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Stromal protein phosphorylation in spinach (*Spinacia oleracea*) chloroplasts

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When intact spinach chloroplasts were supplied with [^{32}P]P_i, stromal protein phosphorylation was found to occur in the dark. On illumination the thylakoid protein kinase was activated and the amount of label found in thylakoid proteins quickly exceeded that incorporated into stromal protein, such that the latter was found to account for only 10–15% of the total radioactivity bound to chloroplast proteins after 5 min illumination. The rate of phosphorylation of stromal polypeptides was unchanged by light. After SDS/polyacrylamide-gel electrophoresis, more than 15 labelled polypeptides of stromal origin were observed. A polypeptide with an M_r of approx. 70000 had the highest specific activity of labelling. Both the large and small subunits of the ribulose-1,5-bisphosphate carboxylase were phosphorylated. The level of phosphorylation of stromal protein was increased by CO_2 fixation in intact chloroplasts. This increase was not observed in the absence of $NaHCO_3$ or in the presence of the phosphoribulokinase inhibitor DL-glyceraldehyde. These effects appeared to be largely due to changes in the phosphorylation state of the large and small subunits of ribulose-1,5-bisphosphate carboxylase. Studies with the reconstituted chloroplast system showed that the thylakoid protein kinase(s) played no part in the phosphorylation of stromal protein. The rate and level of phosphorylation of stromal protein was unaffected by the activation state of the thylakoid protein kinase and was unchanged when thylakoids were omitted from the reaction medium. The phosphorylation of stromal proteins is therefore catalysed by a discrete soluble protein kinase.

INTRODUCTION

Observations of the phosphorylation of stromal polypeptides have been reported in isolated intact chloroplasts and stroma from spinach [1,2] and from maize (*Zea mays*) mesophyll [3,4]. In the latter case the major phosphorylated stromal protein has been clearly shown to be pyruvate,orthophosphate dikinase [3–5], a key enzyme of photosynthesis in species that exhibit the ATP-dependent CO_2-concentrating mechanism known as the 'C_4 cycle' [6]. The regulation of the activity of this enzyme has been shown to involve phosphorylation/dephosphorylation changes of enzyme threonine [4,5] and histidine residues [7]. In contrast, the phosphorylation of stromal polypeptides from 'C_3' species is only poorly characterized, and the nature and regulation of the protein kinases that phosphorylate stromal proteins is unknown. Although the phosphorylation of several stromal polypeptides has been observed in spinach [1], only one major phosphorylated stromal polypeptide with an M_r of 66000 was found with intact spinach chloroplasts fed with [γ-^{32}P]ATP [2]. A cyclic AMP-independent type of protein kinase has been found to be bound to the outer-envelope membrane of spinach chloroplasts [8]. This protein kinase was shown to phosphorylate the mature form of the small subunit of ribulose-1,5-bisphosphate carboxylase and was postulated to have a role in the transport of polypeptides across the chloroplast envelope.

Two protein kinases have been isolated and purified from chloroplast fractions [9,10], but the origin and physiological function of these remains to be clarified. The regulatory properties of the thylakoid protein kinases *in situ* [1,11] appear to be clearly distinct from the Ca^{2+}-dependent protein kinases isolated from the membranes of dark-grown pea (*Pisum sativum*) shoot buds [12] and soluble protein kinases from wheat (*Triticum aestivum*) germ [13,14]. The phosphorylation of membrane and soluble polypeptides from maize (*Zea mays*) coleoptiles has been shown to be regulated by Ca^{2+} and calmodulin [15].

The present study attempts to examine the conditions necessary to promote stromal protein phosphorylation, to determine whether thylakoid protein kinase activity is involved in this process and to assess its involvement with the regulation of carbon metabolism in spinach, a species which exhibits the C_3 type of photosynthesis [6].

MATERIALS AND METHODS

Spinach (*Spinacia oleracea* var. Virtuosa) was grown for 6 weeks in hydroponic culture in a glasshouse as described previously [16]. Intact chloroplasts were prepared by the method of Walker [17]. To ensure maximum labelling of stromal proteins, only chloroplast preparations that had an intactness value of at least 80% in the ferricyanide assay [18] were used in the following experiments. Chlorophyll was estimated in 80% (v/v) acetone extracts by the method of Arnon [19]. CO_2-dependent O_2-evolution measurements and ^{32}P incorporation from [^{32}P]P_i were performed in paired Hansatech oxygen electrodes with intact chloroplasts at a chlorophyll

