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Phosphorylation of a stromal enzyme protein in maize (*Zea mays*) mesophyll chloroplasts

Christine FOYER
Research Institute for Photosynthesis, University of Sheffield, Sheffield S10 2TN, U.K.

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When intact maize (*Zea mays*) mesophyll chloroplasts were illuminated in the presence of [^{32}P]orthophosphate and subsequently subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, a major polypeptide species of M_r 100000 was found to be heavily labelled. This polypeptide was not found in maize mesophyll thylakoid or cytoplasmic fractions, but was localized solely in the chloroplast stroma. No phosphorylation of polypeptides in the 100000-M_r region was observed in the mesophyll chloroplasts from C_3 species (where the primary product of CO_2 fixation is a 3-carbon compound), suggesting that this polypeptide arises from a protein associated with C_4 metabolism (where the first product of CO_2 fixation is a 4-carbon compound). The 100kDa polypeptide was major component of the maize mesophyll chloroplast, comprising 10–15% of the total protein, which banded in an identical position to the apoprotein of the enzyme pyruvate,orthophosphate dikinase, which catalyses a reaction of the C_4 cycle [Edwards & Walker (1983) *C3,C4: Mechanisms, and Cellular and Environmental Regulation, of Photosynthesis*, Blackwell Scientific Publications, Oxford and London]. Phosphorylation in the 100kDa species was prohibited by treatment of lysed chloroplasts with antibody to pyruvate,orthophosphate dikinase (EC 2.7.9.1). These data suggest that the phosphorylated polypeptide observed after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis is the monomeric form of this enzyme. The 100kDa polypeptide was partially phosphorylated in darkness, but a significant increase in the degree of phosphorylation was found on illumination. This polypeptide was found to be dephosphorylated only slowly when the chloroplasts were returned to darkness. Maximum phosphorylation was observed in the presence of pyruvate or dihydroxyacetone phosphate, which also caused maximum activation of pyruvate,orthophosphate dikinase. Phosphorylation of the 100kDa polypeptide did not coincide with deactivation of pyruvate, orthophosphate dikinase, but maximum phosphorylation occurred under conditions that promoted maximum activity of the enzyme, at which time one phosphate group was associated with each enzyme molecule. Protein phosphorylation did not appear to arise from the reaction mechanism of the enzyme.

The regulation of protein kinases leading to an altered state of phosphorylation of specific substrate proteins is considered to be an important mechanism in the control of cellular metabolism (Greengard, 1978). In the chloroplast the presence of Mg^{2+}-dependent thylakoid-bound protein kinase activity has been clearly established (Bennett, 1983; Horton, 1983; Staehelin & Arntzen, 1983).

Abbreviations used: DHAP, dihydroxyacetone phosphate; LHCP, light-harvesting chlorophyll-*a*/*b*-binding protein; OAA, oxaloacetate; PGA, 3-phosphoglycerate; SDS, sodium dodecyl sulphate.

The substrates for this kinase are the light-harvesting chlorophyll-*a*/*b*-binding protein (LHCP) and some of the polypeptides associated with photosystem II (Bennett, 1983; Allen, 1983). Several stromal components are also subject to phosphorylation, but the identity of these phosphorylated species remains to be elucidated. In the present paper the effects of various substrates on the phosphorylation status and activity of the stromal protein, pyruvate,orthophosphate dikinase, *in situ*, in intact maize mesophyll chloroplasts, are described. Pyruvate,orthophosphate dikinase is an enzyme of the C_4 cycle that is localized in the

