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Phosphorylation and dephosphorylation of membrane proteins from the prochlorophyte *Prochlorothrix hollandica* in fixed redox states

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Externally added reductant enhanced phosphorylation of polypeptides in isolated membranes. In the presence of excess NADPH phosphorylation of bands at 88, 68, 64, 52, 48, 46, 42, 37, 34, 29, 26, 24, 16 and 9 kDa could be detected. Oxidizing conditions inhibited phosphorylation of these polypeptides. Although redox-state-dependent protein phosphorylation occurs in *Prochlorothrix*, changes in phosphorylation related to state transitions induced via light/dark or light 1/light 2 transitions could be demonstrated neither in whole cells nor in membranes. The presence of Mg^{2+} (5 mM) was required for phosphorylation, the activity was also stimulated by Mn^{2+}; Ca^{2+} totally inhibited phosphorylation. The (de)phosphorylation of the 29 kDa polypeptide in particular appeared to be very sensitive to the phosphatase inhibitor NaF. The 29 kDa band ran in parallel with the lowest chlorophyll-protein complex on non-denaturing green gels. The prominent phosphorylated band at 29 kDa did not coincide with the 31 kDa band identified as the major polypeptide in the chlorophyll *a*/*b*-protein complex of *Prochlorothrix hollandica*. The apparent analogy between the light-harvesting complex of Photosystem II and the 29 kDa band will be discussed.

Introduction

Protein phosphorylation is a ubiquitous instrument for regulatory processes and signal transduction in biological systems [1,2]. Reversible phosphorylation is achieved by competing kinase and phosphatase enzymes [3–5]. Short-term adaptation to changes in the spectral quality of light were originally measured as changes in oxygen yield, both in green algae and cyanobacteria [6,7]. The role of phosphorylation in this adaptation process involves the regulation of light-energy distribution to PS II and PS I via so-called state transitions [3–5,8]. The activity of the kinase in chloroplasts is controlled by the redox state of plastoquinone [3–5]. The phosphatase is not subjected to a control mechanism [4]. In order to function as a control mechanism, the rate of phosphorylation and dephosphorylation of target polypeptides needs to display appropriate kinetics. Out of the many polypeptides present in pea thylakoid membranes those from PS II and LHC II become phosphorylated in particular, but only LHC II is dephosphorylated rapidly enough to fit with the kinetics of state transitions [9,10]. The dephosphorylation of LHC II is inhibited by NaF [11]. Models on state transitions display that a part of the LHC II pool (peripheral pool) becomes disengaged from PS II and migrates towards the stroma lamellae and PS I [5, 12–14].

In cyanobacteria, lateral heterogeneity is absent and the role of protein phosphorylation in state transitions in these organisms is subjected to dispute [15–18].

Prochlorophytes are an only recently introduced group of oxyphotobacteria [19–21]. Prochlorophytes very much look like cyanobacteria but do lack the extrinsic phycobilisomes. The antennae of these organisms contain chlorophyll *a* and *b* and are intrinsic in the thylakoid membrane just like the LHC complexes in chloroplasts. Lateral heterogeneity of protein complexes and local appression of thylakoid membranes have been demonstrated in both the symbiont *Prochloron* [22] and in *Prochlorothrix hollandica* [23]. *P. hol-*

Abbreviations: Chl, chlorophyll; CP, chlorophyll-protein complex; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; EDTA, ethylenediaminetetraacetic acid; LHC, light-harvesting complex; $NADP^+$, nicotinamide adenine dinucleotide; PS I (II), Photosystem I (II); SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; TCA, trichloroacetic acid;

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landica is a free-living prochlorophyte which in contrast to *Prochloron* can be cultured in the laboratory [20]. The question is whether the chlorophyll *a/b*-protein complexes of prochlorophytes are functionally related to LHC II and whether the mechanism of possible state transitions also involves reversible phosphorylation of these complexes. Schuster et al. [17] reported phosphorylation of a 34 kDa polypeptide associated with CP2 in *Prochloron*, the intensity of phosphorylation, however, was found to be similar in the light and in the dark. The work presented here describes the phosphorylation and dephosphorylation characteristics of polypeptides in membranes from *P. hollandica* in the absence or presence of externally added reductants of the plastoquinone pool.

