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Phosphorylation of the 9 kDa Photosystem II-associated protein and the inhibition of photosynthetic electron transport

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Phosphorylation of chloroplast thylakoid membrane proteins by light-activated protein kinases results in a decreased light-saturated rate of photosynthetic electron transport. The three major phosphorylated proteins have been identified as the 26 kDa and 24 kDa polypeptides of the light-harvesting chlorophyll *a*/*b* complex (LHC II) and a 9 kDa polypeptide of unknown function. In this paper, I report the effect of inhibitors of either the protein kinases, such as 5′-*p*-fluorosulfonylbenzoyladenosine (FSBA) and *N*-ethylmaleimide, or of phosphoprotein phosphatases, such as NaF, on the extent of phosphorylation of the light-harvesting chlorophyll *a*/*b* complex (LHC II) and the 9 kDa polypeptide. These phosphorylation studies were complemented by measurement of the ATP-dependent inhibition of the oxygen evolution rate in chloroplasts treated with FBSA, *N*-ethylmaleimide or NaF. The results indicate that PS II photochemical activity is insensitive to the amount of phosphate incorporated into the LHC II, suggesting that the inhibition may be due to the activity of the protein kinase which can phosphorylate the 9 kDa polypeptide.

Introduction

Several thylakoid membrane proteins are phosphorylated when chloroplasts are incubated under conditions which promote the reduction of the plastoquinone pool [1]. The two major phosphoproteins, with molecular masses of 26 and 24 kDa, have been identified as constituents of the light-harvesting chlorophyll *a*/*b* (LHC II) complex [2]. The reversible phosphorylation of LHC II, which can be conveniently monitored by changes in the chlorophyll *a* fluorescence emission for PS II [3,4], is thought to be the molecular mechanism by which chloroplasts can regulate the distribution of excitation energy between Photosystem II (PS II) and PS I [3–8]. Several PS II-associated proteins can also be phosphorylated under reducing conditions [9]; the most heavily labelled of these has a molecular mass of 9 kDa. This 9 kDa polypeptide is neither the high redox potential form of cytochrome *b*-559 [10,11], nor the dicyclohexylcarbodiimide-reactive subunit of the ATP synthase [12]. Allen and colleagues have proposed that the 9 kDa polypeptide is a chlorophyll-binding protein [13], and suggested that its phosphorylation assists in the lateral repulsion of the phospho-LHC II from the PS II reaction centre [14]. A phosphoprotein with a 9 kDa molecular mass has recently been purified from the thylakoid membrane [15], and its primary

Abbreviations: Chl, chlorophyll *a* and *b*; PS II, Photosystem II of chloroplasts; LHC II, the 26 kDa and 24 kDa polypeptides of the light-harvesting chlorophyll *a*/*b* protein; DTE, 2,3-dihydroxy-1,4-dithiolbutane; AMP-PNP; 5′-adenylylimidodiphosphate; FBSA, 5′-*p*-fluorosulfonylbenzoyladenosine; Mes, 4-morpholineethanesulfonic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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structure deduced from the nucleotide sequence of its gene [16]. The absence of histidine from the sequence would suggest, however, that the 9 kDa phosphoprotein is neither a chlorophyll- nor a haem-binding protein.

Thylakoid membrane phosphoryaltion has been demonstrated to inhibit the oxygen evolution capacity. The degree of inhibition was found to be independent of the actinic light intensity [17,18]. It was therefore deduced that thylakoid membrane phosphorylation must not only decrease the light-harvesting capacity of PS II, which would affect PS II photochemistry at limiting light intensities, but also alters the electron transfer reactions catalysed by PS II. The molecular basis of this 'secondary' effect of phosphorylation could involve either a cyclic electron flow around PS II [19], or an inhibition of electron transfer between the primary, Q_A, and secondary, Q_B, plastoquinone acceptors of the PS II reaction centre [18]. In a recent study of the oscillatory oxygen release pattern from flash-excited chloroplasts it was shown that PS II centres in phosphorylated thylakoid membranes have an increased probability to miss catalysing a light-induced charge transfer (N.K. Packham, M. Hodges, A.L. Etienne and J. Briantais, unpublished data). The origin of the missed S-state transition, which was found not to be due to an increased deactivation kinetics of either the S2 or S3 oxidation states [20], was inferred to result from a decrease in the equilibrium constant for the exchange of the electron between Q_A and Q_B (N.K. Packham, M. Hodges, A.L. Etienne and J. Briantais, unpublished data).