maize mesophyll stroma and undergoes light-mediated activation (Hatch, 1978). This process does not appear to involve reduction/oxidation changes in the enzyme protein (Chapman & Hatch, 1981; Nakamoto & Sugiyama, 1982), even though activation is linked to photosynthetic electron transport (Yamamoto *et al.*, 1974). Orthophosphate is essential in the activation of the enzyme, as is a high-M_r heat-labile protein that acts as a catalyst for the interconversion between the inactive and active species (Burnell & Hatch, 1983). Inactivation requires ADP and low amounts of ATP (Chapman & Hatch, 1981; Nakamoto & Sugiyama, 1982) plus the protein factor and occurs under both anaerobic and aerobic conditions *in vitro* (Nakamoto & Edwards, 1983). The inactivation process has been correlated with phosphorylation of a threonine residue on the enzyme protein, the phosphate group originating from the β-position of ADP (Ashton & Hatch, 1983). Pyruvate,orthophosphate dikinase catalyses the reversible phosphorylation of pyruvate and P_i, utilizing two phosphate groups of a single molecule of ATP in the following reaction:

$$\text{Pyruvate} + \text{ATP} + P_i \rightarrow \text{phosphoenolpyruvate} + \text{AMP} + PP_i \quad (1)$$

Phosphorylation of the enzyme protein from the β-position of ATP is an integral part of the reaction mechanism, in a reaction sequence suggested by Andrews & Hatch (1969) to be as follows:

$$\text{Enzyme} + \text{ATP} + P_i \rightarrow \text{enzyme-P} + \text{AMP} + PP_i \quad (2)$$

$$\text{Enzyme-P} + \text{pyruvate} \rightarrow \text{enzyme} + \text{phosphoenolpyruvate} \quad (3)$$

In micro-organisms, the active site of the enzyme contains a histidine residue that is phosphorylated during the reaction sequence (Spronk *et al.*, 1976). Phosphorylation of the enzyme protein may result, therefore, from either the reaction mechanism of the enzyme or the activation/inactivation process, which has been shown to involve formation of a phosphothreonine residue (Ashton & Hatch, 1983).

Materials and methods

Maize (*Zea mays* var. Kelvedon Glory) was grown in a greenhouse under sunlight with supplemented lighting at a minimum temperature of 20°C. Protoplasts were prepared from secondary leaves of 12–14-day-old plants by the method of Day *et al.* (1981) and chloroplasts were isolated from these protoplasts essentially as described by Edwards *et al.* (1979). The isolation medium consisted of 0.4M-sorbitol, 25mM-Tricine {*N*-[2-hydroxy - 1,1 - bis(hydroxymethyl)ethyl]glycine}/KOH buffer, pH7.4, 10mM-EDTA and 0.3mM-KH_2PO_4. The chloroplasts prepared in this way were over 95% intact as determined by ferricyanide-dependent O_2 evolution before and after osmotic shock. The clear soluble supernatant fraction remaining after the chloroplasts had been pelleted was designated the cytoplasmic fraction.

^{32}P incorporation was carried out in intact chloroplasts incubated at 20°C in Hansatech oxygen electrodes either in darkness or in red light at an irradiance of $300\,W \cdot m^{-2}$. The reaction medium consisted of 0.4M-sorbitol, 25mM-Tricine/KOH buffer, pH8.1, 10mM-EDTA, catalase (400 units), 0.3mM-P_i, 200μCi of [^{32}P]P_i with chloroplasts added to give a chlorophyll concentration of $50\,\mu g \cdot ml^{-1}$ in a final volume of 2ml. Equilibration of [^{32}P]P_i between the stroma and the reaction medium is rapid because of P_i exchange across the chloroplast envelope. Substrates were added as indicated at the following concentrations: 3-PGA (2.5mM), OAA (2.0 or 0.2mM), pyruvate (10mM) and DHAP (0.2mM). Duplicate samples were taken as indicated and rapidly mixed with 25μl of 100% (w/v) trichloroacetic acid. The precipitated proteins were extracted with acetone and the insoluble fraction (approx. 40μg of protein) was subjected to electrophoresis in the presence of sodium dodecyl sulphate on 10–30% (w/v)-polyacrylamide gradient slab gels using the buffers of Laemmli (1970). After electrophoresis the gels were stained with Coomassie Brilliant Blue R, destained, dried and autoradiographed by using Kodak X-Omat RP X-ray film. ^{32}P incorporation into proteins was determined in the excised bands of specific apoproteins by Čerenkov counting in a Beckman LS 7500 liquid-scintillation counter over a 30min period.

