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Correlations between fluorescence and phosphorylation changes in thylakoid membranes of *Chlamydomonas reinhardtii* in vivo: a kinetic analysis

P. Delèpelaire and F.-A. Wollman *

Institut de Biologie Physico-chimique, 13, rue P. et M. Curie, 75005 Paris (France)

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The reorganization of the light-harvesting antenna in the thylakoid membranes upon phosphorylation of some of its apoproteins was further characterized in vivo using the green algae *Chlamydomonas reinhardtii*. To this end we have studied light-to-dark transitions on intact cells placed in the anaerobic state using the F34 mutant strain which lacks PS II centers. We show that the 50% decrease in fluorescence yield in such transitions is accompanied by a 50% increase in PS I antenna size. The half-times of the kinetics of the fluorescence changes in the dark-to-light and light-to-dark transitions are of 320 and 120 s, respectively. The rate-limiting steps in these transitions are attributed to the dephosphorylation and phosphorylation processes themselves rather than to the activation of the kinase or to the diffusion of the phosphorylated complexes in the thylakoid membrane. Accordingly, the changes in phosphorylation of three of the main phosphopolypeptides occur with the same kinetics as those of the fluorescence changes. Different phosphorylation kinetics are observed for two phosphopolypeptides which are, however, also part of the light-harvesting complexes. Possible heterogeneities in the kinase enzymatic activities are discussed. The peculiar status of the phosphopolypeptide D2, associated with the PS II center, is described.

Introduction

We have shown in a previous paper [1] that a reversible phosphorylation of the LHC polypeptides occurs in vivo as well as in vitro in *Chlamydomonas reinhardtii*. In addition, the corresponding changes in the distribution of the light energy collected by the LHC were shown to occur also in the F34 mutant lacking in PS II centers. Changes in the association of LHC complexes with PS I centers can be readily monitored by the fluorescence changes induced by different levels of PQ pool reduction: they are more easily studied in the F34 mutant, since there is no fluorescence modulation by the redox state of Q. During an incubation in the dark under anaerobic conditions, the plastoquinone pool becomes reduced, which in turn activates the kinase, thus leading to a phosphorylation of the LHC and subsequent redistribution of the energy in favour of PS I: under these conditions the fluorescence yield is low, the energy being trapped by PS I centers. In a subsequent illumination the PQ pool is reoxidized because of the functioning of the PS I centers, thus inactivating the kinase: the LHC is dephosphorylated and is no longer associated with PS I: the fluorescence yield is high, since there is no PS II in this mutant. We investigate in the present paper the relation-

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* To whom correspondence should be addressed.

Abbreviations: LHC, light-harvesting complexes; PQ, plastoquinone; DCMU, 3,4-dichlorophenyl-1,1-dimethylurea; F_m, maximum level of fluorescence; PS I, Photosystem I, PS II, Photosystem II; Chl, chlorophyll; A, absorbance.

ship between the kinetics of these fluorescence changes and the activities of the two enzymes, a kinase and a phosphatase, which control this regulation process.

Material and Methods

Two *Chlamydomonas reinhardtii* strains were used throughout this work: a wild-type strain and a mutant strain, F34, lacking in PS II centers. Both strains were grown in Tris-acetate-phosphate medium at a light intensity of 200 lx and used in the mid-exponential phase ($3 \cdot 10^6$ cells/ml) of growth.

Fluorescence measurements

The F34 cells were harvested by centrifugation and resuspended at a final chlorophyll concentration of 20 μg Chl/ml in Tris-acetate medium containing 10% Ficoll, glucose oxydase (2 mg/ml), glucose (20 mM), and ^{32}P (1 μCi/ml). They were then incubated in the dark for 20 min before measuring their fluorescence level: under these conditions (no oxygen present) the functioning of the respiratory-like chain in the thylakoid membrane [2], which transfers electrons from NADPH to O_2 through the PQ pool, reduces the PQ pool, and the LHC is consequently phosphorylated and associated with the PS I unit. Fluorescence changes were recorded during a subsequent illumination which reoxidizes the PQ pool, so as to inactivate the kinase, and dephosphorylates the LHC which is no longer associated with PS I: this corresponds to an 'upward transition', since fluorescence yield increases during this period. The fluorescence yield decreases during the 'downward transition' when the light is shut off and the PQ pool rereduced, thus reactivating the kinase. Fluorescence was monitored using a spectrophotometer as a fluorimeter as described by Joliot et al. [3].