An obvious candidate to mediate the ATP-dependent inhibition of PS II would be the phospho-9 kDa polypeptide. This species is associated with PS II in the appressed regions of the thylakoid membrane [9]. It has, however, been recently shown that the 9 kDa polypeptide is not part of the PS II reaction centre core [11]. Moreover, Horton and Lee [17] have demonstrated that the recovery in PS II photochemical activity matches the recovery of the room temperature chlorophyll *a* fluorescence yield. Changes in the chlorophyll *a* fluorescence yield monitor the reversible phosphorylation of the LHC II [3,4]. It has therefore been proposed that the phospho-LHC II is responsible for the inhibition of PS II at saturating light intensities. In this study I have attempted to establish the relationship between the phosphorylation of the thylakoid membrane polypeptides and the inhibition of the oxygen evolution capacity. I have studied the effects of inhibitors of the LHC II thylakoid membrane kinase (FBSA and *N*-ethylmaleimide (see Refs. 21 and 22)) on the phosphorylation of the thylakoid membrane polypeptides. The results were compared with the effect that these inhibitors have on the ATP-dependent inhibition of oxygen evolution. My conclusion is that inhibition of PS II probably results from the activity of the 9 kDa protein kinase.

Materials and Methods

Osmotically shocked chloroplasts, with Chl *a*/*b* quotients of about 2.5, were prepared from market lettuce following procedures described elsewhere [23]. The thylakoid membranes were suspended, at a chlorophyll concentration of 0.4 mg/ml, in 5 mM $MgCl_2$, 50% glycerol and 20 mM Tricine (pH 7.5). Unless stated otherwise, the thylakoid membranes were supplemented with 5 mM NaF and 0.5 mM ATP. For radioisotope labelling experiments [^{32}P]ATP (specific activity of about 10^6 cpm/nmol ATP) was also added. Phosphorylation of the thylakoid membrane proteins was initiated either by the incubation of dark-adapted chloroplasts with 10 mM DTE or by low-intensity white light (see Ref. 18). The phosphorylation reactions were terminated after 30 min by the addition of ice-cold 0.1 M EDTA.

Electron transfer rates were measured with a Hansatech DW1 oxygen electrode illuminated by saturating white light [23]. The chloroplasts were suspended, at a chlorophyll concentration of about 40 μg/ml, in 0.1 M EDTA, 25 mM Mes (pH 6.0), and supplemented with 2.5 mM potassium ferricyanide, 2.5 mM benzoquinone and 2 μg/ml gramicidin.

Alternatively, the thylakoid membranes (equivalent to 40 μg chlorophyll) were recovered by centrifugation (60 s at 10000 × *g* using an MSE Microcentaur) and resuspended in 50 μl of 1% bromophenol blue, 5% SDS, 50% glycerol, 0.1 M Tris (pH 6.8) and heated at 95 °C for 1 min. The phospho-polypeptides were separated by SDS-polyacrylamide gel electrophoresis on a 12%

acrylamide gel, and detected by autoradiography (Kodak X-Omat S film exposed to the radioactive gel for 48 h). The excised bands were treated with 0.5 ml of 30% hydrogen peroxide at 95 °C for 2 h. The 26 kDa and 24 kDa polypeptides were not normally separated but were combined together as LHC II. The ^{32}P content of the LHC II and 9 kDa polypeptides was determined by Cerenkov counting.

