POST-TRANSLATIONAL PHOSPHORYLATION OF PHOSVITIN

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1. Introduction

The yolk protein, phosvitin, is synthesized in the liver of laying hens and, after estradiol injection, also in rooster liver. It is secreted into the blood as a complex with lipovitellin [1–3]. More than half of the amino acids of phosvitin are serines, which are practically all present as phosphate esters. Phosvitin-phosphate comprises at least 75% of the protein phosphate in the complex with lipovitellin [1].

While the phosphorylation of other proteins is generally assumed to occur after translation [4] some authors have suggested a specific phosphoserine-tRNA as a possible intermediate in the synthesis of phosvitin [5, 6]. We have studied in what phase of the synthesis of phosvitin the serines are phosphorylated: before translation, at the nascent chain, or after completion of the peptide chain. For this purpose we have measured the incorporation of labelled leucine and phosphate *in vitro* (slices) and *in vivo*, and studied the effect of cycloheximide. Our results show that the phosphorylation occurs mainly after the release of the peptide chain from the polysomes, probably during transport to the blood.

2. Materials and methods

2.1. *Materials*

Estradiol was a gift from Organon, Oss, The Netherlands. Cycloheximide was a gift from "De Koninklijke Nederlandse Gist- en Spiritusfabriek", Delft, The Netherlands. Carrier-free [^{32}P]orthophosphate in dilute HCl was obtained from Philips-Duphar, Petten, The Netherlands. It was neutralized before use. L-[4,5-^{3}H]leucine (1 mCi/ml; 52 Ci/mmole) was obtained from the Radiochemical Centre, Amersham, Great Britain. Eagle's Minimum Essential Medium (MEM) with Earle's salts, fetal bovine serum and antibiotics (penicillin/streptomycin, 5000 I.U. of each/ml) were obtained from Flow Laboratories Ltd., Irvine, Great Britain.

2.2. *Animals and injections*

The roosters were 4–12 weeks old hybrids of White Leghorn roosters and Rhode Island Red hens, obtained from the Spelderholt Institute for Poultry Research, Beekbergen, The Netherlands. The animals were fed ad libitum.

Estradiol was dissolved in propylene glycol (25 mg/ml) and injected subcutaneously (1 ml/kg body weight). Cycloheximide was also dissolved in propylene glycol (1 mg/ml) and injected intravenously (1 ml/kg body weight). Radiochemicals were injected intravenously.

2.3. *Incubation of liver slices*

100 ml Eagle's Minimum Essential Medium was supplemented with 10 ml fetal bovine serum and 2 ml antibiotic solution.

Roosters were killed 48 hr after an injection of estradiol. The livers were removed and cut into 1 mm slices. 0.5 g portions were incubated in 5 ml of the medium at 37° with gentle shaking in an atmosphere of O_2–CO_2 (95:5). After 15 min preincubation the medium was replaced by fresh medium which contained L-[4,5-^{3}H]leucine and in one experiment $^{32}P_i$ (see Results, fig. 1). In other experiments $^{32}P_i$ was added 20 min after [^{3}H]leucine. When cycloheximide was used, it was added 15 min after [^{3}H]leucine. After different intervals the incubation was stopped by placing the vessels in ice.

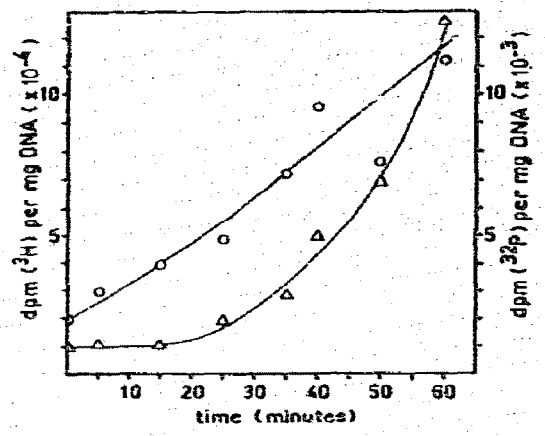

Fig. 1. Incorporation of [^{3}H]leucine (△—△—△) and ^{32}P (○—○—○) in hot-acid precipitable material, secreted into the medium by liver slices. To the incubation mixture 15 μCi [^{3}H]leucine and 50 μCi $^{32}P_i$ were added. For details see sect. 2.3 and 2.4.