Optical cross-section measurements

Optical cross-sections of PS I centers in the F34 mutant under the various conditions of PQ pool redox state were measured by the ratio of the ($\Delta A_{515\ \mathrm{nm}} - \Delta A_{480\ \mathrm{nm}}$) absorption changes induced after a weak flash over that induced by a saturating laser flash and measured 150 μs after the flash according to Joliot and Delosme [4].

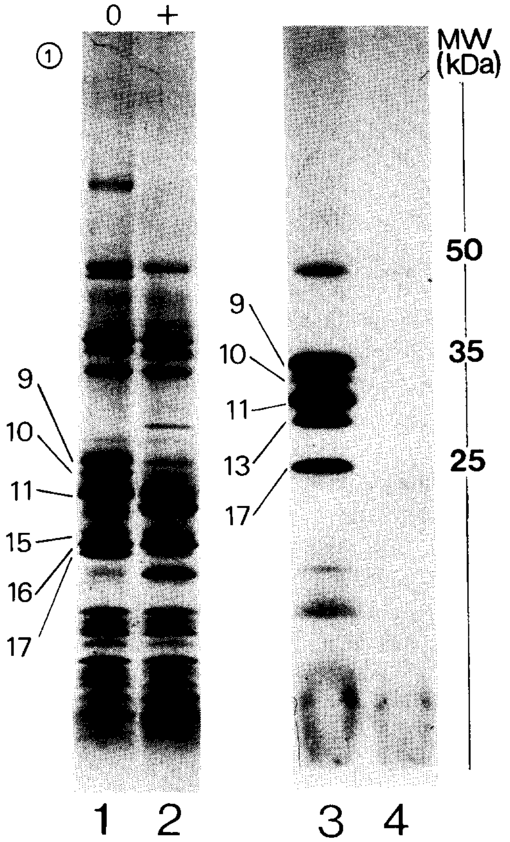
Biochemical techniques

^{32}P labeling of the thylakoid membrane polypeptides, isolation of the thylakoid membranes, gel electrophoresis and autoradiography were carried out as in our previous paper [1]. Urea gels were run according to Piccioni et al. [5].

Results

The phosphorylation pattern of the wild-type strain under reducing conditions is shown in Fig. 1 after analysis of the thylakoid membrane polypeptides on a 7.5–15% polyacrylamide gradient gel (lane 3). Several polypeptides are phosphorylated among which LHC polypeptides: 10, 11, 13 and 17. There are also fainter bands in the high and the low molecular weight range corresponding mainly to PS II polypeptides (see below). Trypsin treatment (lane 2, stained gel and lane 4, autoradiogram) shows that all this label is cleavable and thus exposed to the outer surface of the thylakoid membrane. This treatment allowed us to assign the lower labeled band in the CP II region to polypeptide 17 and not to polypeptide 16 as we misquoted it in our previous paper [1], since polypeptide 17, but not polypeptide 16, is cleavable by trypsin (lane 2).

More refined analysis of the phosphorylation patterns of the wild-type and F34 mutant strain under two distinct conditions (PQ pool reduced or oxidized) are shown in Fig. 2 after polyacrylamide gel electrophoresis in the presence of urea, which allows to study in greater detail the low molecular-weight polypeptides. As already observed, several polypeptides are phosphorylated under reducing conditions and dephosphorylated under oxidizing conditions: polypeptides 9–11, 13 and 17 (10, 11, 13 and 17 are associated with LHC) and several other polypeptides which are associated with the PS II center (and consequently missing in the PS II mutant (see lanes 2 and 5): 6, L5, L6. One polypeptide, D2.1, also associated with the PS II center deserves special attention, since it is more phosphorylated when the PQ pool is oxidized than when it is reduced (compare lanes 3 and 4). Overall, the phosphorylation pattern of the F34 and the wild type is the same for the LHC polypeptides: this allowed us to study in the PS II mutant all the events related to the redistribution



