohydrate chains per molecule was determined by the method of Mega and Ikenaka (9). The 24 h methanolysates were analyzed with an amino acid analyzer (Hitachi KLA-3B) equipped with a column (1 × 15 cm) of Hitachi 2613 resin.

Cleavage with Cyanogen Bromide—White-RBP was dissolved in 70% formic acid, and cleaved with cyanogen bromide (100-fold molar amounts of methionines) at 4°C for 48 h. The reaction mixture was diluted with distilled water and lyophilized (10).

Reduction and S-Carboxymethylation—The material was dissolved in a solution (pH 8.1) containing 6 M guanidine-HCl, 2 mM EDTA, and 0.5 M Tris-HCl, and then reduced with 2-mercaptoethanol (100-fold of half-cystines) at 37°C under nitrogen. After 4 h, iodoacetic acid (an equimolar amount to 2-mercaptoethanol) dissolved in 4 M NaOH was added to the reaction solution,

the pH was adjusted to 8 with 4 M NaOH (11), and the reaction mixture was subjected to gel-filtration.

Separation of Peptides—Peptides obtained by chemical cleavage and enzymatic digestions were separated by gel-filtration, high voltage paper electrophoresis and high performance liquid chromatography (HPLC). Paper electrophoresis was carried out on Whatman No. 1 paper at pH 3.6 (pyridine : acetic acid : water, 1 : 10 : 289, v/v/v) (12) at a voltage of 2,000 V for 2 h. HPLC was carried out with a Hitachi model 638 apparatus using a column (0.4 × 30 cm) packed with C-18 reversed phase gel, TSK LS-410, at a flow rate of 1 ml/min. Chromatography was performed with a 60 min linear gradient using two buffer systems; A, a primary solvent of 0.05% TFA and a secondary solvent of 2-propanol-acetonitrile-0.05% TFA (42 : 18 : 40, v/v/v); B, a primary solvent of 0.01 M ammonium acetate and a secondary solvent of acetonitrile (50%) in 0.01 M ammonium acetate. Peptides were monitored at 220 and 280 nm.

Enzymatic Digestion—Tryptic digestion was performed with TPCK-trypsin in 0.05 M ammonium bicarbonate, pH 8.0, for 4 h at 37°C. The substrate to enzyme ratio was 100 : 1 (w/w).

Digestion with chymotrypsin was performed in 0.05 M ammonium bicarbonate, pH 8.0, with TLCK-chymotrypsin (substrate : enzyme ratio of 200 : 1 (mol/mol)), for 2 h at 37°C.

Digestion with *Staphylococcus* V8 protease (13) was performed in 0.05 M ammonium bicarbonate, pH 8.0, for 24 h at 37°C at a substrate : enzyme ratio of 25 : 1 (mol/mol).

Digestion with acid carboxypeptidase was carried out as follows. Twenty nmol of a peptide was incubated with 0.5 µg of acid carboxypeptidase (14) in 150 µl of 0.05 M sodium acetate, pH 3.7, containing 0.1% Triton X-100 at 37°C. Aliquots of the digestion mixture were taken at intervals, and applied to an amino acid analyzer (Hitachi model 835).

Digestion with pyroglutamyl aminopeptidase of the amino-terminal peptide (30 nmol) was performed with 0.005 units of the enzyme for 16 h at 37°C in 0.05 M sodium phosphate, pH 8.0, containing 5 mM 2-mercaptoethanol and 5 mM EDTA (3).

Sequence Analysis—Manual Edman degrada-

tion was carried out by the method of Iwanaga *et al.* (15). PTH-derivatives of amino acids were identified by HPLC (16).

Analysis of Phosphoserine—Phosphoserine was released by the hydrolysis of proteins or peptides in evacuated, sealed test tubes with twice-distilled HCl at 100°C for 3 h. Identification of phosphoserine was performed by high voltage paper electrophoresis with standards, under the same conditions as described in *Separation of Peptides*. Spots corresponding to phosphoserine or threonine were extracted with water. The extracts were hydrolyzed with 4 N methanesulfonic acid at 110°C for 24 h, and the released amino acids were analyzed with the amino acid analyzer.

Conversion of phosphoserine to β-methylamino alanine (DL-α-amino-β-methylaminopropionic acid) was performed by incubation of peptides in 0.1 M NaOH containing 1 M methylamine at 37°C overnight (17).

RESULTS

Molecular Weights, and Amino Acid and Carbohydrate Compositions of White- and Yolk-RBPs—The molecular weights of the proteins were estimated by SDS polyacrylamide gel electrophoresis using reference proteins. The polyacrylamide gel electrophoretogram of White- and Yolk-RBPs showed that the molecular weights of both RBPs were about 34,000 (Fig. 1). As shown in Table I, White- and Yolk-RBPs have similar amino acid compositions, but White-RBP apparently contains more glutamic acids than Yolk-RBP. The carbohydrate compositions of White- and Yolk-RBPs are remarkably different. High contents of Man and GlcNAc and low contents of Gal and sialic acid in White-RBP suggest that the carbohydrate moieties have the hen egg white ovomucoid type structure. On the other hand, Yolk-RBP might have the complex type structure. The results of analyses of the number of N-glycosidic carbohydrate chains indicate that White- and Yolk-RBPs contain two N-glycosidic carbohydrate chains per molecule, respectively.

