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Modification of photosystem II activity by protein phosphorylation

M. Hodges, N.K. Packham and J. Barber

AFRC Photosynthesis Research Group, Imperial College, London SW7 2BB, England

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Phosphorylation of proteins within pea thylakoid membranes decreases photosystem II (PSII) mediated electron transfer at saturating light intensities which, according to changes in room temperature chlorophyll fluorescence transients, is due to a modification in the electron transfer from Q_A to Q_B. However, a previously reported increase in the ability of DCMU to inhibit PS2 electron flow to DCPIP, as a consequence of protein phosphorylation, was not observed, although changes in DCMU efficacy were found to depend upon the redox state of the plastoquinone (PQ) pool.

Protein phosphorylation *Photosystem II* *Chlorophyll fluorescence* *Herbicide inhibition* *PQ redox state* *Electron transport*

1. INTRODUCTION

In chloroplast thylakoid membranes there is at least one protein kinase which catalyses the ATP-dependent phosphorylation of several PS II-associated polypeptides including 9-, 24–26-, 32- and 45-kDa species [1–3]. The kinase can be activated, in isolated thylakoids, when the PQ pool is reduced either photochemically or with added reductants [4,5]. The light-harvesting chl *a/b*-protein complex (LHC) has been identified to be the major site of phosphate incorporation. The effect of this has been discussed in terms of the regulation of excitation energy distribution between the two photosystems [4,6–10] brought about by the lateral displacement of a 'mobile' pool of phosphorylated LHC [11,12], which alters the photochemical reactions of both PS I and PS II at limiting light intensities [7,13,14]. Several other effects have, however, been reported in the literature which might suggest that protein phosphorylation may modify photosynthetic electron flow by directly altering PS II function; that is, as a consequence of phosphorylating polypeptides within the PS II complex. It has been claimed that protein phosphorylation leads to:

(a) an increase in the ability of the PS II-herbicides, diuron, atrazine and dinoseb to inhibit the Hill reaction [15];

(b) an enhanced stability of the bound semiquinone (Q_B^-) at the two electron gate of PS II [16];

(c) an increase in the negative charge density around the primary, stable electron acceptor of PS II (Q_A) [17];

(d) a stimulation of a hydroxylamine-sensitive cyclic electron transfer pathway around PS II [18];

(e) a decreased rate of PS II electron transfer at saturating light intensities [19], and

(f) a protection of PS II against photoinhibition [19].

In addition to the above it has been found with the cells of *Chlamydomonas reinhardii* that the

Abbreviations: PS, photosystem; LHC, light harvesting chl *a/b* protein complex; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCPIP, 2,6-dichlorophenolindophenol; DPC, diphenylcarbazide; Tricine, *N*-tris(hydroxymethyl)methylglycine; F_o, initial chlorophyll fluorescence; F_m, maximum chlorophyll fluorescence; F_i, chlorophyll fluorescence at i; Q_A, primary stable electron acceptor of PS II; Q_B, secondary stable electron acceptor of PS II

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dephosphorylation of the 32-kDa polypeptide of PS II correlated with a decrease in the binding constant of DCMU and in the number of binding sites [20].

Here, evidence is presented which suggests that protein phosphorylation does not lead to an increase in the ability of DCMU to inhibit the Hill reaction as claimed by others [15], but that it does attenuate the rate of electron transfer from H_2O to DCPIP at saturating light intensities and that this might reflect an altered ability to transfer electrons from Q_A to Q_B after protein phosphorylation.

2. MATERIALS AND METHODS

Chloroplasts were isolated as in [21] from *Pisum sativum* and resuspended as a concentrated stock in 0.33 M sorbitol (pH adjusted to 7.5 with Tris) and 3 mM $MgCl_2$. The chlorophyll concentration was determined as in [22].

