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Relationships between protein phosphorylation and electron transport in the reconstituted chloroplast system

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Phosphorylation of the light-harvesting chlorophyll protein (LHCP) by the thylakoid protein kinase has been examined in the reconstituted chloroplast system. The level of phosphorylation by $[^{32}P]P_i$ was maximal at high light intensity and in the absence of 3-phosphoglycerate; dephosphorylation resulted from a subsequent decrease in light intensity or from the addition of 3-phosphoglycerate. Addition of ribose 5-phosphate, which acts as an ATP 'sink', also caused dephosphorylation. It is concluded that the degree of phosphorylation is dependent on the redox state and energy state of the system, thereby providing a mechanism for adapting light harvesting to the demands of carbon assimilation.

It is now well-established that chloroplast membranes possess a protein kinase that phosphorylates a number of polypeptides, including LHCP (Bennett, 1977, 1979, 1980). Measurements of chlorophyll fluorescence (Bennett *et al.*, 1980; Horton & Black, 1980, 1981*a*; Allen *et al.*, 1981; Chow *et al.*, 1981), electron-transport rates (Horton & Black, 1982; Steinback *et al.*, 1982), the redox state of Q (Telfer & Barber, 1981) and of cytochrome *f* (Horton & Black, 1981*b*) all indicate that phosphorylation controls the distribution of excitation between Photosystems II and I. A number of different approaches (Horton & Black, 1980; Horton *et al.*, 1981; Allen *et al.*, 1981; Allen & Horton, 1981) have indicated that the kinase is activated when the electron acceptor (plastoquinone) pool operating between Photosystems II and I is reduced. This led to the hypothesis that protein phosphorylation can enable balanced rates of excitation of Photosystems II and I to be achieved under low light of different spectral quantity (Bennett *et al.*, 1980; Horton & Black, 1980). However, it is probable that the activity of the kinase will also fluctuate in response to other factors that will influence the redox state of plastoquinone, besides imbalance of Photosystems II and I (Horton & Black, 1980, 1981*a*); thus the kinase activity may change as a function of light intensity, the size of the transmembrane pH gradient and the rate of utilization of the terminal electron acceptor, NADP. Indeed, Allen & Bennett (1981) have shown that the kinase activity is maximal during the induction phase of photosysthesis, but decreases as CO_2 fixation proceeds at the higher steady-state level. Furthermore Baker *et al.* (1982) have shown that the kinase is inhibited by ADP. It was the aim of the present study to examine the degree of phosphorylation of LHCP when both the plastoquinone redox state and the ATP/ADP ratio were varied by manipulation of lysed chloroplasts capable of carbon metabolism. The system used was based on the reconstituted system described by Lilley & Walker (1979).

Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; LHCP, light-harvesting chlorophyll *a*/*b*-binding protein; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Q, the primary electron acceptor of Photosystem II, the redox state of which controls the level of chlorophyll fluorescence.

Experimental

Spinach (*Spinacea oleracea* L. cv. Yates hybrid 102) was grown in pots under glass as described by Lilley & Walker (1974). Peas (*Pisum sativum* L. cv. Feltham First) were grown in trays under glass. Intact spinach and pea chloroplasts were isolated by methods described previously (Walker, 1980). Chloroplasts were osmotically shocked into a medium that finally contained 0.33 M-sorbitol, 1 mM-EDTA, 10 mM-KCl, 5 mM-$NaHCO_3$, 10 mM-$MgCl_2$, 200 units of catalase, 0.4 mM-P_i, 200 μCi of $^{32}PO_4{}^{3-}$, 100 μM-NADP, 200 μM-ADP, 75 μg of ferredoxin/ml, 4 mM-ascorbate, 1 mM-dithiothreitol, 5 μM-9-aminoacridine and 50 mM-Hepes/KOH buffer,

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pH 7.9. Reaction mixtures, containing 50 μg of chlorophyll/ml, were illuminated by red light in Hansatech electrodes at 20°C in which CO_2-dependent O_2 evolution was measured polarographically. Samples (250 μl) were taken at intervals during the incubation and rapidly mixed with 25 μl of 100% trichloroacetic acid. The precipitated proteins were extracted with acetone and the insoluble fraction was examined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis essentially as described previously (Horton *et al.*, 1981). ^{32}P incorporation into LHCP was estimated in the excised bands of LHCP apoprotein by Čerenkov counting in a Beckman LS7500 scintillation counter over a 30 min period.

O_2 evolution, chlorophyll fluorescence and fluorescence from 9-aminoacridine were measured simultaneously in an apparatus detailed elsewhere (Horton, 1982).