Abbreviations used: DTT, dithiothreitol; LHCP, light-harvesting chlorophyll *a*/*b*-binding protein; LS, large subunit, and SS, small subunit, of ribulose-1,5-bisphosphate carboxylase; SDS, sodium dodecyl sulphate; C_3 species, one in which the first stable product of CO_2 fixation is a three-carbon acid.

concentration of 100 $\mu g \cdot ml^{-1}$ in a reaction medium containing 0.33 M-sorbitol, 2 mM-EDTA, 2 mM-$MgCl_2$, 3 mM-pyrophosphate, 0.3 mM-P_i (300 μCi), 1 mM-$NaHCO_3$, 200 units of catalase and 50 mM-Hepes/KOH, pH 7.6. Incubations were performed in darkness or in red light (300 $W \cdot m^{-2}$) at 20 °C. Samples (500 μl) were taken in duplicate at the times indicated and stromal protein was isolated immediately as follows. The chloroplasts were pelleted by centrifugation at 12000 *g* for 15 s in an Eppendorf 5412 bench centrifuge, the supernatant discarded via a vacuum line and the chloroplasts resuspended vigorously in 500 μl of medium containing 5 mM-DTT, 5 mM-EDTA and 5 mM-Hepes/KOH, pH 7.9. The thylakoid and envelope membranes were then pelleted by centrifugation at 12000 *g* for 15 s and the chlorophyll-free supernatant was rapidly mixed with 30 μl of 100% trichloroacetic acid. The total time taken for this separation procedure was less than 60 s. The precipitated stromal proteins (approx. 900 μg of protein/sample) were pelleted by centrifugation. Thylakoid samples were extracted with acetone and subsequently both thylakoid and stromal proteins were subjected to electrophoresis in the presence of SDS on 10–30% ($^{w}/_{v}$) polyacrylamide gradient slab gels by using the buffers of Laemmli [20]. After electrophoresis the gels were stained with Coomassie Brilliant Blue R, destained, dried and autoradiographed by using Kodak X-Omat RP X-ray film. The radioautograms of stromal proteins were left to develop for 5 days, which was more than 5-fold longer than the time required for thylakoid proteins phosphorylated under similar conditions. The image intensity is not directly related to the degree of phosphorylation, but the radioautograms do provide a qualitative picture of relative phosphorylation in individual polypeptide bands. ^{32}P incorporation into proteins was determined in the excised bands of specific apoproteins by Čerenkov counting in a Beckman LS7500 liquid-scintillation counter over a 30 min period. The values given are averages for three separate samples, with a standard error no greater than 10% (no correction has been made for colour quenching).

The M_r values for individual stromal polypeptides were estimated from a calibration curve of standard proteins of known M_r (that is the 'High Molecular Weight Standard Mixture SDS-6H' and the 'Dalton Mark VI SDS-6') purchased from Sigma.

Protein was determined by using a dye-binding protein assay kit (Bio-Rad Laboratories, München, Germany) with bovine γ-globulin and bovine serum albumin as protein standards.

The relative protein content of individual polypeptide bands was based on Coomassie Blue staining. The gels were scanned by using a Gilford spectrometer with a densitometer attachment. ^{32}P incorporation into individual polypeptides was calculated on a mol-of-^{32}P/mol-of-polypeptide basis from the protein measurements and the specific radioactivity in the reaction medium (300 μCi of ^{32}P with 0.6 μmol of P_i). Such measurements allow for comparative estimates of phosphorylation/mol of protein to be made.

Incorporation of ^{32}P from [γ-^{32}P]ATP into stromal polypeptides, in a reconstituted chloroplast system similar to that described by Lilley & Walker [21], was performed in reaction mixtures containing 0.33 M-sorbitol, 1 mM-EDTA, 10 mM-KCl, 10 mM-$MgCl_2$, 4 mM-ascorbate, 1 mM-DTT, 0.2 mM-NADPH, 0.5 mM-[γ-^{32}P]ATP (20 Ci/mmol), 1 mM-$NaHCO_3$, ferredoxin (200 μg/ml), catalase (200 units/ml), 50 mM-Hepes/KOH, pH 7.9, and thylakoids (100 μg of chorophyll/ml) and stromal protein (900 μg of protein/ml) in a final volume of 2 ml. Incubations were performed in oxygen electrodes, and samples (500 μl) were taken and rapidly fractionated into thylakoid and stromal protein by centrifugation at 12000 *g* for 15 s. Thylakoid samples were extracted with acetone and subsequently both thylakoid and stromal proteins were analysed as described for samples from intact chloroplast.

