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THYLAKOID PROTEIN PHOSPHORYLATION DURING STATE 1–STATE 2 TRANSITIONS IN OSMOTICALLY SHOCKED PEA CHLOROPLASTS

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In osmotically shocked pea chloroplasts illuminated with modulated blue-green light (light 2), phosphorylation of the light-harvesting chlorophyll *a*/*b*-protein complex (LHCP) accompanies the slow decrease in modulated fluorescence that indicates adaptation to light absorbed predominantly by Photosystem II (State 2). On subsequent additional illumination with continuous far-red light (absorbed predominantly by Photosystem I; light 1) both effects are reversed: modulated chlorophyll fluorescence emission increases (indicating adaptation towards State 1) and LHCP is dephosphorylated. Net phosphorylation and dephosphorylation of LHCP induced by light 2 and excess light 1, respectively, occur on the same time scale as the ATP-dependent chlorophyll fluorescence changes indicative of State 2 and State 1 transitions. The phosphatase inhibitor NaF (10 mM), stimulates the effect of blue-green light on fluorescence and prevents the effect of far-red light. These results provide a demonstration that light of different wavelengths can control excitation energy distribution between the two photosystems via the plastoquinol-activated LHCP phosphorylation mechanism suggested previously (Allen, J.F., Bennett, J., Steinback, K.E. and Arntzen, C.J. (1981) Nature 291, 25–29; and Horton, P. and Black, M.T. (1980) FEBS Lett. 119, 141–144).

Introduction

The phenomenon of State 1-State 2 transitions was first described for *Chlorella* by Bonaventura and Myers [1] and for *Porphyridium* by Murata [2]. State 1 is induced by light absorbed predominantly by PS I ('light 1') and State 2 is induced by light absorbed predominantly by PS II ('light 2'). Transition from State 1 to State 2 occurs when PS II is overexcited relative to PS I and involves an increase in the relative proportion of absorbed excitation energy being transferred to the PS I reaction centre. This adaptive mechanism is reversed if PS I is subsequently overexcited relative to PS II and hence transition to State 1 involves a redistribution of excitation energy in favour of PS II. The efficiency of overall photosynthetic electron flow is thereby maintained over a range of wavelengths that would otherwise predominantly excite one or other photosystems to the detriment of the overall quantum yield [3].

An explanation of this phenomenon has been put forward by Allen et al. [4] and by Horton and Black [5], who propose that distribution of excitation energy between the photosystems is regulated by the redox state of the plastoquinone pool. The link between plastoquinone and excitation energy distribution is held to be the reversible phosphorylation of the exposed segment of the light-harvest-

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; LHCP, light-harvesting chlorophyll *a*/*b*-protein complex; PS, photosystem; Q, primary acceptor of PS II.

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ing chlorophyll *a/b*-protein complex (LHCP). Phosphorylation of LHCP has been found to cause changes in chlorophyll fluorescence which seem to reflect increased excitation energy transfer to PS I [6]. Both phosphorylation of LHCP and the attendant fluorescence changes occur only if the plastoquinone pool is at least partially reduced [4,5,7–9].

According to this hypothesis [4,5], transition to State 2 is brought about because reduction of plastoquinone by light 2 activates a protein kinase that catalyses phosphorylation of LHCP and this results in a redistribution of excitation energy in favour of PS I [10]. Conversely, the transition to State 1 is caused by the oxidation of plastoquinone under light 1 and inactivation of the protein kinase, thus allowing dephosphorylation of LHCP by a thylakoid-bound phosphatase [11]. In this case there is redistribution of excitation energy in favour of PS II.

Under appropriate conditions, isolated pea thylakoids exhibit changes in modulated chlorophyll fluorescence that are similar to those seen during State 1-State 2 transitions in vivo [12,13]. In vitro the transition to State 2 is dependent on the presence of ATP and occurs under conditions which would be expected to induce phosphorylation of LHCP [12].

Here we report the results of an experimental test of the hypothesis [4,5] that LHCP phosphorylation brings about the transition to State 2 and that its dephosphorylation brings about the transition to State 1: the degree of LHCP phosphorylation was measured in pea thylakoids under conditions inducing fluorescence changes indicative of State 1-State 2 transitions.