Incorporation of ^{32}P from [γ-^{32}P]ATP into polypeptides in cytoplasmic and stromal extract was measured in reaction media (250μl volume) consisting of 5mM-dithiothreitol, 5mM-$MgCl_2$, 2mM-KH_2PO_4, 20mM-Tris/HCl buffer, pH8.1, and 0.4mM-[γ-^{32}P]ATP (125Ci/mmol), to which stromal or cytoplasmic protein was added at an amount equivalent to 50μg of chlorophyll. Duplicate reactions were stopped after 10min by the addition of 25μl of 100% (w/v) trichloroacetic acid, and ^{32}P incorporation into the 100kDa polypeptide was measured as described above. For the antibody-precipitation treatments, various amounts of antibody were added to the stromal incubation medium. After 10min the precipitated material was removed by centrifugation and the amount of ^{32}P incorporation in the 100kDa region was compared with that in untreated controls by the methods described above. The stromal extracts were prepared by rupturing the chloroplasts in a

10-fold dilution with 5mM-dithiothreitol and rapidly removing the insoluble material by centrifugation at 12000*g* for 5min. The stromal extracts were used immediately after preparation.

Pyruvate,orthophosphate dikinase activity was determined spectrophotometrically by the method of Hatch & Slack (1975) in samples taken from duplicate incubations carried out for ^{32}P incorporation, except that [^{32}P]P_i was omitted. Samples (100μl; $\equiv 5\mu$g of chlorophyll) were taken at the times indicated, ruptured with 0.1% Triton X-100 and assayed immediately in a reaction volume of 1ml. Measurements of ^{32}P incorporation and pyruvate,orthophosphate dikinase activity were carried out simultaneously.

Protein was determined by using a dye-binding-protein-assay kit purchased from Bio-Rad Laboratories, München, Germany, with bovine γ-globulin and bovine serum albumin as protein standards. After staining with Coomassie Brilliant Blue the gels were scanned with a Gilford spectrometer with a densitometer attachment. ^{32}P incorporation into the 100kDa polypeptide region was calculated (on a mol-of-^{32}P/mol-of-100kDa-protein basis) from the protein measurements and the c.p.m./mmol of [^{32}P]P_i from the specific radioactivity in the reaction medium (200μCi of ^{32}P with 0.3mM-P_i in 2ml) measured as for the samples by Čerenkov counting.

Results

When intact maize mesophyll chloroplasts were illuminated in the presence of [^{32}P]P_i for 10min, a number of polypeptides were found to be phosphorylated (Fig. 1). The major phosphorylated species were found at positions corresponding to M_r 9000, 32000, 24000–26000 (the latter are the apoprotein monomeric forms of the LHCP) and 100000. The 100kDa polypeptide has previously been shown to be derived from the enzyme pyruvate,orthophosphate dikinase (Ashton & Hatch, 1983). The following observations support this conclusion. Phosphorylation of a polypeptide in the 100kDa region was not observed in C_3 chloroplasts. This polypeptide is a major protein component of the maize mesophyll chloroplast and derives from a soluble protein localized in the stroma (Fig. 1). After SDS/polyacrylamide-gel electrophoresis this polypeptide was found to band in a similar position to purified pyruvate,orthophosphate dikinase (Fig. 2), which is a tetramer of approx. M_r 387000 in maize. These observations suggested that the 100kDa polypeptide was a C_4 component of the mesophyll stroma. A banding position equivalent to that of pure pyruvate,orthophosphate dikinase after SDS/polyacrylamide-gel electrophoresis would suggest that the phosphorylated polypeptide of M_r nearly 100000 is the monomeric form of this enzyme. In support of this, the addition of anti(pyruvate,orthophosphate dikinase) antibody to a stromal fraction incubated with [γ-^{32}P]ATP and subsequently centrifuged at 12000*g* for 5min caused a depletion of ^{32}P incorporation in the 100kDa region.

