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 [^{32}P]orthophosphate or [γ-^{32}P]ATP, respectively, and the presence of phosphorylated polypeptides was detected by autoradiography after membrane fractionation by SDS PAGE. The ^{32}P 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 [^{32}P]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 [^{32}P]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 phos-

THE JOURNAL OF CELL BIOLOGY · VOLUME 93 JUNE 1982 712–718
© The Rockefeller University Press · 0021-9525/82/05/0712/07 $1.00

phatase which was not light dependent (14).

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 [γ-^{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 [^{32}P]orthophosphate and, in purified thylakoid preparations, with exogenous [γ-^{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 T_{44} mutant strains were used in this study. The latter mutant was isolated from a culture of the T_4 strain (22) and is characterized by absence of photosystem II reaction centers at the nonpermissive 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 [^{3}H]acetate with or without [^{32}P]orthophosphate; in such cases the concentrations of these two constituents was reduced to 1 mM, and Tris-HCl (25 mM, pH 7.4) was added as a buffer.

In Vivo Pulse Labeling

Cells were harvested, washed once, and resuspended in growth medium to a concentration of 2×10^7 cells/ml (nondividing conditions). Aliquots of 25 ml were preincubated for 15 min at 25°C with continuous agitation, before the addition of radioisotope. [^{32}P]Orthophosphate was added to give a final specific activity of 0.5 Ci/mmol, and the incubation was continued for a further 30 min. Illumination, when required, was provided by cool fluorescent lamps (1.6×10^4 ergs/cm^2 per sec). In experiments where cells were driven into state I or state II (18) by illumination at different wavelengths, cells were continuously stirred in a water jacket bath maintained at 25°C, and light was provided by a quartz halogen fiber light (Dolan Jenner Industries, Inc., Woburn, MA) passing through a 650-nm or 715-nm interference filter (Baird Atomic half band width, 40 nm), giving incident light intensities of 1.75 and 1.25×10^4 ergs/cm^2 per s, respectively.

Thylakoid membranes were obtained by sonication in an ice bath (3×15 s with intervals of 15 s) in Tris-HCl (50 mM, pH 7.2), followed by centrifugation of the homogenate for 90 min at 80,000 *g* on a step gradient of sucrose in Tris-HCl, (50 mM, pH 7.2) (15:30:60%, wt/vol). Membranes were collected from the 30:60% sucrose interface and were washed and pelleted in the same buffer. All manipulations were carried out at 4°C with sterilized glassware used for the incubations.

In Vitro Labeling

Membranes for the in vitro assay of protein kinase activity were obtained by the procedure described by de Petrocellis et al. (47), with the following modifications. Only one round of centrifugation was carried out, and the discontinuous sucrose gradient included an extra intermediary step of 1.75 M sucrose. Only membranes at the 1.5:1.75 M interface were collected, washed, and pelleted. Labeling of thylakoid membranes was done at 25°C with illumination provided by a quartz halogen fiber light (Dolan Jenner 2.5×10^6 ergs$\cdot$cm$^{-2}\cdot$sec^{-1}). The reaction mixture contained Tris-HCl (50 mM, pH 8), $MgCl_2$ (10 mM), ATP (0.05 M) and [γ-^{32}P]ATP to give a final specific activity of 0.15 Ci/mmol. The reaction was terminated by addition of an equal volume of a solution containing SDS (5%), β-mercaptoethanol (70 mM), EDTA (50 mM), KH_2PO_4 (25 mM), and ATP (25 mM), and aliquots were chromatographed on Whatman DE81 paper with ammonium formate (200 mM, pH 5.5) as the developer (39). For electrophoresis, the reaction was terminated by rapid centrifugation in an Eppendorf microfuge, followed by resuspension in electrophoresis sample mixture (23). The chloroplast thylakoid membrane proteins were fractionated by SDS PAGE according to the methodology of Chua (23). Gels were stained for protein with Coomassie Brilliant Blue R, destained, dried, and exposed to x-ray film (Agfa-Gevaert Curix RP2) with, in some cases, an enhancement screen (Agfa-Gevaert MR400). Chlorophyll was quantitated by the method of Arnon (5), and protein concentration was determined according to Lowry et al. (38) using bovine serum albumin as a standard.