Materials and Methods

Prochlorothrix hollandica originating from the Loosdrecht lakes in The Netherlands [20] was made available by Dr. Tineke Burger-Wiersma. All samples of the mono algal culture of this organism used in studies elsewhere originate from the same isolate. Cells were grown in BG11 medium [24] in aerated Kluyver flasks supplied with a circular cool white fluorescent light of 30 W (Philips) yielding an average limiting light intensity of 20 $\mu E \cdot m^{-2} \cdot s^{-1}$. Batch cultures were harvested about 6 days after inoculation at an absorbance$_{750}$ of 0.3–0.4 (about 1.5 mg Chl/l) the average generation time under these conditions was about 1 day. In some experiments cells were harvested under sterile conditions and resuspended in BG 11 medium without added phosphate. ^{32}P-Orthophosphate (5 μCi/ml; final concentration, 0.1 mM, New England Nuclear) was added and cells were allowed to grow for another 24 h.

Membrane preparation and treatment of these samples to remove RNA, DNA and polyphosphate contamination was done as in Ref. 25. Cells (300 ml) were collected by 5 min centrifugation at 2000 × *g*. The pellets were washed twice and finally resuspended in 20 mM Tricine-NaOH (pH 7.8) and 100 mM sucrose. Where applied, as indicated in the figure legends, a reducing system comprising 1 μl per ml glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49, Boehringer Mannheim from yeast grade II; 140 U/ml), 5 mM glucose 6-phosphate and 1 mM $NADP^+$ was added to all media, prior to mechanical disruption in the French press. (These additions have been shown to support electron supply to respiring cyanobacterial membranes (5–50 μg Chl *a*) for at least 2 h [26]).

Cells (10 ml, corresponding to 0.5 mg Chl) were passed once through the precooled French press at 70 MPa. Unbroken cells and large debris were pelleted at 5000 × *g*. Membranes were collected at 35 000 × *g* and resuspended in the Tricine/sucrose buffer with additions as specified in the results section. The samples (either whole cells loaded with ^{32}P or isolated membranes) were illuminated with white light as indicated in the figure legends, with PS I light (Balzer 710 nm, 8 W/m^2) or PS II light (Schott, 672 nm, 12 W/m^2) or kept in the dark with additions as indicated in the results section. At time zero, [γ-^{32}P]ATP (3 Ci/mMol,

