Phosphorylation of Chlamydomonas reinhardi
Chloroplast Membrane Proteins In Vivo and In Vitro

GEOFFREY C. OWENS and ITZHAK OHAD
Department of Biological Chemistry, The Hebrew University of Jerusalem, Institute of Life Sciences, 91904 Jerusalem, Israel

ABSTRACT Phosphorylation of thylakoid membrane proteins in the chloroplast of wild-type and mutant strains of Chlamydomonas reinhardi has been studied in vivo and in vitro. Intact cells or purified membranes were labeled with [32P]orthophosphate or [γ-32P]ATP, respectively, and the presence of phosphorylated polypeptides was detected by autoradiography after membrane fractionation by SDS PAGE. The 32P was esterified to serine and threonine residues. At least six polypeptides were phosphorylated in vitro and in vivo, and corresponded to components of the photosystem II complex contributing to the formation of the light-harvesting-chlorophyll (LHC) a,b-protein complex, the DCMU binding site (32-35 kdaltons), and the reaction center (26 kdaltons).

In agreement with previous reports (Alfonzo, et al., 1979, Plant Physiol., 65:730-734; and Bennett, 1979, FEBS (Fed. Eur. Biochem. Soc.) Lett., 103:342-344), the membrane-bound protein kinase was markedly stimulated by light in vitro via a mechanism requiring photosystem II activity. Phosphorylation of thylakoid membrane polypeptides in vivo was, however, completely independent of illumination. Similar amounts of phosphate were incorporated into the photosynthetic membranes of cells incubated in the dark, in white light with or without 3-(3,4-dichlorophenyl-1,1-dimethyl urea (DCMU), or in red or far-red light. Different turnovers of the phosphate were observed in the light and dark, and a phosphoprotein phosphatase involved in this turnover process was also associated with the membrane.

Comparison of the amount of esterified phosphate per protein in vivo and the maximum incorporation in isolated membranes revealed that only a small fraction of the available sites could be phosphorylated in vitro.

In contrast to the DCMU binding site, the LHC and 26-kdalton polypeptide were not phosphorylated in vivo when the reaction center II polypeptides of 44-54 kdaltons were missing.

The finding that all the phosphoproteins appear to be components of the photosystem II complex and are only partially dephosphorylated in vivo suggests strongly that protein phosphorylation might play an important role in the maintenance of the organizational integrity of this complex. The observation that the LHC is not phosphorylated in the absence of the reaction center lends support to this idea.

The incidence of protein phosphorylation in animal cell membranes is well known. Phosphoproteins may be found in the nuclear envelope (28, 36), endoplasmic reticulum (34, 37), plasma membranes (29, 43), and mitochondria (54). Recently, the phenomenon has been described in the photosynthetic membranes of higher plants (1, 11, 53) and in Euglena gracilis (10). In isolated intact chloroplasts incubated with [32P]orthophosphate, protein phosphorylation was shown to be dependent upon the ATP generated by photophosphorylation, and was therefore uncoupler and DCMU sensitive (10, 11). The principal phosphoproteins in higher plant chloroplast membranes have been identified as the polypeptide components of the light-harvesting chlorophyll a,b-protein complex (LHC) which constitutes a photon-collecting antenna for photosystem II (1, 13). When isolated pea chloroplasts prelabeled in vitro with [32P]orthophosphate were returned to the dark, an almost complete loss of the label from the LHC was observed (11) due to the presence of a membrane-bound phosphoprotein phospho-
The protein kinase activity was found to be membrane bound, and the light dependency and 3-(3,4-dichlorophenyl-1,1-dimethyl urea) (DCMU) sensitivity remained when isolated thylakoids were labeled with exogenous \([\gamma-^{32}P]ATP\) (1, 12). This implied that electron flow through photosystem II was involved in a process of light activation of the membrane protein kinase. The activated state of the protein kinase in preilluminated spinach thylakoids persisted in the dark for only several minutes (1).

Fluorescence quenching in isolated higher plant chloroplast membranes under putative phosphorylation conditions in vitro has led to the proposal that reversible phosphorylation of the LHC controls intersystem excitation transfer (2, 15, 32). These findings were obtained from experiments that have made exclusive use of either intact chloroplasts or isolated membranes. In the present paper, where wild-type and mutant strains of *Chlamydomonas reinhardi* have been used, phosphorylation of thylakoid membrane proteins has been studied in whole cells using \([\gamma-^{32}P]ATP\) and, in purified thylakoid preparations, with exogenous \([\gamma-^{32}P]ATP\) as a donor.