Thylakoid and stromal protein fractions were isolated in bulk by suspending pelleted intact chloroplast preparations in 5 ml of medium containing 5 mM-EDTA, 5 mM-DTT and 5 mM-Hepes/KOH, pH 7.9. The thylakoids were pelleted at 11000 rev./min (14500 *g*) for 5 min on a Sorvall RC5C centrifuge. The chlorophyll-free supernatant was designated the 'stromal protein fraction' and was removed. Thylakoids were resuspended in the above buffer and adjusted to 10 mM-$MgCl_2$ as was the stromal protein fraction. Incorporation of ^{32}P into isolated thylakoid or stromal polypeptides was carried out in plastic tubes in a simplified reaction medium containing 50 mM-Hepes/KOH, pH 7.9, 5 mM-$MgCl_2$, 0.2 mM-NADPH, ferredoxin (50 μg/ml), 10 mM-NaF, 0.5 mM-[γ-^{32}P]ATP (20 mCi/mmol), catalase (200 units/ml) and thylakoids (100 μg of chorophyll/ml) or stromal protein at a concentration of 450 or 900 μg/ml in a final volume of 1 ml. Samples (500 μl) were taken after 5 min or at the times indicated and treated and analysed in a similar manner to those taken from the reconstituted chloroplast system. In addition, rapid estimates of ^{32}P incorporation into stromal protein were made by taking samples (500 μl) and precipitating protein with addition of 30 μl of 100% (w/v) trichloroacetic acid. The precipitated material was collected by centrifugation and dispersed in 1 ml of 5 mM-Hepes/KOH buffer, pH 7.9, containing 5 mM-DTT and 5 mM-EDTA and pelleted by centrifugation at 12000 *g* for 10 s. The pellets of stromal protein were finally dispersed in 500 μl of 100 mM-Tris base and counted for radioactivity in the Beckman liquid-scintillation counter.

RESULTS

When intact chloroplasts were incubated with [^{32}P]P_i, a large number of stromal polypeptides, with M_r values ranging from 12000 to 147000, were found to be phosphorylated (Fig. 1). Of these the polypeptide species with the highest specific activity of labelling had an apparent M_r of about 70000. This polypeptide accounted for less than 0.5% of the total stromal protein as estimated by relative staining with Coomassie Brilliant Blue. In the conditions used here, the 70000-M_r polypeptide was calculated to be phosphorylated with a specific activity of at least 1 P_i group for every 25 70000-M_r polypeptides on a mol/mol basis.

The procedures used to isolate stromal protein from intact spinach chloroplasts in these experiments yielded approx. 9 mg of soluble stromal protein/mg of chlorophyll. The major polypeptide components of the stromal extracts observed after SDS/polyacrylamide-gel electrophoresis had calculated M_r values of about 56000 and 16000. From the staining intensity with Coomassie Brilliant Blue these polypeptides were estimated to comprise 43% and 8% of the total stromal protein

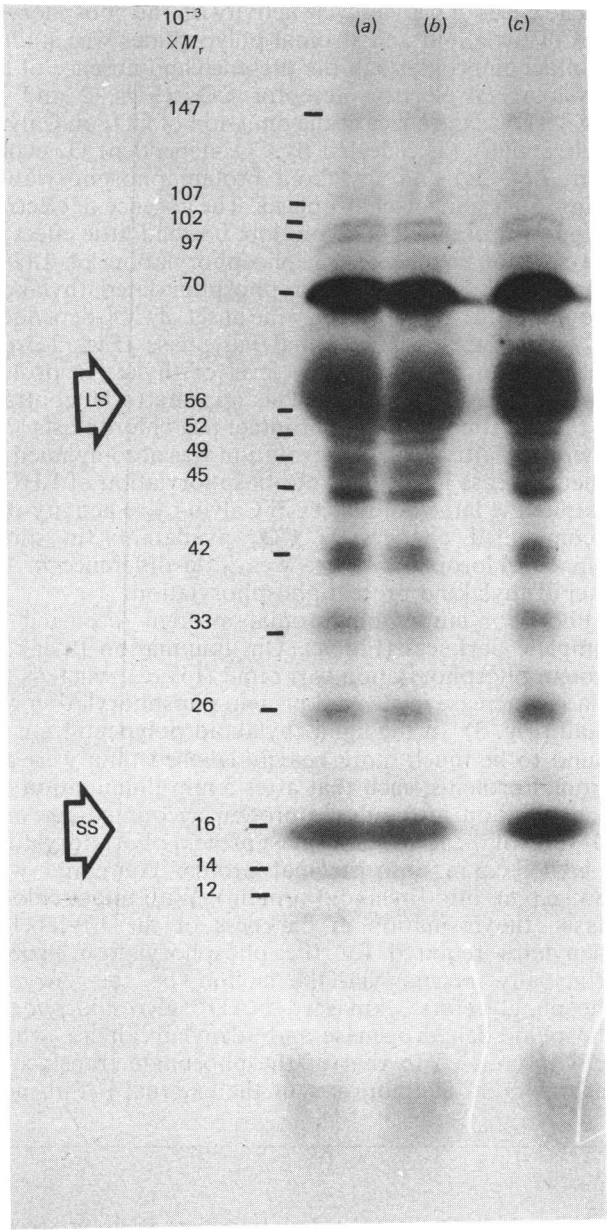
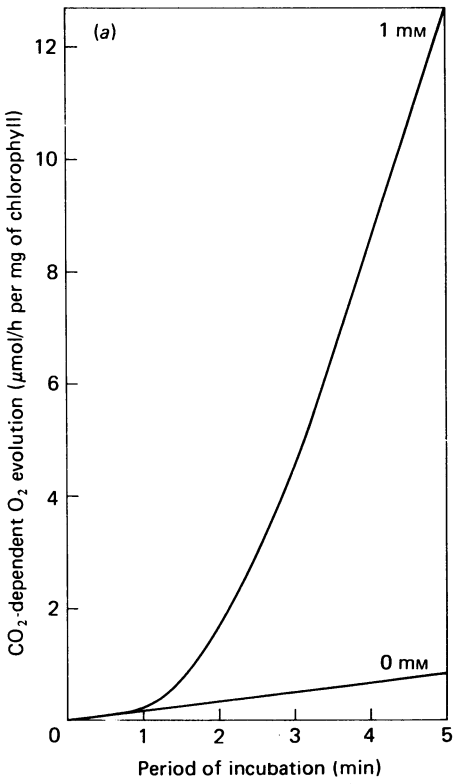


