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ON THE NATURE OF THE FLUORESCENCE DECREASE DUE TO PHOSPHORYLATION OF CHLOROPLAST MEMBRANE PROTEINS

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1. Phosphorylation of chloroplast membranes by illumination in the presence of ATP results in a 15–20% increase in the rate of Photosystem I electron transfer at low light intensity. 2. Phosphorylated membranes when depleted of Mg^{2+} and resuspended in a low salt medium still show a 17% lower yield of Photosystem II fluorescence than do unphosphorylated membranes. A 31% difference is seen after restoration of the maximal yield by addition of Mg^{2+}. 3. The concentration of Mg^{2+} required to induce a half-maximal increase in fluorescence is 0.9 mM for control and 1.8 mM for phosphorylated chloroplasts. Phosphorylation at 1 mM Mg^{2+} can therefore cause more than double the amount of decrease in fluorescence yield from Photosystem II compared to phosphorylation at 5 mM. 4. The above results are discussed in terms of the mechanism of the ATP-induced fluorescence changes and a suggestion is made that the apparent interaction between phosphorylation and Mg^{2+} concentration may be a physiologically important phenomenon.

Introduction

In chloroplasts which are uncoupled and in which the primary acceptor is reduced measurements of the yield of chlorophyll fluorescence at 20°C are generally considered to reflect the rate of excitation of PS II chlorophylls.

It is now well established that phosphorylation of chloroplast membrane proteins by an ATP-dependent, light-activated protein kinase results in a decrease in the maximum yield of chlorophyll fluorescence (F_m) from PS II [1–5]. The light activation is mediated by reduced plastoquinone and this process therefore provides a likely mechanism for controlling the relative rates of excitation of PS II and PS I [1,3–5]. The ability of plants to regulate the relative rates of excitation of PS I and PS II, a phenomenon referred to in terms of the State 1–2 transitions, is probably important in optimizing photosynthetic efficiency under low light intensity [6,7]. In a recent study it has been shown that the steady-state oxidation level of cytochrome *f* in light preferentially absorbed by PS II is increased after phosphorylation [8]; this directly shows the ability of phosphorylation to bring about a change in the balance between PS II and PS I excitation. Similar observations on the redox state of the acceptor pool of PS II have also been made [9].

However, important questions concerning the

Abbreviations: PS II, Photosystem II; PS I, Photosystem I; F_m, maximum fluorescence level when all PS II traps are closed; F_0, minimum fluorescence level when all PS II traps are open; $F_v = F_m - F_0$; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; LHCP, light-harvesting chlorophyll protein; Tricine, *N*-tris(hydroxymethyl)methylglycine; Chl, chlorophyll.

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way by which phosphorylation is controlling the excitation rates of the two photosystems remain unanswered. This may occur by an alteration in distribution of absorbed radiation between the two photosystems, by an increase in transfer of excitation from PS II/LHCP to PS I. Alternatively, there may be a decrease in the rate of PS II excitation not associated with increased energy transfer to PS I.

(The other possibility, an increase in PS I activity alone is unlikely since it is a PS II-associated protein, the LHCP which is the major phosphoprotein and because the absolute yield of fluorescence of PS II chlorophyll at 20°C is seen to decrease upon phosphorylation.) Existing fluorescence data could be accommodated by either mechanism. Clearly measurements of rates of PS I electron transfer are crucial in resolving this question.

Changes in the energy-transferring properties of the thylakoid pigment-protein complexes have been widely studied with respect to the influence of the cation content of the surrounding medium (see, for example, Ref. 6). In terms of understanding the effect of phosphorylation it is therefore worthwhile to study the relationship between it and cation concentration. Thus questions such as whether the decrease in fluorescence yield due to phosphorylation and replacement of Mg^{2+} by low Na^{+} are additive need to be considered. These questions take on additional importance because it has been suggested that the interactions between the pigment-protein complexes are modulated by the balance between membrane surface charge and the screening power of the surrounding cations. Because a surface exposed segment of LHCP is phosphorylated [5] it was therefore proposed that phosphorylation would perturb this charge/screening balance by increasing the density of the surface (negative) charge [2,3,5,6].

