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THE INFLUENCE OF METABOLIC STATE ON THE LEVEL OF PHOSPHORYLATION OF THE LIGHT-HARVESTING CHLOROPHYLL-PROTEIN COMPLEX IN CHLOROPLASTS ISOLATED FROM MAIZE MESOPHYLL

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The activity of the protein kinase that phosphorylates the light-harvesting chlorophyll-protein of Photosystem II (LHCP) has been investigated in intact chloroplasts isolated from maize mesophyll cells. Measurements of ^{32}P incorporation into LHCP, ATP concentration, ATP/ADP ratio, ΔpH, chlorophyll fluorescence and oxygen evolution were made in the presence of different metabolic substrates. Without added substrate a high level of LHCP phosphorylation was observed which was suppressed by addition of oxaloacetate or phosphoglycerate but stimulated by pyruvate. Whereas no correlation was observed between LHCP phosphorylation and adenylate status, a clear effect of redox state on protein kinase activity was observed. A correlation between a highly reduced electron-transfer chain (produced under conditions which favour cyclic electron flow) and the maximum level of protein phosphorylation was observed. The regulation of kinase activity and its dependence on electron transfer and carbon assimilation are discussed.

Introduction

The thylakoid membranes of higher plant chloroplasts show ATP-dependent protein kinase activity which results in phosphorylation of a number of membrane polypeptides including those which comprise LHCP (see Ref. 1 for a review). The physiological role of this protein phosphorylation is to exert control over the rates of photon delivery to the two photosystems [1,2]. Experiments using isolated pea thylakoids clearly demonstrated that the kinase activity was controlled by the redox state of the plastoquinone pool [2–5]. More recently, it has been shown that ADP can act as a kinase inhibitor [6] and subsequently that kinase activity is dependent on energy charge [7]. In the latter work it was suggested that redox transitions merely act as an 'on/off' switch rather than a continuous modulator, the major physiological control being attributed to energy charge [7]. In order to understand fully the role of protein phosphorylation in vivo it is necessary to determine which of these regulators is a major control factor. Experiments using intact pea chloroplasts showed how kinase activity was maximal during the induction period of CO_2-dependent oxygen evolution but declined as the steady state was approached [8]. However, it has been shown that both the redox state and energy state are maximal during induction and both decrease as the rate of CO_2 fixation increases [9,10]. Using a thylakoid system reconstituted for carbon assimu-

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Abbreviations: LHCP, light-harvesting chlorophyll-protein complex; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Q, the primary acceptor of Phtosystem II of which the redox state controls the level of chlorophyll fluorescence; q_Q quenching of chlorophyll fluorescence by oxidized Q; q_e quenching of chlorophyll fluorescence by ΔpH; Tricine, *N*-tris(hydroxymethyl)methylglycine; Chl, chlorophyll; PS, photosystem.

lation, Horton and Foyer [11] demonstrated a correlation between LHCP phosphorylation and redox state as influenced by the presence of 3-phosphoglycerate and the light intensity. Evidence was also found for kinase control by the [ATP]/[ADP] ratio, and it was suggested that perhaps the kinase responds in a concerted manner to the levels of ATP and NADPH [11].

The manipulation of carbon metabolism in isolated intact mesophyll chloroplasts from maize provides an ideal opportunity to distinguish between the regulation of kinase by plastoquinone redox state and by adenylate status.