Results

Table 1 shows results of experiments designed to examine the kinase/phosphatase properties previously characterized by using purified chloroplast thylakoids supplied with [γ-^{32}P]ATP (Bennett *et al.*, 1980; Allen *et al.*, 1981). Pea chloroplasts show light-induced incorporation of [^{32}P]P_i into LHCP, which is reversible in darkness. The phosphatase action is inhibited by NaF, as observed in thylakoids. However, spinach chloroplasts do not behave in the same way; although phosphorylation proceeds normally, it is not dark-reversible to any great extent. At the present time the difference in the behaviour of pea and spinach chloroplasts is not understood. The rest of the experiments were, therefore, performed on pea chloroplasts. Fig. 1 examines the redox state of Q as a function of light intensity. [Q] was determined by using the DCMU method of Krause *et al.* (1982). As predicted by these authors and by Bradbury & Baker (1981), Q can be incompletely reduced during photosynthesis; in fact, light intensities far greater than those required to saturate electron flow are needed to give complete reduction of Q. It has been shown (Amesz *et al.*, 1972) that redox changes in Q and plastoquinone behave in parallel. Redox potentials of the major Q component (approx. 0 mV) and plastoquinone (+50 mV) would also indicate that the redox state of plastoquinone will similarly depend on light intensity (Horton & Croze, 1978; Golbeck & Kok, 1979).

In Fig. 2, results of an experiment designed to examine the effects of light intensity on phosphorylation of LHCP are shown. Light intensities were less than saturating. Phosphorylation was higher in strong light (11992 compared with 7988 counts) after 7 min. The lower fluorescence level in low light (broken lines) is indicative of a more oxidized acceptor pool (Horton, 1982; Krause *et al.*, 1982). Transition from high to low light caused Q oxidation (as shown by the fluorescence decrease) and considerable dephosphorylation occurred (down

Table 1. *Labelling of LHCP with [^{32}P]P_i in the reconstituted chloroplast system*

Pea (*a*) and spinach (*b*) chloroplasts were illuminated in the medium described in the Experimental section. No phosphoglycerate was present. Light intensity was 300 W/m^2 and NaF was present at 10 mM, where indicated. At various periods of light (L) and subsequent darkness (D) samples were taken and assayed for phosphorylation of LHCP.

		Phosphorylation of LHCP (counts/30 min)	
	Time (min)	+NaF	−NaF
(*a*)	0	1072	1907
	2.5 L	9035	6328
	4.5 L	10892	11365
	2.0 D	10651	7230
	10.0 D	9561	1176
(*b*)	0	1018	973
	2.5 L	5349	3921
	4.5 L	8305	9150
	2.0 D	10596	11400
	10.0 D	10692	12669

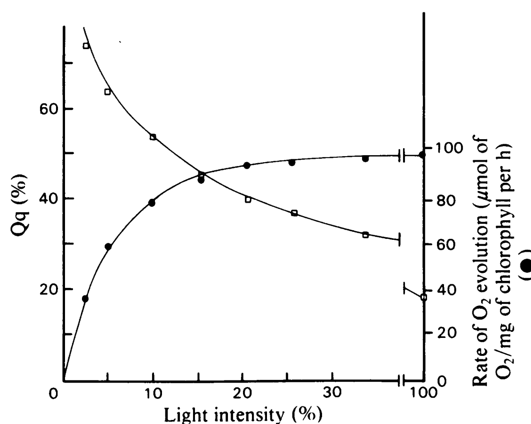

Fig. 1. *Effect of light intensity on the redox state of Q and the rate of O_2 evolution in reconstituted pea chloroplasts*

The redox state of Q (termed 'Qq') was estimated by measuring the proportion of rapid fluorescence increase upon DCMU addition (Krause *et al.*, 1982; Horton 1982). The rate of O_2 evolution was measured in the steady state after addition of phosphoglycerate (2 mM); 100% light intensity was 300 W/m^2.

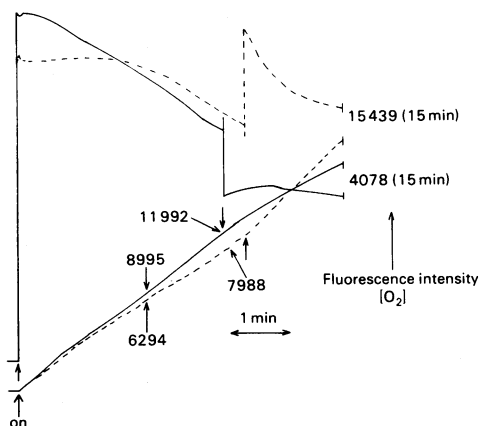

Fig. 2. *Effects of light intensity on the amount of phosphorylation of LHCP*

Upper curves are chlorophyll fluorescence traces and lower curves O_2 evolution. Fluorescence was measured by using a low-intensity modulated measuring beam and photosynthesis excited with a broad-band red light whose intensity could be varied (Horton, 1982). Intensity was 300 W/m² (——) and 150 W/m² (----) initially. At the points shown by arrows the intensities were reversed. Numerals indicate the amount (radioactivity counts) of phosphorylation of LHCP in samples taken at the indicated times. Phosphoglycerate was present initially at 2 mM.