In this paper, therefore, we report measurements of PS I electron transport in phosphorylated chloroplasts and describe the relationship between cation effects and phosphorylation. It is shown that phosphorylation does induce an effect that is analogous to depletion of divalent cations and that an increase in PS I excitation rate results. However, a component of the decrease in fluorescence due to phosphorylation appears to be additive to the cation effect and results from effects different from those due to cation depletion.

Materials and Methods

Chloroplasts were isolated from peas or spinach by methods previously described [3,10]. Chloroplast proteins were phosphorylated by incubation of chloroplasts for 10 min in a medium comprising 0.33 M sorbitol/5 mM $MgCl_2$/50 mM Hepes (pH 7.6 with NaOH)/10 mM NaF after first stripping the chloroplasts of their envelopes by suspension in 5 mM $MgCl_2$ followed by addition of double-strength medium [8]. The chloroplasts were uncoupled with 1 μg/ml ionophore A23187 or 2 μM gramicidin and the incubations contained, where indicated, 0.15 mM ATP. Chlorophyll fluorescence at room temperature was measured 'front-face' using a bifurcated fibre optic mounted on one side of a four-sided, thermostatically controlled vessel containing a stirred 2 ml sample. The measuring beam was defined by a Corning 4-96 filter and was modulated at 620 Hz by a Rofin (Egham, Surrey, U.K.) microchopper and fluorescence was measured at 695 nm (Balzars Interference filter) using an EMI 9558 photomultiplier and a Brookdeal (Bracknell, Berks) Lock-in Amplifier. Actinic light (to activate protein kinase) was provided by a 150 W slide projector filtered through heat absorbing glass and a Corning 2-62 red transmitting filter. Fluorescence emission spectra at −196°C were determined as before [3] except that samples (50 μl) were stored and scanned in glass tubes.

For the investigation of the effect of Mg^{2+} concentration, chloroplasts were diluted with a Mg-free medium at the end of the 10 min incubation period, centrifuged and resuspended in a small volume of the same medium. Fluorescence was then assayed in the above apparatus in the presence of 5 μM DCMU, uncoupler and at a chlorophyll concentration of 20 μg/ml in the presence of different Mg^{2+} concentrations. All media contained 10 mM NaF to inhibit reversal of fluorescence decreases due to phosphatase activity [3,11].

Photosystem I activity was measured using a Hansatech O_2 electrode (King's Lynn, Norfolk) by assaying O_2 uptake in the presence of ascorbate, diaminodurene, methyl viologen and superoxide dismutase [12]. Illumination (red) was provided by

a 150 W slide projector. The intensity was varied using Balzars neutral density filters and measured with a Kipp and Zonen Radiometer.

Results

Fig. 1 shows the results of an experiment in which the rate of Photosystem I electron transport was measured in phosphorylated (i.e., ATP-treated) and control membranes. Only at low light intensities (less than 15 $W \cdot m^{-2}$) was an increase in rate seen for the phosphorylated sample. At 9 $W \cdot m^{-2}$ ($\equiv$ 13% in Fig. 1) values of 90 ± 6 and 76 ± 6 μmol O_2/mg chlorophyll per h were obtained for ATP-treated and control chloroplasts, respectively (Table I). At intensities above 30 $W \cdot m^{-2}$ no differences in rate were seen. For these chloroplasts a maximum rate of approx. 400 μmol O_2/mg chlorophyll per h was obtained at 200 $W \cdot m^{-2}$.

This increase in Photosystem I rate at low light intensity is entirely predictable if phosphorylation of LHCP increases the probability of energy transfer to PS I, as speculated in recent publications [1–5]. A similar increase in PS I activity accompanies depletion of divalent cations from chloroplasts [13]. The latter process is explicable in terms of the ability of divalent cations to screen surface membrane charge, so allowing various interactions between pigment-protein complexes to occur [6].

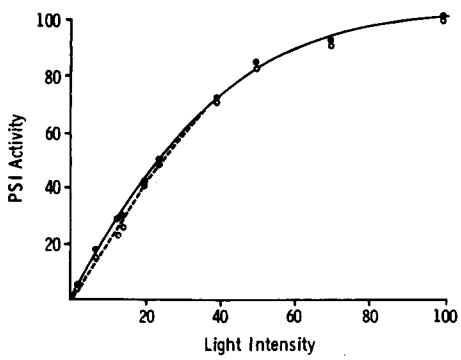

Fig. 1. The rate of PS I electron transport at different light intensities. Spinach chloroplasts were incubated for 10 min with or without ATP under continuous illumination at 25 μg chlorophyll per ml. PS I assay reagents were added and, after 2 min dark, O_2 uptake was measured. 100% intensity was 68 $W \cdot m^{-2}$ of red light. 100%PS I was 290 μmol O_2/mg Chl per h. Phosphorylated, ●; control, ○. 1 μg/ml ionophore A23187 was used as uncoupler.