Results

The time-dependent incorporation of ^{32}P into thylakoid membrane proteins and inhibition of PS II

Fig. 1 shows that incubation of osmotically shocked lettuce chloroplasts with [^{32}P] ATP, under conditions in which the thylakoid membrane protein kinases are activated, results in the incorporation of the phosphate label into three major polypeptides. The reversible phosphorylation of these phosphoproteins, with molecular masses of 26, 24 and 9 kDa, is well established [1–6]. All three polypeptides incorporate phosphate onto threonine residues, as shown by high-voltage electrophoresis of the partial acid hydrolysates of the phosphoproteins (S.J. Yeaman and N.K. Packham, unpublished observations). Under the conditions employed in this report no phosphoproteins with molecular masses in the 30–35 kDa range can be identified, although the alpha and beta CF1 components of the ATP synthetase are labelled.

The time courses for the phosphorylation of the LHC II and 9 kDa polypeptides in illuminated chloroplasts are shown in Fig. 2. The LHC II and 9 kDa polypeptides ahve half-times for ^{32}P incorporation of 6 and 12 min, respectively. Similar time dependences are observed when dark-adapted chloroplasts are pretreated with DTE to activate the kinase before the addition of ATP (data not shown), indicating that the reduction of the plastoquinone pool is not the rate-limiting step in the phosphorylation process. The extents and kinetics of phosphorylation of the LHC II and 9 kDa polypeptides are independent of pH between 6.0 and 8.5 (data not shown).

Fig. 3 shows the time course for the phosphorylation-dependent inhibition of the oxygen evolution rate. A maximal inhibition of 23% could be measured. In this case, and in the other experiments described in this report, the 'phosphorylated' and 'non-phosphorylated' chloroplast samples underwent the same pretreatment. The oxygen evolution rates were measured, and the degree of inhibition caused by thylakoid membrane phos-

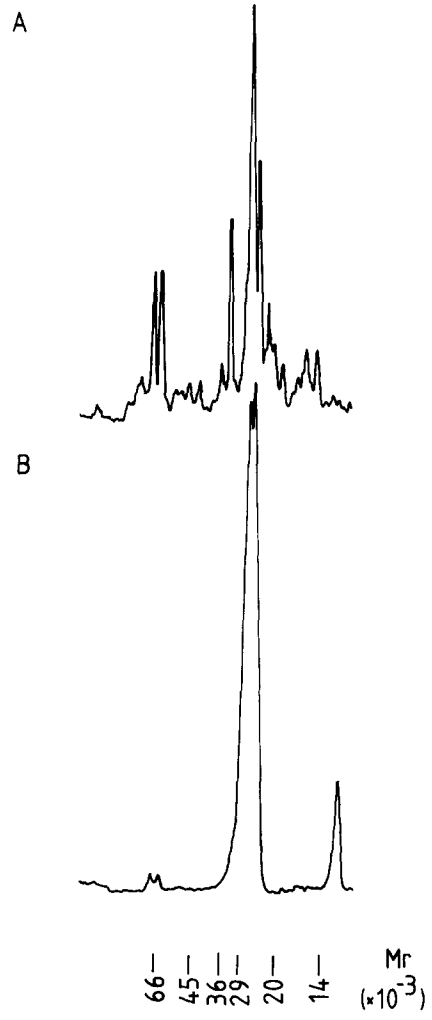

Fig. 1. Phosphorylation of thylakoid membranes from lettuce. Phosphorylation of thylakoid membranes from osmotically shocked chloroplasts from lettuce was as described in Materials and Methods. (A) Densitometer trace of Coomassie blue-stained acrylamide gel of thylakoid membrane proteins. (B) Densitometer trace of exposed autoradiogram of phosphorylated thylakoid membranes. Similar autoradiograms were obtained if the SDS-treated thylakoid membrane proteins were not heated, indicating that the absence of minor phosphoproteins could not be due to the activity of 'heat-activated' proteinases.

