Phytochemistry, 1975, Vol. 14, pp. 1161–1165. Pergamon Press. Printed in England.

PHOSPHORYLATION OF RIBOSOMAL PROTEIN IN SOYBEAN

DAVID A. TEPFER and D. E. FOSKET

Department of Developmental and Cell Biology, University of California, Irvine, Irvine, California 92664, USA

(*Received* 5 *October* 1974)

Key Word Index—*Glycine max*; Leguminosae; soybean; cytokinins; ribosomal protein phosphorylation.

Abstract—Transfer during log phase of a cytokinin-requiring strain of soybean cells to a medium lacking cytokinin brought about a decline in polyribosome content over a 24-hr period. This was correlated with a 3-fold increase in the incorporation of ^{32}P into ribosomal protein. Polyribosome gradients prepared from ^{32}P-labeled cells were fractionated, the RNA hydrolyzed, and the distribution of labeled protein determined. Both the monosome and the polyribosome regions of the gradients contained more ^{32}P-labeled protein in the cytokinin-deprived cells. Polyacrylamide gel electrophoresis of ribosomal proteins from cytokinin-deprived and cytokinin-treated cells did not reveal any consistent qualitative differences. However, 5 distinct peaks of ^{32}P incorporation were found after separation of the ribosomal proteins from labeled cells on polyacrylamide gels, and 3 of these peaks exhibited more than 2-fold enhancement of incorporation into the proteins extracted from the ribosomes of cytokinin-deprived cells.

INTRODUCTION

Since Kabat [1] demonstrated that eukaryotic ribosomal proteins become phosphorylated, this phenomenon has been observed in many organisms, including higher plants [2–15]. The activity of the kinase responsible for ribosomal protein phosphorylation in most animal systems is stimulated by cyclic AMP [5, 7, 10, 15]. The regulatory role of cyclic AMP in plants is not well established, although exogenous cyclic AMP has been shown to act synergistically with some plant hormones [16–19]. Also there is preliminary evidence that the hormone cytokinin may exert its effect on cell division by increasing the cyclic AMP levels of plant tissues [20]. Whether these reports will be substantiated or not, cytokinins are known to influence the activity of a variety of plant kinases [14, 21]. Ralph *et al.* [14] showed that cytokinin inhibits the phosphorylation of proteins associated with ribosomes, but they did not rigorously demonstrate that these phosphorylated proteins were ribosomal proteins, nor did they present any evidence which would suggest a functional role for this phosphorylation.

We have examined the phosphorylation of ribosomal protein in a strain of cultured soybean cells where both cell division and the polyribosom content of the cells have been shown to be controlled by cytokinin [22]. In this paper we present evidence that the withdrawal of cytokinin from log-phase soybean cells brings about a decrease in their polyribosome content and that this decline in polysome formation is correlated with approx. a 3-fold increase in ribosomal protein phosphorylation.

RESULTS

Cells which had been grown on SCF medium with 5×10^{-7} M zeatin for 2–4 days were transferred to liquid SCF medium containing or lacking the cytokinin, zeatin. The polyribosome content of the cells was determined by comparing the area under the monoribosome and polyribosome regions of the A_{260} profiles of the polysome gradients. The polysome gradients typically exhibit two peaks which sediment in the monosome region of the gradient and were shown to contain monosomes by electron microscopy. Data from ribonuclease digestion (not shown) suggest that

the heavier peak represents monosomes complexed with *m*RNA, while the lighter peak represents free monoribosomes. The combined area under both of these peaks was considered to be monosomes for the purpose of computing the polyribosome content of the cells.

After culture in medium lacking cytokinin for 24 hr the levels of polyribosomes declined, as compared to cells which had been grown for 24 hr on liquid medium containing zeatin. A 23% reduction in polysomes was observed in the zeatin-deprived cells. This reduction in polysome content was the first measurable indication of a general decline in growth activities that occurred after log phase cells were transferred to a medium lacking cytokinin. After 48 hr the mitotic index had declined to zero, and after approximately 8 days, the cells were dead. Response to cytokinin withdrawal was not as rapid as that observed when stationary phase cells were transferred to a cytokinin-containing medium, where a 4·5-fold increase in polysome content was observed within 6 hr [22]. Nevertheless it does represent a system in which protein synthesis is declining due to depletion of an essential growth factor.