Fig. 1. Radioautogram of phosphorylated stromal polypeptides

Polypeptides were phosphorylated *in situ* in intact spinach chloroplasts incubated in the dark for 5 min in the presence of [^{32}P]P_i and 1 mM-$NaHCO_3$ (track *a*), and then for either 5 min (track *b*) or 10 min (track *c*) in the light. Stromal protein was separated and treated as described in the Materials and methods section. Protein loading was 900 μg/well. Estimates of M_r values for the phosphorylated polypeptides are given.



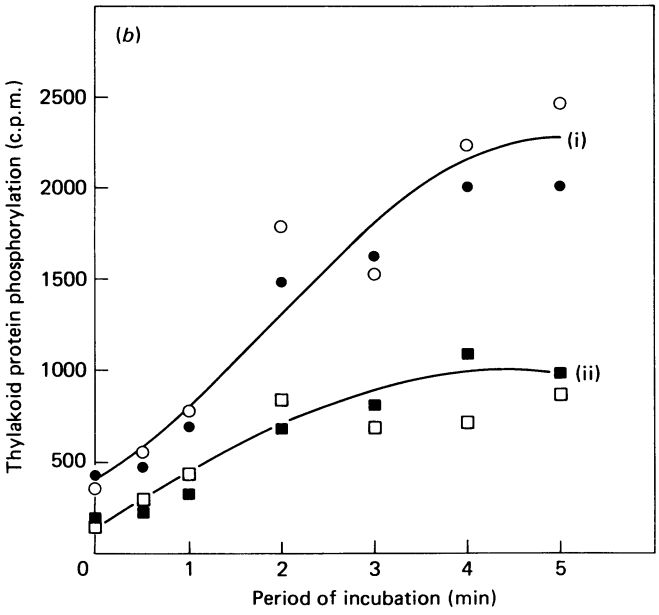


Fig. 2. Effect of CO_2-dependent O_2 evolution on thylakoid protein phosphorylation in illuminated intact chloroplasts

Chloroplasts were incubated in the dark for 5 min. The light was switched on at zero time. Rates of CO_2-dependent O_2 evolution (*a*) were measured simultaneously with ^{32}P-incorporation (*b*) into total phosphorylated thylakoid protein (i, circles) and LHCP alone (ii, squares) measured in the absence of CO_2 (open symbols) and with saturating CO_2 (closed symbols).

respectively and were designated the 'large' and 'small' subunits of ribulose-1,5-bisphosphate carboxylase on this basis. Phosphorylation of both the large and small subunits of ribulose-1,5-bisphosphate carboxylase in intact chloroplasts was apparent (Fig. 1). Both subunits were found to be equally labelled, but the relative maximum specific activity of labelling of these was less than 0.1% of each subunit protein.