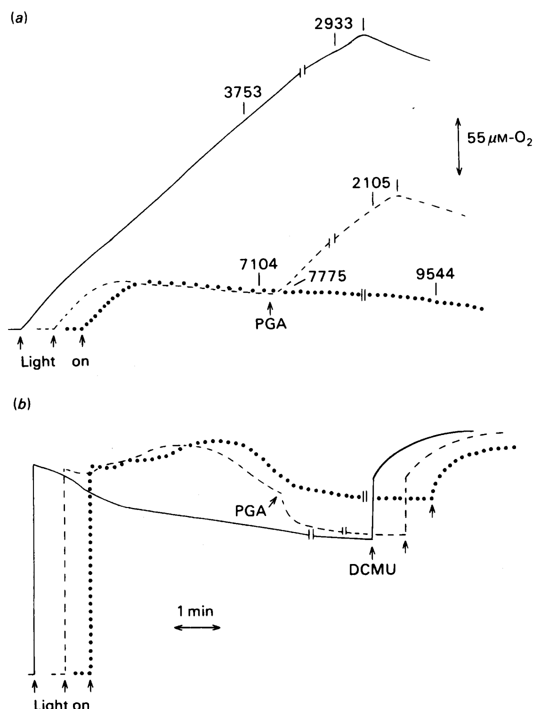

Fig. 3. *Effect of the presence of phosphoglycerate on the amount of phosphorylation of LHCP*

(*a*) O_2 evolution; (*b*) chlorophyll fluorescence. Light intensity was 150 W/m². Phosphoglycerate (PGA) concentration was 2 mM. ——, Phosphoglycerate present initially; ----, phosphoglycerate added after 7 min; ····, no phosphoglycerate. Other details are as described in the legend to Fig. 2.

to 4078 counts after 15 min). Conversely, increasing the light intensity caused Q reduction [as shown by the fluorescence increase; the subsequent decrease is due to development of a larger transthylakoid pH gradient which also causes fluorescence quenching (Krause *et al.*, 1982)]. After 15 min the amount of phosphorylation increased almost 2-fold to 15439 counts. Clearly the activity of the protein kinase is dependent on light intensity in a way that is consistent with alteration in the redox state of plastoquinone.

At constant light intensity, the redox state can be altered by the presence or absence of electron acceptors; in isolated thylakoids, it has been shown that addition of Methyl Viologen or ferricyanide prevents light-activation of the kinase (Horton & Black, 1981*a*; Allen *et al.*, 1981). In the reconstituted system, 3-phosphoglycerate can be used as a more physiological electron acceptor. Fig. 3 shows the effect of phosphoglycerate on the degree of phosphorylation of LHCP. In one experiment phosphoglycerate was present initially and a low level of phosphorylation was recorded (continuous line). In a second, phosphoglycerate was omitted (dotted line). Here, there is an initial burst of O_2 evolution as the added NADP is reduced and during this phase ATP is synthesized (Carver *et al.*, 1983). In the absence of phosphoglycerate a much higher level of phosphorylation is recorded. In a third experiment, phosphoglycerate is added after 7 min. In the subsequent period dephosphorylation of LHCP occurs. Fluorescence measurements show how the redox state of Q is shifted towards reduction when phosphoglycerate is absent. Thus addition of phosphoglycerate caused oxidation of Q as evidenced by the fluoresence decrease (Horton, 1982). Similarly, the presence of a rapid increase in fluorescence when DCMU is added shows incomplete reduction of Q when phosphoglycerate is present (Krause *et al.*, 1982). These experiments were intentionally done in low light; at high light, phosphoglycerate causes only marginal effects on the redox state of Q (Horton, 1982) and in these conditions phosphoglycerate does not cause dephosphorylation.

Addition of 3-phosphoglycerate causes only a small decrease in transthylakoid pH gradient (Horton, 1982) and only a brief and transitory decrease in the ATP/ADP ratio (Carver *et al.*, 1983).