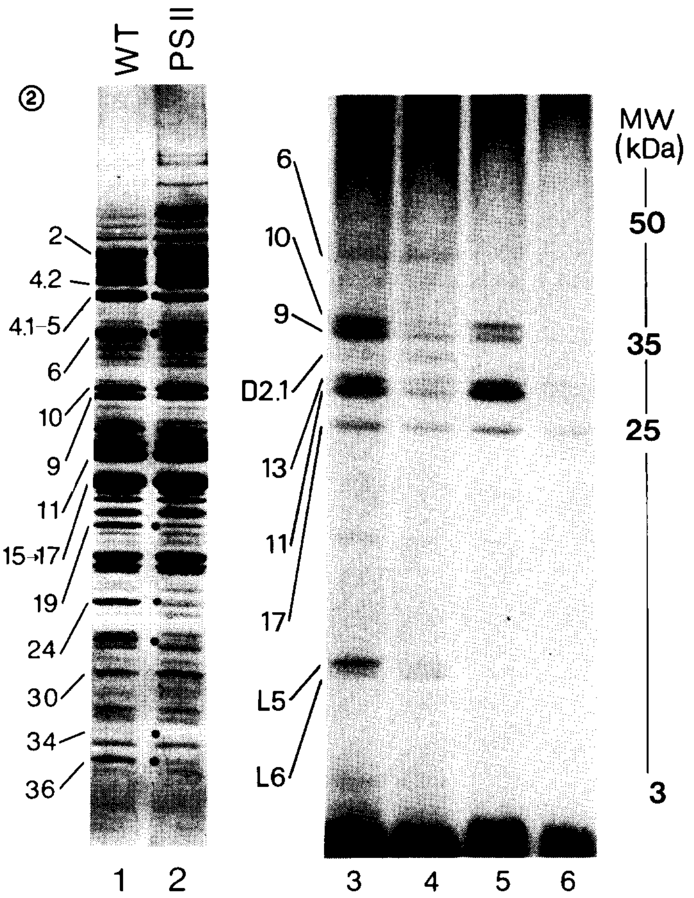


Fig. 1. Effect of trypsin digestion on phosphorylated polypeptides in the thylakoid membrane of the wilde-type (WT) strain. Lane 1, 7.5–15% acrylamide gradient gel of SDS solubilized thylakoid membranes, Coomassie blue staining; lane 2, same as lane 1 using trypsin-treated membranes (50 μg/ml for 30 min at 4°C at [Chl] = 1 mg/ml); lane 3, autoradiogram showing the phosphopolypeptides present in lane 1; lane 4, autoradiogram of trypsin-treated membranes.

Fig. 2. Analysis by polyacrylamide gel electrophoresis in the presence of 8 M urea of the thylakoid membrane polypeptides of the wild-type (lane 1) and the F34 mutant strain devoid of PS II reaction centers (lane 2) and of their phosphorylation under different conditions. Lane 3, wild type, PQ pool reduced under anaerobic conditions; lane 4, wild type, PQ pool oxidized under continuous illumination in the presence of DCMU; lane 5, F34, PQ pool reduced under anaerobic conditions; lane 6, F34, PQ pool oxidized under continuous illumination.

of energy induced by the phosphorylation of the LHC polypeptides with no interference with the redox state of Q, the primary acceptor of PS II.

As already mentioned, the algae display a low fluorescence yield when the PQ pool is reduced and a high fluorescence yield when it is oxidized. These variations actually correspond to changes in the optical cross-sections of the PS I units as shown Table I (see Materials and Methods for this measurement). Under anaerobic conditions in the dark, the algae have a PS I antenna size 50% greater than when they are incubated for 20 min under continuous illumination. We also note that

TABLE I

	$\Delta I/I_{515\,\mathrm{nm}}$ weak flash	–	$\Delta I/I_{480\,\mathrm{nm}}$ laser flash	Ratio weak flash/ laser flash
PQ ox	925		5801	0.16
PQ red	1069		4624	0.23

there is an apparent decrease of the amplitude of the signal induced by a saturating laser flash after incubation under anaerobic conditions.

In Fig. 3A are shown the fluorescence transitions occurring inbetween these two extreme cases in a typical experiment devised to change the repartition of the light-harvesting antenna in the F34 mutant lacking in PS II centers (see Materials and Methods). Several observations can be drawn from Fig. 3A:

(1) there is a lag of variable length in the fluorescence rise which increases with the time of incubation of the algae under anaerobic conditions (in Fig. 3A are shown two extreme cases: one corresponds to an incubation time of 5 min and shows a very small lag, whereas the other which displays a lag of several minutes corresponds to an incubation time of 40 min.);

(2) the half-time of the fluorescence rise (not taking into account the lag phase) is longer than that of the fluorescence decrease (320 versus 120 s);

(3) the semi-log plot of the rise phase (where only the phosphatase is active) is a straight line, showing that this reaction is first-order whereas the semi-log plot of the fluorescence decrease (where both the kinase and the phosphatase are active) shows that this reaction is not a first-order reaction (see Fig. 4).