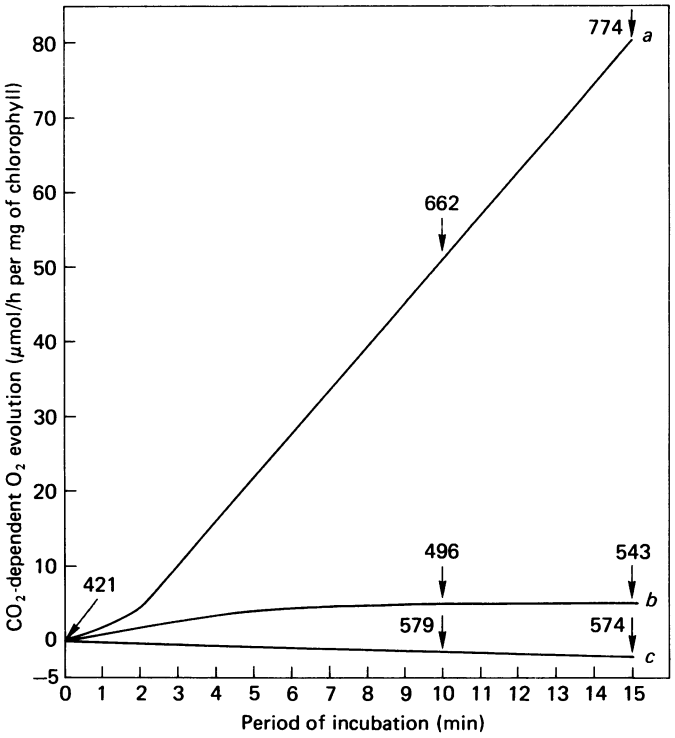


Fig. 3. Effect of photosynthesis on stromal protein phosphorylation in intact spinach chloroplasts

Chloroplasts were incubated for 5 min in the dark and then, at zero time, either illuminated with 1 mM-$NaHCO_3$ (*a*), or illuminated in the absence of added $NaHCO_3$ (*b*), or maintained in the dark with 5 mM-$NaHCO_3$ (*c*). CO_2-dependent O_2 evolution was measured in the oxygen electrode. Values above the arrows indicate the level of total stromal protein phosphorylation (c.p.m.) as estimated by Čerenkov counting of excised protein bands after SDS/polyacrylamide-gel electrophoresis.

The effect of Calvin-cycle activity on the phosphorylation of thylakoid and stromal polypeptides was studied in intact chloroplasts in the presence and absence of the physiological electron acceptor CO_2 (Figs. 2 and 3). Fig. 2 shows the effect of the omission of CO_2 on Calvin-cycle activity (as reflected by CO_2-dependent O_2 evolution; Fig. 2*a*) and thylakoid protein phosphorylation (Fig. 2*b*) in spinach chloroplasts. The absence of electron acceptor prohibited O_2 evolution, but had little effect on the rate or final level of phosphorylation of LHCP [Fig. 2*b*(ii)] or of the other phosphorylated thylakoid proteins [Fig. 2*b*(i)]. Similarly the onset of CO_2-dependent O_2 evolution after the initial lag phase (Fig. 2*a*) did not elicit any change in the level of thylakoid protein phosphorylation (Fig. 2*b*). This appears to be contrary to the situation reported for intact pea chloroplasts [22], where the initiation of O_2 evolution was accompanied by a net decrease in the level of phosphorylation of LHCP. Despite the large differences in Calvin-cycle activity that accompanied changes in CO_2 availability in intact spinach chloroplasts, there was little difference in the level of thylakoid protein phosphorylation.

Phosphorylation of stromal protein occurred in complete darkness (Fig. 1). On illumination thylakoid protein phosphorylation was rapid (Fig. 2), whereas the relative increase in stromal protein phosphorylation was small (Fig. 3). In the light, thylakoid polypeptides were found to be much more heavily labelled than were the stromal proteins, such that after 5 min illumination ^{32}P incorporation into soluble protein accounted for only 10–15% of the total chloroplast protein phosphorylation (e.g. 433 c.p.m. into stromal protein compared with 2855 c.p.m. into thylakoid protein). With intact chloroplasts, the formation in darkness of the ^{32}P-labelled adenylates required for the phosphorylation process principally occurs via the action of the enzymes phosphoglycerate kinase, NADP:glyceraldehyde-3-phosphate dehydrogenase and adenylate kinase, whose activity coupled to that of the phosphate translocator ensures rapid equilibration of the external $[^{32}P]P_i$ pool

Table 1. (*a*) Phosphorylation of stromal proteins in intact chloroplasts during dark (D)/light (L)/dark transitions in the presence of 5 mM-$NaHCO_3$, and (*b*) thylakoid protein phosphorylation in the dark

