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IN ORGANELLO and IN VITRO PHOSPHORYLATION OF CHLOROPLAST RIBOSOMAL PROTEINS

Carole GUITTON, Anne-Marie DORNE and Regis MACHE

Laboratoire de Physiologie Cellulaire Végétale, CNRS ERA 488,
Université de Grenoble I, 38402 St Martin d'Hères Cedex, France

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Summary: Two chloroplast ribosomal proteins are phosphorylated in isolated chloroplast. One in the large subunit (L18) and one in the small subunit (L^S31). The phosphorylation is light dependent and occurs on a serine residue for both ribosomal proteins. These two proteins and other chloroplast ribosomal proteins are also phosphorylated in vitro using [γ^{32}P]-ATP and a cAMP -dependent or a cAMP - independent protein kinase. The existence of a protein-kinase bound to chloroplast 70S ribosomes is also demonstrated, the enzyme is able to phosphorylate almost every chloroplast ribosomal protein.

The phosphorylation in vivo of ribosomal proteins has been observed in a variety of eukaryotic cells (1). The major phosphorylated ribosomal protein identified as S6 is part of the 40S subunit and may contain up to five phosphoryl groups (2). In addition to S6, several other proteins in both the large and small ribosomal subunits have been shown to be phosphorylated (3, 4). The residue most frequently occurring in phosphorylated proteins is phosphoserine (5, 6). The physiological significance of these phosphorylations remains unknown.

Manai and Cozzone (7) have recently reported that E. coli harbors a protein kinase activity that phosphorylates a number of cytoplasmic proteins associated with ribosomes. The ribosomal protein fraction contains a major phosphorylated protein, with an approximate Mr value of 65,000. The only phosphoamino acid is phosphotyrosine. The nature of this protein has not been identified so far.

It was therefore of interest to search for the presence of ribosomal phosphoproteins in chloroplasts. Chloroplast ribosomes appear to be structurally and functionnally similar to prokaryotic ribosomes in many respects (8, 9, 10). It has been found in our laboratory that up to 18 spinach chloroplast ribosomal proteins are encoded in the chloroplast genome (11).

We report here evidence that two spinach chloroplast ribosomal proteins, one in the large subunit and one in the small, are phosphorylated after incubation of intact isolated chloroplasts with ^{32}P. The phosphorylated residue is serine. These phosphoproteins can be phosphorylated in vitro by a protein kinase present on chloroplast 70S monosomes. A short communication has been presented previously (12).

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MATERIALS AND METHODS

Materials. Spinach plants (Spinacea oleracea, var. Géant d'hiver) were grown on soil in a controlled room in the conditions already described (11), or were purchased from a local market. Biochemicals were purchased from Sigma. ^{32}P (orthophosphoric acid) was from New England Nuclear or from C.E.A. [$\gamma^{32}P$]-ATP (3,000 Ci/mmole) was from Amersham. Sorbitol was purified with mixed bed resin (Biorad AG 501-X8) and charcoal.

Chloroplast preparation and incubation conditions for phosphorylation "in organello". Spinach chloroplasts were isolated from washed young leaves (5 cm in length) and were incubated for 45 min as previously described (11). The incubation medium contained 21 L-aminoacids, 5.10^{-6} M each, and ^{32}P(200 µCi).

Ribosomes and ribosomal proteins preparation. Purified chloroplast 70S monosomes, their subunits and ribosomal proteins were prepared as previously described (13). Ribosomal proteins devoided of phosphoryl groups were prepared as follows:: proteins isolated from 6 A_{260} units of 70S monosomes were deposited on a minicolumn containing 0.5 mg (1.5 units) of agarose bound alkaline phosphatase originated from calf intestinal mucosa (Sigma), and were eluted with a 10 mM Tris-HCl pH 9.4 buffer containing 5 mM dithiothreitol.

Electrophoretic separation of ribosomal proteins and autoradiography. Chloroplast ribosomal proteins were analysed by two-dimensional electrophoresis as previously described (10) using the system of Madjar et al. (14) without modification. This system consists in a first acidic dimension followed by a second dimension in the presence of SDS. R 250 Coomassie blue was used for staining. The nomenclature of ribosomal proteins was done as previously (10). Stained gels were dried under vacuum and exposed at -70°C on medical X-Ray films with intensifying screens.

