

Increase in the level of thylakoid protein phosphorylation in maize mesophyll chloroplasts by decrease in the transthylakoid pH gradient

P. Fernyhough, C.H. Foyer and P. Horton*

Research Institute for Photosynthesis, The University, Sheffield S10 2TN, England

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Phosphorylation of the light-harvesting chlorophyll protein (LHCP) has been measured in intact chloroplasts prepared from maize mesophyll protoplasts. Maximum levels of phosphorylation were obtained in the absence of added reducible photosynthetic substrate and the presence of low concentrations of the ΔpH dissipating ionophore, nigericin. Assays of chlorophyll fluorescence indicated a high reduction level of plastoquinone under these conditions. It is suggested that the size of the transthylakoid pH gradient exerts control over the redox activation of the protein kinase and that protein phosphorylation is involved in the regulation of cyclic and non-cyclic electron flow. Further evidence for this view was obtained from stimulation of protein phosphorylation by pyruvate which lowers ΔpH by ATP consumption and by inhibition of phosphorylation in strong light and by low levels of DCMU.

Protein phosphorylation Maize Chloroplast membrane Photosynthesis pH gradient

1. INTRODUCTION

Phosphorylation of the light-harvesting chlorophyll protein (LHCP) by the thylakoid protein kinase causes an alteration in the distribution of absorbed photons so as to decrease the rate of excitation of Photosystem II (PS II) and increase that of Photosystem I (PS I), thereby providing a molecular basis for the state 1-2 transition reviewed in [1]. A series of experiments have demonstrated the control of protein kinase activity by the redox state of plastoquinone [2-5]. Recent work on intact chloroplasts prepared from maize protoplasts confirmed the involvement of the redox state as the major control factor, and did not produce any evidence for the presence of a stimulatory effect of a high ATP/ADP ratio upon kinase activity [6], as had been reported by others for thylakoids [7].

Since the discovery of protein phosphorylation and its resultant effects on chlorophyll fluorescence, it has been suggested that the physiological role of the process might be connected with enhancing the rate of cyclic electron flow so as to maintain the correct ATP/NADPH stoichiometry for carbon assimilation [2,8]. Protein phosphorylation could do this by either enhancing excitation of PS I per se or by establishing better 'redox poise' via control over the rate of electron output from PS II. The author in [8] clearly demonstrated the benefits in terms of quantum efficiency that could result from such a control mechanism.

One important criterion which must be fulfilled if this mechanism is to have any validity is that (contrary to any inhibitory effect of a low ATP/ADP ratio as proposed in [7]) conditions of enhanced ATP demand should stimulate protein phosphorylation. In a recent paper we showed that addition of pyruvate to intact maize mesophyll chloroplasts caused a stimulation of protein phosphorylation [6]; pyruvate causes rapid con-

* To whom correspondence should be addressed

sumption of ATP via the pyruvate- P_i -dikinase reaction and therefore lowers the steady-state value of the transthylakoid pH gradient (ΔpH).

Here, by use of the ionophore nigericin, we demonstrate directly that the level of protein phosphorylation is responsive to ΔpH , thereby providing a mechanism by which an enhanced rate of ATP consumption can be translated into a high level of protein phosphorylation. We have used intact chloroplasts because we wish to understand the physiological responses of protein phosphorylation to changes in metabolism; maize mesophyll chloroplasts are particularly suitable for this study because their internal metabolism is so simply adjusted [6].

2. MATERIALS AND METHODS

Intact maize chloroplasts were prepared exactly as in [6]. Measurement of incorporation of ^{32}P into LHCP was carried out as in [6]. In all cases steady-state levels of labelling were attained within 5–10 min of changing external conditions and, as shown in [6], the labelling was reversed upon dark incubation. Also, ^{32}P incorporation was inhibited by DCMU (a known inhibitor of the light-induced activation of the protein kinase) and stimulated by

addition of the protein phosphatase inhibitor NaF. Together with the associated changes in the fluorescence emission spectra (see fig.4), these characteristics of the observed incorporation of ^{32}P established the validity of this method of measuring protein phosphorylation since these same features have been displayed in studies of the protein kinase using thylakoids [1,8]. Simultaneous assays of O_2 evolution, chlorophyll fluorescence and 9-aminoacridine fluorescence were made as in [9].