(*a*)	Phosphorylation (c.p.m.) in				
Conditions	Total stromal protein	LS	SS	70000-M_r protein	Remainder
5 min D	753	184	89	160	320
+15 min L	1536	574	259	262	497
+10 min D	1061	345	141	206	566
+20 min D	1178	420	153	321	452
+30 min D	1020	263	135	235	487
+45 min D	998	218	99	287	582
+60 min D	937	193	92	298	560

(*b*)	Phosphorylation (c.p.m.) in		
	9000-M_r polypeptide	LHCP	32000-M_r polypeptide
−NADPH	19	35	12
+0.1 mM-NADPH	81	231	73

with the stromal P_i and adenylate pools [3]. The extent of chloroplast protein phosphorylation was somewhat variable between different chloroplast preparations, but was consistent for each preparation. Since ^{32}P incorporation into stromal protein in intact chloroplasts illuminated in the absence of CO_2 (Fig. 3*b*) was comparable with that of similar chloroplasts maintained in darkness (Fig. 3*c*), light alone appeared to have little effect on the rate, or maximum level, of stromal protein phosphorylation. However, the level of stromal protein phosphorylation achieved in chloroplasts carrying out high rates of CO_2-dependent O_2 evolution (Fig. 3*a*) was increased compared with that of chloroplasts kept in the dark (Fig. 3*c*) and that in chloroplasts illuminated in the absence of CO_2 (Fig. 3*b*). This suggests that the increase in the level of phosphorylation was related to carbon assimilation. Significant increases in the level of phosphorylation of both the large and small subunits of the ribulose-1,5-bisphosphate carboxylase accompanied CO_2 fixation, whereas the increase in ^{32}P incorporation into other phosphorylated stromal polypeptides was relatively small (Table 1, *a*). The increase in phosphorylation state of the carboxylase subunits was reversed when CO_2 fixation ceased in darkness, whereas the phosphorylation level of other stromal proteins remained relatively constant (Table 1, *a*). Similarly, when CO_2 fixation in illuminated chloroplasts was inhibited by the addition of DL-glyceraldehyde [23], a significant decrease in the phosphorylation state of the carboxylase subunits was observed (Fig. 4). The pattern of stromal protein phosphorylation in chloroplasts carrying out CO_2-dependent O_2 evolution shown in Fig. 1 may be compared directly with that of chloroplasts in which CO_2 fixation is inhibited by the presence of 10 mM-DL-glyceraldehyde shown in Fig. 4, since these autoradiograms were obtained with chloroplast samples illuminated simultaneously in duplicate oxygen electrodes.

In intact chloroplasts, stromal protein phosphorylation occurred in darkness, suggesting that it was not catalysed by the thylakoid protein kinase. However, the thylakoid protein kinase can be active in the dark under suitable reducing conditions, for example, if the NADPH/NADP ratio is high. Such a situation has been found to occur when intact spinach chloroplasts were returned to the dark after a period of illumination [24]. Protein phosphorylation in isolated thylakoids in the dark was dependent on the presence of the reductant NADPH

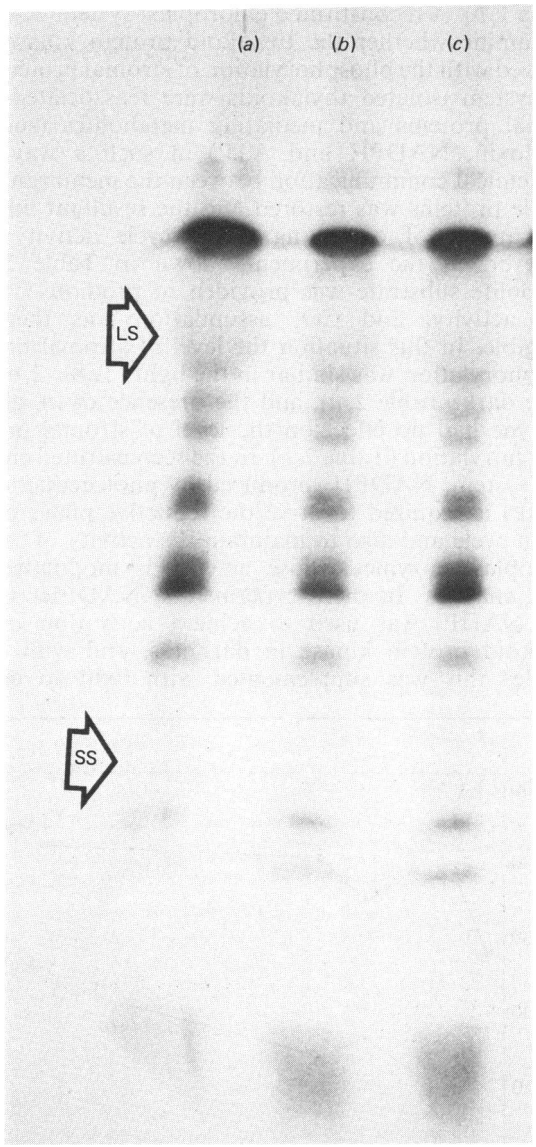


Fig. 4. Radioautogram of phosphorylated stromal polypeptides from chloroplasts incubated as in Fig. 1, except that 10 mM-DL-glyceraldehyde was included in the reaction media