To quantitate the amount of phosphate incorporated in vitro relative to protein, membranes were obtained from cells grown in the presence of [^{3}H]-acetate, using the modified procedure of de Petrocellis et al. (47). The membranes were labeled using [γ-^{32}P]ATP as described above, and the molar ratio of ^{32}P to protein in the relevant polypeptides was compared with that of membranes purified from cells grown in [^{32}P]orthophosphate and [^{3}H]acetate. Bands were excised from the gel, dissolved overnight in hydrogen peroxide at 80°C, and subsequently counted in Triton X-100 enriched scintillation fluid in a Packard Tri-Carb liquid scintillation spectrometer, Model 300C (Packard Instrument Co., Inc., Downers Grove, IL). Corrections were made for quenching and spillover between the ^{32}P and ^{3}H channels.

Fluorescence Measurements

State-I and State-II conditions in whole cells were monitored by measurement 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, Medfield, MA: transmitting between 380 and 680 nm), to give a flux of 4.4×10^3 ergs/cm^2 per s incident 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 α and β 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-

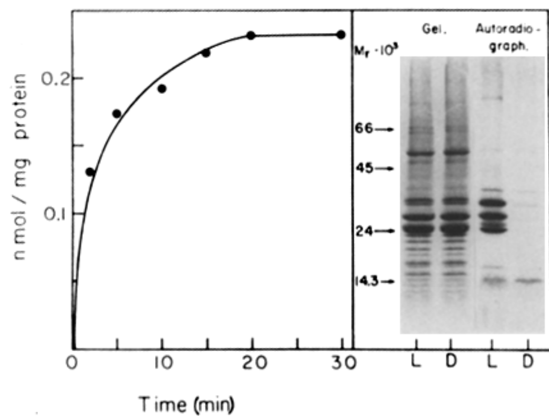

FIGURE 1 Phosphorylation in vitro of isolated chloroplast membranes. Time-course of the phosphorylation of isolated thylakoid membranes using exogenous [γ-^{32}P]ATP (left panel) and the corresponding pattern of incorporation in the light and dark after incubation for 15 min (right panel).

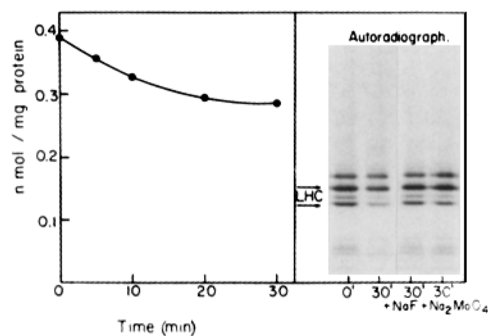

FIGURE 2 Dephosphorylation in vitro of isolated chloroplast membranes. Time-course of dephosphorylation of isolated thylakoid membranes in the dark after labeling in vitro. Membranes were incubated in the dark in Tris-HCl (50 mM, pH 8.0), $MgCl_2$ (10 mM), $ZnSO_4$ (0.1 mM) and benzamidine (2 mM). Samples were taken at intervals of 0, 5, 10, 20, and 30 min (left panel). Autoradiograph of the dephosphorylation profile after 30 min of incubation in the presence and absence of phosphoprotein phosphatase inhibitors (right panel).

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 y-1 cells incorporated [^{32}P]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 complete contradistinction 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 hydrolyzate 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 y-1 cells were pulse-labeled in the light with either [1-^{14}C]acetate or [^{32}P]-orthophosphate and subsequently chased in the light for 1 h and 4 h. The results given in Fig. 4 *a* 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. 4 *b*).

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-

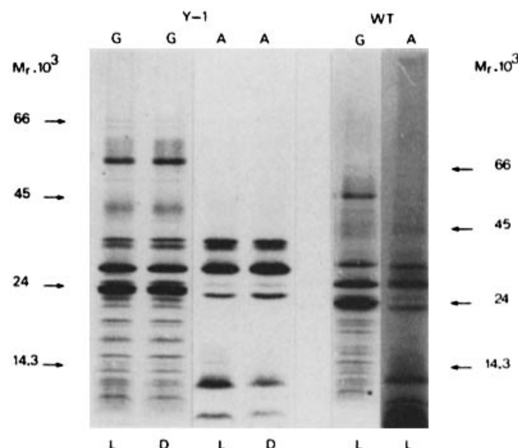

FIGURE 3 Phosphorylation in vivo of whole cells in nondividing conditions. Light-grown wild-type cells (right panel) and Y-1 cells (left panel) were pulse labeled in the light (*L*) or dark (*D*) with [^{32}P]orthophosphate. *G*, Coomassie Brilliant Blue R stain; *A*, autoradiograph.