Fluorescence emission spectra at $-196^\circ C$ were recorded using a chopped blue exciting light (Corning 4-96 and 5-57 filters), emission being collected at an angle of 33° using a fibre optic, and measured using an Applied Photophysics monochromator together with an EMI 9558 photomultiplier. The signal was amplified by a Brookdeal Lockin amplifier and fed to a BBC microcomputer, which also controlled wavelength scanning. The chloroplast samples ($100 \mu l$) were contained in glass tubes totally immersed in liquid nitrogen.

3. RESULTS AND DISCUSSION

Fig.1a shows the response of maize mesophyll chloroplasts to illumination in the absence of add-

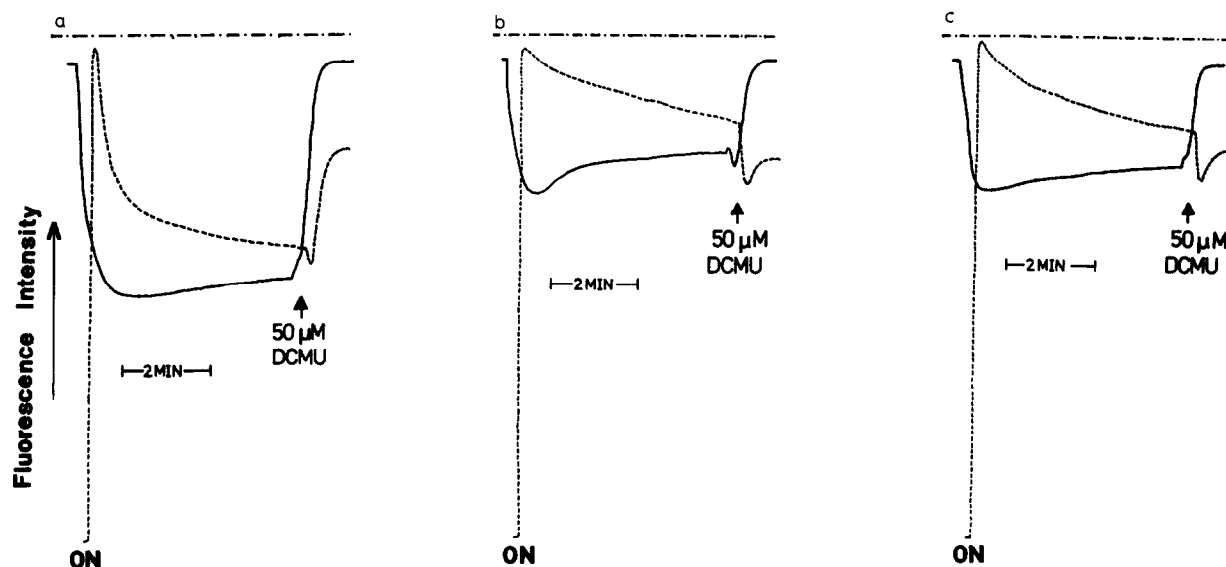


Fig.1. Effect of pyruvate and nigericin on the kinetics of O_2 evolution (-----), chlorophyll fluorescence (----) and 9-aminoacridine fluorescence (—) in maize mesophyll chloroplasts. Light intensity $40 W \cdot m^{-2}$. (a) No substrate. (b) Pyruvate (10 mM). (c) No substrate + nigericin (1 nM). Traces are not corrected for time delay between the 3 recorder channels.