The maize mesophyll chloroplast contains the enzymes of the reductive phase of the Calvin cycle, phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase [12,13], and the C_4 enzymes, pyruvate, orthophosphate dikinase [12, 13] and NADP-malate dehydrogenase (Fig. 1). Addition of pyruvate stimulates ATP utilisation, the addition of oxaloacetate causes the utilization

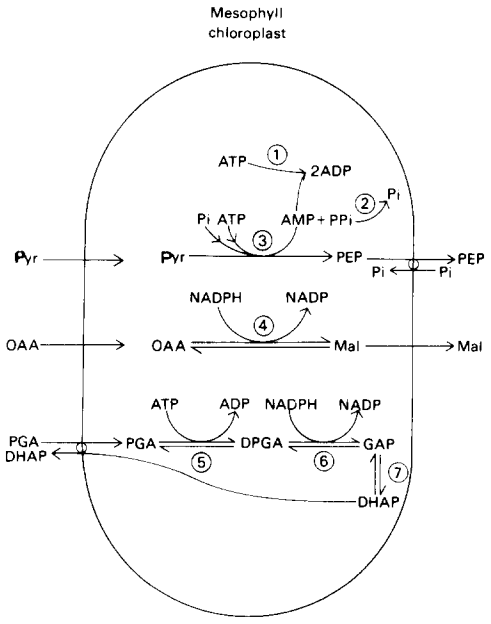

Fig. 1. Diagrammatic representatuon of the metabolism of pyruvate (Pyr), oxaloacetate (OAA) and phosphoglycerate (PGA) by maize mesophyll chloroplasts. (1) Adenylate kinase, (2) pyrophosphatase, (3) pyruvate, orthophosphate dikinase, (4) NADP-malate dehydrogenase, (5) phosphoglycerate kinase, (6) NADP-glyceraldehyde-3-phosphate dehydrogenae, (7) triosephosphate isomerase. GAP, glyceraldehyde phosphate; DHAP, dihydroxyacetone phosphate; PEP, phospho*enol*pyruvate; Mal, malate; DPGA, diphosphoglycerate.

of reducing equivalents without ATP consumption, while the addition of 3-phosphoglycerate requires that both ATP and NADPH are consumed. These metabolites readily enter the chloroplast [12–15], allowing a flexible manipulation of the energy and redox states and of the chloroplast stroma. The response of the thylakoid membrane to the specific demands of the stroma can be measured using 9-aminoacridine fluorescence, chlorophyll fluorescence and oxygen evolution. The nature of the response can then be related to the plastoquinone redox state, [ATP]/[ADP] ratio, ATP concentration and, in turn, to LHCP phosphorylation.

Methods

Maize (*Zea mays*, variety Kelvedon Glory) was grown in a greenhouse under sunlight and supplemented lighting at a minimum temperature of 20°C. Secondary leaves were removed from 12–14-day-old plants and protoplasts isolated by the method of Day et al. [15]. Chloroplasts were isolated from these protoplasts essentially as described by Edwards et al. [16]. The isolation medium consisted of 0.4 M sorbitol, 10 mM EDTA, 25 mM Tricine-KOH buffer (pH 7.4) and 0.3 mM KH_2PO_4. Chloroplast intactness as determined by ferricyanide-dependent O_2 evolution before and after osmotic shock was approx. 95%. The reaction medium contained 0.4 M sorbitol, 10 mM EDTA, 0.3 mM KH_2PO_4, 25 mM Tricine-KOH buffer (pH 8.1), catalase (400 U) and 5 μM 9-aminoacidine, with chloroplasts added to give a chlorophyll concentration of 50 μg Chl/ml. Substrates were added at the following concentrations; 3-phosphoglycerate, 2.5 mM; oxaloacetate, 2.0 mM; and pyruvate, 10 mM. Reactions were terminated using 50 μM DCMU. Red actinic illumination was provided by an Aldiss 2000 150 W projector filtered through a Baltzars K65 interference filter and glass heat filter. Light intensities of 40 and 360 W/m^2 were obtained by using Oriel neutral density filters.

Chlorophyll fluorescence, 9-aminoacridine fluorescence and oxygen evolution were measured simultaneously in a modified Hansatech electrode chamber as described previously [9]. Quenching of chlorophyll fluorescence due to oxidized Q (q_Q)

and the transthylakoid ΔpH (q_e) were distinguished by the relaxation kinetics observed when DCMU is added to illuminated chloroplasts [10]; the fast phase ($t_{1/2}$ approx. 1 s) represented reduction of oxidized Q, whereas the slower relaxation phase ($t_{1/2}$ approx. 15 s) follows the collapse of ΔpH and represents the extent of q_e.