Isolation of Cyanogen Bromide Peptides of White-RBP—Since White-RBP contained seven methionine residues, the protein was oxidized with CNBr. The CNBr degradation products were reduced, S-carboxymethylated and fractionated on

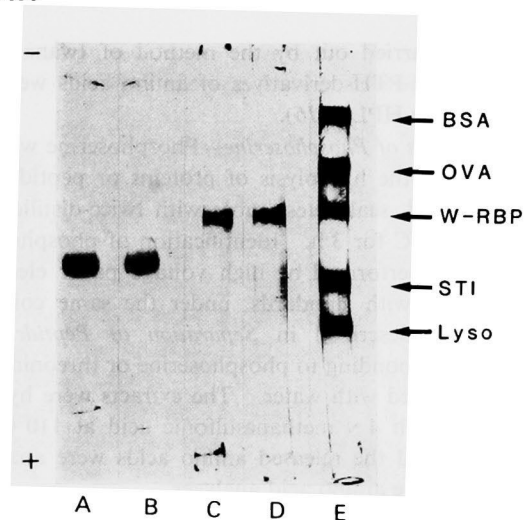


Fig. 1. Polyacrylamide gel electrophoresis of RBPs. The conditions are given in the text. Slots A and B, without SDS; slots C, D, and E, with SDS; A and C, White-RBP; B and D, Yolk-RBP; E, White-RBP (W-RBP) and standard proteins (bovine serum albumin (BSA), ovalbumin (OVA), soybean trypsin inhibitor (Kunitz) (STI), hen egg white lysozyme (Lyso)).

a Sephacryl S-200 column (2×200 cm) equilibrated with 0.05 M ammonium bicarbonate buffer, pH 8.5 (Fig. 2S). Six fractions (A to F) were obtained. The large peak behind fraction F was salts and reagents. Peptides eluted between fractions A and B were incomplete CNBr degradation products, and their yields were very low. The fractions (A to F) were further purified by HPLC (Fig. 3S). Nine peptides, A, B, C, D-1, D-2a, D-2b, E, F-1, and F-2, were obtained. Peptides D-2a and D-2b were separated by rechromatography by HPLC with solvent system B. Peptides F-1 and F-2 were separated by paper electrophoresis at pH 3.6.

The amino acid compositions of the CNBr peptides are shown in Table IIS. The amino acid compositions of D-2a and D-2b were identical except for aspartic acid and lysine. It is thought that these two peptides are located at the same position in the protein. The sum of the amino acid compositions of CNBr peptides was in accord with that of White-RBP. Peptides A and D-1 were glycopeptides.

The Sequences of CNBr Peptides—The sequences of the CNBr peptides were determined by the manual Edman method and acid carboxypep-

TABLE I. Amino acid and sugar compositions of RBPs. The numbers of residues in parentheses are from the established sequence. Values for serine and threonine are extrapolated to zero time of hydrolysis. Values for valine and isoleucine are those of 72 h hydrolysis. Cystine was determined as pyridylethylcysteine.

Amino acids & sugars	White-RBP	Yolk-RBP
Aspartic acid	18.6 (19.5)	18.6
Threonine	6.1 (7)	6.5
Serine	30.0 (30)	28.0
Glutamic acid	34.0 (35)	27.9
Proline	8.3 (8)	8.3
Glycine	6.9 (7)	6.2
Alanine	12.6 (13)	11.6
1/2 Cystine	17.1 (18)	15.4
Valine	5.1 (5)	5.1
Methionine	6.2 (7)	6.1
Isoleucine	6.6 (7)	6.6
Leucine	13.0 (13)	11.0
Tyrosine	8.6 (9)	8.5
Phenylalanine	6.0 (6)	5.0
Lysine	15.4 (15.5)	14.0
Histidine	7.7 (8)	7.6
Tryptophan	5.3 (6)	5.6
Arginine	4.9 (5)	4.8
Mannose	6.0	6.0
Galactose	1.3	4.6
Glucosamine	11.2	8.1
Sialic acid	0.7	4.5
Total of amino acids	219	

tidase digestion. The results are summarized in Fig. 4S. To confirm the sequences of peptides B, D-2a, D-2b, and E, these peptides were digested with TPCK-trypsin or V8 protease, and the peptides in the digests were separated by HPLC (data not shown). Their amino acid compositions completely supported the sequences. The carboxyl-terminal residue of peptide B was Glu. This showed that peptide B was the carboxyl-terminal peptide of White-RBP.

The amino-terminal PTH-amino acids were not obtained from peptides D-2a and D-2b by

the manual Edman method. This suggests that the amino-terminal residues of peptides D-2a and D-2b may be blocked. Assuming the presence of a pyroglutamyl residue at the amino-terminus, the peptides were digested with pyroglutamyl aminopeptidase. The removal of amino-terminal pyroglutamyl residues made it possible to carry out Edman degradation of the peptides. The sequences of D-2a and D-2b were the same except for the fourteenth residue, Lys in D-2a and Asn in D-2b. These results show that both D-2a and D-2b are located at the amino-terminal position with a replacement at the fourteenth amino acid. The amino-terminal amino acid sequence of White-RBP was further confirmed by Edman degradation of R-Cm-White-RBP after pyroglutamyl aminopeptidase digestion.

The Sequence of CNBr Peptide A—Since CNBr peptide A was composed of 123 residues, it was further digested with TPCK-trypsin. Tryptic peptides of peptide A were separated by HPLC (Fig. 5S). Thirteen peptides, AT-a to m, were obtained. Peptides AT-e, AT-f, AT-g, and AT-

h were purified by rechromatography by HPLC with solvent system B. The amino acid compositions of these tryptic peptides are listed in Table IIIS.

