Vol. 147, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 31, 1987 Pages 115-119

PHOSPHORYLATION HETEROGENEITY OF TRYPTIC PHOSPHOPEPTIDES OF CHICKEN RIBOFLAVIN-BINDING PROTEIN

Valerie L. Vaughn*, Rong Wang+, Catherine Fenselau+, and Harold B. White III+,[1]

*Department of Chemistry and Biochemistry,
University of Delaware,
Newark, Delaware 19716

+Department of Pharmacology and Experimental Therapeutics,
Johns Hopkins School of Medicine,
Baltimore, MD 21205

Received July 7, 1987

SUMMARY. The tryptic phosphopeptide of hen egg white riboflavin-binding protein has been found to exist as a mixture of peptides which differ only with respect to the number of covalently bound phosphoryl groups. Anion-exchange chromatography was used to separate homologues of the tryptic phosphopeptide of egg white riboflavin-binding protein. Four peptide peaks were obtained and analyzed using plasma desorption mass spectrometry. Molecular ions obtained agree closely with calculated molecular weight values for phosphopeptides with 8, 7 and 5 phosphoryl groups. Amino acid analyses showed that the octa- and hepta-phosphorylated peptides were pure and had the same amino acid compositions.

In laying hens, riboflavin is transported to the oocyte by a specific phosphoglycoprotein called riboflavin-binding protein (1). This protein has a molecular weight of 30 KDaltons and binds one molecule of riboflavin with a dissociation constant of 1.3 nM (2).

Three forms of riboflavin-binding protein are found in laying hens: serum, yolk and white riboflavin-binding proteins (3). Serum riboflavin-binding protein is synthesized in the liver, transported to the oocyte and deposited (1). Thereafter, this form is referred to as yolk riboflavin-binding protein. Egg white riboflavin-binding protein is synthesized in the oviduct and secreted to become part of the albumen (4). Riboflavin-binding proteins synthesized in the liver and in the oviduct are all products of the same gene (5) and structurally similar (3).

The mechanism of transport across the oocyte membrane is not yet known but appears to require phosphorylation of riboflavin-binding protein. If potato acid phosphatase is used to remove phosphate, the dephosphorylated riboflavin-binding protein is indistinguishable from the native protein by most criteria yet, the efficiency of this transport decreases dramatically with increasing removal of phosphoryl residues (6).

[1]To whom correspondence should be addressed.

0006-291X/87 $1.50

The phosphorylation of riboflavin-binding protein is interesting for reasons other than its role in transport of the vitamin-protein complex. First, phosphorylation occurs on eight of nine serine residues within a fifteen residue sequence that also contains five glutamic acid residues. The numerous serine residues elsewhere in the protein are not phosphorylated. Next, previous research in these laboratories suggested, but did not document, that riboflavin-binding protein exists as several forms which all possess the same amino acid backbone yet differ with respect to the number of phosphorylated serine residues (7). We refer to this as "phosphorylation heterogeneity."

In this paper, we demonstrate that the tryptic peptide of egg white riboflavin-binding protein, which contains the phosphorylated region, is heterogeneously phosphorylated. The phosphorylated region of egg white riboflavin-binding protein exists as several forms - all with the same amino acid backbone but differing with respect to numbers of covalently bound phosphate. Such phosphorylation heterogeneity has been demonstrated previously for bovine α_{S2}-casein (8) and human β-casein (9).

MATERIALS AND METHODS

Riboflavin-binding protein was purified from hen egg white by the method of Miller and White (10). Riboflavin-binding protein was reduced and alkylated (11) and tryptic peptides prepared from 500 mg of protein (12). The phosphopeptides were purified and separated using anion-exchange chromatography (7). Collected fractions were monitored at 220 nm and the phosphopeptides located using a spectrophotometric phosphate assay (13).

Solutions, containing approximately one nanomole of peptide in distilled, deionized water, were made for each of the samples and hydrolyzed for 21 h in 6 N HCl. Amino acid analyses were performed using a Pico Tag Amino Acid Analysis System (Waters). A solution of 2.0 nanomole of the octaphosphorylated phosphopeptide in distilled, deionized water was sequenced using an Applied Biosystems Protein Sequencer (Model 470A).

