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NUCLEAR PHOSPHOPROTEINS

II. METABOLISM OF EXOGENOUS PHOSPHOPROTEIN BY INTACT NUCLEI

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SUMMARY

When ^{32}P-labeled phosphoprotein is added to a suspension of calf thymus nuclei, the protein enters the nuclei and a rapid metabolic 'turnover' of the previously incorporated phosphate groups occurs. The only ^{32}P-labeled product which can be detected in these nuclei is inorganic phosphate. The results indicate that the phosphoprotein is probably not involved in the metabolic transfer of phosphate groups from one molecule to another, but favors the hypothesis that the phosphorylation and dephosphorylation of the nuclear phosphoproteins serve the purpose of modifying the structural and functional characteristics of the protein.

INTRODUCTION

In the previous paper[1] the properties of a phosphoprotein fraction derived from calf thymocyte nuclei were described. In view of the data available at this point, this phosphorylated protein could fit into one of two possible general functional categories. On one hand, it might be serving as an intermediate in some type of phosphoryl group transfer reaction, in which case the phosphate groups would be the primary focus of interest. On the other hand, the addition and removal of phosphate groups might serve the sole function of altering the structural and functional characteristics of the protein moiety itself. In this case, changes in the protein would be the main focus of interest, with the phosphate groups merely serving as agents which modify the protein.

In order to distinguish between these two possibilities, it is necessary to determine the metabolic origin and fate of the phosphate groups. It is already known that the phosphoprotein-phosphate groups are derived from the terminal phosphate of various nucleoside and deoxynucleoside triphosphates[1], but it is not known to what substrate, if any, these phosphoprotein-phosphate groups are transferred during phosphate turnover. This question could not be answered by studying the turnover of [^{32}P]phosphoprotein which had been labeled in the intact nucleus, because there is no known way of specifically labeling the phosphoprotein with ^{32}P without also

simultaneously labeling the nucleic acids, phospholipids, and low molecular weight phosphate compounds. However, by employing the purified phosphoprotein fraction which had been previously labeled with ^{32}P and then adding it to fresh unlabeled nuclei, it was possible to specifically follow the subsequent metabolism of the phosphoprotein-phosphate groups.

In the present paper, it will be shown that when ^{32}P-labeled phosphoprotein is prepared and added back to fresh thymus nuclei, the metabolic turnover of the protein phosphate groups is reestablished. The ^{32}P which is turned over from this labeled phosphoprotein is not transferred to any other stable molecule, but rather appears entirely in the form of inorganic phosphate.

METHODS

Preparation of radioactive phosphoprotein

Thymus nuclei were prepared from 100 g fresh calf thymus according to the procedure of ALLFREY, MIRSKY AND OSAWA[2], and were incubated for 30 min as described previously[3]. Isotopic precursors employed were either $Na_2H^{32}PO_4$ (100 mC/mmole; 0.1 mC/ml final concentration) or [3-^{3}H]serine (4.2 C/mmole; 0.1 mC/ml final concentration). After incubation the nuclei were chilled and collected *via* centrifugation at 1000 × *g* for 5 min, and the phosphoprotein fraction prepared[1]. The final preparations were diluted to contain 200 μg/ml phosphoprotein ([^{3}H]serine-labeled phosphoprotein = 10 000 counts/min per ml; ^{32}P-labeled phosphoprotein = 120 000 counts/min per ml).

Incubation of nuclei with radioactive phosphoprotein

To every 9 ml of phosphoprotein solution was added 1 ml of 1.25 M sucrose containing 40 mg/ml NaCl and 90 mg/ml glucose. Fresh thymus nuclei were prepared as described above and 1 ml of nuclei in 0.25 M sucrose–3 mM $CaCl_2$ added to 1 ml of the phosphoprotein mixture. Incubation was carried out at 37° in a shaking waterbath, and the reaction stopped with cold 16% trichloroacetic acid. Alkali-labile ^{32}P was measured as described previously[3]. [^{3}H]serine-labeled protein was dissolved in 0.3 M NaOH and counted in Bray's scintillation solution[4].

Identification of ^{32}P-labeled nuclear components

Low molecular weight phosphate compounds were fractionated on a column of Sephadex G-15 (1.5 cm × 83 cm) and eluted with 0.14 M NaCl. Nuclei were incubated for 1 h with ^{32}P-labeled phosphoprotein, after which the reaction was stopped with cold 16% trichloroacetic acid. The precipitate was removed by centrifugation and the trichloroacetic acid extract neutralized with NaOH. An aliquot was placed on a Sephadex G-15 column which had been calibrated with markers of ATP, glucose 6-phosphate, pyrophosphate, and inorganic phosphate. Total phosphate in the fractions was determined by the procedure of AMES AND DUBIN[5].