LHCP phosphorylation measurements

Samples for the measurement of ^{32}P incorporation, [ATP] and [ADP] were taken from duplicate oxygen electrodes set up as for fluorescence. [ATP] and [ADP] were measured as described by Carver et al. [17]. Duplicate samples (200 μl) were taken from the reaction medium at the times indicated and were immediately mixed with 25 μl cold 2.5 M $HClO_4$. The soluble fraction was neutralised with 1.25 M K_2CO_3 and [ATP] and [ADP] were assayed in an LKB Wallac Luminometer 1250 with luciferin-luciferase in the form of ATP-monitoring reagent (LKB Wallac). Reactions mixtures for $^{32}P_i$ incorporation were as for the measurement of chlorophyll fluorescence exacept that 250 μCi $^{32}P_i$ were included. Duplicate samples (250 μl) were taken at the times indicated during the incubation and rapidly mixed with 250 μl of 100% trichloroacetic acid. The precipitated proteins were extracted with acetone and the insoluble fraction was subjected to SDS-polyacrylamide gel electrophoresis [11]. ^{32}P incorporation into LHCP was estimated in the excised bands of LHCP apoprotein by Cerenkov counting in a Beckman LS 7500 scintillation counter over a 30 min period. The volume of the isolated intact chloroplasts was taken to be 26 μl/mg Chl.

Results

Figs. 2–4 show the kinetics of oxygen evolution, 9-aminoacridine fluorescence and chlorophyll fluorescence at saturating and low light intensities in chloroplasts incubated with various substrates. In the presence of 3-phosphoglycerate, a rapid quenching of fluorescence is observed both in low and high light (Fig. 2). In low light at steady-state ΔpH is smaller (as judged by the amplitude of quenching of 9-aminoacridine fluorescence and by the size of the q_e component seen upon DCMU addition) and Q is more oxidized (as judged by the extent of q_Q seen upon DCMU addition) compared to values seen at high light (Fig. 2). A similar relationship exists when oxaloacetate is the substrate but here, because of the low rate of ATP consumption, the ΔpH seems very similar at the two light intensities (Fig. 3). Comparing Figs. 2 and 3, it is clear that in the presence of 3-phosphoglycerate, Q is more oxidized and Δ pH smaller compared to oxaloacetate. The extent of Q oxidation correlates with the rate of oxygen evolution.

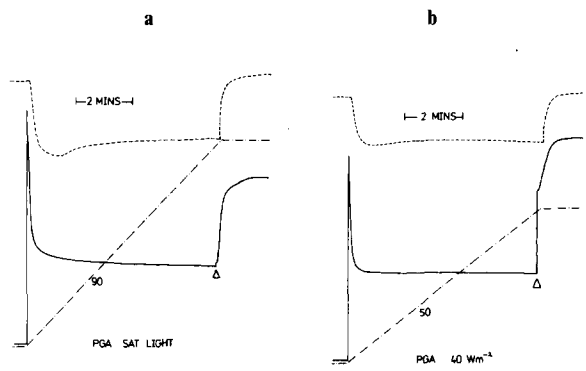

Fig. 2. Measurements on chloroplasts in the presence of 2.5 mM 3-phosphoglycerate (PGA) at (a) saturating (360 W/m^2) and (b) low (40 W/m^2) light intensity. (- - - - -) Fluorescence from 9-aminoacridine, (———) fluorescence from chlorophyll, (– · – · –) oxygen concentration. Numbers indicate the rate of oxygen evolution in μmol O_2/mg Chl per h. The traces are not corrected for a pen gap between each of the above. Δ indicates addition of 50 μM DCMU. Conditions were as described in Materials and Methods.