Further characterization of the process underlying these kinetics is shown Fig. 3B and C. When the upward transition is interrupted at different illumination times (i.e., at different values of the fluorescence yield), the half-times of the downward transitions remain unchanged (Fig. 3B). On the other hand, under different intensities of the

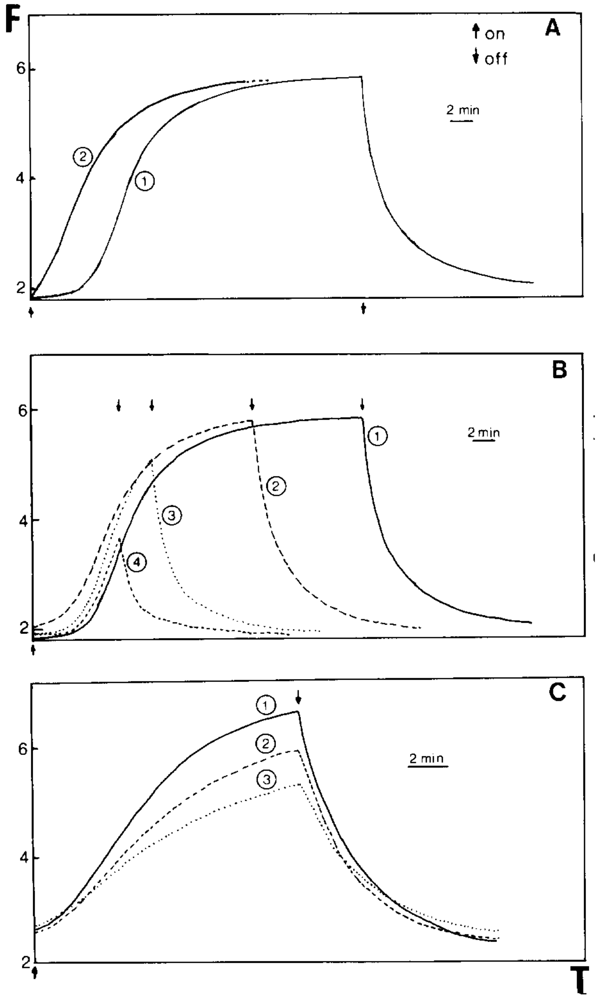


Fig. 3. Fluorescence changes observed on the F34 mutant during 'upward' and 'downward' transitions under different conditions: (A) fluorescence changes during a typical 'upward-downward' transition: the lag in the 'upward' transition can be more or less pronounced (curves 1 and 2); the two arrows point to shutting on and off the light; (B) same experiment as in (A), except that the 'upward' transition was interrupted at different times, corresponding to the different curves (dots); (C) same experiments at in (A), except that the light intensity used to reoxidize the PQ pool in the 'upward' transition was variable. (1), 100%; (2), 8.1%; (3), 3.2%.

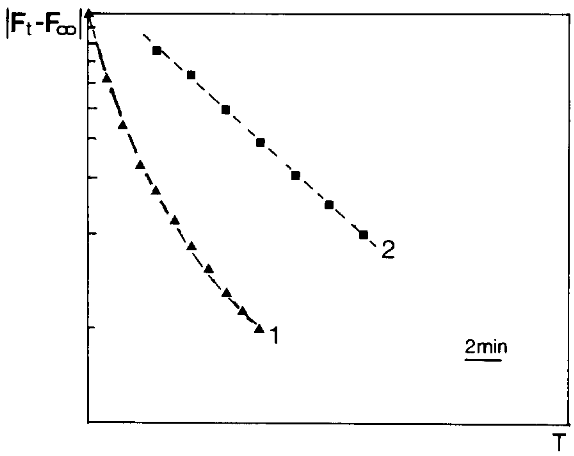


Fig. 4. Semi-log plots of the 'upward' and 'downward' transitions corresponding to Fig. 3A. Curve (1), 'downward' transition; curve (2), 'upward' transition.

continuous illumination, to ensure different levels of PQ pool reduction, we observe a decrease in the maximal fluorescence yield reached in the upward transition with no parallel changes in the half-times of either the upward or downward transitions (Fig. 3C).