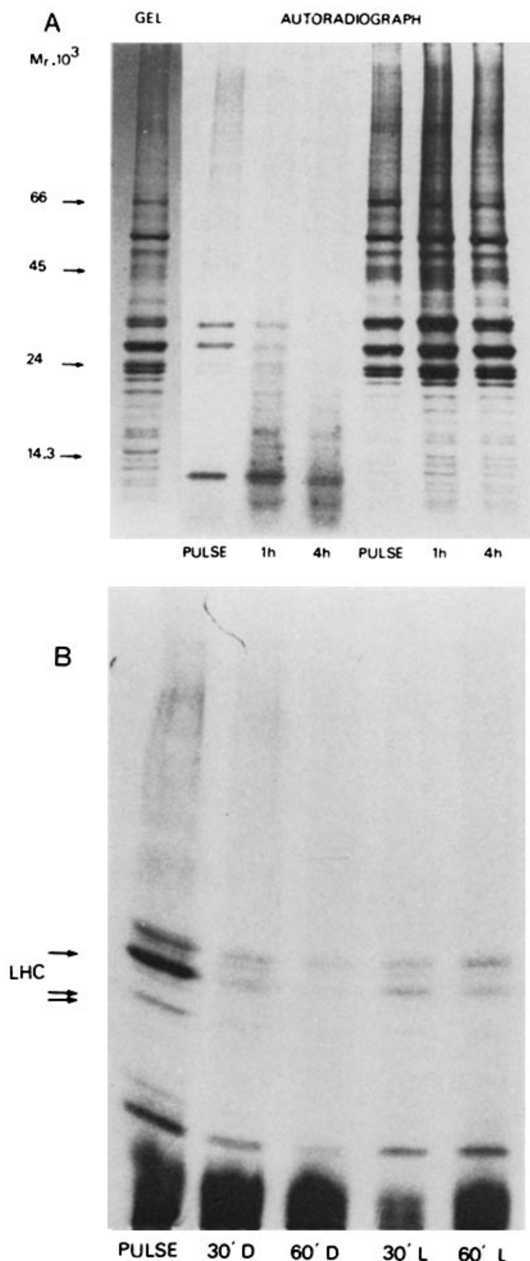

FIGURE 4 (*A*) Turnover of the esterified phosphate in comparison to protein turnover. Autoradiograph obtained from an experiment in which light-grown y-1 cells were pulse labeled with [^{32}P]orthophosphate or [1-^{14}C]acetate, and subsequently chased for 1 and 4 h. First three lanes, [^{32}P]labeled membranes; last three lanes, [^{14}C]-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.

ature-sensitive mutant was used, which specifically lacks the reaction center polypeptides of photosystem II, when grown at the nonpermissive temperature.

Inhibition of electron flow through photosystem II in vitro reduced the level of phosphorylation of the thylakoid to that of the dark level (Fig. 5). However, DCMU had no effect on phosphorylation of the photosynthetic membrane in vivo, which is in agreement with the observed phosphorylation in the dark in whole cells (Fig. 5).

It is generally accepted that, in cells illuminated by light 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 T_{44}, 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 T_{44} 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

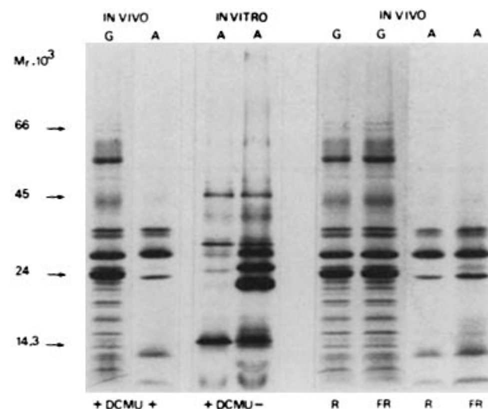

FIGURE 5 The effect of inhibition of linear electron flow in vivo and in vitro, and modulation of the redox state of the electron transfer chain by red and far-red light on membrane protein phosphorylation. Pulse-labeling light-grown y-1 cells in the presence of DCMU (10^{-5} M) (first panel on the left) in comparison to the effect of the same concentration of DCMU on phosphorylation in vitro (second panel). Third panel, phosphorylation pattern obtained from light-grown y-1 cells pulse-labeled in red (*R*) and far-red (*FR*) light; the maximum fluorescence levels of these cells after 15 min of preincubation before addition of label were 4.8 and 5.8 v/mg chlorophyll, respectively. *G*, Coomassie Brilliant Blue stain; *A*, autoradiograph. The molecular weight markers refer to the first panel.