The sequences of these peptides were determined by the manual Edman method and digestion with acid carboxypeptidase. The results are summarized in Fig. 6S. To confirm the sequences, the peptides were further digested with TPCK-trypsin, TLCK-chymotrypsin, and/or V8 protease, if necessary. The results of these enzymic cleavages support the sequences.

Free lysine was found in the tryptic digest of peptide A and the sequence of AT-j was identical with that of AT-i except for the lack of the amino-terminal lysine residue. These results indicate that AT-i and AT-j are located at the same position.

The sequence of AT-k was identical with the amino-terminal sequence of peptide A, so AT-k was the amino-terminal peptide of peptide A.

The amino acid composition of AT-l was identical with the total of those of AT-b and AT-m, and the amino-terminal sequence of AT-l was



Fig. 11. The complete amino acid sequence of White-RBP. <Glu represents pyroglutamyl residue; CHO, carbohydrate chain; Ser, phosphoserine; Ser, indefinite phosphoserine.



identical with that of AT-m. Based on these results, AT-m and AT-b were arranged in this order.

In order to determine the alignment of the tryptic peptides of peptide A, the peptide was digested with V8 protease, and the peptides were separated by HPLC (Fig. 7S). Thirteen peptides, AV-a to m, were obtained. Peptide AV-l and AV-m were purified by rechromatography with solvent system B. The amino acid compositions of these V8 peptides are shown in Table IVS. The amino-terminal sequences of some peptides were determined by the manual Edman method.

Since the amino-terminal sequences and the compositions of AV-i and AV-j were the same, both peptides are the same peptides but separated into two peaks. These peptides contain tryptophan residues. It was sometimes observed that a peptide containing tryptophan residue(s) was separated into two peaks on HPLC. Peptide AV-h was probably formed by the non-specific cleavage at the Ser(29)-Asn(30) bond of peptide A.

The amino acid composition of AV-k was identical with the total of those of AV-l and AV-c, and the amino-terminal sequence of AV-k was identical with that of AT-l. Therefore, peptides AV-l and AV-c were arranged in this order.

Taking into account the amino acid compositions and amino-terminal sequences of these V8 peptides, the tryptic peptides of CNBr peptide A were arranged as shown in Fig. 8S.

The Complete Amino Acid Sequence of White-RBP—In order to complete alignment of the CNBr peptides, R-Cm-White-RBP was digested with TPCK-trypsin. The peptides were separated by HPLC with solvent system B (Fig. 9S). Five methionine containing peptides, T-a to e, were isolated. They were purified by rechromatography with solvent system A. The amino acid compositions of these peptides are shown in Table VS. T-e is a mixture of two peptides in which a Lys-Asn replacement occurred. The amino-terminal sequences of these tryptic peptides gave the alignment of the CNBr peptides, as shown in Fig. 10S. Consequently, the complete amino acid sequence of White-RBP was determined to be as shown in Fig. 11.

Detection of Phosphoserine—An anionic amino acid having more acidity than aspartic acid was detected when the 3 h acid hydrolysates of White- and Yolk-RBPs were electrophoresed on paper.

The mobility of the acidic amino acid was identical with that of phosphoserine (Fig. 12). After complete hydrolysis of the acidic amino acid, only serine was detected. These results show that both White- and Yolk-RBPs contain phosphoserine and not phosphothreonine.

Detection of phosphoserine in the CNBr peptides of White-RBP indicated that CNBr peptides B and C contained phosphoserine. The abnormally low serine contents in CNBr peptides B and C (Table IIS), and tryptic peptide T-a (Table VS) reflect the low recovery (about 70–75%) of serine from phosphoserine by acid hydrolysis (18). For quantitative analysis of phosphoserines in peptides B and C, the peptides were treated with 0.1 M

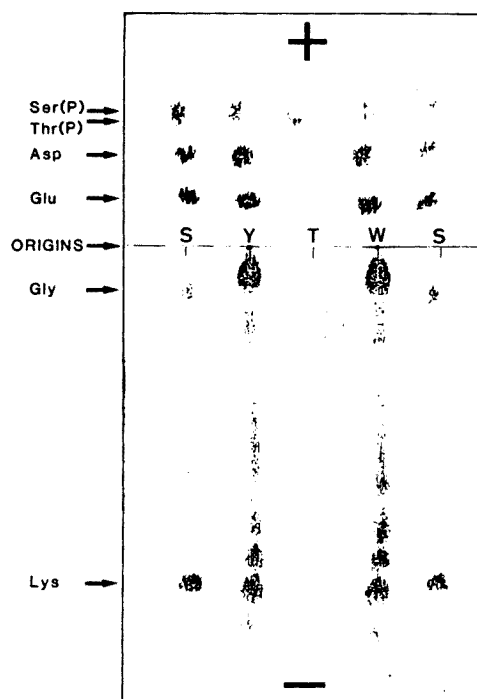


Fig. 12. High voltage paper electrophoresis of the hydrolysates of White- and Yolk-RBPs. The proteins were hydrolyzed in evacuated, sealed test tubes with twice-distilled HCl at 100°C for 3 h. Electrophoresis was carried out on Whatman No. 1 paper at pH 3.6 (pyridine : acetic acid : water, 1 : 10 : 289, v/v/v) at a voltage of 2,000 V for 2 h. The arrows indicate the origins and the positions of the amino acids. S, a mixture of phosphoserine, Asp, Glu, Gly, and Lys; T, phosphothreonine; W, hydrolysate of White-RBP; Y, hydrolysate of Yolk-RBP.