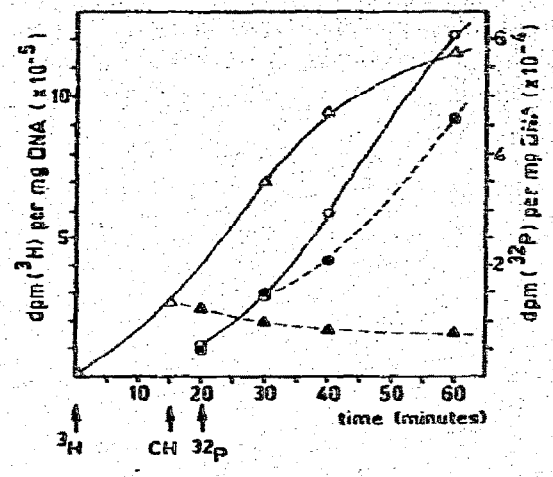

Fig. 2. The effect of cycloheximide on the incorporation of [^{3}H]leucine and ^{32}P in hot-acid precipitable material in liver slices. 15 μCi [^{3}H]leucine was added at time zero and 20 min later 50 μCi $^{32}P_i$. 5 min before the ^{32}P 2 mg cycloheximide was added. For details see sect. 2.3 and 2.4. △—△—△, ^{3}H control; ▲-▲-▲, ^{3}H with cycloheximide; ○—○—○, ^{32}P control; ●-●-●, ^{32}P with cycloheximide.

2.4. *Measurement of radioactivity in total proteins and the phosvitin–lipovitellin complex*

The medium and slices were separated by centrifugation of the cooled incubation mixture (15 min; 2000 *g*; 2–4°). The slices (0.5 g) were homogenized in 10 ml 0.9% NaCl. 2-ml samples of this homogenate and 2-ml samples of the medium were mixed with an equal volume 1 M $HClO_4$, containing 0.02 M leucine and 0.1 M NaH_2PO_4. The precipitate was washed three times with 2 ml 0.5 M $HClO_4$ (containing leucine and phosphate) and then heated for 1 hr at 80° in 4 ml 0.5 M $HClO_4$. DNA was determined in the supernatant [7]. The precipitate was washed with 3 ml diethylether–ethanol–chloroform (2:2:1, v/v/v). The dried residue was dissolved in 1.3 ml Soluene-100 (Packard) and 50 μl water. 10 ml toluene with PPO (4 g/l) and *p*-bis-(*O*-Methylstyryl)-benzene (80 mg/l) were added and the radioactivity was measured in a liquid scintillation counter (Nuclear Chicago Mark I).

Labelled phosvitin–lipovitellin complex was isolated from the media after addition of carrier. To 2 ml medium 1 ml plasma of a rooster, estrogenized 5 days before [2, 3], was added. The complex was isolated by DEAE-cellulose chromatography as described by Bergink et al. [3]. The recovery was calculated by determining the acid-insoluble non-lipid phosphorus [3]. After addition of 0.1 ml carrier plasma the complex was precipitated by adding $HClO_4$ (containing leucine and phosphate) to 0.5 M. The radioactivity in the hot-acid precipitable material was determined as described above.

To an aliquot of the complex fractions 0.1 ml carrier plasma was added and the alkali-labile phosphate (mainly from phosvitin) was isolated according to Beuving and Gruber [2]. Isobutanol–xylene (65:35, v/v) was used instead of isobutanol–benzene. Radioactivity was measured by Cerenkov radiation.

All data are expressed as dpm/mg DNA.