The characteristics of the in vitro system are in general agreement with previous work on higher plants, but in whole cells phosphorylation occurs independent of illumination and photosynthetic electron flow. In addition to the polypeptide components of the LHC, several other polypeptides associated with the photosystem II complex were found to be phosphorylated. The LHC was not phosphorylated in cells in which the organization of photosystem II was altered. From a comparison of the total amount of phosphate incorporated into membrane polypeptides during growth with the amount incorporated in vitro, it can be concluded that at least 80% of the membrane phosphoproteins accessible to the protein kinase are in a continuously phosphorylated state.

**MATERIALS AND METHODS**

**Cultivation of Cells**

*C. reinhardi* wild-type (+) and y-1 as well as Tm mutant strains were used in this study. The latter mutant was isolated from a culture of the Tm strain (22) and is characterized by absence of photosystem II reaction centers at the nonpermisive temperature, and inability to synthesize chlorophyll in the dark. Cells were cultivated, as previously described, at 25°C with or without acetate as a carbon source (45). In some experiments, cells were grown from a single inoculum loop in \([\text{H}]\)-acetate with or without \([\text{P}]\)orthophosphate; in such cases the concentration of the steady-state level of fluorescence at 25°C in the presence of DCMU (0.01 mM). Excitation was provided by a tungsten-halogen light source passing through a 496-nm filter (Corning Medical, Medford, MA; transmitting between 380 and 680 nm), to give a flux of 4.4 X 10^{-11} ergs/cm²/sec on the sample cuvette, and the photomultiplier was protected by a 685-nm interference filter (Baird Atomic half band width 40 nm; Baird Corp., Bedford, MA). The overall set up was as described by Cahen et al. (21).

**RESULTS**

**Phosphorylation and Dephosphorylation of Isolated Thylakoids**

Purified thylakoids from *C. reinhardi* possess a protein kinase activity that is markedly stimulated by light (Fig. 1). The progress curve of phosphorylation in the light reached a plateau after 15 min (Fig. 1). At least six polypeptides are phosphorylated in vitro, including components of the LHC a,b-protein complex (9, 17, 24) and several other polypeptides in the molecular weight range of 12–20 and 32–35 kdaltons. A higher molecular weight band coinciding with the location of the \(\alpha\) and \(\beta\) subunits of the coupling factor (42) was occasionally labeled. This could be due to tight binding of ATP to these components, which is known to occur (42) and, in fact, specific labeling of this band at 0°C was sometimes observed.

In addition to protein kinase activity, the isolated thylakoids contained a phosphoprotein phosphatase activity. The kinetics of dephosphorylation of in vitro labeled membranes is given in Fig. 2. It has been shown that the LHC and 32-kdalton region polypeptides are very sensitive to proteolysis by trypsin (49), and the incidence of a membrane-bound protease has been reported (35). Therefore, benzamidine (2 mM) was used in all the dephosphorylation experiments to avoid loss of phospho-
peptides due to proteolysis. Only about 25% of the incorporated phosphate was removed during the 30 min of incubation. The phosphoprotein phosphatase activity of the membrane was at least partially inhibited by fluoride (20 mM) or molybdate (20 mM), which are known to be specific inhibitors of phosphoprotein phosphatases (7).

**Phosphorylation and Desphosphorylation In Vivo**

Pulse-labeled light-grown wild-type of \( \gamma-1 \) cells incorporated \( \left[^{32}P\right]\)orthophosphate into the same polypeptides that are phosphorylated in vitro (Fig. 3). This implies that the membrane-associated protein kinase might be solely responsible for the observed pattern of phosphorylation in whole cells. However, in contrast to the results of the in vitro experiments, membrane protein phosphorylation in vivo was independent of light and incorporation was at least equal if not slightly higher in the dark (Fig. 3). Identical results were obtained with photoautotropically grown cells.

To ascertain whether these \( ^{32}P \)-labeled membrane polypeptides were indeed phosphoproteins, gel slices containing labeled polypeptides were treated with TCA (10%, 10 min, 90°C), NaOH (1 M, 10 min, 90°C) and hydroxylamine-succinate (1 M, pH 7.0, 30 min, 37°C) (6). The radioactivity was removed only from the slices treated with NaOH, as expected for esterified phosphate. Label was also completely removed following treatment with *Escherichia coli* alkaline phosphatase (15 U/mg membrane protein) and bovine pancreatic trypsin (0.5 mg/mg membrane protein). Phosphorylated membranes were hydrolyzed in HCl (6 N) in vacuo for 3 h (20), and the hydrolysate was chromatographed on Whatman 3 MM paper with butanol:acetic acid:water (60:15:25, vol/vol/vol) as the developer (57). Autoradiography revealed the presence of phosphothreonine and a smaller amount of phosphoserine as determined by use of appropriate markers.