Characterization of the phospho amino acids. Ribosomal proteins, obtained from chloroplasts incubated with ^{32}P for protein phosphorylation, were hydrolysed in HCl 6M at 110°C for 2 h, in a final volume of 500 µl. The identification of liberated phospho amino acids was done by one dimension high voltage electrophoresis as described by Hunter and Sefton (15), except that migration was for 120 min at 1.3 kV, at pH 1.9, in acetic acid/ formic acid (88% by vol) /H_2O, 78:25:897 (vol/vol). Samples (about 5 µg each) of authentic phosphoserine, phosphothreonine and phosphotyrosine were run simultaneously and revealed by ninhydrin staining. Labelled phospho amino acids were detected by autoradiography as indicated above.

In vitro phosphorylation of ribosomal proteins. Ribosomal subunits (1.5 A_{260} units) prepared following the method described by Dorne et al. (11) were phosphorylated for 30 min in a reaction volume of 0.05 ml containing 50 mM Tris HCl, pH 7.4 at 30°C ; 3 mM $MgCl_2$; 1 mM dithiothreitol ; 0.025 mM [$\gamma^{32}P$]-ATP (5 µCi) ; 0.0014 mM cAMP. Protein kinase was added according to Del Grande et al. (16) using 20 units of 3'5' cAMP dependent protein kinase (holoenzyme) from rabbit muscle (Sigma) or a cyclic nucleotide independent protein kinase (G type casein kinase) from bovine adrenal cortex (17). The measurement of the ribosome bound protein kinase activity was done in the same conditions but no exogenous protein kinase was added. In some experiments 70S ribosomes were prepared in the presence of 30 mM KCl instead of 0.5 M KCl.

Preparation of $ChlPK_1$ and $ChlPK_2$ protein kinases from spinach chloroplasts. The two protein kinases, $ChlPK_1$ and $ChlPK_2$, were isolated from Spinach chloroplasts according to the methods of Lin et al. (18).

RESULTS AND DISCUSSION

Incorporation of ^{32}P "in organello". Spinach chloroplasts isolated from young leaves were incubated with ^{32}P in a light driven protein synthesis system, as described in Materials and Methods. After 45 min of incubation, chloroplast ribosomes were isolated and ribosomal proteins were analyzed by two dimensional