Methods

Intact chloroplasts were isolated from 12–14-day-old pea shoots (*Pisum sativum* var. Feltham First) as described previously [14] except that in the experiment of Fig. 1 the washing procedure was omitted. In all cases the chloroplasts were finally resuspended in 0.33 M sorbitol, 3 mM $MgCl_2$, 50 mM Hepes, brought to pH 7.5 with KOH (final K^+ concentration 25 mM). The chloroplast suspension was kept on ice as a concentrated stock. Chlorophyll concentration was determined by the method of Arnon [15].

Modulated chlorophyll fluorescence was measured in a modified version of the apparatus described by Telfer and Barber [12]. A stirred 1 cm^2 glass cuvette, maintained at 22°C, was illuminated with blue-green light modulated at 90 Hz by an Ortec Brookdeal 9479 light chopper. This light served as both the exciting beam and the modulated, actinic light 2. Schott glass filters BG18 (4 mm) and BG38 (2 mm) defined the spectral composition of the exciting light, which had an intensity of 1.3 $W \cdot m^{-2}$. Modulated fluorescence was measured at 180° with an EM1 9558 photomultiplier and an Ortec Brookdeal 9501 lock-in amplifier (time constant of 1 s). The photomultiplier detected fluorescence emission through a Balzer 689 nm interference filter and 4 mm of Schott RG 645 glass filter. Non-modulated light 1 (Balzer 710 nm, 8 $W \cdot m^{-2}$) was introduced at right angles to the exciting beam.

The intact chloroplasts were subjected to an osmotic shock for 15 s in 6 mM $MgCl_2$ and ferredoxin (purified from *Spirulina maxima*). Double-strength medium was then added to give final concentrations as follows: 0.33 M sorbitol, 3 mM $MgCl_2$, 50 mM Hepes, brought to pH 7.5 with KOH (final K^+ concentration 25 mM). Other additions were as noted in the Figure legends.

In the experiments where the degree of incorporation of ^{32}P into LHCP was measured, high specific activity [γ-^{32}P]ATP was added to the cuvette immediately after the unlabelled ATP. For measurement of ^{32}P incorporation, 100- or 200-μl samples were removed at the times indicated and the reaction was terminated by addition of the samples to ice-cold trichloroacetic acid (final concentration 10%). Protein samples were acetone precipitated and prepared for SDS-polyacrylamide gel electrophoresis as previously described [16]. The degree of protein phosphorylation (^{32}P incorporation) was measured by Cerenkov counting of LHCP bands excised from the gel.

Results

The ATP dependence of the transition to State 2 in osmotically shocked chloroplasts was demonstrated by Telfer and Barber [12], using ferredoxin as an auto-oxidisable electron acceptor. Fig. 1a

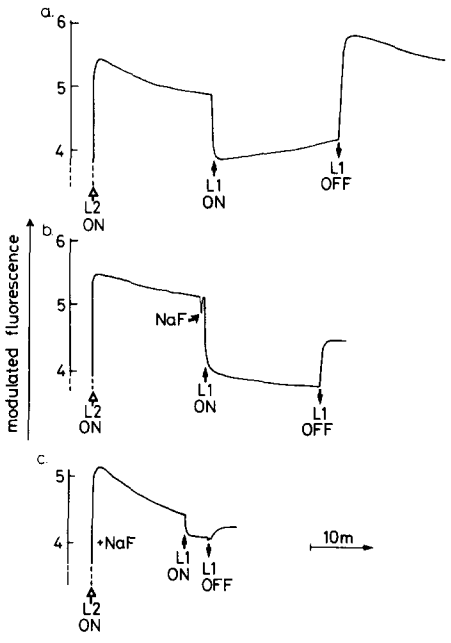

Fig. 1. State 1-State 2 transitions monitored as changes in the modulated fluorescence of chlorophyll *a* showing the effect of NaF. The experimental procedure was as described in Methods and Results. The reaction mixture included (volume 3 ml) 27 $\mu g/ml^1$ chlorophyll, 2.5 μM ferredoxin, 0.166 mM $NADP^+$, 27 nM nigericin, 0.15 mM ATP. In addition a and b contained 10 mM NaCl and c 10 mM NaF. NaF was added as indicated in trace b to give a final concentration of 10 mM. L1 and L2 refer to light 1 and light 2, respectively.

shows the results of a similar experiment which was carried out using $NADP^+$ as the terminal electron acceptor. In osmotically shocked pea chloroplasts supplied with ATP in the dark, modulated light 2 gives an initially high fluorescence emission which slowly decreases with time. This suggests that the chloroplasts are in State 1 in the dark and that light 2 then brings about the transition towards State 2.