RESULTS

Turnover of exogenous phosphoprotein phosphate groups by intact nuclei

When purified radioactive phosphoprotein is added to suspensions of freshly

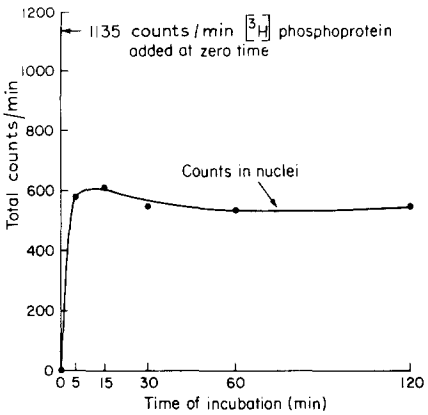

Fig. 1. Time course of uptake of [^{3}H]serine-labeled phosphoprotein by isolated calf thymus nuclei. After incubation with the radioactive phosphoprotein, nuclei were chilled, sedimented, and washed five times with cold incubation medium. The sediment was precipitated with 16% trichloroacetic acid and washed as previously described[3]. The dried protein residue was dissolved in 0.3 M NaOH and counted in Bray's scintillation solution[4].

prepared calf thymus nuclei, the phosphoprotein is readily taken up by the nuclei. The kinetics of uptake of [^{3}H]serine-labeled phosphoprotein are summarized in Fig. 1. Maximum labeling of the nuclei occurs within 5 min, after which the level of radioactive phosphoprotein reaches a steady plateau.

Since the radioactive phosphoprotein was found to be taken up by the nuclei, the next question which arose was whether the phosphate groups of this exogenous phosphoprotein are turned over under these conditions. When ^{32}P-labeled phosphoprotein was prepared and added back to fresh unlabeled nuclei, it was found that a rapid loss of the ^{32}P counts from the phosphoprotein occurred (Fig. 2). The rate of this ^{32}P turnover is of the same order of magnitude as that seen when the phosphoprotein is labeled and subjected to a cold chase directly in the intact nuclei[3]. Furthermore,

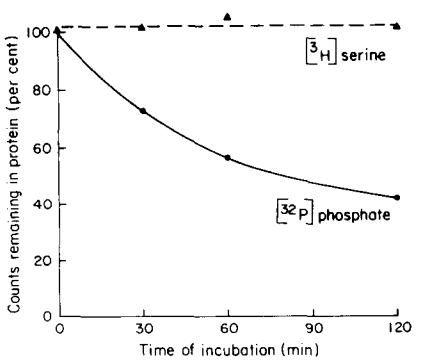

Fig. 2. Metabolic turnover of phosphate groups from ^{32}P-labeled phosphoprotein which has been added to suspensions of isolated calf thymus nuclei. Nuclei were incubated at 37° in the presence of phosphoprotein labeled with either [^{3}H]serine or [^{32}P]phosphate. The retention of label is plotted as a function of time of incubation. Note that [^{3}H]serine is retained while [^{32}P]-phosphate is rapidly lost.

during the rapid turnover of the phosphate groups from the exogenous phosphoprotein, [^{3}H]serine counts in the phosphoprotein were found to be completely stable (Fig. 2). This indicates that the rapid loss of ^{32}P counts from the phosphoprotein is not due to proteolysis and release of phosphorylated peptides.

When ATP synthesis is blocked in these nuclei by the addition of iodoacetate, the turnover of these phosphoprotein-phosphate groups is inhibited (Fig. 3). This

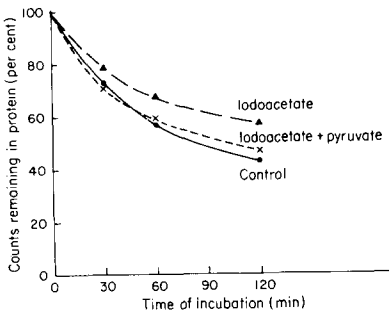

Fig. 3. Effect of iodoacetate (1 mM) on turnover of [^{32}P]phosphate groups from phosphoprotein which has been added back to calf thymus nuclei. Note that iodoacetate causes an inhibition of the turnover reaction which is reversible by the addition of pyruvate (0.02 M).

dependence of the turnover reaction on energy metabolism is similar to that observed when phosphoproteins are labeled directly in the intact nucleus[3], although the degree of inhibition by iodoacetate is considerably less in the present case. In both systems the inhibition caused by iodacetate is reversible by the addition of pyruvate, which bypasses the block in glycolysis and reestablishes ATP formation *via* its direct metabolism in the Kreb's cycle[6].