To establish whether the turnover of the phosphate esterified to the thylakoid membrane proteins was independent of protein turnover, the same population of light-grown \( \gamma-1 \) cells were pulse-labeled in the light with either \( \left[^{14}C\right]\)acetate or \( \left[^{32}P\right]\)orthophosphate and subsequently chased in the light for 1 h and 4 h. The results given in Fig. 4a clearly show that turnover of the esterified phosphate is not coupled to protein turnover per se. The half-life of the esterified phosphate appeared to be <60 min in the light, and, when chase of \( ^{32}P \)orthophosphate pulse-labeled cells was carried out in the dark for 30 min and 60 min, the turnover appeared to be even faster (Fig. 4b).

**Thylakoid Membrane Protein Phosphorylation in Relation to Photosynthetic Electron Flow**

It has been demonstrated that the stimulation by light of the protein kinase in vitro is mediated by the redox state of photosystem II (2). The data presented thus far show that the phosphorylation of thylakoid membrane polypeptides in whole cells is independent of photosynthetic electron flow. To further investigate this apparent independence, membrane protein phosphorylation was tested in whole cells under conditions where electron flow was blocked by a photosystem II-specific inhibitor, DCMU, and where the redox state of the electron transfer chain was affected by far-red and red illumination (State I and State II, respectively) (18). In addition, a temper-
absorbed preferentially by photosystem I, the electron transfer chain is more oxidized than in cells exposed to light preferentially absorbed by photosystem II (26). The redox state of photosystem II can be estimated from the steady-state fluorescence level induced in cells by red or far-red light and subsequently exposed to light absorbed by both photosystems (18). The result of a pulse-labeling experiment of cells previously driven into State I or State II is also given in Fig. 5, and it can be seen that far-red-illuminated cells (State I) incorporated at least the same amount of label in an analogous manner to dark-adapted cells, as compared to those pulse-labeled in red light (State II).

When thylakoid membrane protein phosphorylation was examined in the temperature-sensitive T₄, which lacks the reaction center of photosystem II when grown at the nonpermissive temperature of 37°C, differences in the pattern of phosphorylation in vivo and in vitro were observed, as compared with cells grown at 25°C (Fig. 6). The components of the LHC were not phosphorylated in cells grown at 37°C in either the light or the dark, and the same result was obtained when membranes, prepared from a culture grown at 37°C, were phosphorylated in vitro. Nevertheless, the polypeptides in the 32- to 35-kdalton region and the low-molecular weight polypeptides were labeled in vivo and in vitro, although in the latter case phosphorylation was not stimulated by light. This was expected since activation of the protein kinase in isolated thylakoids is mediated by photosystem II. The pattern of phosphorylation of thylakoid membranes in vivo and in vitro of T₄ cells grown at 25°C, which possess photosystem II reaction centers, was indistinguishable from that of wild-type (+) and y-1 (Fig. 6).

Stoichiometry of Membrane Protein Phosphorylation In Vivo and In Vitro

The fact that thylakoid membrane proteins can be phosphorylated in vitro implies that these proteins are partially dephosphorylated in vivo due to continuous turnover. This

![Figure 4](image-url)

**Figure 4** (A) Turnover of the esterified phosphate in comparison to protein turnover. Autoradiograph obtained from an experiment in which light-grown y-1 cells were pulse labeled with [32P]orthophosphate or [1-14C]acetate, and subsequently chased for 1 and 4 h. First three lanes, [32P]labeled membranes; last three lanes, [14C]-labeled membranes. (B) Dephosphorylation of the thylakoid membrane phosphoproteins in the light and dark. Light-grown y-1 cells were pulse-labeled and subsequently chased for 30 and 60 min in the light or dark.
the maximum incorporation of phosphate in vitro was limited by substrate availability. However, it cannot be excluded that some inactivation of the protein kinase occurred during the phosphorylation reaction. Calculation of the relative stoichiometries of phosphate per polypeptide revealed that, with the exception of the lower molecular weight components of the LHC (22-24 kdaltons), at least one mole of phosphate per mole protein is present in vivo. It should be noted that in the region of the gel from which the 22- to 24-kdalton polypeptides were excised there are closely adjacent nonphosphorylated polypeptides, leading to an overestimation of the protein content. Furthermore, since polypeptide pairs were taken, it is not possible to state whether each polypeptide is equally phosphorylated.