The phosphorylation patterns of the thylakoid membrane polypeptides during the upward and downward transitions are shown Fig. 5A and B. As already observed [1] the extent of dephosphorylation (i.e., the decrease of phosphate bound to a given polypeptide normalized over the phosphate bound when the PQ pool is reduced) during the upward transition is not the same for all polypeptides, the greatest extent being observed for polypeptides 10 and 11 in this experiment. We can distinguish two classes of polypeptides in terms of kinetics of phosphorylation and dephosphorylation: 9–11 whose kinetics of dephosphorylation and rephosphorylation are very comparable to those seen for the fluorescence changes: the half-times of dephosphorylation for polypeptides 9–11 are 330 and 160 s for their rephosphorylation (Fig. 5B). On the other hand, polypeptide 17 (as well as polypeptide 13) does not follow this pattern (see Fig. 5B): its extent of dephosphorylation is much smaller and its kinetics shows transitory overphosphorylation during the upward transition. However, as noted earlier [1], the extent of dephosphorylation is not the same for all polypeptides under different experimental conditions.

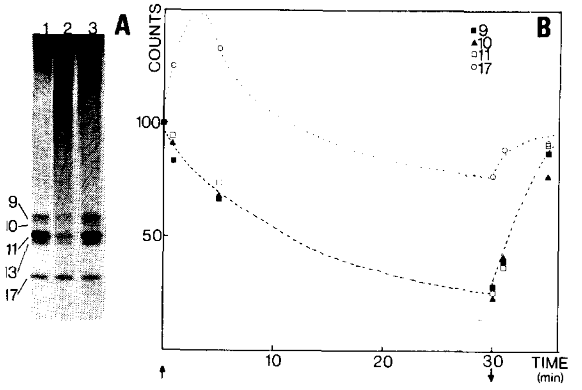


Fig. 5. Kinetics of phosphorylation and dephosphorylation of the thylakoid membrane polypeptides during ‘upward’ and ‘downward’ transitions (A) patterns of the phosphorylated polypeptides; lane 1, control under anaereobic conditions; lane 2, +20 min light; lane 3, +20 min light+5 min dark. (B) Radioactivity bound to the distinct polypeptides (9–11,17) during the ‘upward/downward’ transition as estimated by scanning the autoradiogram; three polypeptides follow a similar kinetics (9–11) whereas 17 and 13 (not shown in the figure) do not.

Discussion

During an upward transition in the F34 mutant cells the fluorescence yield increases by more than a factor 2. This is accompanied by an extensive change in the optical cross-section of the PS I units which is 50% larger when the PQ pool is reduced (see Table I). In addition, we have previously shown in a similar experiment that the phosphorylated LHC polypeptides become dephosphorylated and that the F_{715}/F_{682} ratio at 77 K increases by a factor 2. These data clearly prove that, even in the absence of active PS II centers, LHC complexes are reversibly associated with PS I centers in an LHC phosphorylation dependent fashion. We can make a rough estimate of the number of chlorophyll molecules actually involved in the reversible association of LHC with the PS I centers. A decrease by a factor 2 of the fluorescence yield in the F34 mutant upon phosphorylation of the LHC is consistent with the analysis by Haworth et al. [6] who suggested that about half of the total LHC could be reversibly phosphorylated. Knowing that there are about 400–500 chlorophyll molecules per electron-transfer chain, half of which are located on LHC complexes, then 100–125 chlorophyll molecules would be reversibly transfered to a PS I unit upon phosphorylation of the LHC. In view of the corresponding 50% increase in the PS I antenna size, we can calculate there are about 200–250 chlorophyll molecules in the PS I unit in the dephosphorylated state and 300–375 in the phosphorylated state.

The phosphorylation-dependent changes in the PS I antenna size that we observe using the F34 mutant cells in vivo are of much greater amplitude than those reported with higher plant chloroplasts in vitro (50% vs. 17%) [7,8] and reach those obtained in the latter organisms during high-to-low salt transitions [8]. Similar observations can be made from the comparison of the F_{max} levels in the three types of experiments. This supports the validity of fluorescence experiments at room temperature to study the reorganization of the antenna induced by phosphorylation of thylakoid membrane polypeptides. The larger effects observed upon phosphorylation of the F34 thylakoid in vivo might arise from a greater mobility of the LHC complexes due to the absence of PS II centers in this mutant. However, it has been shown that, besides the migration of phosphorylated LHC from PS II to PS I regions, changes in the surface charge density upon phosphorylation will mimic cation effects and produce significant destacking and intermixing of membrane complexes if the external concentration of cations is low enough [9]. Then the cation concentration at the external surface of F34 thylakoids in vivo might be lower than that used in vitro (5 mM) on pea chloroplasts [8].