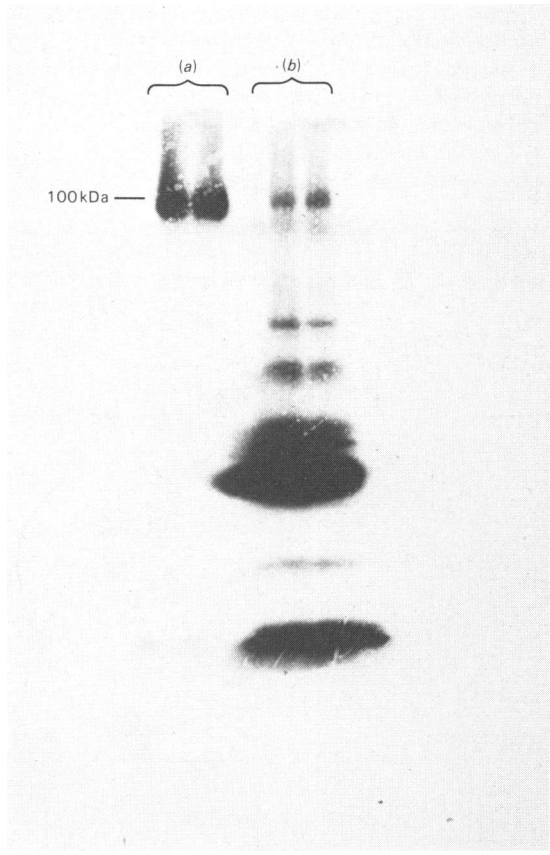

Fig.1. *Autoradiogram of stromal and thylakoid fractions from maize mesophyll chloroplasts (100μg of chlorophyll) that had been illuminated for 10min in the absence of added organic substrates*

After illumination the chloroplasts were rapidly (12000*g*, 2min) pelleted and lysed in 10mM-EDTA. The stromal (*a*) and thylakoid (*b*) fractions were separated by centrifugation and subjected to SDS/polyacrylamide-gel electrophoresis.

In contrast with the phosphorylated thylakoid polypeptides, which require light for plastoquinone-mediated activation of the thylakoid protein kinase, and thus phosphorylation, the 100kDa polypeptide showed significant phosphorylation in darkness (Table 1). However, it was evident that when the maximal level of phosphorylation achieved in darkness was obtained (after

approx. 15min incubation), a considerable increase in the extent of phosphorylation could be achieved during illumination. This light-stimulated phosphorylation was slowly reversed when the chloroplasts were returned to the dark (Table 1).

Carbon metabolism in isolated maize mesophyll chloroplasts can be modulated by the addition of metabolites such as pyruvate, OAA, PGA and DHAP. These metabolites readily enter the chloroplast and allow manipulation of the energy and redox status of the stroma by promoting the activities of certain component enzymes (Fernyhough *et al.*, 1983). The addition of pyruvate to the chloroplast reaction medium stimulated increased phosphorylation of both the LHCP and the 100kDa polypeptide in the light above the level measured when no metabolite was added (Table 2). The increase in phosphorylation status of LHCP under these conditions may be explained in terms of redox regulation of the thylakoid protein kinase (Fernyhough *et al.*, 1983). The stimulatory action of pyruvate on the phosphorylation of the 100kDa polypeptide was observed both when this substrate was present from the beginning of the experiment and when pyruvate was added during illumination (Table 3). When pyruvate was added after 5min illumination (Table 3*b*) the level of enzyme activity and extent of phosphorylation of the 100kDa polypeptide were both substantially increased compared with that measured in chloroplasts illuminated in the absence of substrate (Table 3*a*).