**DISCUSSION**

The results presented in this paper demonstrate that the photosynthetic membranes of *C. reinhardi* are characterized by a number of phosphoproteins due to the activity of a membrane-bound protein kinase. These phosphorylation polypeptides can be divided into three groups according to molecular weight:

(a) Two and possibly three polypeptides in the molecular weight range of 32-35 kdaltons corresponding to polypeptides D1, 9/10 (the individual polypeptides are designated by the numbers proposed by Chua [23]).

(b) Four polypeptides in the molecular weight range of 22-28 kdaltons probably corresponding to 11, 12, 14, 15, and 17 (23).

(c) At least one polypeptide in the molecular weight range of 12-20 kdaltons, possibly 24 (23).

Recent results have shown that a polypeptide of 32 kdaltons, possibly equivalent to *C. reinhardi* D1, constitutes the binding site for herbicides (44, 48) and, in addition, polypeptides 10, 14, and 15 have been shown to belong to the antenna of photosystem II (24). It is well established that polypeptides 11 and 17 are polypeptides participating in the formation of the LHC (8, 17), and phosphorylation of polypeptide 12 is revealed after chloroform:methanol extraction of polypeptides 11, 14, 15, 16, and 17 (46, and data not shown). Polypeptide 12 appears to be a component of photosystem II as evidenced by its absence in the *Chlamydomonas* F34 mutant lacking photosystem II (22), and in membranes lacking photosystem II due to inhibition of chloroplast translation during greening of the y-1 mutant used in the present study (8). Furthermore, polypeptide 12 is a major component of a photosystem II particle recently

![Table 1](image)

**Table 1**

| M, of bands x 10^3 | Phosphate per protein amoles per mg protein | mole phosphate per mole of protein |
|-------------------|---------------------------------------------|-----------------------------------|
| 22 and 24         | 26.7 2.29 0.62 0.052 0.084                 |                                   |
| 26 and 28         | 18.1 1.69 0.09 0.08 0.09                   |                                   |
| 32 and 35         | 34.2 4.1 2.3 0.28 0.12                     |                                   |
| Total membrane    | 26 2.4                                      | 0.092                             |

Membranes were obtained from cells grown in the presence of 32P orthophosphate and 32P acetate alone. The latter material was incubated with [γ-32P]ATP for 20 min in the light in the presence of protease and phosphoprotein phosphatase inhibitors. Polypeptides were fractionated by SDS PAGE, and gel slices containing the principal phosphorylated polypeptides were excised and counted for 3H and 32P. For full experimental details, see Materials and Methods.
isolated from a mutant lacking chlorophyll-protein complex I and thylakoid membrane ATPase (25). Similarly, a low-molecular-weight polypeptide found in this photosystem II particle could be synonymous with the phosphorylated protein in the 12–20 kdalton molecular weight range (25). It may be tentatively concluded, therefore, that all the major phosphoproteins constitute components of photosystem II. It is tempting to speculate that the protein kinase is associated in the membrane with the photosystem II complex. Moreover, the phosphorylation of these polypeptides depends upon the state of the organization of the complex. When the reaction center is missing, as in the case of the T₄₄ mutant grown at the nonpermissive temperature, the LHC and polypeptide 12 are no longer phosphorylated. This might imply that the LHC is spatially disconnected from the disorganized photosystem II. Freeze-fracture electron microscopy has revealed that in the absence of reaction center II the large 140 Å EF face particles, which are considered to represent the total photosystem II complex including LHC (4), are depleted and reduced in size (40, 52, 56).

In addition to the protein kinase, the presence of a phosphoprotein phosphatase activity has also been demonstrated in isolated thylakoids. Both activities are independent of light in vivo, and it appears that the turnover rate is faster in the dark.

Based on observations from in vitro studies alone, two conclusions have been reached by other groups: (a) The protein kinase can be either active or inactive, depending upon the redox state of the plastoquinone pool (2, 31). When the ratio of reduced to oxidized plastoquinone increases above a certain level as yet undetermined, activation of the protein kinase ensues, bringing about phosphorylation of the LHC. (b) Phosphorylation of the LHC results in an increase of spillover to photosystem I, as evidenced by a correlation between LHC phosphorylation in vitro and ATP-dependent quenching of fluorescence in uncoupled membranes (15, 31, 32).