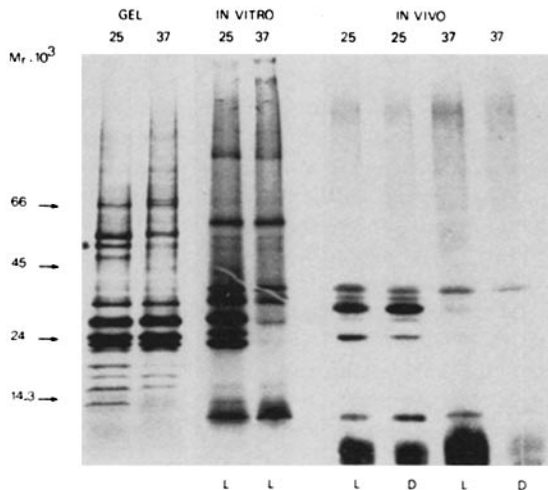

FIGURE 6 Comparison between thylakoid membrane protein phosphorylation in vitro and that in vivo in the T_{44} mutant. The first panel (left) shows the polypeptide pattern of the mutant grown at the permissive (25°C) and nonpermissive (37°C) temperatures. The reaction center II polypeptides which are absent in the latter condition are indicated by an asterisk. Autoradiograph of the pattern of phosphorylation of isolated thylakoids obtained from cells grown at 25° or 37°C is given in the center panel, and in the third panel the result of a pulse-labeling experiment in vivo is presented. Cells grown at 25°C were pulse-labeled at 25°C in the light (*L*) and dark (*D*), and cells grown at 37°C were pulse-labeled at 37°C in the light (*L*) or the dark (*D*).

assumes negligible desphosphorylation during membrane preparation for the in vitro assay due to the precaution taken to keep the material at 4°C at all times before the experiments.

To estimate the amount of phosphate esterified to each polypeptide resulting from maximum phosphorylation in vivo and in vitro, the following experiment was carried out. Cells were grown from a single inoculum loop in medium containing [^{3}H]acetate and [^{32}P]orthophosphate or [^{3}H]acetate alone. After 8–10 cell divisions, it was reasonable to assume that a steady-state level of phosphorylation had been attained reflecting the total sites available, and that the specific radioactivity of the intracellular phosphate was equal to that of the medium. Furthermore, after this number of cell divisions, all thylakoid membrane polypeptides could be assumed to be equally labeled with [^{3}H]acetate. The major phosphorylated polypeptides were resolved in SDS PAGE, and the amounts of phosphate and protein in the relevant gel slices were determined from the counts of ^{32}P and ^{3}H, respectively. The ^{3}H counts were related directly to the protein concentration of the samples which was determined before the electrophoresis, and the amount of phosphate was calculated from the known specific radioactivity of the isotope. Isolated ^{3}H-labeled membranes were phosphorylated in vitro using [γ-^{32}P]ATP of known specific radioactivity, and the amount of phosphate incorporated was measured in the same way. Using the values for the molecular weights of the major phosphorylated polypeptides derived from the relative mobility of electrophoretic markers, it was possible to obtain an estimate of the molar ratio of phosphate to protein.

The results shown in Table I indicate that ~10% of the sites available in vivo are phosphorylated in vitro, implying that the majority of these polypeptides are phosphorylated under steady-state conditions in whole cells. Dephosphorylation was minimized during the preparation of the [^{3}H]-labeled membranes and incubation with [γ-^{32}P]ATP, which suggests that 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 I

Molar Ratio of Phosphate to Protein Incorporated In Vivo and In Vitro into the Major Phosphorylated Polypeptides

M_r of bands × 10^3	Phosphate per protein nmoles per mg protein		mole phosphate per mole of protein		
	in vivo	*in vitro*	*in vivo*	*in vitro*	*in vitro*/*in vivo*
22 and 24	26.7	2.29	0.62	0.052	0.084
26 and 28	18.1	1.69	0.98	0.091	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 [^{32}P]orthophosphate and [^{3}H]acetate alone. The latter material was incubated with [γ-^{32}P]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 ^{3}H and ^{32}P. 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 T_{44} 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 side 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. reinhardi* 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. reinhardi*, 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 system 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.