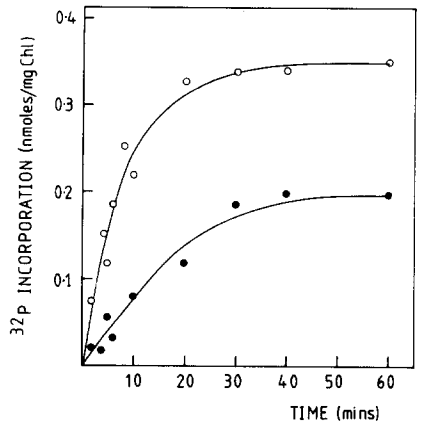

Fig. 2. Time course of the light-induced ^{32}P incorporation into the LHC polypeptides (○) and the 9 kDa polypeptide (●). Experimental conditions were as described in Materials and Methods.

phorylation was determined. Fig. 3 shows that the ATP-dependent inhibition was complete within 15 min of pre-illumination, and had a half-time of about 5 min. The kinetics of the ATP-dependent inhibition would appear to fit with the labelling of the LHC II and not the 9 kDa polypeptide.

The observation of an inhibition of the oxygen evolution rate upon addition of ATP (Fig. 4B) is not necessarily a direct consequence of thylakoid membrane phosphorylation. Incubation of osmotically shocked chloroplasts with millimolar concentrations of AMP-PNP has, however, no effect on the oxygen evolution rate (Fig. 4C), indicating that hydrolysis of the terminal phosphoanhydride bond of ATP is indeed needed for the inhibition.

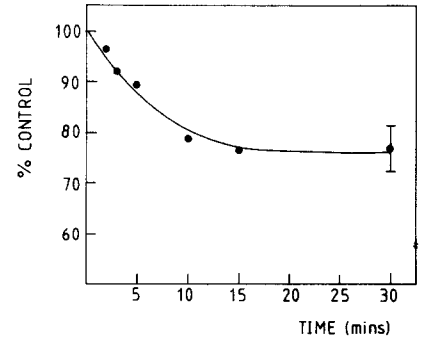

Fig. 3. Time course of the ATP-dependent inhibition of the oxygen evolution rate. The experimental conditions were as described in Materials and Methods. The rates are the means (±S.D.) of seven separate preparations. Maximal activity in the non-phosphorylated control chloroplasts was 165 μmol/mg Chl per h.

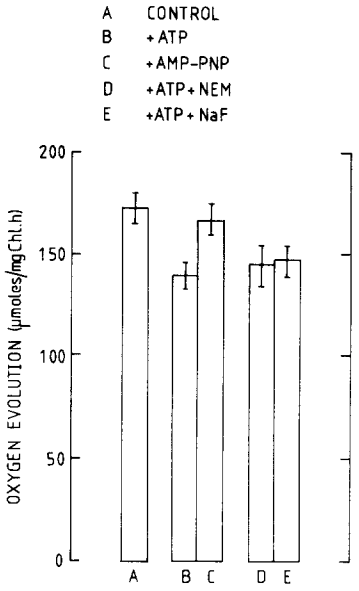

Fig. 4. The effect of kinase and phosphatase inhibitors on the ATP-dependent inhibition of the oxygen evolution rate. The oxygen evolution rates were measured as described in the Materials and Methods. The chloroplasts were pre-treated either in the presence or absence of 0.4 mM ATP, and with the following inhibitors: 2.5 mM *N*-ethylmaleimide (NEM) (D) or 5 mM NaF (E). The oxygen evolution rate in chloroplasts treated with ATP (or, as in (C), 2.5 mm AMP-PNP) was plotted against the normalised 'uninhibited' sample. The rates are the means (±S.D.) of at least seven independent measurements.

The ATP concentration dependence of ^{32}P incorporation into the thylakoid membrane proteins and the inhibition of oxygen evolution

Fig. 5 shows the ATP concentration requirement for ^{32}P incorporation into the LHC II and 9 kDa polypeptides. The LHC II and 9 kDa polypeptides have a similar ATP concentration dependence; the half-maximal stimulation is observed at about 0.1 mM ATP. A similar ATP concentration dependence has been reported by Horton and colleagues [24] for chloroplasts isolated from intermittent-light-grown pea plants. The inhibition of the oxygen evolution rate also has a similar ATP concentration dependence (data not shown).