Tracks are labelled as in Fig. 1.

Table 2. Phosphorylation of stromal enzyme protein in the reconstituted chloroplast system

Thylakoids were omitted from reaction mixtures (*d*)–(*g*).

Conditions	Phosphorylation (c.p.m.) of total stromal enzyme protein after 10 min incubation
(*a*) Complete system, 10 min dark	342
(*b*) Complete system, 5 min dark + 5 min light	324
(*c*) −NADPH, 10 min dark	365
(*d*) −Thylakoids, 10 min dark	347
(*e*) −Thylakoids, +10 mM-DL-glyceraldehyde, 10 min dark	340
(*f*) −Thylakoids, −NADPH −ferredoxin, 10 min dark	289
(*g*) −Thylakoids, +10 mM-NaF, 10 min dark	303

(Table 1, *b*). A reconstituted chloroplast system was used to examine whether the thylakoid protein kinase was involved with the phosphorylation of stromal proteins. In this system isolated thylakoids were reassociated with stromal proteins and mediating metabolites (such as ferredoxin, NADPH and ATP) in such a way that biochemical communication between the membrane and soluble proteins was restored and the resultant mixture was capable of catalysing Calvin-cycle activity [21]. However, in the experiment shown in Table 2, no metabolite substrate was provided to promote Calvin-cycle activity, and CO_2 assimilation was therefore negligible. In this situation the level of stromal protein phosphorylation was similar in the light (Table 2, *b*) and in the dark (Table 2, *a*), and the presence of DL-glyceraldehyde had no effect on the level of stromal protein phosphorylation (Table 2, *e*). In the reconstituted chloroplast system, NADPH (produced by photoreduction of NADP) is required to drive the reductive phase of the Calvin cycle and also to maintain the activity of certain chloroplast enzymes whose activity is modulated via redox changes. In these experiments, NADPH (rather than NADP) was used to achieve activation of the thylakoid protein kinase in darkness, and with some samples this was supplemented with light to ensure maximum activation of the enzyme. There was little difference in the level of stromal protein phosphorylation under these conditions, which would promote maximum thylakoid protein kinase activity (Table 2, *a*, *b*) and that obtained in the dark in the absence of NADPH (Table 2, *c*), where thylakoid protein kinase activity would be expected to be minimal, or even in the absence of thylakoids (Table 2, *d*). This suggests that the thylakoid protein kinase was not involved in stromal protein phosphorylation. The presence of fluoride, a general inhibitor of phosphatase activity, did not significantly increase the measured rate or maximal level of stromal protein phosphorylation (Table 2, *g*). This may suggest that protein phosphatase activity in the stroma is low.

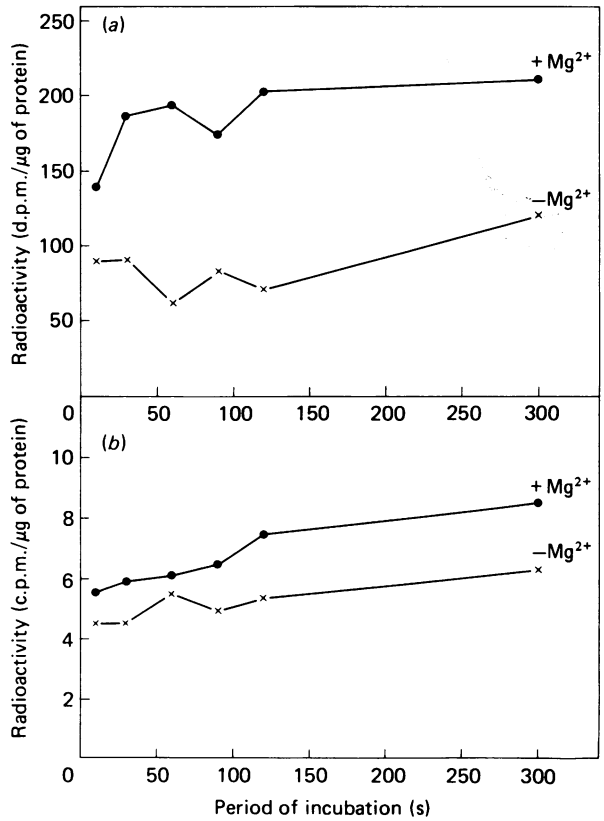


Fig. 5. Phosphorylation of stromal enzyme protein with [γ-^{32}P]ATP in the presence and absence of 10 mM-$MgCl_2$