This research was supported by BARD grant 79-80. G. C. Owens is the recipient of a NATO-SRC postgraduate studentship.

Received for publication 25 August 1981, and in revised form 6 January 1982.

REFERENCES

1. Alfonzo, R., N. Nelson, and E. Racker. 1979. A light-dependent protein kinase activity of chloroplasts. *Plant Physiol.* 65:730–734.
2. Allen, J. F., J. Bennett, K. E. Steinback, and C. J. Arntzen. 1981. Chloroplast protein phosphorylation couples plastoquinone redox state to distribution of excitation energy between photosystems. *Nature (Lond.).* 291:25–29.
3. Anderson, B., and J. M. Anderson. 1980. Lateral heterogeneity in the distribution of chlorophyll-protein complexes of the thylakoid membranes of spinach chloroplasts. *Biochim. Biophys. Acta.* 593:427–440.
4. Armond, P. A., L. A. Staehelin, and C. J. Arntzen. 1977. Spatial relationship of photosystem I, photosystem II, and the light-harvesting complex in chloroplast membranes. *J. Cell Biol.* 73:400–418.
5. Arnon, D. I. 1949. Copper enzymes in isolated chloroplasts. Polyphenol oxidase in *Beta vulgaris. Plant Physiol.* 24:1–15.
6. Avruch, J., G. R. Leone, and D. B. Martin. 1976. Identification and subcellular distribution of adipocyte peptides and phosphopeptides. *J. Biol. Chem.* 251:1505–1510.
7. Barnett, C. A., J. J. Schmidt, and G. Litwack. 1980. Effects of calf intestinal alkaline phosphatase, phosphatase inhibitors, and phosphorylated compounds on the rate of activation of glucocorticoid-receptor complexes. *Biochemistry.* 19:5446–5455.
8. Bar-Nun, S., and I. Ohad. 1977. Presence of polypeptides of cytoplasmic and chloroplastic origin in isolated photoactive preparations of photosystems I and II in *Chlamydomonas reinhardi* y-1. *Plant Physiol.* 59:161–166.
9. Bar-Nun, S., and I. Ohad. 1977. Appearance and composition of chlorophyll protein complexes I and II during chloroplast membrane biogenesis in *Chlamydomonas reinhardi* y-1. *Biochim. Biophys. Acta.* 459:451–467.
10. Beliveau, R., and G. Bellemare. 1979. Light-dependent phosphorylation of thylakoid membrane polypeptides. *Biochem. Biophys. Res. Commun.* 88:797–803.
11. Bennett, J. 1977. Phosphorylation of chloroplast membrane proteins. *Nature (Lond.).* 269:344–346.
12. Bennett, J. 1979. Chloroplast phosphoproteins. The protein kinase of thylakoid membranes is light-dependent. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 103:342–344.
13. Bennett, J. 1980. Chloroplast phosphoproteins. Phosphorylation of polypeptides of the light-harvesting chlorophyll protein complex. *Eur. J. Biochem.* 99:133–137.
14. Bennett, J. 1980. Chloroplast phosphoproteins. Evidence for a thylakoid-bound phosphoprotein phosphatase. *Eur. J. Biochem.* 104:85–89.
15. Bennett, J., K. E. Steinback, and C. J. Arntzen. 1980. Chloroplast phosphoproteins. Regulation of excitation energy transfer by phosphorylation of thylakoid membrane polypeptides. *Proc. Natl. Acad. Sci. U. S. A.* 77:5253–5257.
16. Bennoun, P., and H. Jupin. 1974. The relationship between thylakoid stacking, state I, and state II phenomena in whole cells and the cation effects in chloroplasts of *Chlamydomonas reinhardi. In:* Proceedings of the 3rd International Congress on Photosynthesis M. Avron, editor. Vol. 1. Elsevier, Amsterdam. 163–169.
17. Blomberg, F., and N.-H. Chua. 1979. Immunochemical studies of thyalkoid membrane polypeptides from spinach and *Chlamydomonas reinhardtii.* A modified procedure for crossed immunoelectrophoresis of dodecyl sulphate protein complexes. *J. Biol. Chem.* 254:215–223.
18. Bonaventura, C., and J. Myers. 1969. Fluorescence and oxygen evolution from *Chlorella pyrenoidosa. Biochim. Biophys. Acta.* 189:366–383.
19. Breton, J., and N. E. Geacintov. 1980. Picosecond fluorescence kinetics and fast energy transfer processes in photosynthetic membranes. *Biochim. Biophys. Acta.* 594:1–32.