TABLE I

RATES OF PS I ELECTRON TRANSPORT IN PHOSPHORYLATED AND CONTROL CHLOROPLASTS

Conditions as in Fig. 1. Light intensity 9 $W \cdot m^{-2}$. Values are means of ten experiments $\pm$95% confidence limits.

	Rate of PS I electron transport. (μmol O_2/mg Chl per h)
Phosphorylated	90 ± 6
Control	76 ± 6

Attachment of the negatively charged phosphate to the surface segment of LHCP may influence this cation screen/charge balance and hence decrease fluorescence by the same process as cation depletion [3,6]. The interaction between [Mg^{2+}] and phosphorylation was therefore investigated.

Phosphorylated and control chloroplasts were prepared by illumination for 10 min with or without ATP. The fluorescence yield was recorded throughout the incubation (Fig. 2a) and decreased by approx. 25% during the incubation when ATP was present. After 10 min the chloroplasts were depleted of Mg^{2+} as described in Materials and Methods. Chlorophyll fluorescence was assayed in the low fluorescence state and after addition of

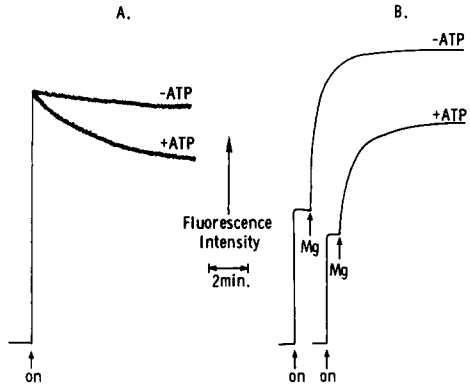

Fig. 2. Mg^{2+} effects on fluorescence of control and phosphorylated pea chloroplasts. A. Fluorescence decrease during incubation with or without ATP at 100 μg chlorophyll per ml. B. Fluorescence from chloroplasts pretreated as in A, depleted of Mg^{2+} and resuspended in a 'low salt' medium comprising 0.1 M sucrose/10 mM NaF/10 mM Tricine (pH 7.8 with NaOH)/5 μM DCMU. Chlorophyll 20 μg/ml. Both A and B contained 1 μM gramicidin as uncoupler. Mg^{2+} concentration is 5 mM when added. 'On' refers to simultaneously turning on of measuring and actinic light sources.

Mg^{2+} (Fig. 2b). Mg^{2+} clearly increases the F_m of both control and phosphorylated chloroplasts. Similarly, but of greater significance, phosphorylation decreases the yield when assayed either plus or minus Mg^{2+}. In Table II are shown data taken from 13 separate experiments involving five different incubations with or without ATP. The F_m level was found to be less by, on average, 17% after phosphorylation when assayed in the absence of Mg^{2+}. When assayed after addition of Mg^{2+}, phosphorylation was seen to have decreased the F_m by 31%

As expected, the decreases in fluorescence from PS II at room temperature are also expressed as decreases in the F_{685}/F_{735} ratios observed after cooling samples to −196°C. Table III shows exactly the same pattern as Table II. Thus, the lowest ratio was seen in phosphorylated membranes depleted of Mg^{2+} and the highest for control membranes in the presence of Mg^{2+}. A decrease due to phosphorylation of 15% persisted even after Mg^{2+} depletion.

The partially additive nature of Mg^{2+} depletion and phosphorylation is evidence that they may influence chlorophyll fluorescence by different mechanisms. The persistence of a fluorescence decrease both at zero and saturating Mg^{2+} would not be expected if both processes were working only by influencing the surface charge/cation screening balance. However, evidence that phosphorylation does influence the cation effect is revealed when the F_m values of phosphorylated and control chloroplasts are titrated as a function of Mg^{2+} concentration (Fig. 3). At saturating Mg^{2+} (5 mM), the difference due to phosphorylation

TABLE II

THE EFFECT OF Mg^{2+} ON THE FLUORESCENCE YIELD OF PHOSPHORYLATED CHLOROPLASTS

Conditions as in Fig. 2. Values are means of 13 experiments ±95% confidence limits in arbitrary units.