Subsequent addition of actinic light 1 (Fig. 1a) causes a rapid decrease in fluorescence intensity due to oxidation of Q, the primary acceptor of PS II, thus indicating the degree of imbalance of distribution of the excitation energy of light 2 between the photosystems. The rapid decrease in fluorescence intensity is followed by a slow increase during the period of illumination with the additional light 1. When light 1 is turned off there is a rapid increase in fluorescence intensity to a level higher than that achieved with prolonged light 2 illumination in the absence of light 1. This indicates that during illumination with light 1 there has been a change in the degree of imbalance of excitation energy in favour of PS II, i.e., Q becomes relatively more reduced by light 2 alone and the treatment with additional light 1 has brought about the transition towards State 1. This is a reversible adaptation, since continued illumination with light 2, after switching off light 1, brings about a slow decrease in fluorescence indicative of a readaptation towards State 2.

If the transition to State 1 requires dephosphorylation of LHCP by the protein phosphatase then it should be prevented by the phosphatase inhibitor, NaF [11]. Fig. 1b shows that this is the case; addition of NaF to thylakoids approaching State 2 (induced by illumination with light 2) prevents the subsequent transition to State 1 (induced by illumination with light 1). The rapid decrease in fluorescence intensity due to oxidation of reduced Q is in fact followed by a further slow decline in fluorescence intensity, indicating that the thylakoids still continue to approach State 2. This is confirmed by the increase in fluorescence intensity seen when light 1 is removed which is small compared to the increase in Fig. 1a in the absence of NaF.

In Fig. 1c it can be seen that NaF added before illumination increases the rate of the fluorescence decline during the transition to State 2. This is consistent with the idea that normally phosphatase activity reduces the net rate of transition towards State 2. The effect of NaF on the transition from State 1 to State 2 cannot be an effect of increased cation concentration as it was not observed in either experiment a or b where 10 mM NaCl was added as a control.

Fig. 2a shows changes in modulated fluorescence in pea thylakoids supplied with [γ-^{32}P]ATP in order to permit measurement of the degree of LHCP phosphorylation under conditions which induce State 1 or State 2. In these experiments electron transport was catalysed by ferredoxin alone, as in the experiments of Telfer and Barber [12]. Pea thylakoids were dark adapted for 2 min before addition of [γ-^{32}P]ATP. After a further 2 min incubation a dark sample was taken for measurement of the degree of phosphorylation of