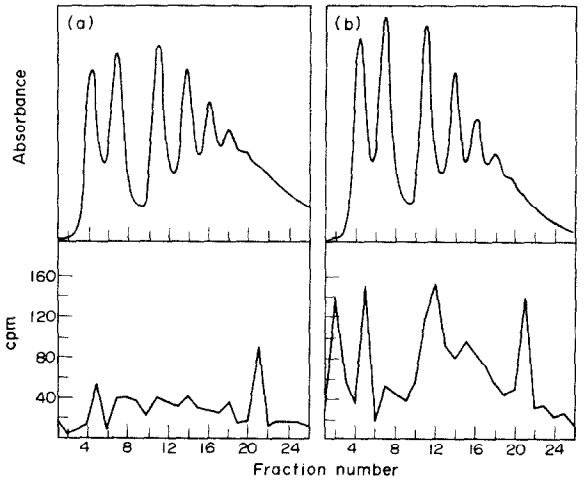

Fig. 1. Polyribosome profiles and ^{32}P incorporation into the protein component of fractions taken from polysome gradients. Cells were exposed to ^{32}P during the last 3 hr of a 24 hr culture period in media containing 5×10^{-7} M zeatin (a) or lacking a cytokinin (b). Ribosomes were extracted from samples of these cells for analysis by sucrose density gradient centrifugation. Ten drop fractions were collected from the polysome gradients. Each fraction was treated sequentially with RNase and hot TCA before the precipitated proteins were collected on glass fiber filter discs and counted.

An experiment was performed to examine ribosomal protein phosphorylation during the period of cytokinin deprivation. Again the log phase cells were transferred to liquid SCF medium either containing or lacking zeatin. After 21 hr, ^{32}P was added to the medium and the cells were harvested after exposure to the ^{32}P for 3 hr. Ribosomes were extracted from the cells, separated on sucrose gradients, and the gradients were fractionated. After the RNA in these fractions was removed by a combined RNAse digestion and hot TCA hydrolysis, ^{32}P was found to be associated with the protein that remained. The zeatin-deprived cells exhibited nearly 3-fold more radioactivity bound to the protein than the cytokinin-grown cells. This enhanced protein phosphorylation was not confined to any one region of the polysome gradient. Both monosome peaks were extensively labeled, but radioactivity was distributed throughout the polysome region as well (Fig. 1). This difference in ^{32}P incorporation into protein cannot be explained by differences in the uptake of ^{32}P in zeatin-treated and zeatin-deprived cells. The uptake of ^{32}P, as determined by the radioactivity in the post-mitochondrial supernatant, differed by only 2·5% in the 2 treatments.

The incorporation of labeled phosphate into ribosomal protein was examined after fractionating these proteins on 1-dimensional gels. The cells were exposed to ^{32}P throughout the 24 hr period of zeatin deprivation or stimulation. Before the ribosomal protein was extracted and subjected to electrophoresis on polyacrylamide gels, the ribosomal pellets were put through another purification cycle by centrifugation through a second discontinuous sucrose gradient. Approximately 28 bands could be counted in the stained gels. The pattern of bands seen in the absorbance scans of ribosomal proteins from zeatin-treated and zeatin-deprived cells exhibited some quantitative differences, but not consistent qualitative differences (Fig. 2).

Measurement of radioactivity in 1 mm slices of the gels revealed that phosphorylated ribosomal proteins were confined to 5 distinct peaks (Fig. 2). Furthermore it confirmed that phosphorylation of ribosomal proteins was stimulated in the zeatin-deprived cells. To further quantify these data, the area under each peak of radioactivity was determined and expressed as specific activity (Fig. 3.). In the zeatin-deprived cells, ^{32}P incorporation was