Several steps can be formally distinguished in the process leading from the reduction of the PQ pool to the actual effect on the optical cross-section of the PS I units:

external redox potential
reduction of the PQ pool
activation of the kinase
phosphorylation of the LHC

Migration of the phosphorylated LHC towards the PS I unit from the granal to the stromal parts of the hylakoid membrane [10,11]:

Connection of the phosphorylated LHC with the PS I unit

The whole process is kinetically controlled by one or several of these steps which are the rate-limiting ones.

(1) Changes in the redox state of the PQ pool are completed within a few seconds and are thus much faster than the kinetics of the fluorescence changes.

(2) If the rate-limiting step were the diffusion in the membrane plane of the LHC, then the mean distance between LHC and the PS I centers would be an essential parameter: this is not what we observe, since the kinetics of the downward transition are independent of the level at which the upward transition is interrupted (see Fig. 3B), then independent, in this hypothesis, of the mean distance between LHC and PS I.

(3) If the reversible activation of the kinase were to occur through binding of reduced or oxidized PQ molecules with different affinities to some binding site, then a change in the relative concentration of oxidized and reduced plastoquinone molecules should change the rate in the activation process. Since the kinetics of the upward and the downward transitions are independent of the amplitude of the change in the redox steady-state of the PQ pool, we can exclude than an activation process occurring with the above characteristics is rate-limiting in our experiments.

(4) As the amount of phosphate incorporated into several LHC subunits varies with the same kinetics as the fluorescence change, we are led to conclude that the rate-limiting step is most likely due to the catalytic activities of the enzymes themselves.

From our experiments at least two parameters distinguish the kinase from the phosphatase: the apparent $t_{1/2}$ of the kinase is faster than that of the phosphatase, a condition required to observe actual phosphorylation by the activated kinase, even though the phosphatase is still active. The phosphatase has an apparent first-order kinetics whereas the kinase has not: the reported inhibition of the kinase activity by the reaction products, ADP [12] and phosphorylated LHC (Bennett. J., personal communication), is consistent with the non first-order kinetics of the downward transition. Telfer et al. [13] already reported that, in vitro, the phosphatase kinetics is slower than that of the kinase but did not report on the difference in the order of the reaction.

The lag of variable length which we observe in the upward transition could be due to several phenomena:

(1) since it is longer when the incubation time in anaerobic conditions is longer it could be due to an effect on redox species more difficult to reduce;

(2) an inactivation of the phosphatase during long periods of reducing conditions: this seems unlikely, since the kinetics of the phosphatase do not change during the upward transition;

(3) an overshoot in ATP formation induced by cyclic electron transfer around PS I at the onset of illumination even though the kinase is getting inactivated.

However, the latter hypothesis cannot account for the similar lag in the fluorescence changes upon oxidation of the PQ pool which has been observed in vitro by Steinback et al. [14]: in their experiment the PQ pool was reoxidized in the dark after reduction in the light. Therefore, a light-independent parameter must be involved in some instances in the inactivation of the kinase or in an activation of the phosphatase.

As compared with the individual kinetics of dephosphorylation of the different polypeptides there seems to be an apparent matching between the transitory overphosphorylation of 17 and the lag: this might be indicative of a special function of polypeptide 17 among the LHC polypeptides and of an heterogeneity either of the kinase or of the substrates susceptible to phosphorylation.

A possible heterogeneity in the kinase activities associated with the thylakoid membranes is also supported by the peculiar status of the PS II associated polypeptide D2 (see Fig. 2) as compared to the other phosphopolypeptides: the phosphorylated form of D2 (D2.1) only appears in the wild-type cells in vivo when the PQ pool is oxidized by an illumination in the presence of DCMU and disappears when it is reduced. This is in favour of the existence of several distinct kinases whose activation mechanisms are different: Lin et al. [15] have actually isolated two distinct kinases from the thylakoid membrane.

On the other hand, the phosphorylation of the PS II polypeptides could be involved in the integration of a functional PS II complex in the thylakoid membrane as it has been proposed by Owens and Ohad [16,17]: indeed one of us has shown that during the biosynthesis of the polypeptides of the PS II reaction center, some of them undergo posttranslational changes corresponding to phosphorylations (polypeptides 6′, D2.1, L5 and L6) [18].

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