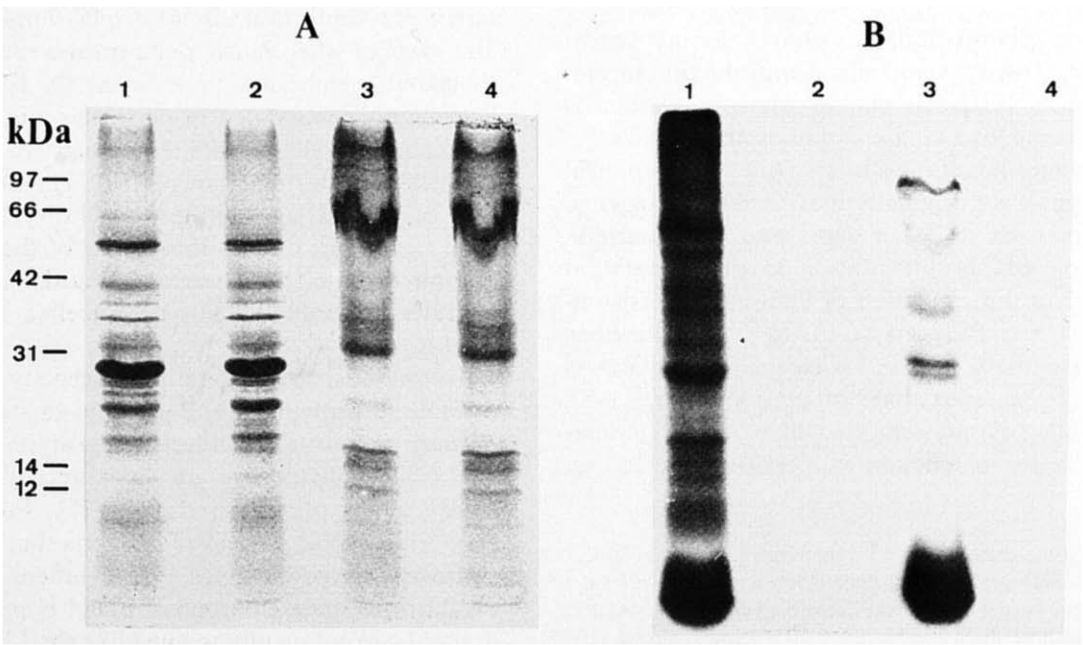

Fig. 1. Phosphorylation of pea thylakoid and *Prochlorothrix hollandica* membranes under oxidizing and reducing conditions. Isolated membranes in tricine/sucrose buffer plus 10 mM NaF and 5 mM $MgCl_2$ were labelled during 10 min in the dark in the presence of 2 mM ferricyanide (oxidizer) or 10 mM freshly prepared sodium dithionite (reducer). The membranes were precipitated with 10% TCA and subsequently dissolved in electrophoresis sample buffer without prior solubilization in maltoside. (A) Coomassie stained gel; (B) Autoradiogram. Lane 1: pea, reduced; lane 2: pea, oxidized; lane 3: *P. hollandica*, reduced, lane 4: *P. hollandica*, oxidized.

New England Nuclear) was added to 100 μl aliquots of the membranes (5 μg Chl) and allowed to react at room temperature (other conditions specified in the results section). The final ATP concentration was 0.1 mM and the radioactivity used in each incubation was 10 μCi. The experiments on pea and on *P. hollandica* presented in Fig. 1 followed standard procedures as in Ref. 8, the solubilization of the prochlorophyte membranes was improved in later experiments. To this end, the phosphorylation was stopped with EDTA (5 mM, final concentration) and the membranes were subsequently dissolved in 0.5% dodecyl-β-D-maltoside (Calbiochem). Non-dissolved material, about 10% of the starting content of chlorophyll, was precipitated by a 5 min spin in an Eppendorf centrifuge. The supernatant (90 μl) was precipitated with 10% trichloroacetic acid (TCA), washed once with 5% TCA and twice with 100% acetone (all steps on ice). The pellets were dried on the air and dissolved in 20 μl 100 mM Tris-HCl (pH 8.8), 3.5 μl 10% SDS and 45 μl sample buffer (0.4 M Tris-HCl (pH 6.8), 6.5% glycerol (v/v), 5% β-mercaptoethanol, 0.004% bromophenol blue) by heating at 70 °C during 10 min. SDS-PAGE proceeded according to Laemmli [27] on 15% acryl-/0.2% bisacrylamide slabgels. The gels (16 cm long, 1.5 mm thick) were run overnight at room temperature at constant current (11 mA). The tracking dye front with excess label was removed and the gel was fixed in 5% acetic acid, after two changes to fresh acetic acid (15 min while shaking) most of the background label was removed. The gels were stained in 12.5% TCA and 0.1% Coomassie Brilliant Blue G 250 (28), destained in 5% acetic acid, dried and autoradiographed on Kodak X-ray film at room temperature. No intensifying screens were used in order to allow estimations of the relative density of bands on the autoradiograms with a gel scanner (Bio-Rad model 1650). The area of peaks was determined by weight.

Molecular mass markers (Bio-Rad, 97.4, 66.2, 42.7, 31.0, 21.5 and 14.4 kDa) were indicated with small dashes of radioactive ink. Non-denaturing green gels were run as in Ref. 29. Fluorescence measurements were done as in Ref. 30, using a HansaTech modulated fluorescence measurement system apparatus with broad blue 'Chl *b* light' (455–485 nm, combination of two Schott BG18 and one BG 38, 2 W/m^2), 425 nm (combination of Corning 5-60 and 4-96 at an intensity of 9 W/m^2, and 710 nm (as above) as different sources of actinic light.

Chlorophyll content of the membrane preparations and of whole cells (after one passage through the French press) was estimated in 80% acetone [31].