NaOH containing 1 M methylamine as described in "MATERIALS AND METHODS," to convert phosphoserines into β -methylamino alanines (DL- α -amino- β -methylaminopropionic acids). Then the peptides were hydrolyzed and analyzed with an amino acid analyzer (Table VI). *S*-Carboxymethylcysteines in the peptides were also completely converted into β -methylamino alanines on the treatment, moreover, β -methylamino alanine was eluted between histidine and ammonia, overlapping these peaks, with the present amino acid analysis system. As the exact determination of β -methylamino alanine was impossible, phosphoserine contents were estimated from the decrease in the amount of serine with the conversion. Non-phosphorylated serine does not change with

the conversion. It was found from the decreased amounts of serine that about three or four of six serines in peptide C were phosphorylated, and all three serines in peptide B were phosphorylated. The exact phosphate binding site in peptide C could not be elucidated, since both serine and phosphoserine did not give their PTH-derivatives on manual Edman degradation.

DISCUSSION

Both White- and Yolk-RBPs isolated from hen eggs were glycoproteins. The carbohydrate contents of both RBPs were about 15%, but their carbohydrate compositions were different from each other (Table I). The values agreed with the reported values (5, 19-21). It was suggested from the carbohydrate compositions of RBPs that White-RBP has ovomucoid type sugar chains and Yolk-RBP complex type sugar chains. The number of carbohydrate chains in White- and Yolk-RBPs was estimated to be two chains by the method of Mega and Ikenaka (9). In the present study, the molecular weights of the RBPs were determined to be 34,000 by SDS polyacrylamide gel-electrophoresis. This value is similar to the reported values (22-24). But the molecular weight of White-RBP calculated from the amino acid, carbohydrate and phosphoric acid compositions was 29,200. This observation is consistent with the fact that the molecular weights of glycoproteins estimated by SDS polyacrylamide gel electrophoresis are usually overestimated.

White-RBP comprised 219 amino acid residues, and had a pyroglutamyl residue as the amino-terminus. The presence of this type of blocked terminal amino acid had not been observed in other egg white proteins. Both lysine and asparagine were detected at the fourteenth residue from the amino-terminus, indicating the presence of a microheterogeneity in the peptide moiety. Carbohydrate chains were linked to Asn(36) and Asn(147). The amino acid sequences followed by these asparagine residues satisfied the general rule for the *N*-glycosidic carbohydrate chain attaching site, Asn(CHO)-X-Ser/Thr. White-RBP had a peculiar carboxyl-terminal sequence, Gln-Gln-Glu-Glu-Gly-Glu-Glu. The amino acid compositions of White- and Yolk-RBPs were almost the same, except for the contents of glutamic

TABLE VI. Amino acid compositions of two phosphopeptides before and after the conversion of phosphoserines to β -methylamino alanines.^a

Amino acid	Peptide B ^b		Peptide C	
	Before	After	Before	After
Cm-Cys	1.0 (1)	0.0		
Asp				
Thr				
Ser	2.4 (3)	0.26	4.4 (6)	1.63
Hse			0.5 (1)	0.7
Glu	9.8 (10)	9.9	2.9 (3)	3.0
Pro				
Gly	1.0 (1)	1.1		
Ala	2.0 (2)	2.0	1.0 (1)	1.0
Val			0.9 (1)	0.9
Ile			0.8 (1)	0.8
Leu	3.0 (3)	3.0	1.9 (2)	1.9
Tyr				
Phe	1.0 (1)	1.0		
Lys	2.6 (3)	2.8	0.8 (1)	0.9
His	0.9 (1)	0.8	0.9 (1)	0.7
Trp				
Arg				
β -Methylamino alanine	—	+	—	+

^a The numbers in parentheses are from the established sequence. ^b The conversion was performed after acetylation of amino-terminus by acetic anhydride.