The experiment of Fig. 5 was also performed in the presence of 2.50 mM *N*-ethylmaleimide. This sulphydryl-directed reagent has been shown to inhibit the incorporation of ^{32}P into thylakoid membrane polypeptides, and it has been argued that the sulphydryl group(s) are involved in the

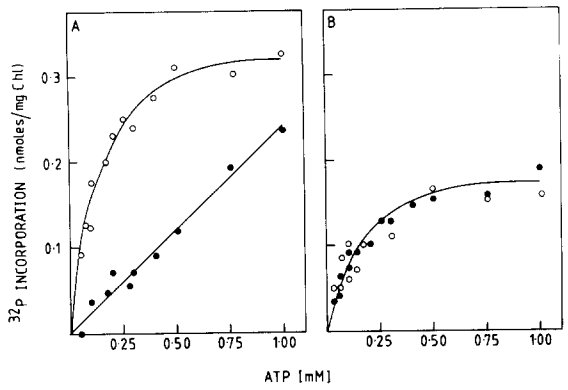

Fig. 5. The effect of ATP concentration on the incorporation of ^{32}P into the LHC II (A) and 9 kDa (B) polypeptides. Experimental conditions were as described in Materials and Methods, except that the ATP concentration was varied between 0 and 1.00 mM (○). The experiments were also undertaken in the presence of 2.50 mM *N*-ethylmaleimide (●).

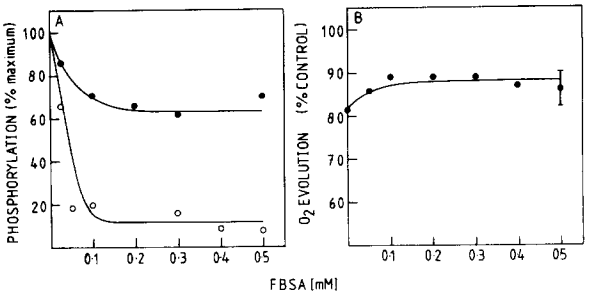

Fig. 6. FBSA-dependent inhibition of the phosphorylation of the LHC II (○) and 9 kDa (●) polypeptides (A) and the degree of inhibition of the oxygen evolution rate (B). Dark-adapted chloroplasts were pre-treated at room temperature with varying amounts of FBSA for 20 min. The chloroplasts were collected by centrifugation and were treated as described in Materials and Methods (except for the absence of NaF). The oxygen evolution rates are the averages (±S.D.) of three separate experiments. The maximal oxygen evolution rate was measured to be 150 μmol O_2/mg Chl per h.

regulation of the protein kinases by redox potential [21]. Fig. 5 shows that the presence of *N*-ethylmaleimide in the incubation medium decreases the extent of LHC II phosphorylation (Fig. 5A), but has no effect on the labelling of the 9 kDa polypeptide (Fig. 5B). A differential sensitivity of LHC II and 9 kDa phosphorylation to *N*-ethylmaleimide may indicate that the sulphydryl group of the 9 kDa protein kinase is less accessible to the water-soluble inhibitor. The presence of *N*-ethylmaleimide in the incubation medium, however, has little effect on the degree of ATP-dependent inhibition of the oxygen evolution capacity (Fig. 4D).

The effect of FBSA on the extent of ^{32}P incorporation into the thylakoid membrane proteins and the inhibition of oxygen evolution

Fig. 6A shows that the extent of phosphorylation of the LHC II, but not the 9 kDa polypeptide, is inhibited by the pretreatment of chloroplasts with millimolar concentrations of FBSA. Similar results were first obtained by Farchaus and colleagues [22], who also showed that the inhibition by FBSA could be overcome by the protection of the thylakoid membrane kinases by ATP. The effect of pre-treating chloroplasts with FBSA on the ATP-dependent inhibition of the oxygen evolution rate is shown in Fig. 6B. There is little effect of FBSA on the degree of inhibition, indicating that the degree of ATP-dependent inhibition of PS II matches the extent of phosphorylation of the 9 kDa polypeptide.