Metabolic fate of phosphoprotein phosphate

Since the turnover of ^{32}P is reestablished when radioactive phosphoprotein is

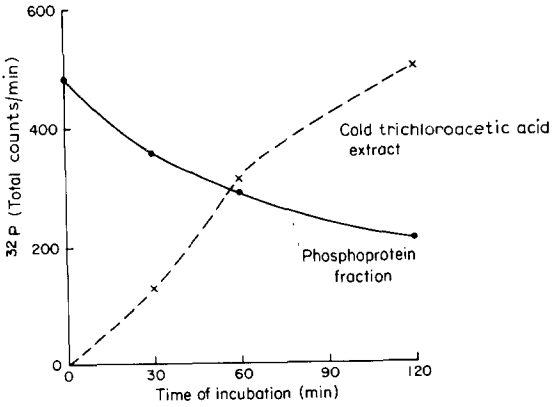

Fig. 4. When ^{32}P-labeled phosphoprotein is added back to a fresh nuclear suspension, the loss of ^{32}P counts from the phosphoprotein fraction coincides with the appearance of counts in a cold trichloroacetic acid extract of the nuclei. The total number of counts in each fraction is plotted as a function of time of incubation.

added back to fresh nuclei, it was possible to determine the fate of these phosphoprotein-phosphate groups by simply examining the various nuclear fractions for the appearance of ^{32}P counts. Of all the fractions examined, the only one in which counts were found to appear was in a cold trichloroacetic acid extract of the nuclei (Fig. 4).

Since this acid-soluble fraction contains most of the low molecular weight phosphate compounds of the nucleus, a Sephadex G-15 column was employed to fractionate the components of the extract. Nuclei were first incubated for one hour in the presence of ^{32}P-labeled phosphoprotein in order to allow turnover of the phosphate

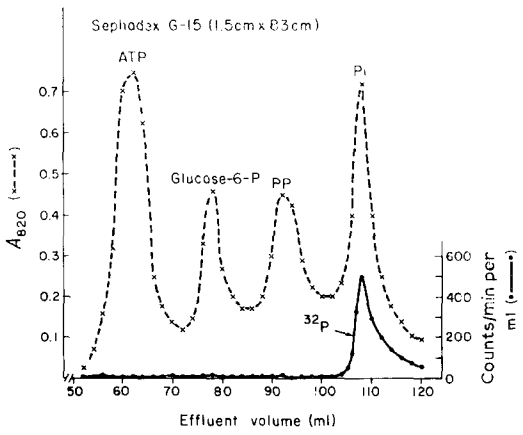

Fig. 5. The cold trichloroacetic acid extract from the experiments summarized in Fig. 4 was analyzed on a column of Sephadex G-15 which had been calibrated with markers of ATP, glucose 6-phosphate, pyrophosphate, and inorganic orthophosphate. Note that all the ^{32}P counts appear in the inorganic phosphate peak.

groups to occur. The nuclei were then extracted with cold trichloroacetic acid and the extract fractionated by gel filtration on Sephadex G-15. The results, summarized in Fig. 5, indicate that all the ^{32}P counts are present in the form of inorganic phosphate.

DISCUSSION

The present experiments indicate that during the turnover of phosphoprotein-phosphate groups, the end product which is detectable is inorganic phosphate. Although it is conceivable that some highly unstable phosphate linkage is formed as an intermediate which would be subsequently destroyed during incubation or the subsequent trichloroacetic acid extraction, this alternative seems unlikely in view of the fact that even the N–P bond, which is very labile in acid solution, would not be completely hydrolyzed under these conditions[7]. The fact that the protein–phosphate bonds do not appear to be of very high phosphoryl group transfer potential[1] also argues against the formation of a highly unstable product.

Since the ^{32}P is apparently released from the phosphoprotein as inorganic phosphate, it does not appear that this phosphoprotein fraction is involved in the metabolic transfer of phosphoryl groups to other acceptors. Thus the net effect of the metabolic reactions in which these phosphoproteins are involved is that the free

energy stored in the terminal phosphate bonds of nucleoside triphosphates is used in the process of phosphorylating the protein; this energy is apparently not recovered in the second step of the reaction in which the phosphate group is released in the relatively low energy state of inorganic phosphate. One possible interpretation of this phenomenon is that the phosphorylation of the protein causes structural and functional changes in the state of the phosphoprotein, and that the energy stored in the terminal pyrophosphate bonds of nucleoside triphosphates is utilized to induce these changes. An example of such a phenomenon is known to occur in the case of phosphorylase and phosphorylase kinase, where the function of the phosphorylation reaction is to induce structural changes leading to the activation of enzyme activity[8]. In the case of the nuclear phosphoproteins, the effect of the structural changes induced by protein phosphorylation might be to modify the structure of the chromatin and the physical interaction between histones and DNA. The rapid changes in phosphorylation of lymphocyte phosphoproteins which occur when resting cells are induced to grow and divide by the addition of phytohemagglutinin[9] lend support to this hypothesis.

ACKNOWLEDGEMENT

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