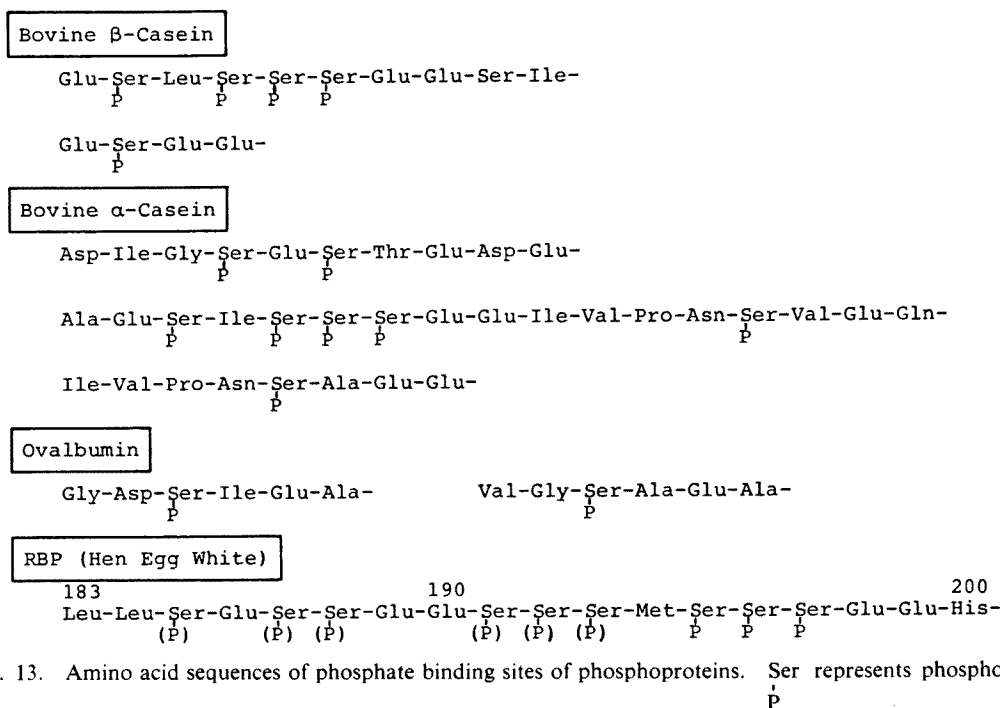


Fig. 13. Amino acid sequences of phosphate binding sites of phosphoproteins. Ser represents phosphoserine; P

Ser, indefinite phosphoserine.

(P)

acid (Table I). The low glutamic acid content in Yolk-RBP may suggest that Yolk-RBP lacks such a glutamic acid group in the carboxyl-terminus.

The presence of phosphate in White- and Yolk-RBPs had been found by Rhodes *et al.* (22), and by Zak and Ostrowski (25), respectively. In the present study, these phosphates were found to be attached to six or seven serine residues located at the carboxyl-terminal region as shown in Fig. 13. Three serine residues, Ser(195), Ser(196), and Ser(197), are phosphorylated. But the other six serines, Ser(185) to Ser(193), seem to be partially phosphorylated, and the exact phosphate binding sites were not determined. In Figs. 11 and 13, the phosphates of indefinite phosphoserines are shown in parentheses. Figure 13 also shows the amino acid sequences around phosphoserine residues of phosphoproteins, bovine α - and β -caseins (26, 27), and ovalbumin (28), whose amino acid sequences were determined. There is a common characteristic structure in the amino acid sequence around the phosphate bound serine, that is, Ser-X-Glu or Ser-X-Ser(P). In the case of White-RBP, the three established phosphate binding sites,

Ser(195), Ser(196), and Ser(197), had the same characteristic structure. Serine residues between Ser(185) and Ser(193) except Ser(192) do not conflict with the characteristic structure. This indicated that RBP kinase may have the same specificity as those of casein and ovalbumin kinases. And it is speculated that serines in the Ser-X-Glu sequence, for example, Ser(196) and Ser(197), are first phosphorylated and then other serines in the Ser-X-Ser(P) sequence, for example, Ser(195), become phosphorylated by acquisition of the structure that can possibly interact with the kinase. There are other serines in the Ser-X-Glu sequence, Ser(186) and Ser(129), but these serines were not phosphorylated. These serines may be buried in the molecule and the kinase can't approach them. The function of phosphates in RBPs is unknown. In our laboratory, the exact phosphate binding site and the structures of hen egg Yolk- and Plasma-RBPs are being investigated for comparison with those of White-RBP and to determine the mechanism of the selective uptake of Plasma-RBP into the yolk.

The authors are grateful to Prof. D. Tsuru, Nagasaki University, Faculty of Pharmaceutical Sciences, for the kind gift of pyroglutamyl aminopeptidase.

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Supplemental
MaterialsTable IIS. Amino acid compositions of CNBr peptides of White-RBP^a.

Amino acid	A	B	C	D-1	D-2a	D-2b	E	F-1	F-2	Total	W-RBP
Cm-Cys	12.9(13)	1.0(1)			1.0(1)	1.0(1)	1.9(2)	0.9(1)		18	18
Asp	12.6(13)			1.9(2)	2.1(2)	2.7(3)			2.0(2)	19.5 ^b	19.5
Thr	4.8(5)			0.9(1)	0.9(1)	0.9(1)				7	7
Ser	14.0(15)	2.4(3) ^c	4.4(6) ^c		0.9(1)	0.9(1)	3.7(4)	1.1(1)		30	30
Hse	0.7(1)		0.5(1)	0.9(1)	0.4(1)	0.3(1)	0.8(1)	0.9(1)	1.0(1)	7	7
Glu	13.9(14)	9.8(10)	2.9(3)		3.5(4)	3.4(4)	2.8(3)	1.1(1)		35	35
Pro	4.7(5)				3.0(3)	2.9(3)				8	8
Gly	2.3(2)	1.0(1)		1.2(1)	2.1(2)	2.0(2)	1.1(1)			7	7
Ala	8.0(8)	2.0(2)	1.0(1)	1.0(1)	1.0(1)	1.0(1)				13	13
Val	2.8(3)		0.9(1)				1.0(1)			5	5
Ile	4.1(6)		0.8(1)							7	7
Leu	5.1(5)	3.0(3)	1.9(2)		1.1(1)	1.0(1)	2.0(2)			13	13
Tyr	6.3(7)			1.0(1)	1.1(1)	1.0(1)				9	9
Phe	3.8(4)	1.0(1)					1.0(1)			6	6
Lys	5.3(7)	2.6(3)	0.8(1)		1.7(2)	1.1(1)	1.1(1)	1.5(2)		15.5 ^b	15.5
His	4.5(5)	0.9(1)	0.9(1)		1.0(1)	1.0(1)				8	8
Trp	4.7(5)						1.0(1)			6	6
Arg	4.6(5)									5	5
Sugar	+			+						++	++
Total	123	25	17	7	21	21	17	4	5	219	219
Yield(%)	91	40	45	55	31	33	53	28	37		

a, The numbers of residues of White-RBP and those in parentheses are from the established sequences.

b, Amino acid replacements in peptides D-2a and D-2b were considered in the calculation.

c, The low value estimated reflects the low recovery of serine on the hydrolysis of phosphoserine.