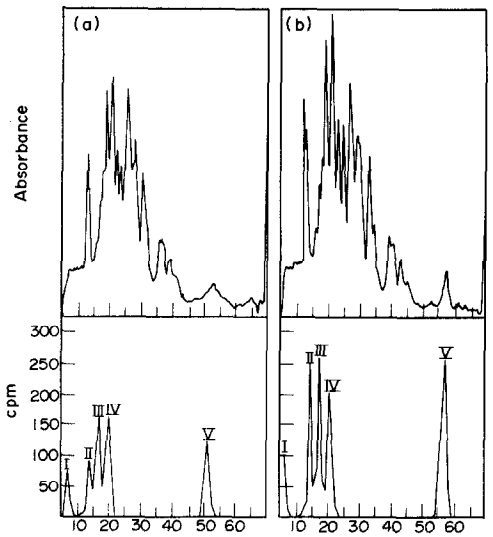

Fig. 2. Absorbance scans of ribosomal proteins separated on polyacrylamide gels and ^{32}P incorporation into proteins contained in 1 mm slices of these gels. Soybean cells were cultured for 24 hr in media containing (a) or lacking (b) cytokinin, both of which contained ^{32}P, before ribosomal proteins were extracted and separated on the gels.

greatly enhanced in 3 or the 5 radioactivity peaks, while in peaks numbers I and IV, there were less dramatic differences in radioactivity in the zeatin-deprived and control tissues.

DISCUSSION

A number of studies have found a correlation between *in vivo* ribosomal protein phosphorylation and ribosome activity. In some cases phosphorylation appears to be associated with the inactivation of ribosomes [4], while in other systems it is correlated with enhanced protein synthesis [15]. Similarly, attempts to determine the activity of phosphorylated ribosomes *in vitro* have been inconclusive. Monier *et al.* [23] found that protein kinase, when added to a cell-free system, inhibited amino acid incorporation by rat liver ribosomes. However, they did not show that the kinase phosphorylated ribosomal proteins under the conditions used to measure ribosomal activity. In the most complete study so far, Eil and Wool [8] phosphorylated purified ribosomes *in vitro* with a partially purified cytosol kinase and then tested the activity of these ribosomes in a cell-free system. They found no convincing difference in the functions of phosphorylated and unphosphorylated ribosomes. However, this work must be considered inconclusive since the ribosome appears to be a very dynamic structure whose configuration changes considerably during the different phases of translation [24]. Thus, even though the relevant protein kinase lacks substrate specificity, the availability of ribosomal protein to the enzyme could determine which proteins become phosphorylated. For example, different proteins might be available for phosphorylation during elongation than during initiation. If this were the case, the phosphorylation of isolated, purified ribosomes *in vitro* might have a different consequence than the phosphorylation of functioning ribosomes *in vivo*. Indeed, Majumder and Turkington [15] found that the pattern of ribosomal protein phosphorylation differed both quantitatively and qualitatively in ribosomes phosphorylated *in vitro* and *in vivo*.

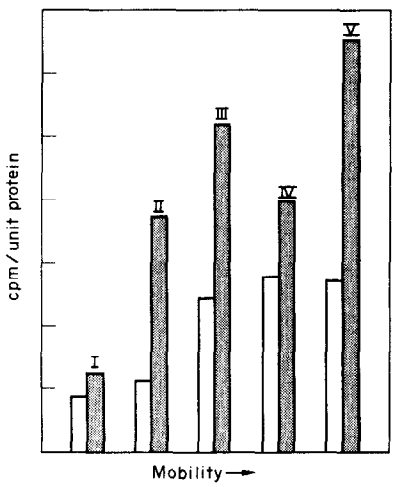

Fig. 3. Graphic representation of the effect of cytokinin upon the specific activity of 5. ^{32}P-containing, peaks observed after separating ribosomal proteins on polyacrylamide gels. The numbers refer to the peaks as designated in Fig. 2: ▨ = cytokinin-deprived cells; □ = cytokinin-grown cells.

It would appear that the significance of ribosomal protein phosphorylation could be determined more reasonably by examining the activity of ribosomes *in vitro* after they have been phosphorylated *in vivo* under conditions where phosphorylation is correlated with changes in protein synthesis. While we have not carried our work this far, we have described a system where such a study might be attempted, and we have presented evidence which strengthens the correlation between ribosomal protein phosphorylation and the inactivation of the ribosome. Ribosomal protein phosphorylation was markedly enhanced when soybean cells were deprived of cytokinin, a condition also shown to

bring about a reduction in the polyribosome content of the cells.