	Fluorescence intensity		Increase by Mg^{2+} (%)
	$-Mg^{2+}$	$+Mg^{2+}$	
Control	18.7±0.9	40.8±1.8	118
Phosphorylated	15.6±0.7	28.3±1.4	81
Decrease (%)	17	31	

TABLE III

THE EFFECT OF Mg^{2+} ON THE RATIO OF PS II TO PS I FLUORESCENCE AT −196°C IN PHOSPHORYLATED CHLOROPLASTS

Conditions as in Fig. 2. The PS II/PS I ratio was determined from emission intensities at 685 nm and 735 nm. Values are means of five experiments with ranges given in brackets.

	PS II/PS I		Increase by Mg^{2+} (%)
	$-Mg^{2+}$	$+Mg^{2+}$	
Control	0.41 (0.38–0.50)	0.81 (0.73–0.95)	98
Phosphorylated	0.35 (0.27–0.44)	0.61 (0.58–0.63)	74
% decrease	15	25	

shows no indication that it diminishes at above saturating levels; the lack of effect of up to 20 mM Mg^{2+} was reported previously [3]. However, the Mg^{2+} concentration required for half-maximum increase in F_m is significantly higher for phosphorylated membranes; values of 0.9 mM and 1.8 mM were measured for control and ATP-treated samples, respectively. This difference has the effect of making the extent of the ATP-induced fluorescence decrease extremely sensitive to change in Mg^{2+} concentration. Thus at 1 mM, a 40% decrease in fluorescence is seen in phosphorylated membranes compared to approx. 20% at 5 mM. Because the kinase itself is Mg^{2+}-dependent [14], the result could be merely an experimental curios-

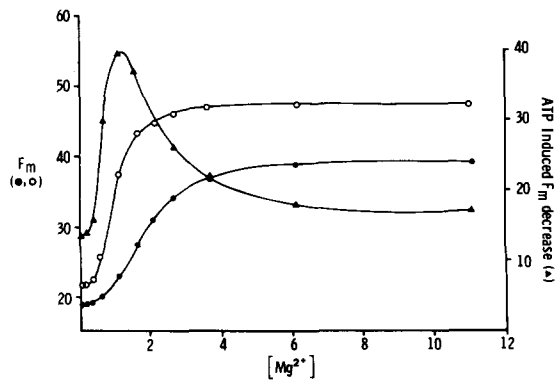

Fig. 3. Effects of $[Mg^{2+}]$ on the F_m of control (○) and phosphorylated (●) chloroplasts. Conditions as in Fig. 2. The ATP-induced decrease is ▲. Mg^{2+} was added as $MgCl_2$.

TABLE IV

THE EFFECT OF Mg^{2+} CONCENTRATION ON THE ATP-INDUCED DECREASE IN FLUORESCENCE

Chloroplasts were incubated for 10 min with or without ATP at different initial Mg^{2+} concentrations. The fluorescence was assayed as usual and expressed as $([F_{+ATP} - F_{-ATP}]/F_{-ATP}) \times 100\%$

Mg^{2+} concn. (mM)	ATP-induced fluorescence decrease
1.0	42
1.5	28
2.5	21
5.0	16

ity, without any physiological significance. Therefore we tested the ability of ATP to induce a fluorescence decrease after illumination in the presence of different Mg^{2+} concentrations; in this way any limitation on kinase activity in the concentration range of interest could be assessed. As shown in Table IV, results very similar to those of Fig. 3 are seen. Thus a 42% decrease is seen at 1 mM Mg^{2+}, decreasing to less than 20% at 5 mM Mg^{2+}.

A consistent and curious difference between phosphorylated and control chloroplasts was noted in the course of these experiments. In Fig. 2b the rate of fluorescence increase on adding Mg^{2+} to depleted chloroplasts is noticeably slower in phosphorylated chloroplasts. In Fig. 4 the normalised fluorescence increases seen after addition of 11 mM Mg^{2+} are shown. The rate of increase observed for control chloroplasts was always 50–100% faster than for phosphorylated ones.