Table IIIS. Amino acid compositions of tryptic peptides of CNBr peptide A^a.

Amino acid AT-a	b	c	d	e	f	g	h	i	j	k	l	m	Total ^b	A	
Cm-Cys	1.2(1)	1.0(1)	1.0(1)	1.0(1)	1.0(1)			1.1(1)	1.1(1)	2.8(3)	5.0(5)	4.1(4)	13	13	
Asp	2.0(2)			1.0(1)		1.1(1)	1.8(2)			1.2(1)	7.4(8)	5.7(6)	13	13	
Thr					0.9(1)					1.7(2)	1.9(2)	1.9(2)	5	5	
Ser	0.9(1)	1.0(1)	1.0(1)	1.0(1)	0.9(1)		1.1(1)			3.3(4)	3.7(4)	2.9(3)	15	15	
Hse					0.7(1)								1	1	
Glu	2.0(2)		1.0(1)	1.0(1)	0.9(1)			1.1(1)	1.1(1)	3.9(4)	5.9(6)	4.3(4)	14	14	
Pro		1.0(1)			1.0(1)	1.1(1)				1.3(1)	1.2(1)	1.0(1)	5	5	
Gly	1.0(1)		1.0(1)								0.9(1)		2	2	
Ala		2.0(2)								2.0(2)	4.0(4)	4.0(4)	8	8	
Val					0.9(1)		0.8(1)				1.0(1)	1.0(1)	3	3	
Ile						1.1(1)		0.9(1)	1.0(1)	1.0(2) ^c	1.9(2)	1.8(2)	6	6	
Leu			1.0(1)							1.8(2)	2.0(2)	2.0(2)	5	5	
Tyr					1.0(1)		0.8(1)	1.1(1)	1.0(1)	1.8(2)	2.1(2)	2.0(2)	7	7	
Phe					1.0(1)			1.0(1)	1.0(1)	0.8(1)	1.0(1)	1.0(1)	4	4	
Lys	1.0(1)	1.0(1)		1.0(1)	1.0(1)			1.0(1)		1.0(1)	2.0(2)	1.1(1)	7	7	
His		1.0(1)	1.0(1)							1.8(2)	1.9(2)	1.1(1)	5	5	
Trp						1.0(1)	0.9(1)				2.6(3)	2.6(3)	5	5	
Arg			1.0(1)			1.0(1)	1.0(1)	1.0(1)	1.0(1)		1.0(1)	1.0(1)	5	5	
Sugar										+			+	+	
Total	2	9	7	6	7	7	5	8	7	6	27	47	38	123	123
Yield(%)	83	33	90	79	83	76	76	76	52	48	86	47	38		

a, The numbers of residues of CNBr peptide A and those in parentheses are from the established sequences.

b, The amino acid compositions of AT-j and l are not included.

c, The low value reflects the incomplete hydrolysis of the Ile-Ile bond.

Table IVS. Amino acid compositions of V8 protease peptides of CNBr peptide A^a.

Amino acid	AV-a	b	c	d	e	f	g	h	i	j	k	l	m	Total ^b	A
Cm-Cys				2.1(2)		1.1(1)	2.0(2)	1.8(2)	1.9(2)	2.2(2)	1.8(2)	2.0(2)	4.0(4)	13	13
Asp			1.1(1)	1.2(1)	1.1(1)		1.1(1)	1.7(2)	2.0(2)	2.4(2)	4.8(5)	3.8(4)	2.9(3)	13	13
Thr					1.0(1)	0.9(1)	1.0(1)				1.0(1)	1.0(1)	0.9(1)	5	5
Ser		0.9(1)				1.0(1)	2.0(2)	2.8(3)	4.5(5)	4.5(5)	0.9(1)	0.9(1)	2.8(3)	15	15
Hse				0.7(1)										1	1
Glu	1.0(1)	1.0(1)	1.0(1)	1.1(1)	1.0(1)	1.0(1)	1.3(1)	2.0(2)	2.9(3)	3.1(3)	2.1(2)	1.1(1)	3.0(3)	14	14
Pro				0.8(1)					1.3(1)	1.1(1)			3.2(3)	5	5
Gly		1.0(1)						1.1(1)	1.0(1)	0.9(1)				2	2
Ala							1.0(1)		1.2(1)	1.1(1)	2.3(2)	2.3(2)	4.5(4)	8	8
Val				1.0(1)					1.1(1)	1.0(1)			1.0(1)	3	3
Ile					0.9(1)				1.0(2) ^c	1.1(2) ^c	0.9(1)	0.9(1)	2.0(2)	6	6
Leu						0.9(1)		1.0(1)	2.0(2)	2.0(2)	1.0(1)	1.0(1)	1.0(1)	5	5
Tyr			1.0(1)			0.9(1)	1.0(1)	1.0(1)	1.0(1)	1.1(1)			3.0(3)	7	7
Phe					0.9(1)		0.7(1)						2.0(2)	4	4
Lys				2.1(2)	2.0(2)				1.0(1)	1.9(2)	2.2(2)	1.1(1)	1.0(1)	7	7
His	1.0(1)			1.0(1)					1.0(1)	1.0(1)	1.0(1)	1.0(1)	1.0(1)	5	5
Trp								0.9(1)	1.0(1)	0.8(1)	1.7(2)	1.7(2)	1.8(2)	5	5
Arg			0.9(1)					1.0(1)	0.9(1)	1.0(1)	1.0(1)		3.1(3)	5	5
Sugar							+							+	+
Total	2	3	3	13	7	6	10	15	26	26	20	17	36	123	123
Yield(%)	76	86	45	76	84	95	85	12	45	18	39	46	86		

a, The numbers of residues of CNBr peptide A and those in parentheses are from the established sequences.