Light and substrate modulation of pyruvate,orthophosphate dikinase activity in isolated intact maize mesophyll chloroplasts was observed (Tables 1, 3 and 4). In the absence of added substrates the activity of the enzyme was low in darkness but increased severalfold on illumination via an activation process that was slowly reversed by a subsequent dark period (Table 1). Light-dependent activation of pyruvate,orthophosphate dikinase in intact maize mesophyll protoplasts and chloroplasts was complete after 10–15min illumination. The addition of the substrate pyruvate to chloroplast incubation medium caused enzyme activation in the dark (Tables 1 and 3) and frequently promoted an increased level of activation in the light (Table 3). However, when chloroplasts had been incubated with pyruvate for a long period in the dark before illumination, the subsequent increase in activity in the light was not significant when compared with that attained

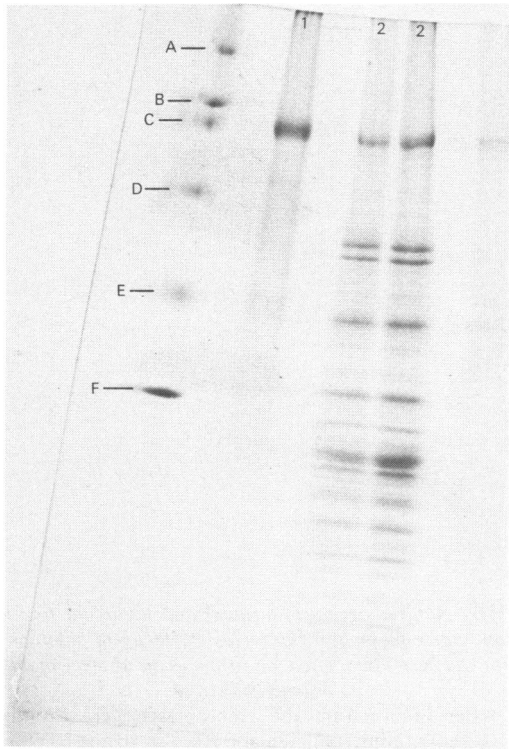

Fig. 2. *Slab gel of purified pyruvate,orthophosphate dikinase (1) and maize mesophyll chloroplasts (2) stained for protein with Coomassie Brilliant Blue after SDS/polyacrylamide-gel electrophoresis*

Marker proteins were: A, myosin (M_r 205000); B, β-galactosidase (116000); C, phosphorylase *b* (97400); D, bovine serum albumin (66000); E, ovalbumin (45000); and F, carbonic anhydrase (29000).

Table 1. *Pyruvate,orthophosphate dikinase activity and extent of phosphorylation of the 100kDa polypeptide, during dark–light–dark transitions, with maize mesophyll chloroplasts*

Conditions	Pyruvate,orthophosphate dikinase [μmol·h^{-1}·(mg of chlorophyll)$^{-1}$]	Extent of phosphorylation of the 100kDa polypeptide (c.p.m.)
(*a*) In the absence of substrate		
30min dark	23	232
10min light	60	343
15min dark	14	375
(*b*) In the presence of 10mM-pyruvate		
30min dark	43	313
10min light	63	1294
15min dark	37	624

Table 2. *Effect of substrates on the extent of phosphorylation of LHCP and the 100kDa polypeptide in intact maize mesophyll chloroplasts in the light*

Substrate	Phosphorylation (c.p.m.) in: LHCP	100kDa polypeptide
None	803	208
Pyruvate (10mM)	1003	379
Oxaloacetate (2mM)	290	149
3-Phosphoglycerate (2.5mM)	154	168

Table 3. *Effect of pyruvate on pyruvate,orthophosphate dikinase activity and extent of phosphorylation of the 100kDa polypeptide in maize mesophyll chloroplasts*