ed substrate. In these chloroplasts endogenous substrate levels are low and there is also no capacity for autocatalytic build-up of reducible acceptors. Therefore, no net O_2 evolution is observed and, because rates of ATP consumption are also low, a high ΔpH is formed, as revealed by the quenching of 9-aminoacridine fluorescence. Most of the chlorophyll fluorescence quenching is therefore due to the formation of this ΔpH . Upon addition of DCMU, ΔpH relaxes slowly; chlorophyll fluorescence is initially quenched and then rises with similar kinetics to the ΔpH relaxation. This DCMU-induced quenching of chlorophyll fluorescence is due to oxidation of reduced plastoquinone [10]. Pyruvate addition, as in [6], reduces ΔpH and resultant chlorophyll fluorescence quenching (fig. 1b) and stimulates phosphorylation of LHCP (table 1). Plastoquinone is highly reduced in the presence of pyruvate, as shown by the large amplitude of DCMU-induced quenching of chlorophyll fluorescence [10].

The stimulation of protein phosphorylation by pyruvate could be due to either a decrease in the level of ATP or the resultant decrease in ΔpH . Previous work on the protein kinase has provided no evidence for a stimulation in activity at low ATP concentration [11]. This suggests therefore, that it is the decrease in ΔpH that causes the increased protein phosphorylation. To investigate this further we have looked at the effect of decreasing ΔpH by addition of the uncoupler nigericin on the level of phosphorylation of LHCP. Nigericin is an H^+/K^+ ionophore which dissipates the ΔpH component but not the $\Delta \psi$ component of the pro-

tonmotive force. In the presence of 1 nM nigericin, the ΔpH and the extent of quenching of chlorophyll fluorescence were very similar to those seen in the presence of pyruvate (fig. 1c). A large decrease in chlorophyll fluorescence was seen upon adding DCMU, indicating a highly reduced plastoquinone pool.

Table 1 shows that the degree of the phosphorylation of LHCP can be stimulated by addition of nigericin. Thus, after 10 min illumination in the presence of nigericin the incorporated counts increased from 157 cpm to 3819 cpm (a 24-fold increase) whilst the incorporation with neither nigericin nor pyruvate showed only an 8-fold increase, from 183 to 1405 cpm. The stimulatory effect of pyruvate, a 30-fold increase from 80 to 2396 cpm, is shown for comparison. In another experiment addition of nigericin after 5 min illumination was seen to enhance phosphorylation from approx. 1000 to 3000 cpm in 5 min compared with an increase of only 400 cpm in its absence.

The data in table 1 clearly show that the level of incorporation of ^{32}P into LHCP can be enhanced by a decrease in ΔpH , induced either by an uncoupler or by increasing the rate of ATP consumption. The fact that both pyruvate and nigericin, which would have opposite effects on ATP turnover, caused similar increases in labelling, tends to rule out artefactual changes in the specific activity of the ATP pool being responsible for the changes in levels of ^{32}P incorporation; instead, the data indicate a real stimulation of phosphorylation upon decrease in ΔpH . An enhanced degree of reduction of plastoquinone would be the simplest explanation for the observed effect of ΔpH on protein phosphorylation since the protein kinase has been shown to be controlled by changes in the plastoquinol/plastoquinone ratio [1-5]. The data in fig. 1 indeed suggest that both pyruvate and nigericin induce a more reduced state of plastoquinone, but it is not clear whether this would be sufficient to account for the increase in phosphorylation observed. In maize chloroplasts it has been shown that the slow phase of the P518 absorbance change is enhanced in the presence of either pyruvate or nigericin [14]. Although the exact basis of this response is also not completely understood it most likely reflects a ΔpH effect on an electrogenic electron transfer in the cytochrome *b-f* complex [15]. Possibly, therefore, plastoquinone may be more

Table 1

The effects of nigericin and pyruvate on the level of phosphorylation of LHCP after 5 min dark incubation followed by 5 and 10 min illumination

Conditions	Phosphorylation of LHCP (cpm)		
	5 min D	5 min L	10 min L
No substrate	183	934	1405
No substrate			
+ nigericin	157	1865	3819
Pyruvate	80	1245	2396

Radioactivity of excised gel bands were expressed as cpm/band and are averages of 3 determinations, each counted for 30 min. For details see fig. 1

reduced and the protein kinase more active when this ΔpH -sensitive electrogenic step is allowed.