Results

Redox-state-dependent phosphorylation of polypeptides in membrane preparations from pea chloroplasts and from *P. hollandica* is demonstrated in Fig. 1. Phosphorylation occurred only under reducing conditions. Complete oxidation of the membranes with ferricyanide completely abolished phosphorylation. Comparison of staining density of pea and *P. hollandica* membranes (lanes 1 and 3) of the autoradiogram displays the relatively poor ^{32}P-incorporation in the latter in in vitro phosphorylation studies with [γ-^{32}P]ATP. Incorporation of label in *P. hollandica* membrane polypeptides occurred at apparent molecular masses of 88, 68, 64, 52, 48, 46, 42, 37, 34, 29, 26, 24, 16 and 9 kDa (dominant labeling in the underlined bands), as judged

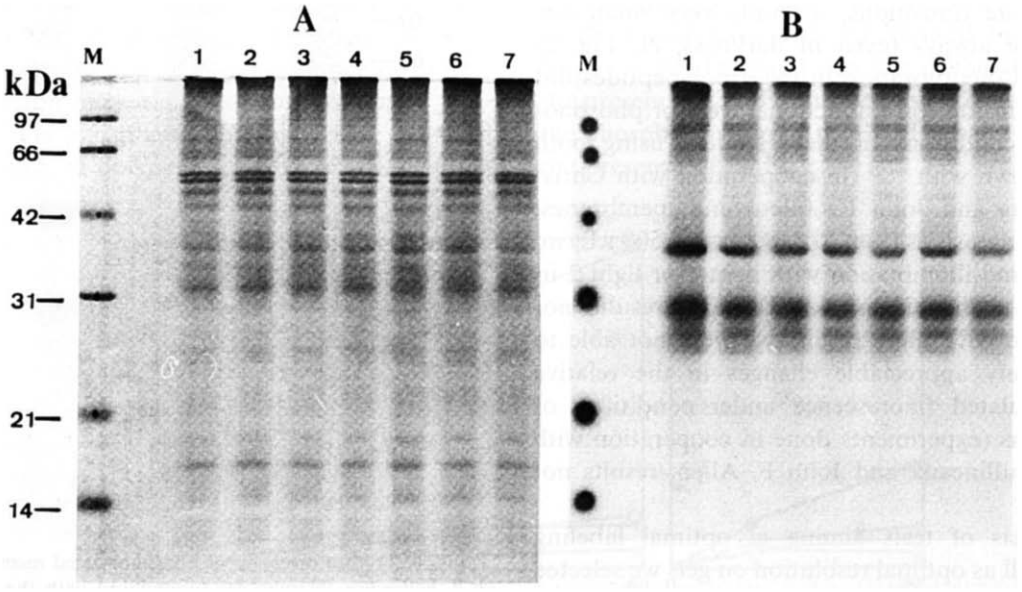

Fig. 2. The effect of light intensity on kinase activity in membranes from *P. hollandica*. Phosphorylation proceeded while illuminating with white light at an intensity of (from left to right) 0, 25, 50, 80, 100, 200 and 500 $\mu E \cdot m^{-2} \cdot s^{-1}$ during 30 min in the presence of 10 mM NaF and 5 mM $MgCl_2$. M, molecular mass markers. A, gel; B, autoradiogram.