The fact that the protein kinase is active in dark-adapted cells, and in the light in the presence of DCMU, appears to contradict the idea of reversible activation of the enzyme by redox modulation of the plastoquinone pool by photosystem II. This apparent discrepancy can be explained if one considers that the electron transfer chain is not completely oxidized in dark-adapted cells. Wollman has shown that the secondary acceptor of photosystem II after Q is more reduced in dark-adapted Chlorella cells, as compared with isolated spinach thylakoids (55). This author postulated that a soluble reductant might be involved which is lost during thylakoid membrane preparation. Further, reduction of the acceptor site of photosystem II in the dark by NADPH in a ferredoxin-dependent reaction has been demonstrated in vitro (41), and energization of isolated thylakoid membranes in the dark can induce back flow of electrons from cytochrome f to Q (50). The fact that chloroplast protein and lipid synthesis continues in dark-grown wild-type C. reinhardtii cells and in dark-incubated y-1 cells (27) using acetate as a carbon source indicates that reducing equivalents and ATP are supplied to the chloroplast in the dark.

Dark phosphorylation is not, however, specific to mixotrophic cells, but also occurs in photoautotrophic cells. In fact, mixotrophic cultures evolve oxygen at comparable rates to obligate photosynthetic cultures and exhibit the same state-I and state-II behavior.

One may therefore conclude that the thylakoid protein kinase can be found in vivo in a permanently active state. This does not contradict the idea that regulation of the active condition is via the redox state of a component close to photosystem II, possibly plastoquinone.

To date, the only physiological significance ascribed to thylakoid membrane protein phosphorylation has been in regulating the distribution of excitation energy between the photosystems (15, 32, 33). The data presented here indicate that the maximum level of phosphorylation obtained in vitro, where prior dephosphorylation was minimized, is only ∼10% of the maximum found in vivo. This might be due to the fact that most of the available sites are phosphorylated in whole cells and that phosphorylation in isolated thylakoids is limited. In addition, it is also possible that full protein kinase activity is not manifest in vitro. It is interesting to note that, in erythrocytes, 90% of the spectrin is continuously phosphorylated in vivo (30). Estimation of the stoichiometry of phosphate to particular phosphorylated polypeptides in vivo revealed that there is <1 mole of phosphate per mole of polypeptide pair only in the case of the lower-molecular-weight components of the LHC. The possibility that part of the LHC is completely unphosphorylated and not accessible to the protein kinase cannot therefore be excluded at present. In isolated thylakoids from Pisum sativum, levels of phosphorylation of the LHC can be obtained which, under the appropriate conditions, approach those reported here for the steady-state level of LHC phosphorylation in vivo (J. Bennett, personal communication). This could be accounted for by more active protein kinase and phosphoprotein phosphatase activities in vitro than found in thylakoid preparations of C. reinhardtii, and the occurrence of dephosphorylation during preincubation of the membranes in vitro.

Net dephosphorylation of all the LHC under conditions of low intersystem transfer is inconsistent with data which indicate that only a fraction of the LHC might interact with photosystem I in the regulation of spillover (3, 19). In contrast to photosystem I, the LHC is predominantly located in the grana, and not more than a 20% decrease in stacking has been reported in intact cells of Chlamydomonas driven from State I to State II (16). The incorporation of phosphate into LHC in vivo is similar in conditions of State I (no spillover) and State II (spillover); however, the turnover rate of esterified phosphate appears to be faster in the former condition.

Insufficient attention has been paid to the fact that, in addition to the phosphorylated polypeptides of the LHC, other components which appear to participate in the formation of the photosystem II complex are also phosphoproteins. The fact that the majority of these proteins are continuously phosphorylated in vivo implies that this is a necessary condition for maintenance of the organizational integrity of the Photosystem II complex. This view is supported by the data presented in this paper as well as by the observation that preferential dephosphorylation of the 32- to 35-kdalton region results in a loss of the high-affinity binding site for DCMU (51). Recent findings by Horton and Black (33) also favor the possibility that phosphorylation affects primarily the interaction between LHC and photosystem II.

In conclusion, one might envisage the existence of two populations of the LHC, characterized by different levels of phosphorylation, one which interacts with phosphorylated Photosystem II units in the grana, and a second, possibly confined to the stroma lamellae, in which phosphorylation promotes interaction with photosystem I units. This latter fraction may be predominantly phosphorylated in the in vitro systems, resulting in the observed fluorescence changes.
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