The possible association between redox poise, ΔpH and protein phosphorylation was examined by two further experiments. Low concentrations of DCMU increase the oxidation state of the plastoquinone pool by decreasing the rate of electron input from PS II. In this way cyclic electron flow, as measured by an antimycin-sensitive increase in ΔpH , is increased [12,13]. Fig.2 shows that $0.3\ \mu\text{M}$ DCMU increased the ΔpH in the presence of pyruvate and also decreased the amount of high DCMU-induced fluorescence quenching indicating a higher oxidation state of plastoquinone. Under these conditions protein phosphorylation is partially suppressed (table 2) but whether this effect was caused mostly by plastoquinone oxidation or the ΔpH increase cannot be stated.

The dependence of protein phosphorylation on the redox balance of the system can also be probed by observation of the light intensity dependence for kinase activity since an increase in light intensity will lead to an increase in ΔpH and a more reduced plastoquinone pool. In the presence of pyruvate, when no O_2 evolution is observed, a change from 360 to $40\ \text{W}\cdot\text{m}^{-2}$ causes no detectable change in the redox state; the changes in the chlorophyll fluorescence signal are correlated only with the

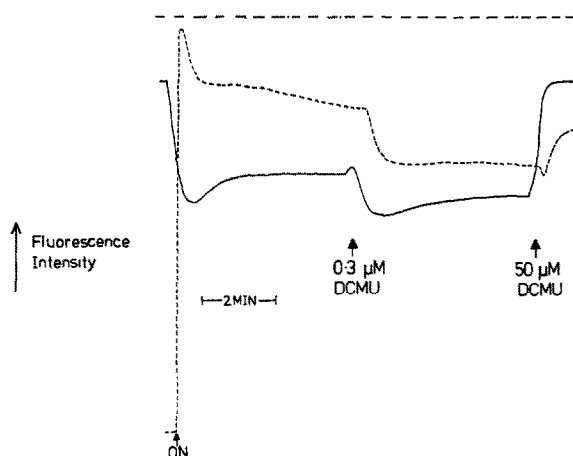


Fig.2. Effect of low concentrations of DCMU ($0.3\ \mu\text{M}$) on the kinetics of O_2 evolution (-----), chlorophyll fluorescence (---) and 9-aminoacridine fluorescence (—) in maize mesophyll chloroplasts. Light intensity $40\ \text{W}\cdot\text{m}^{-2}$.

Table 2

Reversal of phosphorylation of LHCP by DCMU addition

Conditions	Phosphorylation of LHCP (cpm)
5 min D	135
5 min L	1491
5 min L + DCMU	1006
10 min L + DCMU	908

Chloroplasts were initially incubated in darkness for 5 min and then illuminated (L) for 5 min in the presence of pyruvate; DCMU ($0.3\ \mu\text{M}$) was added and illumination continued for a further 10 min

ΔpH change as indicated by the quenching of 9-aminoacridine fluorescence (fig.3). In table 3a it can be seen that a transition from high to low light intensity resulted in stimulation of phosphorylation with an increase in incorporated counts from 1141 to 3633 cpm while subsequent incubation at high light intensity resulted in a decreased incorporation to 2155 cpm. Exactly the same trend was seen when a low light interval was given between two periods of high light (table 3b). The decrease in phosphorylation seen on increase in light intensity strongly suggests an effect of ΔpH on LHCP phosphorylation that is not due to a change in the redox state.