In the absence of substrate (Fig. 4a) no net O_2 evolution is observed and chlorophyll fluorescence is quenched only by ΔpH. Upon DCMU addition

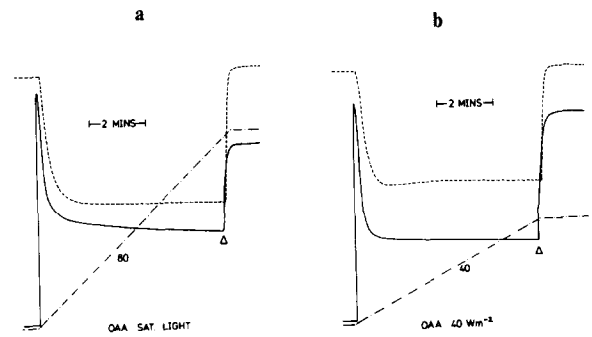

Fig. 3. Measurements on chloroplasts in the presence of 2 mM oxaloacetate (OAA) at saturating (a) and low (b) light intensity. For details see Fig. 2.

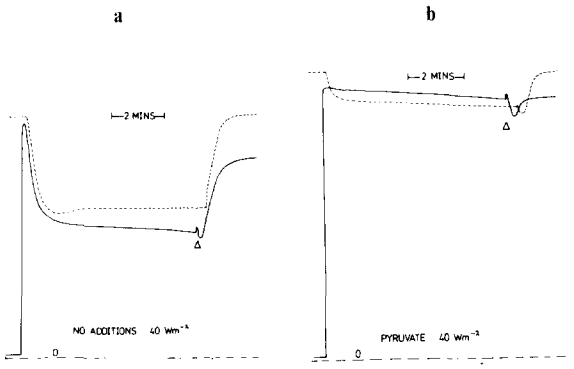

Fig. 4. Measurements on chloroplasts with no added substrate (a) and in the presence of 10 mM pyruvate (b). Light intensity was 40 W/m^2. For details see Fig. 2.

a rapid quenching is seen following a small rise before the relaxation of q_e. This quenching component seen upon DCMU addition has been interpreted as being due to PS I-induced oxidation of plastoquinol [18]. Observation of this phenomenon indicated that plastoquinone is highly reduced in the steady state.

When pyruvate is present the ΔpH seen in Fig. 4a is largely dissipated due to ATP consumption and hence fluorescence quenching is minimal in either high or low light (Fig. 4b). Upon addition of DCMU the transient fluorescence quenching was again observed but in this case to an even larger extent than in Fig. 4a. A transient increase in ΔpH was also seen. The much larger extent of this DCMU-induced quenching indicates a highly reduced plastoquinone pool in the presence of pyruvate.

In Table I, the levels of phosphorylation of LHCP, and the [ATP]/[ADP] and [ATP] values are shown for the experiments described in Figs. 2–4. With no added substrate the [ATP]/[ADP] ratio is doubled in steady-state illumination either in high or low light. A large amount of ^{32}P incorporation was observed which was partially reversed by phosphatase activity in darkness. Addition of oxaloacetate, which produced an even higher steady-state ATP/ADP ratio, suppressed protein phosphorylation, even in saturating light (Table I, B). An even larger suppression of LHCP phosphorylation occurred in the presence of 3-phosphoglycerate (Table I, C). On the other hand, pyruvate either maintained or even increased the amount of phosphorylation (Table I, D). This high level of protein phosphorylation was observed despite an ATP concentration of only approx. 100

TABLE I

THE EFFECT OF SUBSTRATES ON LHCP PHOSPHORYLATION AND ADENYLATE STATUS

Experiments were carried out as described in Methods with the substrates present throughout the experiments. The added levels were: oxaloacetate, 2 mM; 3-phosphoglycerate, 2.5 mM; and pyruvate, 10 mM. Each block of 3 data refers to a separate experiment on a different batch of freshly prepared chloroplasts. LHCP phosphorylation was determined by counting excised bands for 30 min. Each count is the mean of two separate chloroplast samples. ATP and ADP measurements were the means three samples.