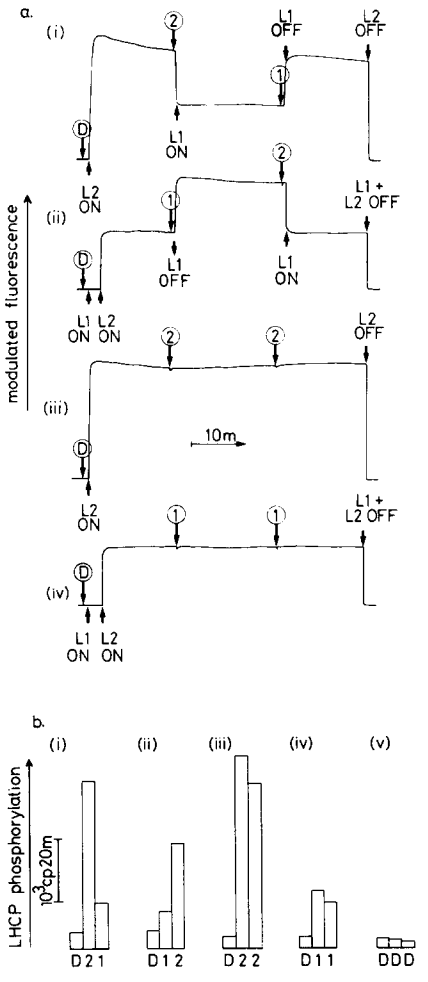

Fig. 2. Light-induced changes in modulated fluorescence and phosphorylation of LHCP induced in isolated thylakoids by various sequences of light 2 and light 1. (a) Changes in modulated fluorescence of chlorophyll *a* which indicate transition towards State 1 (light 1 plus light 2) or State 2 (light 2 alone). The points at which 200-μl samples were taken for SDS-polyacrylamide gel electrophoresis are indicated by an encircled letter or number; D, dark sample; 1, State 1 sample; 2, State 2 sample. All reaction mixtures included 50 μg/ml[1] chlorophyll, 5 μM ferredoxin, 0.5 μM nigericin, 0.5 μM valinomycin, 0.1 mM ATP, 50 μCi/3 ml [γ-^{32}P]ATP. Other conditions were as described in Methods and Results. (b) The incorporation of ^{32}P into the LHCP bands of SDS-polyacrylamide gel electrophoresis tracks of the samples taken as shown in a. Expt. v shows the incorporation of ^{32}P into LHCP in samples from a reaction mixture incubated with labelled ATP for 2, 18 and 38 min.

LHCP. The pea thylakoids were then incubated for 1 min in the dark before illumination with modulated light 2 $\pm$continuous light 1 as indicated in the figure. Further samples for measurement of LHCP phosphorylation in chloroplasts adapted to light 1 or light 2 were taken as indicated.

Fig. 2b depicts the total radioactivity of the LHCP bands of the SDS-polyacrylamide gel electrophoresis tracks corresponding to the three samples taken during the time course of each experiment in Fig. 2a. In Expts. i–iv it is clear that the degree of LHCP phosphorylation was greater following illumination with light 2 alone than with light 1 plus light 2. Expt. i shows that subsequent illumination with additional light 1 after light 2 alone brings about dephosphorylation as compared to Expt. iii where illumination with light 2 alone is continued. On the other hand, Expt. ii showed that there is an increase in phosphorylation on removal of light 1 compared to Expt. iv where illumination with light 1 plus light 2 is continued. Expt. v indicates the very low level of

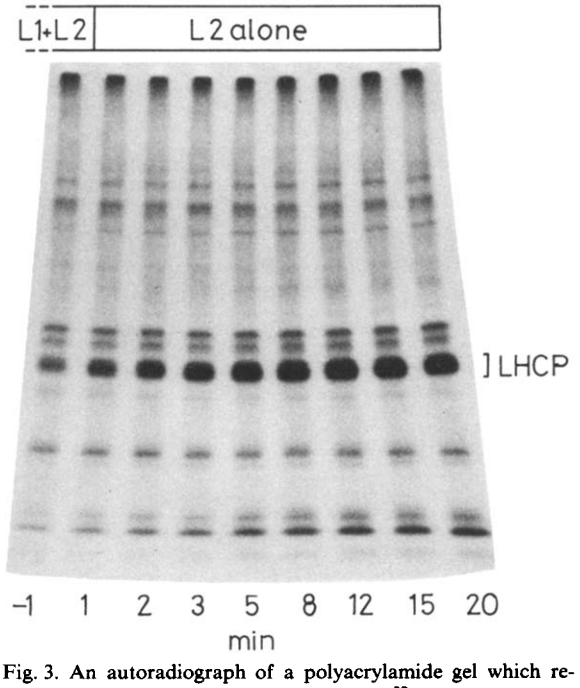

Fig. 3. An autoradiograph of a polyacrylamide gel which records the time course of incorporation of ^{32}P into chloroplast polypeptides during transition from State 1 (induced by 20 min light 1 plus light 2) towards State 2 (induced by light 2 alone). Conditions were as described in Fig. 2 except that 100-μl samples were taken for SDS-polyacrylamide gel electrophoresis at the times (after switching off L1) indicated in the diagram.

LHCP phosphorylation which occurs in samples incubated in the dark.

The absolute changes in fluorescence yield induced by lights 1 and 2 in this experiment were small compared to those reported by Telfer and Barber [12]. It is possible that in this experiment the changes in the redox state of the plastoquinone pool induced by lights 1 and 2 were small and that the ^{32}P incoporation represents only a low level of LHCP phosphorylation. Nevertheless, the phosphorylation data are in agreement with the predictions of the hypothesis [4,5].

Fig. 3 shows the pattern of ^{32}P incorporation into chloroplast polypeptides during the transition from State 1 towards State 2. This experiment was carried out under exactly the same conditions as those of Fig. 2. The changes in modulated fluorescence emission were monitored and were found to follow the pattern typical of State 1-State 2 transitions. It can be seen that LHCP becomes phosphorylated during the time course of the transition, as do a number of unidentified polypeptides of lower molecular weight.