Phosphorylated and control thylakoids were prepared as follows: intact chloroplasts were osmotically shocked in 12 mM $MgCl_2$ for 15 s and double strength medium was added to give final concentrations in the reaction medium of 50 μg chl/ml, 10 mM Tricine (KOH, pH 8.2), 0.33 M sorbitol, 6 mM $MgCl_2$ and 10 mM NaF. To generate phosphorylated samples, 0.4 mM ATP was also included in the medium. Phosphorylated and control membranes were obtained after a 30 min treatment time which involved either an illumination under broad band blue-green light transmitted, at an intensity of 10 $W \cdot m^{-2}$, by a Schott BG38 filter or a dark adaptation in the presence or absence of 5 μM *Spirulina maxima* ferredoxin (Fd) and 0.5 mM NADPH. All treatments were carried out at room temperature. Both the phosphorylation treatments used produced a 15–20% attenuation in PS II fluorescence yield indicative of protein phosphorylation [7,9].

The DCMU titration and light intensity dependence curves of PS II photochemical activity were assayed by the rate of DCPIP reduction ($\Delta\epsilon_{560-520nm}$ = 6.8 $mM^{-1} \cdot cm^{-1}$) using phosphorylated and control samples suspended at 50 μg chl/ml in the reaction media supplemented with 0.05 mM DCPIP. In some experiments 0.5 mM diphenylcarbazide (DPC) was present, while in others 2 mM $NADP^+$ was added, prior to DCMU addition, to oxidise the PQ pool (see [9]). The light-induced absorption changes were performed as in [23] with the 100% light intensity (55 $W \cdot m^{-2}$) being used for the DCMU titrations.

PS II electron transfer rates from H_2O to silicomolybdate were measured, at saturating light, in control and phosphorylated membranes suspended at 50 μg chl/ml in reaction media supplemented with 100 μM silicomolybdate and 20 μM DCMU, using a Rank Brothers (Clarke-type) oxygen electrode.

Room temperature chlorophyll fluorescence transients were measured as in [9] (except in the absence of DCMU) by diluting the phosphorylated and control thylakoids to the following final concentrations: 3.3 μg chl/ml, 10 mM Tricine (KOH, pH 8.2), 0.33 M sorbitol, 6 mM $MgCl_2$ and 10 mM NaF (26 μM ATP was also present in all the phosphorylated samples while 0.3 μM Fd and 33 μM NADPH were both present when the Fd/NADPH method was used).

3. RESULTS

Fig.1 shows the titration with DCMU of PS II electron transfer rate, as measured from H_2O to DCPIP, for non-phosphorylated (fig.1A) and phosphorylated (fig.1B) thylakoids which had been treated with only Fd and NADPH (curves (b), (d)). It can be seen that the chemically induced protein phosphorylation did not have any effect on the ability of DCMU to inhibit photosynthetic electron flow to DCPIP (I_{50} = 0.04 μM DCMU for bulk phosphorylated and non-phosphorylated membranes). This observation was also found for the PS II inhibitor, atrazine, and for DCMU when phosphorylation was brought about photochemically (not shown). When excess $NADP^+$ was added to the samples, before the DCMU titration, the ability of DCMU to inhibit PS II electron flow was decreased with no difference between phosphorylated and non-phosphorylated samples (curves (a),(c)). As can be seen in fig.1A, dark-adapted thylakoids, in the absence of reductants, also showed a similar sensitivity for DCMU to the chemically oxidised membranes (i.e., with excess $NADP^+$).

We observed that protein phosphorylation resulted in a lower rate of PS II electron transfer to DCPIP at saturating light intensities, confirming the observations made by Horton and Lee [19].

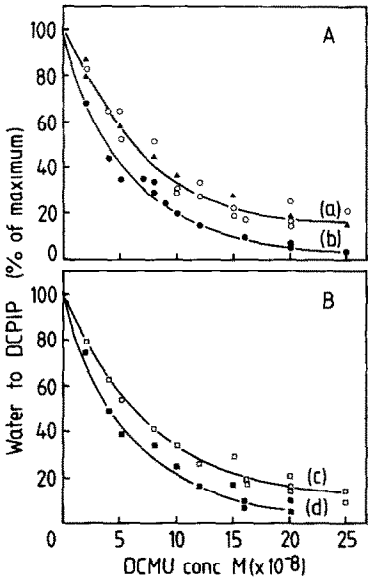

Fig.1. The effect of DCMU concentration on the inhibition of PS II electron transfer to DCPIP in (A) non-phosphorylated and (B) phosphorylated thylakoids suspended in the presence (open symbols) or absence (closed symbols) of excess $NADP^+$. Round and square symbols signify samples containing ferredoxin and NADPH while triangles represent dark-adapted thylakoids in the absence of reductants. Protein phosphorylation was carried out for 30 min using the Fd/NADPH method. NaF was present at all times and excess $NADP^+$ was added to samples in order to oxidise the PQ pool. The mean maximum rates were: non-phosphorylated, 228 μmol DCPIP/mg chl per h and phosphorylated, 186 μmol DCPIP/mg chl per h.