Conditions	LHCP phosphorylation (cpm)	[ATP]/[ADP]	[ATP] (mM)
(A) No added substrate			
5 min dark	79	0.8	0.62
10 min low light	766	1.6	0.78
15 min dark	394	0.8	0.71
5 min dark	75	0.7	0.10
10 min saturating light	1043	1.4	0.41
15 min dark	287	0.2	0.30
(B) + oxaloacetate			
5 min dark	21	0.4	0.32
10 min low light	174	1.5	0.60
15 min dark	38	1.0	0.38
5 min dark	29	0.3	0.24
10 min saturating light	198	2.3	0.64
15 min dark	93	0.4	0.15
(C) + 3-phosphoglycerate			
5 min dark	99	1.0	0.15
10 min low light	70	1.0	0.34
15 min dark	216	0.3	0.14
5 min dark	11	0.6	0.10
10 min saturating light	149	0.7	0.37
15 min dark	215	0.5	0.17
(D) + pyruvate			
5 min dark	22	0.17	0.02
10 min low light	1941	0.10	0.03
15 min dark	338	0.06	0.02
5 min dark	16	0.22	0.08
10 min saturating light	1049	0.11	0.11
15 min dark	183	0.01	0.15

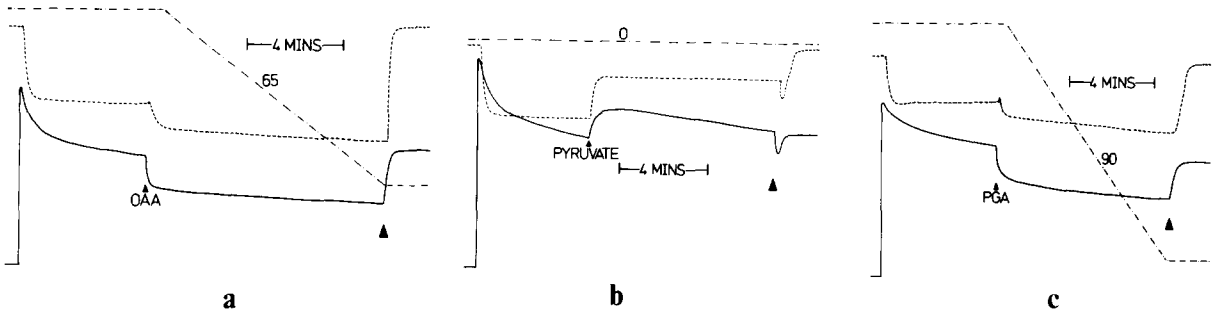

Fig. 5. Effects of substrate addition to chloroplasts preilluminated without substrate addition. (a) 2 mM oxaloacetate (OAA), (b) 10 mM pyruvate, (c) 2.5 mM 3-phosphoglycerate (PGA). Light intensity was 360 W/m^2. For other details see Fig. 2.