	(*a*) In the absence of pyruvate		(*b*) With pyruvate added after 5min illumination	
Conditions	Pyruvate,orthophosphate dikinase activity [μmol·h^{-1}·(mg of chlorophyll)$^{-1}$]	Phosphorylation (c.p.m.)	Pyruvate,orthophosphate dikinase activity [μmol·h^{-1}·(mg of chlorophyll)$^{-1}$]	Phosphorylation (c.p.m.)
15min dark	16	232	14	370
5min light	27	491	28	510
10min light	31	818	50	1004
20min light	38	912	90	1724

Table 4. *Effect of added substrates on pyruvate,orthophosphate dikinase activity and phosphorylation in maize mesophyll chloroplasts*

	Pyruvate,orthophosphate dikinase activity [μmol·h^{-1}·(mg of chlorophyll)$^{-1}$]		Phosphorylation (c.p.m.)	
Conditions	Expt. 1	Expt. 2	Expt. 1	Expt. 2
(*a*) No added substrate				
15min dark	18	33	36	35
10min light	70	104	69	102
(*b*) +200μM-DHAP				
15min dark	193	57	195	84
10min light	251	143	138	112
(*c*) +200μM-OAA				
15min dark	22	47	70	71
10min light	86	111	123	129

without substrate (Table 1). The lack of pyruvate-enhanced activation in these circumstances may possibly be caused by loss of pyruvate due to decarboxylation exacerbated by the alkaline pH of the reaction medium. However, a significant increase in the level of phosphorylation of the 100kDa polypeptide was still promoted in these circumstances. Such experiments also demonstrate that there is no clear correlation between increased enzyme activity and the level of phosphorylation, even though the activity is always high when the extent of phosphorylation is maximum. Nevertheless, it was evident that no correlation between enzyme inactivation and an increase in the extent of phosphorylation could be demonstrated in these studies.

The amount of protein present in the 100kDa region was calculated to be approx. 5μg and was found to account for 11–13% of the total chloroplast protein as determined by Coomassie Brilliant Blue staining. During illumination, the level of phosphorylation was of the same order as LHCP phosphorylation (Table 2). From the phosphorylation data obtained in the presence of pyruvate in the light, it was calculated that approx. one phosphate group was bound per four 100kDa polypeptides. If it is assumed that the holoenzyme is a tetramer comprising four subunits of M_r 100000, then this would suggest that one phosphate group was bound per enzyme molecule in these circumstances.

Incorporation of ^{32}P into the 100kDa polypeptide was observed when a stromal fraction from maize mesophyll chloroplasts was incubated with [γ-^{32}P]ATP. The incorporation of ^{32}P into the 100000-M_r species was linear for at least 15min

and was not significantly inhibited by the presence of the adenylate kinase inhibitor P^1,P^5-di-(adenosine-5′)pentaphosphate (Ap_5A; Lienhard & Seceniski, 1973), suggesting that ATP and not ADP was the phosphorylating substrate in this reaction.

Maize mesophyll chloroplasts contain the enzymes of the reductive phase of the Calvin cycle, phosphoglycerate kinase and glyceraldehyde-phosphate dehydrogenase ($NADP^+$). When intact chloroplasts were incubated in darkness with $[^{32}P]P_i$, the $[\gamma\text{-}^{32}P]ATP$ required for the phosphorylation process may be produced by the action of phosphoglycerate kinase (eqn. 4). This enzyme, together with glyceraldehyde-phosphate dehydrogenase (eqn. 5) catalyses a freely reversible reaction:

$$[^{32}P]\text{Diphosphoglycerate} + \text{ADP} \rightarrow [\gamma\text{-}^{32}P]\text{ATP} + \text{3-phosphoglycerate} \quad (4)$$

$$\text{Triose phosphate} + \text{NADPH} + [^{32}P]P_i \rightarrow \text{NADP} + [^{32}P]\text{diphosphoglycerate} \quad (5)$$

In support of this mechanism of $[\gamma\text{-}^{32}P]ATP$ formation, the addition of the triose phosphate DHAP was found to increase the extent of phosphorylation of the 100kDa polypeptide in darkness (Table 4). In this situation no further phosphorylation was observed on illumination. In the presence of DHAP, activation of pyruvate,orthophosphate dikinase was also promoted in darkness, suggesting that there is possibly a relationship between phosphorylation status and enzyme activation (Table 4). When chloroplasts were incubated in darkness with DHAP, the activity of the enzyme was severalfold greater than that measured in chloroplasts incubated in the absence of substrate.