This difference was evident in thylakoids which had undergone either the light- or chemically-induced phosphorylation treatments. Fig.2A shows the light intensity dependence of the rate of DCPIP reduction in phosphorylated and non-phosphorylated thylakoids. It can be seen that at all intensities investigated protein phosphorylation led to a lower rate of PS II electron transfer. An analysis of the data is shown in fig.2B which suggests that protein phosphorylation generates a constant degree of PS II inhibition over the range of light intensities used. Such an inhibition, at saturating light, cannot be explained by a phosphorylation-induced decrease in PS II antenna size [9,11,12]. The site of inhibition does not appear to be at the oxygen-evolving complex as the addition of DPC did not relieve the inhibitory effect. The attenuated PS II electron transfer rate measured with phosphorylated thylakoids at saturating light was eliminated when electron flow was measured using silicomolybdate, which accepts electrons directly from Q_A in a DCMU-insensitive reaction [24]. This observation infers that the inhibitory site is located after Q_A.

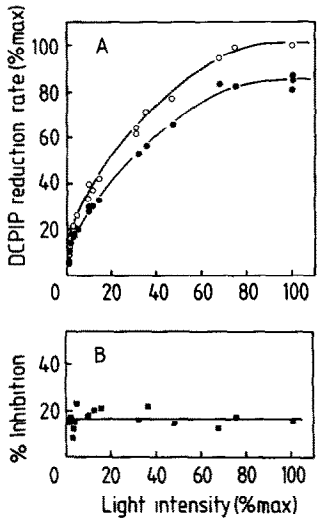

Fig.2. (A) The effect of protein phosphorylation on the PS II electron transfer rate, measured using DCPIP as electron acceptor, as a function of excitation light intensity (100% = 55 $W \cdot m^{-2}$). Non-phosphorylated (○); phosphorylated (●). (B) The percentage inhibition of PS II electron transfer rate brought about by protein phosphorylation as a function of excitation light intensity. The data are calculated from fig.2A and the line is the least squares fit to the experimental data points. Protein phosphorylation was carried out using the Fd/NADPH method.

PS II electron transfer reactions were also investigated by measuring the chlorophyll fluorescence signal exhibited at room temperature by the illumination of dark-adapted thylakoids. Fig.3 shows the normalised fluorescence transients produced from phosphorylated and control membranes. It can be seen that, although the F_m/F_o ratio was the same for both phosphorylated and control thylakoids indicating no change in the yield of photochemistry, the phosphorylated samples gave rise to a larger F_o to F_i component in the fluorescence signal which was followed by a slower increase to the F_m level. These differences were again observed in both light- and reductant-treated thylakoids.

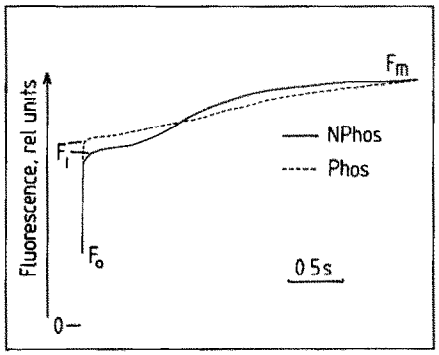

Fig.3. Normalised room temperature chlorophyll fluorescence transients exhibited by phosphorylated (Phos) and non-phosphorylated (NPhos) thylakoids. Protein phosphorylation was carried out using the Fd/NADPH method. The F_o was determined by recording the transients on a faster time scale and both sets of membranes were given identical dark treatments before measurement.