μM and an [ATP]/[ADP] ratio of 0.1. In the presence of pyruvate a consistently higher level of phosphorylation was seen at low light compared to high light. In contrast, in all other situations (Table I, A–C) more labelling was measured in high light. In order to examine the reversibility of these metabolic states on the level of LHCP phosphorylation successive additions of different substrates were made during illumination. This approach would also help overcome the variabilities often found both in the total adenylate pool size and absolute amount of phosphorylation seen in different chloroplast preparations. In all cases 5 min of saturating actinic illumination proceeded substrate addition at which time a consistently high level of LHCP phosphorylation was achieved. Figs. 5 and 6 show the changes in O_2 evolution, chlorophyll fluorescence and 9-aminoacridine fluorescence, whilst in Table II associated measurements of adenylates and protein phpsphorylation are given. As expected addition of either 3-phosphoglycerate or oxaloacetate elicits dephosphorylation which is accompanied by an increase in ΔpH, chlorophyll fluorescence quenching and the rate of oxygen evolution (Fig. 5a and b and Table II, A–C) The DCMU-induced quenching normally seen for 'no additions' (Fig. 4a) is removed by these substrates. Addition of pyruvate causes a decrease in ΔpH and fluorescence quenching but causes a substantial elevation in LHCP phosphorylation (Fig. 5c and Table II, D). It should be noted that in Fig. 6c a large DCMU-induced quenching is observed after pyruvate has been added. The relationship between pyruvate-induced stimulation and oxaloacetate-induced suppression of LHCP phosphorylation is examined in Fig. 6. If oxaloacetate addition is made first (Fig. 6a and Table II, E) dephosphorylation occurs and pyruvate is no longer able to increase phosphorylation. Pyruvate under these conditions can decrease ΔpH and the [ATP]/[ADP] ratio (from 2.1 to 0.9) but cannot restore a high redox state as evidenced from the absence of a DCMU-induced quenching. Thus, neither pyruvate directly nor its effect on the [ATP]/[ADP] ratio can be the sole causative factors in its stimulation of the protein kinase. A

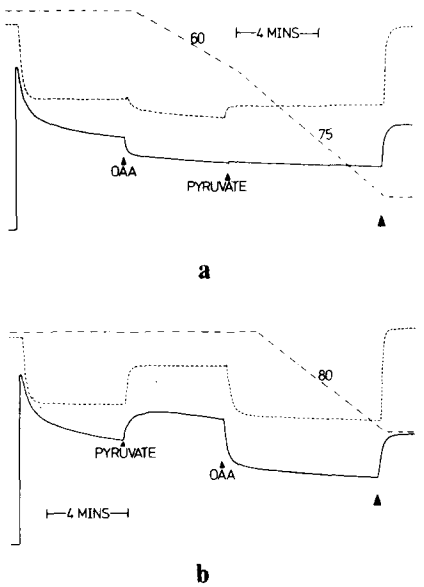

Fig. 6. Effects of sequential additions of pyruvate and oxaloacetate to chloroplasts pre-illuminated without substrate addition. (a) 2 mM oxaloacetate (OAA) followed by 10 mM pyruvate, (b) 10 mM pyruvate followed by 2 mM oxaloacetate. Light intensity was 360 W/m^2. For other details see Fig. 2.

TABLE II

EFFECT OF SUBSTRATE ADDITION ON LHCP PHOSPHORYLATION AND ADENYLATE STATUS

Experiments were carried out as described in Methods. After 5 min of saturating light 3-phosphoglycerate (2.5 mM), oxaloacetate (2 mM) or pyruvate (10 mM) were added. After a further 10 min illumination samples were taken for analysis. In E and F a second substrate was added 5 min after the first addition and a further 5 minutes illumination given in the presence of both substrates. LHCP counts were obtained as in Table I.

Conditions	LHCP phospho-rylation (cpm)	[ATP]/[ADP]	[ATP] (mM)
(A) No additions			
5 min dark	73	0.6	0.32
5 min light	2026	1.6	0.58
15 min light	2603	1.4	0.62
(B) + 3-phosphoglycerate			
5 min dark	90	0.2	0.21
5 min light	1596	1.5	0.45
10 min light + 3-phospho-glycerate	1049	1.3	0.26
(C) + oxaloacetate			
5 min dark	47	0.7	0.30
5 min light	1868	1.4	0.94
10 min light + oxaloacetate	599	2.4	1.16
(D) + pyruvate			
5 min dark	282	0.3	0.44
5 min light	1132	1.5	1.38
10 min light + pyruvate	1749	0.7	0.75
(E) + oxaloacetate + pyruvate			
5 min dark	56	0.3	0.32
5 min light	1397	1.7	0.71
5 min light + oxaloacetate	985	2.1	1.20
5 min light + oxaloacetate + pyruvate	587	0.9	0.56
(F) + pyruvate + oxaloacetate			
5 min dark	63	0.7	0.37
5 min light	748	1.2	0.87
5 min light + pyruvate	3199	0.7	0.50
5 min light + pyruvate + oxaloacetate	2665	0.9	0.72

complication arises if oxaloacetate addition is made following pyruvate (Fig. 6b and Table II, F). Here oxaloacetate, despite raising ΔpH and oxidising the intersystem carriers, is unable to induce the expected degree of dephosphorylation.