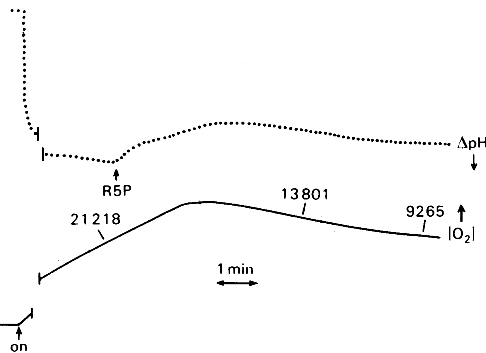

Fig. 4. *Effect of ribose 5-phosphate (R5P) on the amount of phosphorylation of LHCP*

Steady-state phosphoglycerate reduction was obtained as in Fig. 2 at an intensity of $150 W/m^2$. Ribose 5-phosphate (0.5mM) was added and its effect on the relative transmembrane pH gradient as measured by using 9-aminoacridine fluorescence (Horton, 1982) and O_2 evolution is shown. Other details were as described in the legend to Fig. 2.

Addition of ribose 5-phosphate has a more pronounced effect on these two parameters (Carver *et al.*, 1983; Horton, 1982). Ribose 5-phosphate, by conversion into ribulose 1,6-bisphosphate, acts as an ATP 'sink'; the lowering of the ATP/ADP ratio would then cause a cessation of reduction of 3-phosphoglycerate (Robinson & Walker, 1979). The effect provides a physiological way of looking at the influence of the ATP/ADP ratio on phosphorylation of LHCP. The experiment was performed in low light when recovery of the ATP/ADP ratio and ΔpH is extremely slow. In Fig. 4 simultaneous measurements of ΔpH and O_2 evolution were made, together with an assay of the amount of phosphorylation of LHCP. Addition of ribose 5-phosphate caused dephosphorylation of LHCP. An inhibitory effect of ADP on the protein kinase in the pea chloroplasts used in these experiments was found. In the presence of ADP the level of phosphorylation with [γ-^{32}P]ATP after 10 min was reduced from 12873 counts to 4872.

Discussion

Experiments with thylakoids have clearly demonstrated control of kinase activity by the redox state of plastoquinone (Allen *et al.*, 1981; Horton *et al.*, 1981; Allen & Horton, 1981). The present results clearly show that kinase activity is dependent on light intensity and the capacity for turnover of NADPH and ATP by phosphoglycerate reduction. These changes in kinase activity are consistent with the notion that control is exerted through the redox state of plastoquinone, since alteration in kinase activity is associated with changes in the redox state of the acceptor side of Photosystem II. Kinase activity also depends on ATP concentration (Bennett *et al.*, 1980) and the ATP/ADP ratio (Baker *et al.*, 1982) and it is likely that changes in these will occur as the light intensity is reduced. Measurements of 9-aminoacridine fluorescence (not shown) and chlorophyll fluorescence (Fig. 2) indeed show an energization of the membranes as the light intensity is increased. In the case of phosphoglycerate-induced perturbation it has been shown in this laboratory that the effect on the ATP level and ΔpH is slight and transitory (Carver *et al.*, 1982; Horton, 1982) and would not explain the decreased level of protein phosphorylation. The data do not allow us to assess fully the relative importance of redox compared with adenylate control of the kinase. It seems that perhaps these two parameters concertedly control the activity.

The experiments described here implicate the possibility that thylakoid protein kinase has an important regulatory role in photosynthesis. This role is not merely to balance Photosystems II and I (the State I/II transition), but to provide a means by which the thylakoid membrane can respond to the demands of stromal CO_2 metabolism (Horton & Black, 1980, 1981*a*; Markwell *et al.*, 1982). Thus kinase activity is maximal when light intensity (in Photosystem II) exceeds the ability of the stromal enzymes to consume NADPH and ATP. Conversely the kinase activity will be minimal when NADPH and ATP consumption exceeds the rate of photosynthetic electron transport and photophosphorylation. This suggests that phosphorylation/dephosphorylation could provide a mechanism to tune the rate of Photosystem II excitation to the capacity for carbon assimilation. This mechanism is brought about by the synergistic effects of high redox state and high ATP/ADP ratio on the one hand and low redox state and low ATP/ADP ratio on the other. Such a mechanism would imply that the rate of excitation of Photosystem II is limiting in the phosphorylated membranes and that electron transport could be speeded up by increasing its rate of excitation by dephosphorylation. Viewed in this way, the dark dephosphorylated state would be the end result of a process that at low light (when there is excess carbon assimilation capacity) strives for maximal Photosystem II excitation. Conversely phosphorylation brought about by rates of excitation in excess of carbon assimilation (such as in Fig. 1 in high light or Fig. 2 before 3-phosphoglycerate addition) is a way of preventing 'overexcitation' of the system, which may have some protective value.

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