For mass spectral analysis each phosphopeptide isolated was desalted and converted to the ammonium salt via size exclusion chromatography (Sephadex G-15, Pharmacia) using 2.5% $NH_4(CO_3)_2$ as the eluant. Mass spectra were measured on a BIO-ION Nordic (Uppsala, Sweden), BIN-10K time of flight mass spectrometer fitted with a RSX-11M-PLUS data system. Positive ion spectra were recorded with an accelerating potential at 18 kV during 2,000,000 to 9,000,000 primary events. H^+ and Na^+ were used for calibration. Spectra were measured with resolution of 1 nsec/channel.

RESULTS

Anion-exchange chromatography of the tryptic peptides of egg white riboflavin-binding protein resulted in four peptide peaks, two of which contained significant amounts of phosphate (Figure 1). After pooling and conversion to the ammonium salt, peaks A - D were submitted for mass spectral analysis. Molecular ions were determined for each peak fraction using plasma desorption mass spectrometry (Table 1). The molecular ions obtained agree

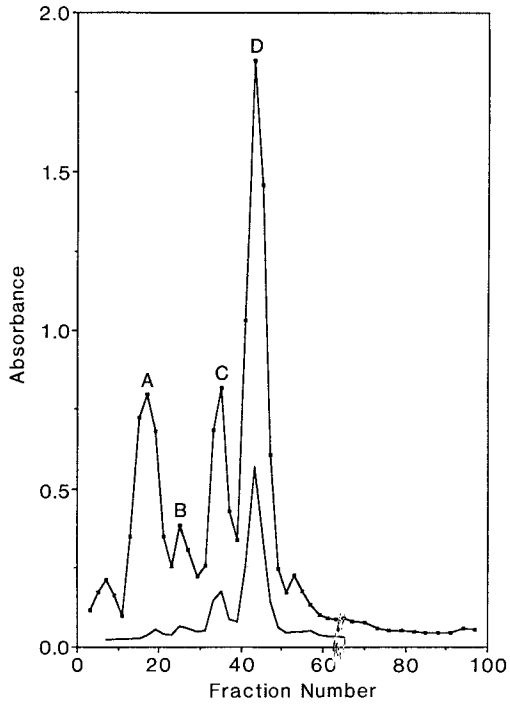

Figure 1. Phosphopeptide purification for mass spectral analysis. The partially purified phosphopeptides from egg white riboflavin-binding protein were applied to a DEAE-cellulose column (1.27 x 12.0 cm) in 0.1 M NaOAc, pH 3.5 and eluted with a linear NaCl gradient (0.1 M - 0.4 M). The column was monitored at 220 nm (—•—) and 50-µl aliquots were analyzed for phosphate (820 nm, ——). The phosphopeptides were labeled A, B, C, and D to simplify identification.

closely with calculated molecular weights of phosphopeptides with the same amino acid composition but different numbers of covalently bound phosphate. Phosphopeptides with 8, 7, and 5 phosphorylated serine residues are indicated.

Amino acid composition data was obtained on homogeneous peptide fractions C and D to confirm that the phosphopeptides had the same amino acid composition and differed only by the number of phosphates. The compositions

Table 1. Plasma Desorption Mass Spectrometry Analysis of wRBP Phosphopeptides

chromatography peak	$(M + H)^+$	number of phosphorylated serine residues	calculated MW of phosphopeptide
A	2967.5	5	2965.8
B	3127.5	7	3125.8
C	3125.4	7	3125.8
	3210.7	8	3205.7
D	3206.8	8	3205.7

Table 2. Amino Acid Analysis of wRBP Phosphopeptides[a]

amino acid	Peak C (ratio, Glu = 6)	Peak D (ratio, Glu = 6)
serine	7.46 (9)[b]	7.26 (9)[b]
glutamic acid	6.00 (6)	6.00 (6)
histidine	1.63 (2)	1.95 (2)
leucine	1.79 (2)	1.98 (2)
methionine	.88 (1)	.83 (1)
alanine	1.41 (1)	1.02 (1)
lysine	.92 (1)	.93 (1)

[a] The tryptic phosphopeptide of wRBP has an amino acid composition of Ser_9, Glu_5, His_2, Leu_2, Met, Ala, Cys, Gln, Lys. [b] The numbers in parentheses are the nearest integer value for each of the components. Serine was corrected for 20% loss during hydrolysis before assigning the integer value.

agree with the known amino acid backbone of the phosphorylated tryptic peptide of riboflavin-binding protein (Table 2). Edman degradation analysis proceeded through the fifth amino acid of the octaphosphorylated peptide (Peak D) and yielded the expected N-terminal sequence - His-Leu-Leu-Ser-Glu. The amino acid analysis data obtained for peaks A and B indicated that these samples contained peptide mixtures.