In isolated thylakoids incubated with [γ-^{32}P]ATP the protein kinase is inhibited by ADP in a manner similar to that of the thylakoid protein kinases from spinach and pea [11]. Conversely, none of the added substrates had a significant direct action on protein kinase activity in isolated thylakoids.

Discussion

The data presented in this paper indicate that it is the redox state of the intersystem electron carriers which controls the activity of the thylakoid protein kinase. This is in support of the previously held notion that plastoquinol is the kinase activator [1–5]. The presence of the electron acceptors 3-phosphoglycerate and oxaloacetate suppresses protein kinase activity in maize chloroplasts. Pyruvate, which drastically reduces the [ATP]/[ADP] ratio, induces a level of phosphorylation higher than that observed with no added substrate. The stimulation of kinase activity by pyruvate is somewhat surprising although fluorescence induction data have previously indicated that plastoquinone is more highly reduced in maize chloroplasts incubated with pyruvate [19]. The same conclusion was reached here to explain the DCMU-induced fluorescence quenching observed most dramatically when pyruvate is present. The mechanism of the quenching remains to be elucidated but it is likely that oxidation of plastoquinol by PS I, which would induce non-photochemical quenching by plastoquinone, is involved. Thus, the observation of the phenomenon would be associated with a highly reduced electron-transfer chain and would be correlated with protein kinase activity. Our data support this notion; hence, with 3-phosphoglycerate and oxaloacetate, which causes little kinase action, no DCMU-induced quenching is seen whilst with no added substrate an effect similar to (but smaller than) that seen with pyruvate is observed.

It is of interest to speculate why the system is more reduced when pyruvate is present. Pyruvate has been shown to simulate a high rate of cyclic electron transfer [19] and this may lead to a greater reduction of plastoquinone through this pathway. The fact that the pyruvate effect on redox state (and on kinase activity) is prevented by oxaloace-

tate (Fig. 6a) is evidence for this view. Thus, our data seem to indicate that high rates of cyclic electron flow are necessary to induce maximum kinase activity. The resulting change in energy distribution in favour of PS I may serve to augment the effect of a high ATP demand and further stimulate cyclic electron flow.

The present results obtained using maize chloroplasts give no indication of a role for adenylate levels in controlling kinase activity [6,7]. During the comparison of strong and weak light in the presence of oxaloacetate a slightly increased level of phosphorylation does accompany an elevated [ATP]/[ADP] ration. Similarly, 3-phophoglycerate suppresses phosphorylation and lowers the [ATP]/[ADP] ratio. However, in both cases redox changes are involved and within the general context of our data it is perhaps more appropriate to ascribe these data to alteration in redox state.

The effect of pyruvate in maize chloroplasts is to be contrasted with the effect of ribose 5-phosphate on pea chloroplasts in the reconstituted system [11]. Ribose 5-phosphate mimics pyruvate in being an ATP sink but in so doing causes dephosphorylation rather than phosphorylation. It is possible that the difference may lie in the relative capacities of the two systems for cyclic electron flow. Thus, the effect of pyruvate on redox state may be large enough to 'overrule' an opposing effect of the adenylate status.

The possible influence on kinase activity of cyclic electron flow suggests that some revision needs to be made to the simple scheme in which protein phosphorylation is determined only by the rates of electron flow into (from PS II) and out of (to PS I) the plastoquinone pool [1–5].

This adds further support to the notion that protein phosphorylation functions at the interface between electron transport and carbon assimulation and not just in the State 1–State 2 transition [1,11].

Acknowledgements

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