The $MgCl_2$ concentration dependence of ^{32}P incorporation into the thylakoid membrane proteins and the inhibition of oxygen evolution

Fig. 7 shows the $MgCl_2$ dependence on the extent of phosphorylation of both the LHC II and 9 kDa proteins. The LHC II and 9 kDa polypeptides have a similar Mg^{2+} requirement; 0.4 mM is sufficient to elicit a half-maximal stimula-

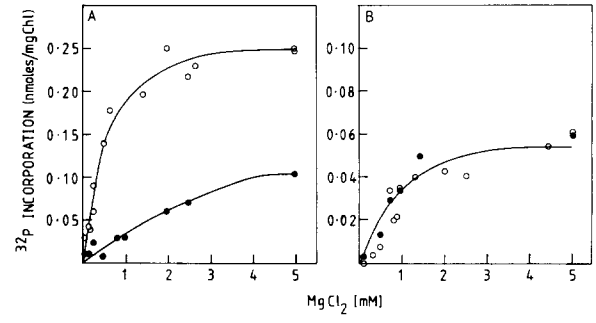

Fig. 7. The effect of $MgCl_2$ concentration on the phosphorylation of the LHC II (A) and 9 kDa (B) polypeptides. The dark-adapted chloroplasts (washed in $MgCl_2$-free buffer) were incubated at the indicated $MgCl_2$ concentrations for 20 min prior to the addition of ATP and illumination (○). The experiments were also undertaken without the addition of NaF (●).

tion the extent of phosphorylation. Similar values were first reported by Bennett [25]. No further stimulation in the degree of phosphorylation of either the LHC II or the 9 kDa polypeptide was observed as the $MgCl_2$ concentration was increased to that needed to induce the appression of the thylakoid membranes (however, see Ref. 26 and 27). The degree of inhibition of the oxygen evolution capacity of PS II caused by ATP has a similar $MgCl_2$ dependence to that needed for the phosphorylation of the thylakoid membrane polypeptides (data not shown).

The labelling experiment of Fig. 7 was also performed in the absence of NaF. Under these conditions, the steady-state extent of the phosphorylation of the LHC II and 9 kDa polypeptides is expected to be decreased, due to the competing action of their respective phosphoprotein phosphatase(s). A decreased extent of phosphorylation could be observed for the LHC II polypeptides (Fig. 7A), but not for the 9 kDa polypeptide (Fig. 7B). The degree of inhibition of the oxygen evolution rate is also unaffected by the presence of NaF (Fig. 4E).

Fig. 8 shows the degree of dephosphorylation of the LHC II against the background $MgCl_2$ concentration. The LHC II phosphatase has a $MgCl_2$ requirement, 0.2 mM is sufficient to cause a half-maximal stimulation in the extent of dephosphorylation. There was, however, no noted effect on the extent of phosphorylation of the 9 kDa polypeptide. This may indicate either that the

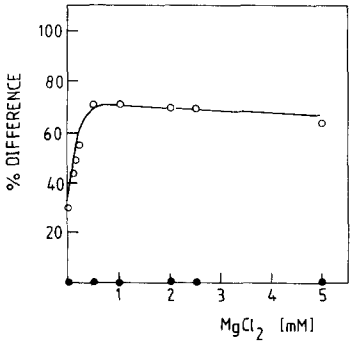

Fig. 8. The effect of NaF on the extent of phosphorylation of the LHC II (○) and 9 kDa (●) polypeptides. The decreased extent of ^{32}P incorporation into chloroplast samples by the depletion of NaF from the incubation medium was plotted against the background $MgCl_2$ concentration.

9 kDa phosphoprotein phosphatase is less active than the phospho-LHC II phosphatase or that it is inhibited by the conditions employed in this experiment. Preliminary observations in my laboratory indicate that the activity of the phospho-9 kDa phosphatase, unlike the phospho-LHC II phosphatase, is inhibited by submicromolar concentrations of ATP (M. Coulthard and N.K. Packham, unpublished observations).