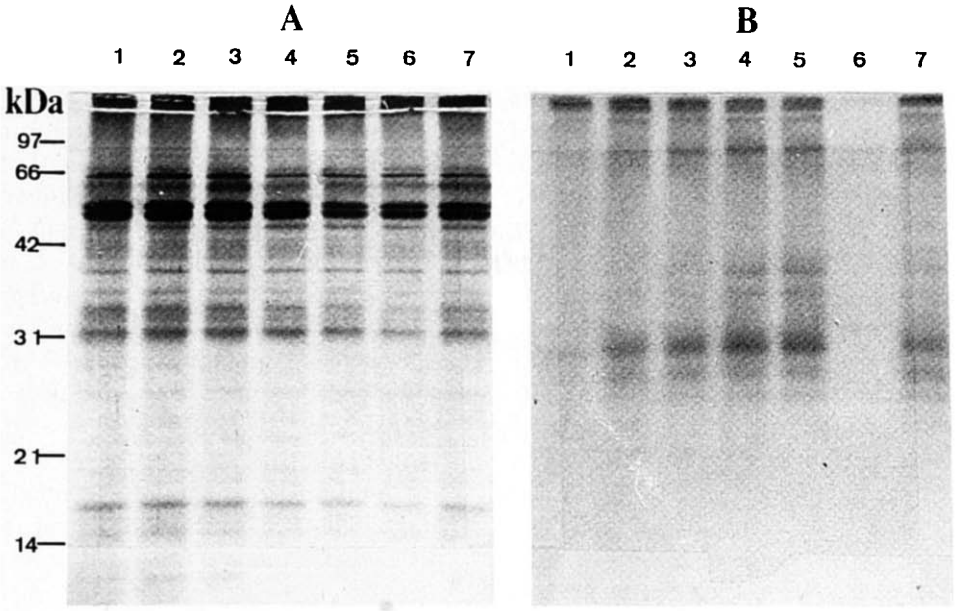

Fig. 3. Cation specificity of the membrane-bound kinase. Phosphorylation of *P. hollandica* membranes (prereduced with the NADPH-generating system) progressed during 30 min in the dark in the presence of 10 mM NaF and (from left to right) 0, 1, 2, 5, 10 mM $MgCl_2$, 5 mM $CaCl_2$ (lane 6) and 5 mM $MnCl_2$ (lane 7). A, gel; B, autoradiogram.

from gels in a number of experiments. The mass of the single so-called 29 kDa band could range between 30 and 27 kDa in different experiments. Bands lighting up on the autoradiograms in this area could not be visualized in Coomassie staining. In order to elucidate a functional role for protein phosphorylation in *P. hollandica* we have determined the kinetics of incorporation during conditions of state transitions or alternatively after imposing of an artificial redox poise. We have focussed on phosphorylation of the 37, 29 and 26 kDa bands.

Studying state transitions, the only very small differences in the always (even in darkness, cf. Fig. 2) occurring label incorporation in these polypeptides did not allow conclusions on a functional role for phosphorylation. The conditions of these studies, using both intact cells grown with ^{32}P (in cooperation with Christine E. Sanders and John F. Allen) and membranes, included light intensities from 0 (darkness) to 500 $\mu E/m^2$ per s (Fig. 2) and illumination with light 1 or light 2 in the presence or the absence of DCMU (results not shown). In line with these results we were not able to demonstrate any appreciable changes in the relative yield of modulated fluorescence under conditions of state transitions (experiments done in cooperation with Conrad W. Mullineaux and John F. Allen, results not shown).

On the basis of tests aiming at optimal labeling intensity as well as optimal resolution on gels we selected membrane preparation via mechanical disruption in the presence of the reducing system with NADPH (cf. Materials and Methods). Comparison of Figs. 1 and 2 demonstrates the advantage of solubilization of the membranes in maltoside prior to electrophoresis.

Phosphorylation of *P. hollandica* membranes was dependent on cations, an Mg^{2+} concentration of 5 mM was sufficient, Mn^{2+} could replace Mg^{2+} but Ca^{2+} totally inhibited phosphorylation (Fig. 3). The ^{32}P at-

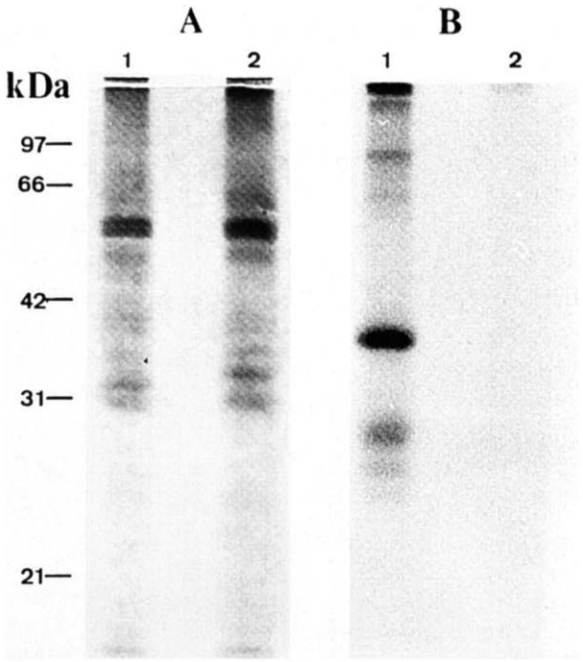

Fig. 4. Trypsin digestion of phosphorylated membrane proteins from *P. hollandica*. Membranes (prereduced with the NADPH-generating system) were labeled during a period of 45 min, with 5 mM $MgCl_2$ but without NaF. After 30 min 1 μg trypsine was added to the sample in lane 2. A, Gel; B, autoradiogram.