The maximum levels of phosphorylation of

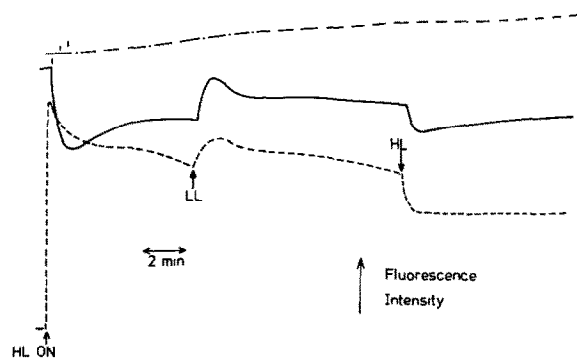


Fig.3. Effect of changes in light intensity on the kinetics of O_2 evolution (-----), chlorophyll fluorescence (---) and 9-aminoacridine fluorescence (—) in maize mesophyll chloroplasts. HL and LL are 360 and $40\ \text{W}\cdot\text{m}^{-2}$, respectively.

Table 3

Effects of light intensity on the level of phosphorylation of LHCP in chloroplasts illuminated in the presence of pyruvate

Light	Phosphorylation of LHCP (cpm)
(a) 5 min D	164
+ 7.5 min HL	1141
+ 7.5 min LL	3633
+ 7.5 min HL	2155
(b) 5 min D	37
+ 7.5 min LL	1566
+ 7.5 min HL	939
+ 7.5 min LL	2415

High light (HL) and low light (LL) are 360 and 40 $\text{W} \cdot \text{m}^{-2}$, respectively, given successively; a and b are two separate experiments

LHCP reported here correspond to approx. 1 μmol phosphate/12 μmol LHCP. The data in fig.4 demonstrate that this is sufficient to bring about a marked change in excitation distribution between PS II and PS I. Thus, phosphorylation induced a 15–20% decrease in the relative fluorescence emission intensity at 685 nm, which was reversed upon dephosphorylation in darkness.

The data described here show that the level of protein phosphorylation and the distribution of excitation between PS II and PS I are controlled at least in part by the transthylakoid ΔpH . Under physiological conditions ΔpH is low either in low light intensity (when proton uptake is slow) or when the rate of ATP synthesis is high (when proton release through the ATP synthase is fast). Rapid steady-state ATP synthesis will occur only when there is a high rate of ATP consumption. This means that protein phosphorylation will be enhanced under conditions where the rate of ATP consumption exceeds the rate of its production by electron transfer. Consumption of NADPH by reduction of glyceralate-1,3-bisphosphate depends upon the supply of ATP for its synthesis and therefore ATP limitation would be associated with accumulation of NADPH. Under these conditions (low ΔpH and reduced electron transfer chain) protein phosphorylation is maximal and it is these same conditions that would promote cyclic elec-

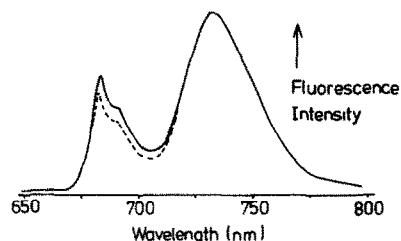


Fig.4. Fluorescence emission spectra of maize mesophyll chloroplasts illuminated for 10 min with pyruvate (---), followed by 10 min darkness (—). Spectra are normalised to the PS I emission maximum.

tron transfer around PS I as a means of making up the ATP deficit. Protein phosphorylation may contribute to the regulation of the relative rates of cyclic and non-cyclic electron flow by causing a change in energy distribution and redox poise in response to both the ATP demand and the redox state of the plastoquinone pool. It is therefore possible that the explanation of the state 1–2 transition may reside not just in an improvement in the efficiency of non-cyclic electron flow but also in the efficiency with which ATP and NADPH are produced in the required stoichiometry for carbon assimilation.

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