20. Bylund, B. B., and T.-S. Huang. 1976. Decomposition of phosphoserine and phosphothreonine during acid hydrolysis. *Anal. Biochem.* 73:477–485.
21. Cahen, D., S. Malkin, S. Shochat, and I. Ohad. 1976. Development of photosystem II complex during greening of *Chlamydomonas reinhardi. Plant Physiol.* 58:257–267.
22. Chua, N.-H., and P. Bennoun. 1979. Thylakoid membrane polypeptides of *Chlamydomonas reinhardi*: wild-type and mutant strains deficient in photosystem II reaction center. *Proc. Natl. Acad. Sci. U. S. A.* 72:2175–2179.
23. Chua, N.-H. 1980. Electrophoretic analysis of chloroplast protein. *In:* Methods in Enzymol. San Pietro, A. editor. Vol. 60c. Academic Press, Inc., NY. 434–446.
24. Delepelaire, P. and N.-H. Chua. 1981. Electrophoretic purification of chlorophyll a/b protein complexes from *Chlamydomonas reinhardtii* and spinach and analysis of their polypeptide compositions. Manuscript submitted to J. Biol. Chem.
25. Diner, B. A., and F. A. Wollman. 1980. Isolation of highly active photosystem II particles from a mutant of *Chlamydomonas reinhardi. Eur. J. Biochem.* 110:521–526.
26. Duysens, L. N. M., J. Amesz, and B. M. Kamp. 1971. Two photochemical systems in photosynthesis. *Nature (Lond.).* 190:510–511.
27. Eytan, G., and I. Ohad. 1972. Biogenesis of chloroplast membranes. VII. The preservation of membrane homogeneity during development of the photosynthetic lamellar system in an algal mutant of *Chlamydomonas reinhardi* y-1. *J. Biol. Chem.* 247:112–121.
28. Gerace, L., and G. Blobel. 1980. The nuclear-envelope lamina is reversibly depolymerized during mitosis. *Cell.* 19:277–287.
29. Greengard, P. 1978. Distinguished Lecture Series of the Society of General Physiologists, Vol. 1 Cyclic Nucleotides, Phosphorylated Proteins and Neuronal Function.
30. Harris, H. W., N. Levun, and S. E. Lux. 1980. Comparison of the phosphorylation of human erythrocyte spectrin in the intact red cell and in various cell-free systems. *J. Biol. Chem.* 255:11521–11525.
31. Horton, P., and M. T. Black. 1980. Activation of adenosine 5'-triphosphate-induced quenching of chlorophyll fluorescence by reduced plastoquinone. The basis of state I-state II transitions in chloroplasts. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 119:141–144.
32. Horton, P., J. F. Allen, M. T. Black, and J. Bennett. 1981. Regulation of phosphorylation of chloroplast membrane polypeptides by the redox state of plastoquinone. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 125:193–196.
33. Horton, P., and M. T. Black. 1981. Light-induced redox changes in chloroplast cytochrome f after phosphorylation of membrane proteins. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 132:75–77.
34. Katz, A. M. 1979. Role of the contractile proteins and sarcoplasmic reticulum in the response of the heart to catecholamines: a historical review. *Adv. Cyclic Nucleotide Res.* 11:303–344.
35. Lagoutte, B., and J. Duranton. 1978. A new enzymatic activity associated with thylakoid membranes. *In:* Chloroplast Development G. Akoyunoglou and J. H. Argyroudi-Akoyunoglou, editors. Elsevier, Amsterdam. 229–234.
36. Lam, K. S., and C. B. Kasper. 1979. Selective phosphorylation of a nuclear envelope polypeptide by an endogenous protein kinase. *Biochemistry.* 18:307–311.
37. Lam, K. S., and C. B. Kasper. 1980. Selective endogenous phosphorylation of two liver microsomal polypeptides in the presence of micromolar levels of magnesium ion. *J. Biol. Chem.* 255:259–266.
38. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with Folin phenol reagent. *J. Biol. Chem.* 193:265–275.
39. Loyter, A., R. Ben-Zaquen, R. Marash, and Y. Milner. 1977. Dephosphorylation of human erythrocyte membranes induced by Sendai virus. *Biochemistry.* 16:3903–3909.