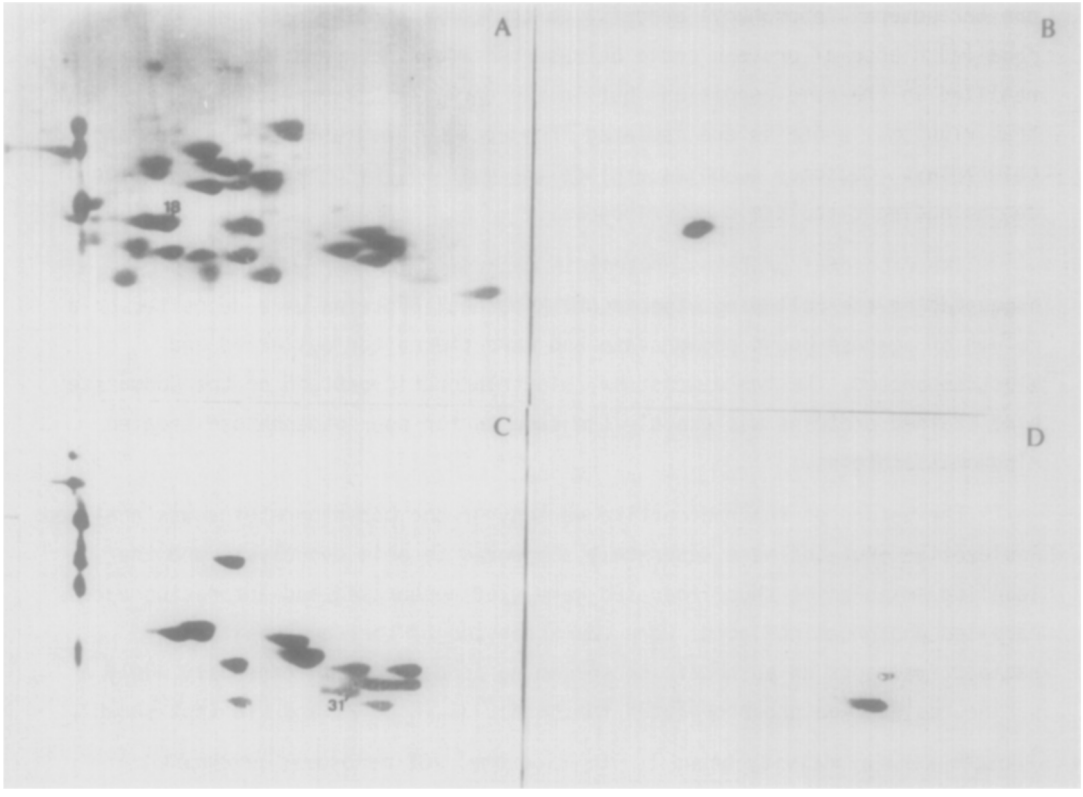

Figure 1. Two dimensional gel patterns of chloroplast 50S and 30S ribosomal proteins. ^{32}P was incorporated by isolated chloroplasts in the presence of L-aminoacids and under a red light in conditions allowing protein synthesis. (A) and (C): Coomassie blue stained proteins from 50S and 30S ribosomal subunits, respectively. 5 A_{260} units of carrier ribosomal subunits were added to labelled ribosomes (ca 1 A_{260} unit). (B) and (D) autoradiograms of slab gels shown in (A) and (C), respectively, after an exposure time of one week.

electrophoresis, after the addition of cold ribosomal proteins. Labelled proteins were detected by autoradiography.

A coincidence between stained and radioactive spots was observed for two ribosomal proteins, one in the large subunit, one in the small subunit (Fig. 1). The radioactive spot for the 50S subunit was on protein L18. The radioactive spot for the 30S subunit was in coincidence with a stained spot first considered as a contamination of a ribosomal protein from the large subunit (10). This protein, which appears in both subunit preparations, is probably situated at the interface of the two subunits. The fact that we observed a large radioactive spot in the 30S subunit and a weak or non-existent radioactive spot in the 50S indicates that the protein is certainly a small subunit ribosomal protein. This protein will be indicated L^S31 instead of L31 as called previously (10). The superposition of the autoradiograph with the corresponding stained map showed that the radioactive spots were slightly extended toward the upper left of the stained spots, as expected by the modification of charge due to the addition of

one (or several) phosphoryl group(s) to the corresponding ribosomal proteins. No labelled ribosomal protein could be observed when incorporation of ^{32}P was realized in the same conditions but in the dark. This observation and the fact that ribosomal proteins are isolated from sucrose gradient purified 30S and 50S chloroplast ribosomal subunits exclude the possibility of a phosphorylation of contaminating cytoplasmic 80S ribosomes.

The ribosomal proteins present in monosomes are not phosphorylated as suggested by the following experiment. Ribosomal proteins were deposited on a column of agarose-bound phosphatase and were thereafter collected and electrophoresed. The two dimensional electrophoretic pattern of the Coomassie blue stained proteins was exactly the same as for non- phosphatase treated ribosomal proteins.

The nature of the radioactive variety of the phosphoproteins was analysed. Radioactive proteins were separately subjected to acid hydrolysis and the labelled amino-acids thus liberated were electrophoresed and autoradiographed. Labelled phosphoamino- acids were characterized by the superposition of radioactive spots on authentic phosphoamino acids run simultaneously. Only a serine residue was phosphorylated for both L^S31 (Fig. 2) and L18 (not shown).

Protein kinase activity bound to 70S ribosome. 70S monosomes prepared as described in Materials and Methods were shown to contain a protein kinase activity. This kinase used $[\gamma^{32}P]$-ATP as phosphorylating donor. Almost all the ribosomal proteins were labelled after incubation for 30 min (Fig. 3). An autoradiograph exposed for a short time showed that the spots corresponding to L4, L18 and L^S31 were the most radioactive. The small subunit of ribulose bisphosphate carboxylase, present as contaminant, was phosphorylated. The radioactive spots were slightly displaced to the upper left by comparison with the stained spots. In order to confirm that ribosomal proteins were really phosphorylated, labelled proteins were subjected to an acid hydrolysis: the radioactive moiety liberated was identified as phosphoserine (not shown). The protein kinase activity which was present on the 70S monosomes prepared at low ionic strength (30mM) was not released in 70S monosomes prepared in the presence of 0.5 M KCl.