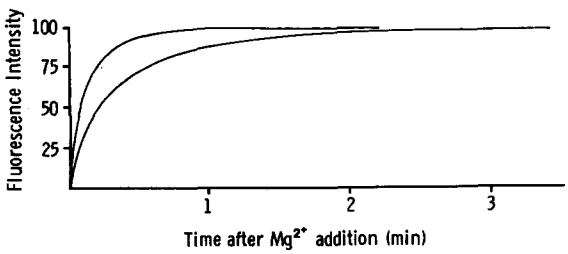

Fig. 4. The rate of fluorescence increase upon adding 11 mM Mg^{2+} to control (upper curve) and phosphorylated (lower curve) chloroplasts. The fluorescence increase was normalised and the conditions are as in Fig. 2.

Discussion

It has previously been shown that phosphorylation of chloroplast membranes results in a decreased yield of chlorophyll fluorescence at room temperature and a decrease in the ratio of emission from PS II:PS I at −196°C [1–5]. These results were interpreted in terms of phosphorylation causing an increase in the fraction of absorbed energy being transferred to PS I [1–7]. In the present paper this argument is strengthened considerably in that an increase in the rate of electron transport through PS I under light-limiting conditions is induced by phosphorylation. Previously, we had shown an ATP-induced decrease in the rate of fluorescence induction in the presence of DCMU, an indication of a decreased rate of excitation of PS II [3]. In both cases the changes are of the order of 15–20%. The changes are consistent with observations made on the redox state of carriers in the electron transport chain connecting PS II and PS I. Thus Telfer and Barber [9] showed that ATP caused a decreased reduction level of the electron acceptor pool of PS II in chloroplasts illuminated with PS II light. Similarly, Horton and Black [8] showed an increased oxidation level of cytochrome *f* after phosphorylation.

The study of the interaction between $[Mg^{2+}]$ and phosphorylation suggests that phosphorylation has more than one effect on the interactions between the chlorophyll proteins. The requirements for a higher $[Mg^{2+}]$ for elevation of the fluorescence yield and the associated antagonism between phosphorylation and increasing $[Mg^{2+}]$ from 1 to 6 mM indicates phosphorylation does interfere with cationic screening, presumably by an increase in surface charge density. This effect makes a large contribution to the ATP-dependent fluorescence decrease at $[Mg^{2+}] < 5$ mM.

However, it is clear that phosphorylation has an effect that is different from and is in addition to that due to cationic screening effects. Thus, phosphorylation induces a significant decrease in PS II fluorescence at saturating $[Mg^{2+}]$. At zero Mg^{2+} an effect of phosphorylation persists. Barber's working hypothesis to explain cation-induced changes in PS II fluorescence is based on the notion that the LHCP/PS II and PS I complexes are randomized at zero Mg^{2+} and segregated when

Mg^{2+} is added [6]. Phosphorylation may allow a more complete randomization perhaps by causing a change in association between LHCP and PS II. The fact that the increase in fluorescence caused when Mg^{2+} is added to phosphorylated membranes is slower is consistent with a more randomized condition after phosphorylation. An effect on the association between LHCP and PS II could hence provide an explanation for the persistence of phosphorylation effects at both zero and saturating levels of Mg^{2+}.

The requirement for higher Mg^{2+} concentration for maximum PS II fluorescence may not be without physiological significance. A large difference in the expression of phosphorylation (in terms of a functional effect on light harvesting and energy transfer) is seen between 1 mM and 5 mM Mg^{2+}. During photosynthesis, stromal Mg^{2+} levels probably fluctuate within this concentration range [15], depending amongst other factors on the size of ΔpH across the thylakoids. At 1 mM Mg^{2+}, phosphorylation of LHCP causes a change nearly equivalent to complete depletion of divalent cations from unphosphorylated membranes. Low Mg^{2+} levels may be expected to occur under low light conditions when ΔpH is low. It is under these conditions that correct balancing of the rates of excitation of PS II and PS I are essential [7], and, accordingly, that full expression of the effects of reversible phosphorylation of LHCP should be most necessary. Conversely, under high light conditions there may be less need to regulate quantum distribution between PS II and PS I (unless the aim is to regulate cyclic electron flow). In fact, in over-saturating light (when the protein kinase will be maximally active due to plastoquinone reduction), over-excitation of PS I may be deleterious.

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