4. DISCUSSION

The data of fig.1 suggests that protein phosphorylation does not lead to an enhanced ability for DCMU to inhibit PS II electron flow to DCPIP. This conclusion is contrary to the interpretation of Vermaas and coworkers [15], who proposed that protein phosphorylation enhanced herbicide inhibitory action at the Q_B-protein. In their experiments they compared the ability of various PS II herbicides to inhibit electron transfer to DCPIP using thylakoids subjected to light-induced protein phosphorylation with dark-adapted, non-phosphorylated control membranes. The data of fig.1 are not inconsistent with their results, however, since we also detect changes in the DCMU sensitivity when comparing non-phosphorylated thylakoids with an oxidised PQ pool (fig.1, curve a) with phosphorylated thylakoids with a reduced PQ pool (fig.1, curve d). Such a difference in the redox state of the PQ pool, between phosphorylated and non-phosphorylated membranes, would be anticipated under the conditions used by Vermaas et al. [15]. We therefore propose that the change in DCMU effectiveness arises from an alteration in the redox state of the PQ pool and is not a direct consequence of protein phosphorylation. The sensitivity of DCMU inhibition to the redox state of the PQ pool could reflect the belief that this herbicide competes with PQ for the quinone-binding site in the Q_B-protein [25,26]. It is thought that the reduction of PQ, at this site, to the semiquinone leads to a more tightly bound molecule and hence to a reduction of the binding affinity of DCMU [26,27]. In this way it would not be unreasonable to assume that the inhibitory action of DCMU will be dependent on the redox state of the PQ pool. Intuitively, when most of the PQ is in the fully reduced form (plastoquinol) the probability for DCMU to bind will be expected to be high because plastoquinol interaction at that site will be poor. Whereas, when the PQ pool is highly oxidised an increased competition with DCMU for the Q_B-binding site would be expected, thereby producing a less effective PS II inhibitor. This appears to be borne out by the data presented in fig.1 and is consistent with the recent findings of Vermaas and Arntzen [28]. They showed that quinone analogues were more effective in their oxidised state at binding to the Q_B-site and competing with herbicides.

Protein phosphorylation, however, does appear to modify PS II functioning, as seen from fig.2A, where electron transfer to DCPIP is partially inhibited even at saturating light intensities, with the degree of inhibition remaining relatively constant over the range of light intensities used (fig.2B). The inhibition at saturating light cannot be due to the decrease in PS II pigment-bed size and it does not appear to represent an inhibition at the site of oxygen evolution, as DPC did not remove the effect. This was also observed by Horton and Lee [19], who discussed the inhibition as being a consequence of a stimulated hydroxylamine-sensitive cycling of electrons around PS II in phosphorylated thylakoids (see [18]). If such a mechanism is accelerated by protein phosphorylation then it would be expected to lead to an enhanced deactivation of the S-states. This is not borne out by the reported inability of protein phosphorylation to modify S_2- and S_3-state relaxation [16]. It is suggested, by the similar rates of electron transfer to silicomolybdate in phosphorylated and non-phosphorylated thylakoids, reported in this work, that the site of inhibition may be located after Q_A. Such a conclusion is strengthened by the differences observed in the chlorophyll fluorescence transients between phosphorylated and non-phosphorylated membranes

(fig.3). The larger F_o-F_i component followed by a slower rise to F_m, seen after phosphorylation, is similar to the differences in fluorescence transients observed when comparing atrazine-resistant with atrazine-susceptible plant species [28,29], where the resistant chloroplasts show the same changes to those exhibited by the phosphorylated thylakoids. It has been proposed that the modified fluorescence signals produced by atrazine-resistant thylakoids arise from an altered equilibrium between $Q_AQ_B^-$ and $Q_A^-Q_B$, which produces a slower rate of electron transfer from Q_A to Q_B [30] or from an altered ability for PQ to bind to the Q_B-protein [28,30]. It is suggested from fig.1, in which DCMU inhibitory action was not seen to be changed by protein phosphorylation, that the latter proposal may not be the case. Therefore, the attenuation in PS II electron flow to DCPIP, seen after protein phosphorylation, may represent a slower transfer of electrons from Q_A to Q_B brought about by an increase in the negative charge placed on one or more of the PS II polypeptides which could lead to a stabilisation of Q_A^- in the light.

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