Eil and Wool [8] were critical of the work of Kabat and others on the grounds that they had not used a sufficiently rigorous isolation and purification procedure to rule out the possibility that the phosphorylated proteins they had observed were casual contaminants which became associated with the ribosomes during extraction. We used ribosome extraction conditions designed to minimize both polysome degradation and contamination by non-ribosomal proteins. Cells were broken in 0·4 M KCl and ribosomal pellets were resuspended in 0·2 M KCl. These conditions are more stringent than the "high salt" buffers of Kabat [1–3], but somewhat less so than those of Eil and Wool [8]. We believe that our isolation procedure is consistent with the view, discussed by Kurland [24], that the isolated ribosome might be defined as a fully functional particle, that is, with the protein factors that are required for translation, which are washed off in 0·5–1·0 M KCl. We believe that the isolation conditions we have employed produced a particle which is reasonably free from contamination by nonribosomal proteins, and that the phosphorylated protein species we have separated on polyacrylamide gels represent ribosomal proteins.

EXPERIMENTAL

Cells of *Glycine max* cv Sodifuri, derived from the cotyledons, were maintained in continuous culture on a chemically defined medium designated SCF (25), with weekly/biweekly routine transfers. Cells were grown at 23° ± 2° under subdued light. For exptl purposes, stationary phase cells (10 days old or older) were trasferred to liquid SCF containing cytokinin zeatin at 5×10^{-7} M for 2–4 days to bring the cells into logarithmic growth with a high level of cytoplasmic ribosomes. Cells were then transferred to liquid medium which either contained or lacked cytokinin for an additional 24 hr. In labeling expts, ^{32}P was added to Pi-free culture medium at final concn of 25 μ Ci/ml, after it was neutralized with sterile 0·02 M NaOH and dild with sterile H_2O.

Ribosome isolation. All operations were carried out at 0–4°. The cells were broken in an extrn medium (250 mM nuclease-free sucrose, 50 mM Tris–HCl at pH 8·5, 400 mM KCl, 20 mM Mg acetate, and 5 mM β-mercaptoethanol) using a motor-driven glass–Teflon homogenizer. The resulting suspn was filtered through Miracloth and centrifuged at 12000 rpm for 15 min in the Sorvall SS-34 rotor. The supernatant was layered onto a discontinuous sucrose gradient of 1 ml of 1·5 M sucrose and 1 ml of 0·5 M sucrose and centrifuged at 130000 g for 7 hr. The surface of the resulting ribosomal pellet was washed with 1 ml of resuspn medium (50 mM Tris–HCl, pH 7·8, 200 mM KCl and 10 mM Mg acetate) and then resuspended in the same soln.

Polyribosome gradients. Aliquots of resuspended ribosomes were layered onto 10–35% continuous sucrose gradients (nuclease-free sucrose in resusn buffer). Gradients were centrifuged at 40000 rpm for 1 hr in the Beckman SW-40 rotor, and fractionated from the top by pumping 70% sucrose into the bottom of the centrifuge tube. A at 260 nm was monitored continuously using a flow cell. For some expts, 10 drop fractions of the gradient were collected.

Extraction of ribosomal protein from sucrose gradient fractions. Each of the 10-drop fractions from the sucrose gradients was treated with ribonuclease A (Worthington Biochemicals) at a concn of 10^{-9} g/ml for 10 min at 37°. Then 0·05 mg of BSA was added to each fraction as a carrier, followed by TCA at a final concn of 10%. Samples were heated to 90° for 20 min, cooled to 0° for 20 min, and the pptd ribosomal protein collected on glass-fiber filters. Each filter was washed 4× with 5 ml of 10% TCA, dried, and counted in 10 ml of toluene-based scintillation fluid.