Lin et al. (24) have recently isolated and characterized two membranous protein kinases from Spinach chloroplasts ($ChlPK_1$ and $ChlPK_2$). Both protein kinases acted on casein or histone III as substrate and phosphorylated a serine residue. We have isolated $ChlPK_1$ and $ChlPK_2$ as indicated by the authors: the assays of $ChlPK_1$ and $ChlPK_2$ phosphorylating activity on ribosomal proteins, attempted in our laboratory, were not positive. We suggest that the protein kinase present on isolated chloroplast ribosomes is different from the two already characterized membranous protein kinases.

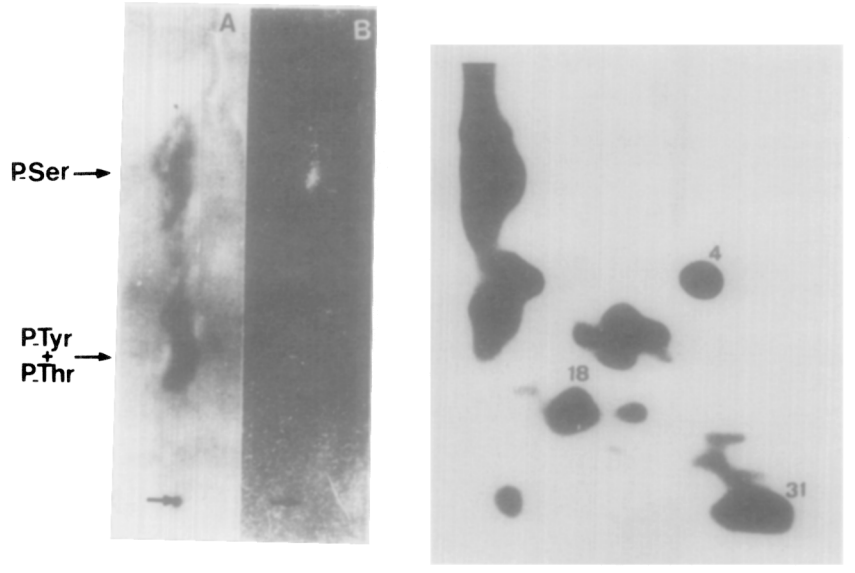

Figure 2. Separation and identification of phosphoamino acids in the ribosomal protein L^S31. (A) Acid hydrolysate of radioactive protein L^S31 was subjected to one- dimensional electrophoresis. Authentic P-ser, P-Thr and P-Tyr were run simultaneously and revealed by ninhydrin staining. (B) Autoradiogram after an exposure time of one week. The origins are indicated by an arrow.

Figure 3. Autoradiogram of two-dimensional gel electrophoretic pattern of 70S ribosomal proteins after in vitro incubation with [γ^{32} P]-ATP, at 30° C for 30 min. No exogenous protein kinase was added. Exposure time: 24 hours at -70° C

In vitro phosphorylation of ribosomal proteins by 3'5'-cAMP dependent protein kinase or by cyclic nucleotide independent casein kinase. Chloroplast ribosomes isolated from spinach were incubated with [γ^{32} P]-ATP in the presence of 3'5' cAMP dependent protein kinase (from rabbit muscle) or cyclic nucleotide independent casein kinase (from adrenal cortex).

Ribosomal proteins were separated by two dimensional gel electrophoresis. When the 3'5'-cAMP dependent protein kinase was used, the autoradiograph revealed 7 spots for the 30S subunit: S1, S6, S11, S13, S14, S17 and L^S31, and 6 spots for the 50S subunit: L3, L4, L11, L13, L18 and a spot corresponding to L^S3 present as contaminant (Fig. 4). When the cyclic nucleotide independent casein kinase was used, the autoradiograph revealed that the most radioactive proteins were L18 and L^S31, besides other more weakly labelled ribosomal proteins (Fig. 5).

These results showed that the two ribosomal proteins phosphorylated in organello (L^S31 and L18) can be phosphorylated by the two kinds of protein kinase and that a cyclic independent protein kinase mainly phosphorylates the two ribosomal proteins phosphorylated in organello.