b, The amino acid compositions of AV-h, j and k are not included.

c, The low value reflects the incomplete hydrolysis of the Ile-Ile bond.

Table VS. Amino acid compositions of methionine containing tryptic peptides of R-Cm-White-RBP ^a .					
Amino acid	T-a	T-b	T-c	T-d	T-e ^b
Cm-Cys	1.2(1)		2.2(2)	2.0(2)	3.2(3)
Asp		1.1(1)	1.0(1)	2.0(2)	2.5(2.5)
Thr				0.9(1)	1.8(2)
Ser	6.9(9) ^c		2.7(3)	2.6(3)	4.4(5)
Glu	5.9(6)		2.0(2)	3.0(3)	5.0(5)
Pro				0.9(1)	3.6(4)
Gly				1.8(2)	
Ala	1.0(1)	1.0(1)		1.1(1)	2.9(3)
Val		1.1(1)	1.0(1)		
Met	0.7(1)	0.9(1)	0.8(1)	2.1(3)	0.7(1)
Ile		1.0(1)			1.2(2) ^d
Leu	2.0(2)		2.0(2)		2.0(2)
Tyr				1.9(2)	2.0(2)
Phe				0.8(1)	0.9(1)
Lys	1.0(1)	1.0(1)	1.0(1)	1.0(1)	1.5(1.5)
His	1.8(2)				1.8(2)
Trp				0.9(1)	
Arg					
Sugar				+	+
Total	23	6	13	24	36
Yield(%)	65	93	73	95	93

- a, The numbers in parentheses are from the established sequences.
b, T-e is a mixture of two peptides in which one amino acid replacement occurred.
c, The low value estimated reflects the low recovery of serine on the hydrolysis of phosphoserine.
d, The low value reflects the incomplete hydrolysis of the Ile-Ile bond.



Fig.2S. Gel-filtration of R-Cm-White-RBP degraded by CNBr. A Sephacryl S-200 column (2.0 X 200 cm) was equilibrated with 0.05M ammonium bicarbonate buffer, pH 8.5. Fractions of 3 ml were collected. Peptides were monitored at 220 nm (—) and 280 nm (-----).

Fig.3S. Separation of fractions A to F in Fig.2S by HPLC. A TSK-Gel LS 410 column, 5μ, 0.4 X 30 cm, was used. Chromatography was performed with a 60 min linear gradient at a flow rate of 1 ml/min using solvent system A, a primary solvent of 0.05% TPA and a secondary solvent of 2-propanol - acetonitrile - 0.05% TPA (42:18:40,V/V/V). Peptides were monitored at 220 and 280 nm.

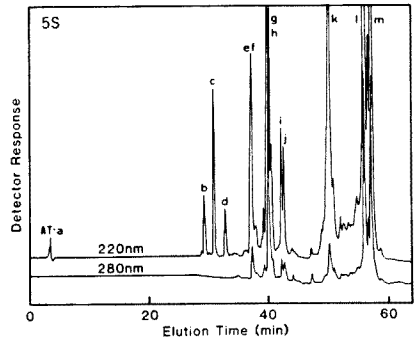
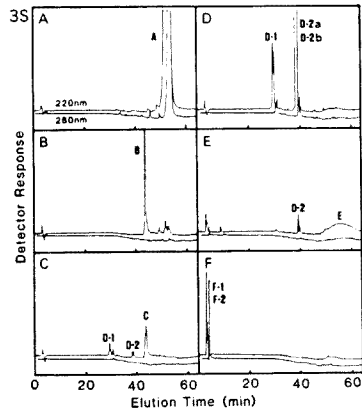
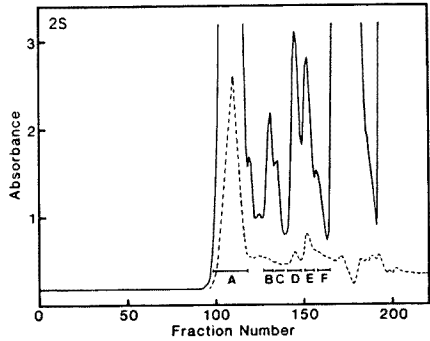


Fig.4S. The amino acid sequences of CNBr peptides of White-RBP. Prefixes T and V show tryptic peptides and V8 protease peptides, respectively. CMC represents carboxymethyl-cysteine; <Glu, pyrroglutamyl residue; CHO, carbohydrate chain. —>, manual Edman degradation; <—, acid carboxypeptidase digestion.

Fig.5S. Separation of tryptic peptides of CNBr peptide A by HPLC. Chromatographic conditions were the same as in Fig.3S.