2.5. *Labelling in vivo*

70 hr after estradiol injection two roosters (each 1450 g body weight) received an injection of 1.5 mCi [^{3}H]leucine. At the same time one rooster received cycloheximide while the control was injected with propylene glycol. 5 min later 5 mCi $^{32}P_i$ was administered to the animals. After different intervals blood samples were taken from the wing vein. Phosvitin–lipovitellin complex was isolated from 1 ml plasma and treated as described under sect. 2.4.

3. Results and discussion

Liver slices incorporate [^{3}H]leucine and [^{32}P]phosphate into protein. Fig. 1 shows a typical example of

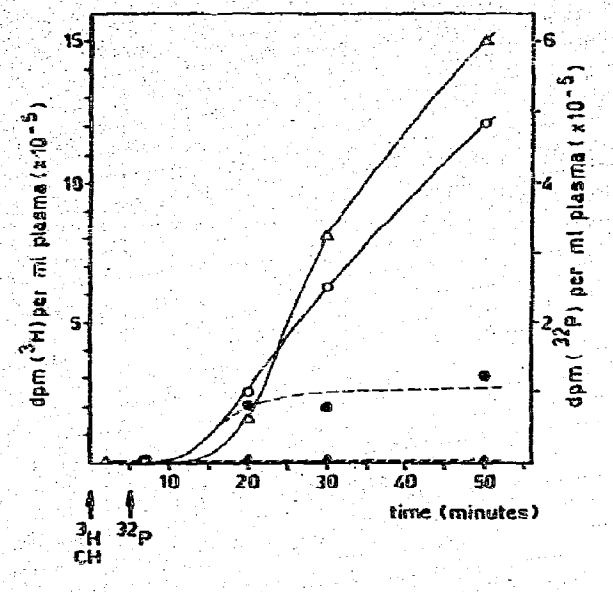

Fig. 3. The effect of cycloheximide on the incorporation *in vivo* of [^{3}H]leucine and ^{32}P in phosvitin–lipovitellin complex secreted into the blood. ^{32}P was determined after isolation of the alkali-labile phosphate. [^{3}H]leucine and cycloheximide were administered at time zero, $^{32}P_i$ at 5 min. For details see sect. 2.4 and 2.5. △—△—△, ^{3}H control; ▲—▲—▲, ^{3}H with cycloheximide; ○—○—○, ^{32}P control; ●-●-●, ^{32}P with cycloheximide.

the labelling of the secreted proteins, when both radioactive precursors are added simultaneously. It is evident that the secreted proteins are labelled earlier with ^{32}P than with [^{3}H]leucine. This difference is not due to a much slower uptake of [^{3}H]leucine since the proteins *inside* the slices contain ^{32}P and ^{3}H in a constant ratio within 5 min (data not shown). The most obvious explanation is to assume that the proteins are phosphorylated after their synthesis.

This conclusion was tested in experiments with cycloheximide. We found that 0.4 mg/ml cycloheximide completely blocks protein synthesis in our system (fig. 2). $^{32}P_i$, given after complete inhibition of protein synthesis, is still incorporated for some time to the same extent as in the control (fig. 2). To make sure that the phosphate was really incorporated into newly synthesized phosvitin we isolated complex from the medium and found that this contained alkali-labile ^{32}P. Per mg tissue DNA 5100 dpm were incorporated 45 min after cycloheximide versus 26500 dpm in the control. Taking into account that in the control synthesis of protein, and therefore supply of phosphorylation substrate, proceeds linearly during the whole period of 60 min one has to conclude that a considerable part of the phosphate enters phosvitin after complete translation.

To establish the validity of our conclusions we studied the incorporation of labelled leucine and phosphate *in vivo*. Fig. 3 shows that secreted phosvitin–lipovitellin complex is phosphorylated at nearly the control level for 20 min after blocking synthesis of the peptide chain. We can therefore conclude that most, if not all, phosphate is incorporated into the phosvitin–lipovitellin complex after peptide chain release, probably during transport to the blood.

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