During the preparation of this manuscript, results concerning the phosphorylation of spinach chloroplast ribosomal proteins appeared (19). The

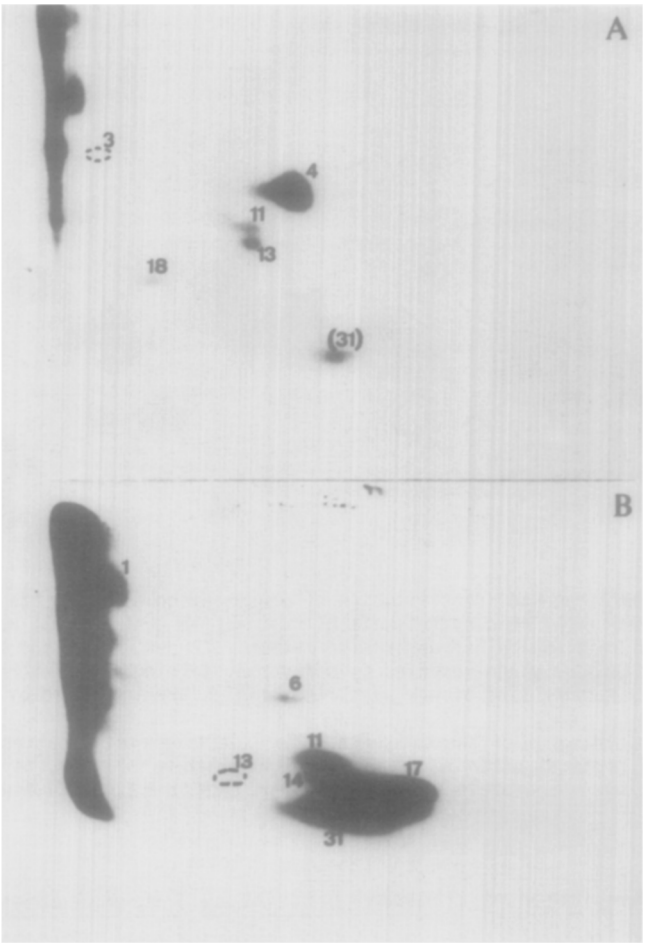

Figure 4. Autoradiograms 50S (A) and 30S (B) ribosomal proteins separated by 2D-electrophoresis after in vitro incubation with [γ^{32}P]-ATP, at 30°C for 30 min, in the presence of 3'5' cAMP dependent protein kinase. Exposure time: 80 hours at -70°C.

authors have shown that only one ribosomal protein L18 (L16 in their nomenclature) was phosphorylated in organello. In our case the light driven incorporation of ^{32}P was made in the presence of L-amino acids, allowing a protein synthesis (11). These labelling conditions could explain that a second ribosomal protein (L^S31) was phosphorylated in organello (see Fig. 1) and suggest that the phosphorylation is dependent on protein synthesis. Our observation that ribosomal proteins present in monosomes are not phosphorylated is in accordance with the hypothesis of a phosphorylation- dephosphorylation process acting on an unknown control system.

The results presented in this study also show that the phosphoryl group is incorporated in a serine residue and that a ribosome bound protein kinase is able to phosphorylate several ribosomal proteins including L18 and L^S31.

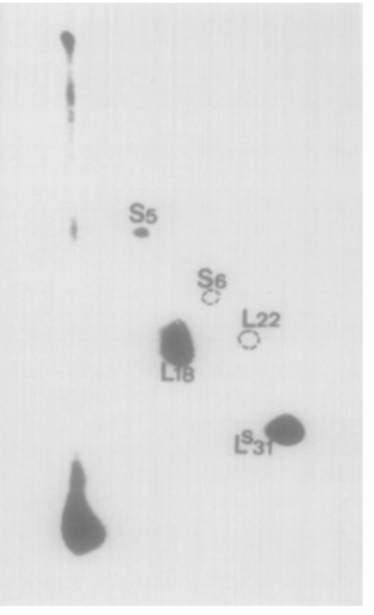

Figure 5. Autoradiogram of 70S ribosomal proteins separated by 2D-electrophoresis after in vitro incubation with [γ^{32}P]-ATP, at 30°C, for 30 min in the presence of cyclic nucleotide independent casein kinase isolated from adrenal cortex. Exposure time: 10 hours.

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