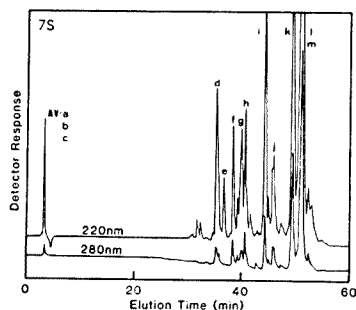
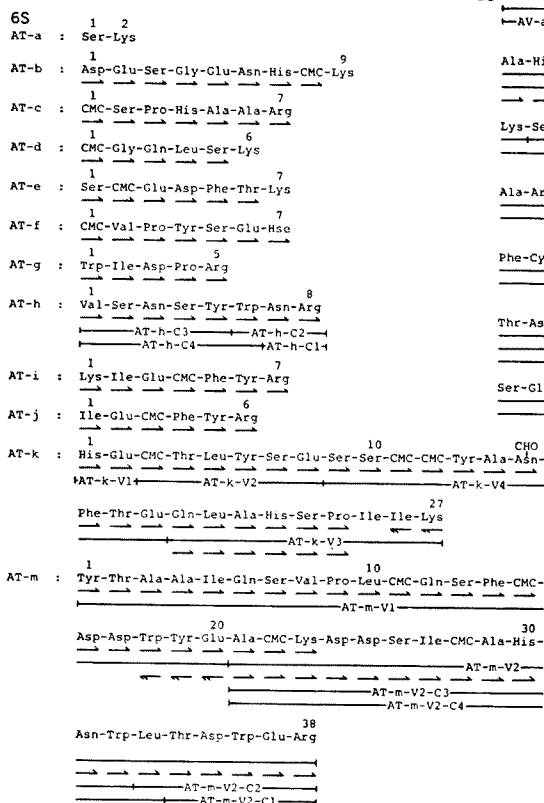


Fig. 6S. The amino acid sequences of tryptic peptides of CNBr peptide A. Prefixes C and V show chymotryptic peptides and V8 protease peptides, respectively. $\longrightarrow$, manual Edman degradation; $\longleftarrow$, acid carboxypeptidase digestion.

Fig. 7S. Separation of V8 protease digests of CNBr peptide A by HPLC. Chromatographic conditions were as in Fig. 3S.

Fig. 8S. Summary of the results of sequence analyses of CNBr peptide A. CHO represents carbohydrate chain. $\longrightarrow$, manual Edman degradation. Cys was identified as carboxymethylcysteine.

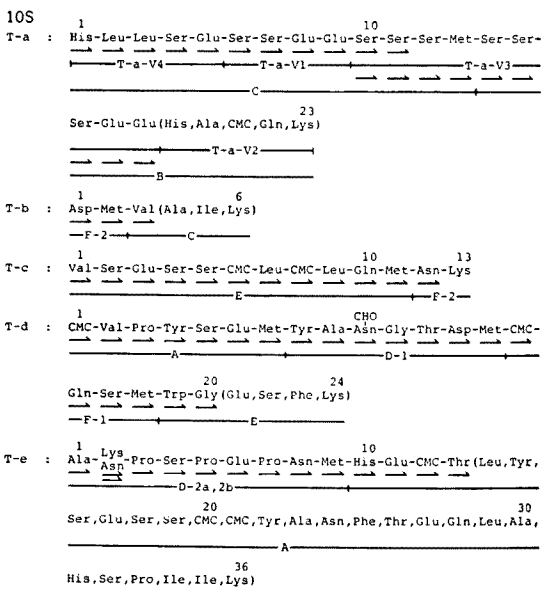
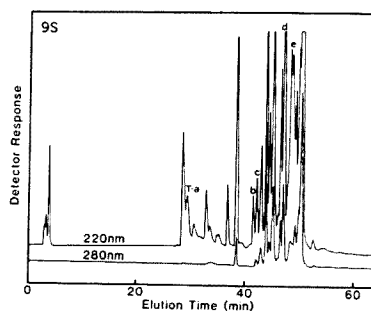
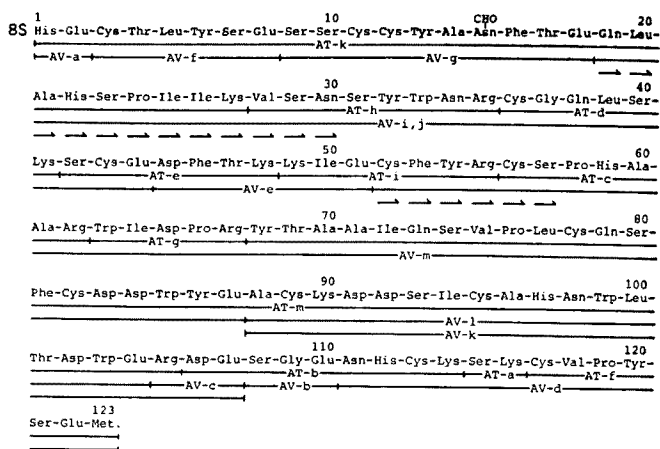


Fig. 9S. Separation of tryptic peptides of R-Cm-White-RBP by HPLC. Chromatography was performed using solvent system B, a primary solvent of 0.01M ammonium acetate and a secondary solvent of acetonitrile (50%) in 0.01M ammonium acetate. Other conditions were the same as in Fig. 3S.

Fig. 10S. The amino acid sequences of methionine containing tryptic peptides of R-Cm-White-RBP. Prefix V shows V8 protease peptides. $\longrightarrow$, manual Edman degradation.