Reactions were stopped at the times indicated by the addition of 6% (w/v) trichloroacetic acid and protein phosphorylation [in d.p.m. (*a*) or c.p.m. (*b*)] estimated either directly by liquid-scintillation counting of radioactivity in the washed protein extracts (*a*) or by Čerenkov counting of excised protein bands after electrophoresis.

The phosphorylation of isolated stromal proteins was found to occur in a simple reaction medium consisting of isolated protein, buffer and ATP. The addition of NADPH and ferredoxin, which are necessary catalysts and activators for carbon metabolism, did not change the total level of protein phosphorylation. Stromal protein phosphorylation was more rapid and attained a higher maximum level in the presence of Mg^{2+} than in its absence (Fig. 5). This was most clearly demonstrated when the level of ^{32}P incorporation was measured in washed total protein extracts directly after phosphorylation (Fig. 5*a*), but was less apparent when the total level of phosphorylation was estimated from the sum of the radioactivity in individual excised polypeptide bands after SDS/polyacrylamide-gel electrophoresis (Fig. 5*b*).

DISCUSSION

The procedures used here allow the measurement of stromal protein phosphorylation, which is the net result of stromal protein kinase and phosphatase activity. The results obtained both with intact chloroplasts and with the reconstituted chloroplast system suggest that the stroma contains protein kinase activity and that stromal protein phosphorylation is a discrete event not requiring the participation of the thylakoid protein kinase. The thylakoid protein kinase is light-activated [25] via a process involving the redox state of plastoquinone [1,10,26] and also the transthylakoid ΔpH [27]. The stromal protein kinase activity is not modulated by light or light-generated reductants such as NADPH and reduced ferredoxin. The mature form of the small subunit of ribulose-1,5-bisphosphate carboxylase has previously been shown to be phosphorylated by a protein kinase bound to the outer envelope membrane of the choloroplast [8]. With the procedures used here in intact chloroplasts the [γ-^{32}P]ATP required for phosphorylation of stromal proteins is generated within the chloroplast stroma from added [^{32}P]P_i and endogenous ADP. Stromal ATP is not transported across the envelope in mature spinach chloroplasts at a significant rate. This would suggest that protein kinase activity associated with the outer chloroplast envelope membrane [8] is not involved with the phosphorylation of stromal protein in intact chloroplasts reported here. Similarly the envelope proteins, which represent 0.7–1.0% of total chloroplast protein in spinach [28], would be largely removed from the stromal extracts by the procedures used.

The function of stromal protein phosphorylation is unknown, but may be related, at least in part, to the synthesis and assembly of chloroplast proteins. The

stromal polypeptide of approx. M_r 70000 had the highest specific activity of labelling and is probably analogous to the major labelled stromal protein of M_r 66000 reported previously [2], which was suggested to possibly be an assembly protein for ribulose-1,5-bisphosphate carboxylase. Some of the other phosphorylated species may originate from chloroplast ribosomal proteins. The results presented here suggest that the function of phosphorylation of most labelled stromal polypeptides is largely unrelated to photosynthesis. However, in the case of both subunits of ribulose-1,5-bisphosphate carboxylase, stimulation of enzyme protein phosphorylation was found to occur during CO_2 fixation and may therefore be involved with enzyme activity. The results also suggest that there was little, if any, competition between the thylakoid and stromal protein kinases and the Calvin cycle for ATP. In contrast with the situation with intact chloroplasts, the phosphorylation of the subunits of ribulose-1,5-bisphosphate carboxylase was not readily observed in stromal extracts phosphorylated in the reconstituted chloroplast system or in isolated stroma incubated with [γ-^{32}P]ATP. This may be because of the loss or dilution of an endogenous regulatory intermediate. The ribulose-1,5-bisphosphate carboxylase accounted for about 50% of the total stromal protein. The labelling of this protein might therefore be considered to be the circumstantial result of non-specific phosphorylation. However, the significant variations in the level of phosphorylation of both subunits of ribulose-1,5-bisphosphate carboxylase, which occurred in parallel with changes in carbon assimilation in intact chloroplasts, suggest that phosphorylation of this enzyme protein could be related to function.

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