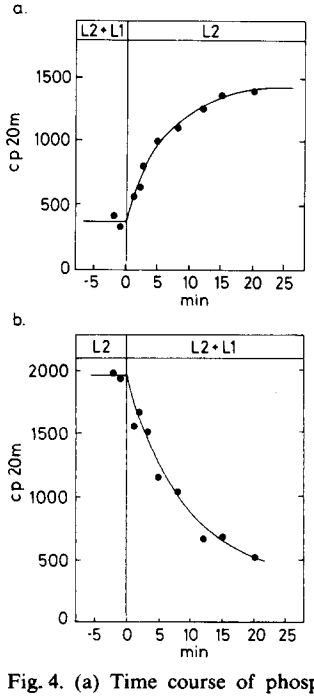

Fig. 4. (a) Time course of phosphorylation of LHCP during transition from State 1 to State 2 in pea chloroplasts (data from the experiment of Fig. 3). The transition was initiated by switching off light 1 after 20 min preillumination with light 1 and 2. (b) Time course of dephosphorylation of LHCP during transition from State 2 to State 1. The transition was initiated by switching on light 1 after 20 min preillumination with light 2 alone. Conditions were as decribed for Fig. 3.

The time course of LHCP phosphorylation during the transition from State 1 towards State 2 is plotted in Fig. 4a. A semilogarithmic plot gives a half-time of 4 min for net phosphorylation. A time course for net dephosphorylation accompanying the transition from State 2 towards State 1 is plotted in Fig. 4b. The half time for this reaction was 6 min.

Discussion

The results presented here are in complete agreement with the hypothesis under test [4,5]: transition to State 2 involves phosphorylation of LHCP while transition to State 1 involves dephosphorylation of LHCP (Figs. 2 and 3). In addition, transition to State 1 is inhibited by NaF which is an inhibitor of the protein phosphatase [11] that catalyses dephosphorylation of LHCP (Fig. 1).

While the data presented here do not exclude the possibility that some other polypeptide is involved, the role of LHCP in distribution of excitation energy between the two photosystems is now well established [17,18] and it seems likely that it is the phosphorylation of LHCP that brings about the required redistribution of excitation energy in favour of PS I in vivo [4,6,7].

Control of LHCP phosphorylation by the redox state of plastoquinone also has adequate experimental support. Site-specific electron donors and electron-transport inhibitors have the expected effects on LHCP phosphorylation and on associated room-temperature and 77 K fluorescence emission [5,7,9]. Potentiometric redox titration confirms that thylakoid protein kinase activity [8] and its attendant chlorophyll fluorescence decrease [7,8] are controlled by a two-equivalent carrier with the midpoint potential of plastoquinone.

State 1-State 2 transitions may also be measured as changes in oxygen yield [1,3]. In isolated, osmotically shocked chloroplasts, an enhancement of the rate of modulated oxygen evolution has been observed on addition of a continuous beam of light 1 [19]. A large enhancement of this kind

indicates an imbalance in excitation distribution in favour of PS II, i.e., the chloroplasts are in State 1. Decreased enhancement of oxygen yield brought about by light 1 indicates a transition to State 1: this transition has been shown to be ATP-dependent by Sinclair and Cousineau [20] though no direct measurements of LHCP phosphorylation have yet been made during observation of changes in oxygen yield. Although Sinclair and Cousineau [20] saw an ATP-dependent decrease in the yield of fluorescence and in the enhancement ratio the absolute yield of oxygen did not increase as expected. In these experiments neither uncoupler nor the reagents required for photophosphorylation were present, thus the rate of oxygen evolution may have been limited by the back-pressure of the proton-motive force.

The adaptive significance of the capacity to perform State 1-State 2 transitions is presumably that the quantum yield of photosynthesis is thereby held high and constant over regions of the spectrum in which excitation of the two photosystems would otherwise be unequal [3]. It has been argued that rapid physiological adaptation to changing wavelength is of selective value in both algae and higher plants [21]. It has also been suggested [22] that plastoquinone-mediated excitation redistribution increases the quantum yield of photophosphorylation by supplying the optimal proportion of excitation energy during changes in the relative rate of PS I-driven cyclic electron transport.

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