DISCUSSION

Here, we have shown that egg white riboflavin-binding protein is heterogeneously phosphorylated as was first suspected for the protein isolated from egg yolk. ^{31}P NMR spectra of egg white and egg yolk riboflavin-binding protein are identical (7). The combination of these two observations indicate that the process of phosphorylation is the same in different tissues.

Clearly the tryptic phosphopeptide of egg white riboflavin-binding protein exists as both the hepta- and octa-phosphorylated species. Peak A yielded a minor component with a molecular ion which corresponds to the pentaphosphorylated peptide. Because peak A is a mixture of peptides, confirmation of this identification by amino acid composition was not possible. Nevertheless, it seems likely that this represents a third homologue of the phosphopeptide. Components with six or nine phosphoryl groups have not been detected.

Mass spectrometry is an extremely sensitive method capable of detecting trace components in samples. Therefore, the presence of both the hepta- and octa-phosphopeptides under peak C is probably due to the incomplete separation of the components in the pooled fractions. The octaphosphopeptide is present only in trace quantities, thus, peak C is primarily the heptaphosphorylated peptide.

The existence of the heptaphosphorylated phosphopeptide in both peaks B and C is not so easily explained. One possibility is that the heptaphosphorylated peptide exists as a mixture of structural isomers with different symmetries of charge distribution which results in separation of the isomers as they are eluted from the anion-exchange column. Alternatively, incomplete separation again allowed the molecular ion of a trace component to be observed.

The identity of the octaphosphorylated species was confirmed by automated Edman analysis. Sequencing progressed through ser 185 which is not phosphorylated but stopped five residues from the N-terminus at ser 187 which is phosphorylated (14, 15). The acidic nature of the tryptic phosphopeptide allows it to be easily isolated but the phosphorylated serine residues prevent it from being sequenced by conventional methods.

We have established that riboflavin-binding protein exists as a mixture of forms which differ with respect to the number of covalently bound phosphoryl groups. An additional level of heterogeneity may also exist in which there are different distributions of phosphate within a phosphorylation class, e.g. structural isomers. Currently, we are attempting to resolve such isomers and determine their sites of phosphorylation.

ACKNOWLEDGEMENTS

We thank Surrendra Batra for the amino acid analysis and peptide sequencing and The Mid-Atlantic Regional Mass Spectrometry Facility. This work was supported in part by NIH Grant AM 27873.

REFERENCES

1. Blum, J. C. (1967) Le Metabolisme de la Riboflavine chez la Poule Pondeuse, Documentation Roche.
2. Becvar, J. E., and Palmer, G. (1982) J. Biol. Chem. **257**, 5607-5617.
3. White, H. B., III (1987) J. Exptl. Zool. Supple. 1, 53-63.
4. Mandeles, S., and Ducay, E. D. (1962) J. Biol. Chem. **237**, 3196-3199.
5. Winter, W. P., Buss, E. G., Clagett, C. O., and Boucher, R. V. (1967) Comp. Biochem. Physiol. **22**, 897-906.
6. Miller, M. S., Benore-Parsons, M., and White, H. B., III (1982) J. Biol. Chem. **257**, 6818-6824.
7. Miller, M. S., Mas, M. T., and White, H. B., III (1984) Biochemistry **23**, 569-576.
8. Brignon, G., Ribadeau-Dumas, B., and Mercier, J.-C. (1976) FEBS Letters **71**, 111-116.
9. Greenberg, R., Groves, M. L., and Dower, H. J. (1984) J. Biol. Chem. **259**, 5132-5138.
10. Miller, M. S., and White, H. B., III (1986) Meth. Enz. **122**, 227-234.
11. Huang, T.-S., and DeLange, R. J. (1971) J. Biol. Chem. **246**, 686-697.
12. Smyth, D. G. (1969) Meth. Enzymol. **11**, 214-231.
13. Ames, B. N. (1966) Meth. Enzymol. **8**, 115-118.
14. Fenselau, C., Heller, D. N., Miller, M. S. and White, H. B., III (1985) Anal. Biochem. **150**, 309-314.
15. Mega, T., Hamazume, Y., Nong, Y.-M., and Ikenaka, T. (1986) J. Biochem. (Tokyo) **100**, 1109-1116.