Discussion

The differential sensitivity of phosphorylation of the 9 kDa and LHC II polypeptides to *N*-ethylmaleimide and FBSA support the earlier studies and conclusions of Cramer, Dilley and colleagues [21,22] that two distinct thylakoid membrane protein kinases are involved in the phosphorylation of the 9 kDa and LHC II polypeptides. Both protein kinases are activated upon the reduction of the plastoquinone pool and have similar Mg^{2+} and ATP concentration dependencies. The LHC II kinase differs from the 9 kDa protein kinase in its sensitivity to *N*-ethylmaleimide and FBSA. This difference between the two thylakoid membrane protein kinases has been exploited to examine the role of the 9 kDa phospho-polypeptide in the regulation of PS II photochemical activity. My results show that the degree of ATP-dependnet inhibition is relatively insensitive to the incubation of the thylakoid membranes with either FBSA (Fig. 6), *N*-ethylmaleimide (Fig. 4D) or NaF (Fig. 4E). All three treatments, however, are shown to have a dramatic effect on the extent of phosphorylation of the LHC II. These results would therefore identify the activity of the 9 kDa protein kinase as being the probable cause for the inhibtion of the light-saturated rate of oxygen evolution.

It should be noted that the evidence in this report cannot exclude the alternative possibility that the inhibition of PS II activity results from the phosphorylation of a pool of LHC II by the 9 kDa protein kinase. Horton and Lee [17] have observed an apparent match in the kinetics of recovery of oxygen evolution with the dephosphorylation of the LHC II and not with the (slower) 9 kDa. These authors concluded that the partial inhibition of PS II could arise from the

decoupling of phospho-LHC II from the reaction centre. However, preliminary studies in my laboratory indicate that the kinetics of dephosphorylation of the phospho-9 kDa polypeptide can be accelerated upon depletion of ATP. Thus, a comparison of the dephosphorylation kinetics of the LHC II and 9 kDa phosphoproteins with the recovery of the oxygen evolution capacity of PS II must be made either under strictly identical conditions or after the addition of a sufficient amount of NaF to inhibit all phosphatase activity

Since the degree of ATP-dependent inhibition of the oxygen evolution rate is observed at saturating actinic light intensities, thylakoid membrane phosphorylation must directly affect the electron transfer reactions [17,18]. The site of this inhibition is presumed to be on the acceptor side of the PS II reaction centre [18], and recent studies have suggested an effect of phosphorylation on the $Q_A^- \cdot Q_B \leftrightharpoons Q_A \cdot Q_B^-$ equilibrium constant (N.K. Packham, M. Hodges, A.L. Etienne and J. Briantais, unpublished data). The molecular basis of this inhibition could arise from the presence of additional negative charge on the acceptor side of PS II [26], which might either alter the binding of plastoquinone ot the Q_B site or decrease the pK_a of the Q_B^- (N.K. Packham, M. Hodges, A.L. Etienne and J. Briantais, unpublished data). The autoradiogram of Fig. 1 shows that the phosphorylation conditions employed prevented the labelling of the PS II-associated 32 kDa ('D1'), 34 kDa ('D2') and 44 kDa polypeptides. I therefore conclude that the ATP-dependent inhibition of PS II photochemistry probably results from the labelling of the 9 kDa polypeptide, and not from a covalent modification of the PS II reaction centre.

The absence of histidine residues in the predicted amino acid sequence of the phospho-9 kDa polypeptide [16] would indicate that this protein is not involved in light-harvesting, since crystal structure studies have shown that the magnesium atoms of either the bacteriochlorophyll *a* molecules in the reaction centre of *Rhodopseudomonas viridis* [29] or of the bacteriochlorophyll *s* molecules of the light-harvesting protein of *Prosthecochloris aestuarii* [30] are liganded to histidine. Although the function of the 9 kDa protein remains unclear it is interesting to speculate that it might be analogous to the 'H' subunit of the bacterial reaction centre. The 'H' subunit, which is not needed for light-harvesting or for the primary photochemical reactions, is thought to affect the binding of quinone to the Q_B pocket of the reaction centre and assist in the transfer of the reducing equivalents from the reaction centre to the quinone pool [29]. If a similar role existed for the 9 kDa polypeptide in PS II then one could predict that a conformational change of this polypeptide by protein phosphorylation could lead to a decreased affinity of the PS II reaction centre for plastoquinone and an observed decrease in the light-saturated rate of photosynthetic electron transfer.

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