The presence of either OAA or 3-PGA in the chloroplast incubation medium had little effect on the activity of the enzyme or on the phosphorylation state of the protein (Tables 2 and 4). When the concentration of OAA was low (200mM), no effect on phosphorylation or enzyme activity was observed, but in the presence of high (2mM) concentrations of OAA, some inhibition of phosphorylation was produced (Table 2). The activity of pyruvate,orthophosphate dikinase could not be measured in the presence of high concentrations of OAA (2mM) or PGA (2.5mM), because at these concentrations the substrates caused 'artefactual' rates in the spectrophotometric assay.

After 10min illumination in the absence of substrate, significant activation of pyruvate,orthophosphate dikinase and phosphorylation of the 100kDa polypeptides were observed. If such phosphorylated chloroplasts were rapidly pelleted after illumination and lysed with a medium consisting of 10mM-pyruvate and 10mM-dithiothreitol, then little decrease in the extent of phosphorylation of the 100kDa polypeptide was subsequently found. These data suggest that the observed phosphorylation did not result from the formation of an enzyme–phosphate complex in the reaction sequence, since the phosphate group would be used in the formation of phosphoenolpyruvate in these circumstances (eqns. 2 and 3).

Discussion

The light-activation of pyruvate,orthophosphate dikinase has been shown to have a requirement for P_i, whereas the de-activation requires ADP and ATP (Hatch, 1978; Chapman & Hatch, 1981; Burnell & Hatch, 1983). The de-activation has been correlated with the phosphorylation of a threonine residue on a 100kDa polypeptide in chloroplast extracts, which was identified as a subunit of pyruvate,orthophosphate dikinase (Ashton & Hatch, 1983). In the present study, intact maize mesophyll chloroplasts have been used to examine the light modulation of pyruvate,orthophosphate dikinase activity and the incorporation of $[^{32}P]$orthophosphate into the 100kDa polypeptide *in situ*. Considerable variation was found in both the maximum activity of pyruvate,orthophosphate dikinase and the absolute amount of phosphorylation in different chloroplast preparations, but these were constant for a given batch of intact chloroplasts. No direct correlation between the extent of enzyme activation and the level of phosphorylation was found in intact chloroplasts. However, maximum phosphorylation of the 100kDa polypeptide was always observed in parallel with high levels of pyruvate,orthophosphate dikinase activity. This is clearly demonstrated in the presence of the metabolites pyruvate (Tables 1 and 3) and DHAP (Table 4). No evidence was found to suggest that an increase in the activity of the enzyme occurred in parallel with the dephosphorylation of the enzyme protein, as was found by Ashton & Hatch (1983) with the isolated enzyme and chloroplast extracts. Indeed, the converse was observed in all experiments with intact chloroplasts, the degree of phosphorylation of the 100 kDa polypeptide being generally highest when the activity of the enzyme was highest.

In these experiments with intact maize mesophyll chloroplasts no direct relationship between phosphorylation and enzyme activity could be demonstrated, and the function of the phosphorylation of the 100kDa polypeptide was not apparent. Indeed, when the chloroplasts were incubated with pyruvate in darkness,

pyruvate,orthophosphate dikinase was activated, but the extent of phosphorylation of the 100kDa polypeptide, although greater than that in the absence of substrate, was not increased proportionally to pyruvate,orthophosphate dikinase activity. Enzyme activity and phosphorylation were maximum during illumination in the presence of pyruvate, even though the activity of the enzyme provided an effective sink for ATP such that ATP concentrations (50–100mM) and [ATP]/[ADP] ratios (0.1–0.2) were very low (Fernyhough *et al.*, 1983).

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