Extraction of ribosomal protein for analysis on polyacrylamide gels. Ribosomal pellets were resuspended vigorously with a Teflon–glass homogenizer in resuspn medium. The resulting suspn was layered over a discontinuous sucrose gradient consisting of 1·5 M and 0·5 M nuclease-free sucrose (in resuspension buffer) and centrifuged at 300000 g for 4 hr in the Beckman SW 50·1 rotor. The surface of the ribosomal pellet was washed and the pellet resuspended in resuspn medium. The suspn was made 0·25 M with HCl and stirred for 30 min. The pptd RNA was pelleted by centrifugation for 20 min at 20000 rpm in a Beckman SW 50·1 rotor. Ribosomal protein in the supernatant was pptd with 5 vol of cold Me_2CO and allowed to stand for 1 hr. The pptd ribosomal protein was pelleted and washed sequentially with Me_2CO, 95% EtOH and Et_2O. After drying, the ribosomal protein was dissolved in 8 M urea.

Polyacrylamide gel electrophoresis of ribosomal proteins. Ribosomal protein samples were subjected to electrophoresis in 15% polyacrylamide gels for 4 hr at 2 mA/tube. The gels contained 6 M urea and were buffered at pH 4·3. After electrophoresis, the proteins were stained with 0·1% Amido Black in 7% HOAc. Gels were destained by soaking for 48 hr in several changes of 7% HOAc. The stained gels were scanned at 550 nm in a recording spectrophotometer with a gel scanning attachment. Gels containing ^{32}P-labeled ribosomal proteins were sliced into 60 × 1 mm discs, the slices dried in scintillation vials, and radioactivity determined by scintillation counting in a toluene-based scintillation fluid.

Acknowledgement—We wish to thank Connie Holm for her generous assistance and advice.

REFERENCES

1. Kabat, D. (1970) *Biochemistry* **9**, 4160.
2. Kabat, D. (1971) *Biochemistry* **10**, 197.
3. Kabat, D. (1972) *J. Biol. Chem.* **247**, 5338.
4. Bitte, L. and Kabat, D. (1972) *J. Biol. Chem.* **247**, 5345.
5. Loeb, J. E. and Blat, C. (1970) *FEBS Letters* **10**, 105.
6. Blat, C. and Loeb, J. E. (1971) *FEBS Letters* **18**, 124.
7. Eil, C. and Wool, I. G. (1973) *J. Biol. Chem.*, **248**, 5122.
8. Eil, C. and Wool, I. G. (1973) *J. Biol. Chem.* **248**, 5130.
9. Walton, G. M., Gill, G. M., Abrass, I. B. and Garren, L. D. (1971) *Proc. Nat. Acad. Sci. U.S.* **68**, 880.
10. Walton, G. M. and Gill, G. N. (1973) *Biochemistry* **13**, 2604.
11. Li, C.-D. and Amos, H. (1972) *Biochem. Biophys. Res. Comm.* **45**, 1398.
12. Correze, C., Pinnell, P. and Nunez, J. (1972) *FEBS Letters* **23**, 87.

13. Trewavas, A. (1972) *Plant Physiol.* **51,** 760.
14. Ralph, R. K., McCombs, P. J. A., Tener, G. and Wojcik, S. J. (1972) *Biochem. J.* **130,** 901.
15. Majumder, G. C. and Turkington, R. W. (1972) *J. Biol. Chem.* **247,** 7207.
16. Galsky, A. G. and Lippincott, J. A. (1969) *Plant Cell Physiol.* **10,** 607.
17. Pollard, C. J. (1971) *Biochem. Biophys. Acta* **252,** 553.
18. Salomon, D. and Mascarenhas, J. P. (1971) *Life Sci.* **10,** 879.
19. Wood, H. and Braun, A. C. (1973) *Proc. Nat. Acad. Sci. U.S.* **70,** 447.
20. Wood, H., Lin, M. and Braun, A. C. (1972) *Proc. Nat. Acad. Sci. U.S.* **69,** 409.
21. Tuly, V., Dilley, D. R. and Wittwer, S. H. (1964) *Science* **146,** 1477.
22. Short, K. C., Tepfer, D. A. and Fosket, D. E. (1974) *J. Cell Sci.* **15,** 75.
23. Monier, D., Santhanam, K. and Wagle, S. R. (1972) *Biochem. Biophys. Res. Commun.* **46,** 1881.
24. Kurland, C. G. (1972) *Ann. Rev. Biochem.* **41,** 377.
25. Fosket, D. E. and Torrey, J. G. (1969) *Plant Physiol.* **44,** 871.