40. Miller, K. R., and I. Ohad. 1978. Chloroplast membranes biogenesis in *Chlamydomonas*: correlation between the function of membrane components and membrane structure. *Cell Biol. Int. Reports* 2:537–549.
41. Mills, J. D., D. Crowther, R. E. Slovacek, G. Hind, and R. E. McCarty. 1979. Electron transfer pathways in spinach chloroplasts, reduction of the primary acceptor of PSII by reduced nicotinamide adenine dinucleotide phosphate in the dark. *Biochim. Biophys. Acta.* 547:127–137.
42. Nelson, N. 1976. Structure and function of chloroplast ATPase. *Biochim. Biophys. Acta.* 456:314–338.
43. Nilsen-Hamilton, M., and R. J. Hamilton. 1979. Fibroblast growth factor causes an early increase in phosphorylation of a membrane protein in quiescent 3T3 cells. *Nature (Lond.).* 279:444–446.
44. Oettmeier, W., K. Masson, and U. Johanningmeer. 1980. Photoaffinity labeling of the photosystem II herbicide binding protein. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 118:267–270.
45. Ohad, I., P. Siekevitz, and G. E. Palade. 1967. Biogenesis of chloroplast membranes. I. Plastid dedifferentiation in a dark-grown algal mutant (*Chlamydomonas reinhardi*). *J. Cell Biol.* 35:521–552.
46. Owens, G. C., and I. Ohad. 1980. Phosphorylation of thylakoid membrane proteins in the chloroplast of *Chlamydomonas reinhardi. In:* Proc. Fifth International Congress on Photosynthesis (in press).
47. de Petrocellis, B., P. Siekevitz, and G. E. Palade. 1970. Changes in the chemical composition of thylakoid membranes during greening of the y-1 mutant of *Chlamydomonas reinhardi. J. Cell Biol.* 44:618–634.
48. Pfister, K., K. E. Steinback, G. Gardner, and C. J. Arntzen. 1981. Photoaffinity labeling of an herbicide receptor protein in chloroplast membranes. *Proc. Natl. Acad. Sci. U. S. A.* 78:981–985.
49. Regitz, G., and I. Ohad. 1974. Changes in the protein organization in developing thylakoids of *Chlamydomonas reinhardi* y-1 as shown by sensitivity to trypsin. *In:* Proceedings of the Third International Congress on Photosynthesis (M. Avron, editor) Elsevier, Amsterdam. 1615–1625.
50. Reinitis, K. G., H. Hardt, and M. Avron. 1974. Energy dependent reverse electron flow in chloroplasts. *Eur. J. Biochem.* 43: 291–298.
51. Shochat, S., G. C. Owens, P. Hubert, and I. Ohad. 1981. Binding sites for herbicides in *Chlamydomonas reinhardi* thylakoids. Ann. Mtg. Is. Biochem Soc. Abstr. p. 24. Isr. J. Med. Sci. (in press).
52. Simpson, D., B. L. Møller and G. Høyer-Hansen. 1978. Freeze-fracture structure and polypeptide composition of thylakoids of wild type and mutant barley plastids. *In:* Chloroplast Development G. Akoyunoglou and J. H. Argyroudi-Akoyunoglou, editors. Elsevier, Amsterdam. 507–512.
53. Steinback, K. E., J. E. Mullet, and C. J. Arntzen. 1979. Phosphorylation of chloroplast membrane proteins. *Plant Physiol.* 63 abs. 147:p. 27.
54. Vardanis, A. 1977. Protein kinase activity at the inner membrane of mammalian mitochondria. *J. Biol. Chem.* 252:807–813.
55. Wollman, F. A. 1978. Determination and modification of the redox state of the secondary acceptor of PSII in the dark. *Biochim. Biophys. Acta.* 503:263–273.
56. Wollman, F. A., J. Olive, P. Bennoun, and M. Recouvreur. 1980. Organization of the photosystem II centers and their associated antennae in the thylakoid membranes: a comparative ultrastructural, biochemical, and biophysical study of *Chlamydomonas* wild type and mutants lacking in PSII reaction centers. *J. Cell Biol.* 87:728–735.
57. Zahlten, R. N., A. A. Hochberg, F. W. Stratman, and H. A. Lardy. 1972. Glucagon stimulated phosphorylation of mitochondrial and lysozomal membranes of rat liver in vivo. *Proc. Natl. Acad. Sci. U. S. A.* 69:800–804.