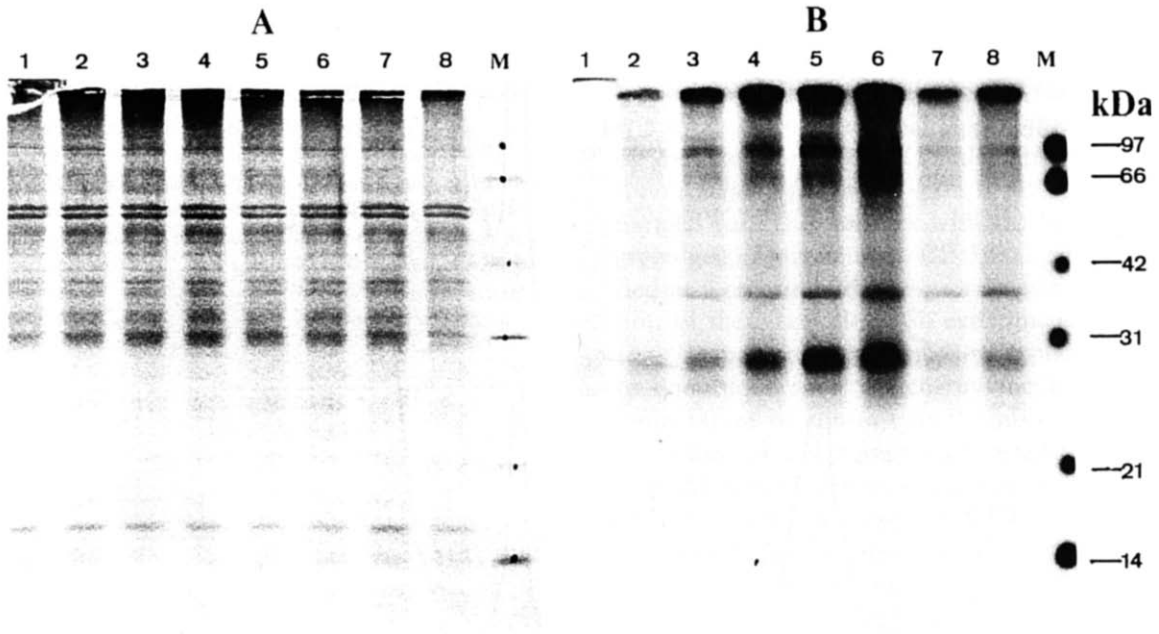

Fig. 5. NADPH enhanced phosphorylation of *P. hollandica* membrane proteins. Phosphorylation was done in the presence of 5 mM $MgCl_2$ and 10 mM NaF during (from left to right, lanes 1–6) 2, 5, 10, 20, 30 and 60 min in the presence of the $NADP^+$-reducing system (Materials and Methods). Lane 7 (10 min) and lane 8 (30 min) were phosphorylated with omission of externally added glucose-6-phosphate dehydrogenase; M, molecular mass markers; A, gel; B, autoradiogram.

tached to the membrane polypeptides could be released with mild trypsin digestion, the resulting fragments ran in the electrophoresis tracking dye front (Fig. 4). A time course of phosphorylation is demonstrated in Fig. 5. Omission of glucose-6-phosphate dehydrogenase reduced the phosphate incorporation by approx. 70%. Optimal reducing conditions as applied in lanes 1–6 preferentially enhanced phosphorylation of the 29 kDa band, using the less reducing conditions applied in the lanes 7 and 8 predominantly diminished incorporation in this band. The relative absorbance changes of bands shown in the autoradiogram of Fig. 5 have been plotted in Fig. 6. The 24, 26, 29 and 37 kDa bands demonstrate different extents and rates of label incorporation.

The effect of the phosphatase inhibitor NaF on the (de)phosphorylation of polypeptides from *P. hollandica* membranes is demonstrated in Table I. Pronounced differences in the relative incorporation of label in the 29 and 37 kDa bands have been revealed. Omission of NaF clearly reduced incorporation in the 29 kDa band by about 50%, label incorporation in the 37 kDa increased in the absence of NaF. The phosphorylation of the 26 kDa band was only slightly affected by NaF, it was hard to individually study the weakly labeled 26 kDa band on the autoradiograms as it showed up as a shoulder on the 29 kDa band in the scans of the autoradiograms. A chase with cold ATP after labeling of the membranes with ^{32}P showed that label release from the 29 kDa band was fast in the absence of NaF, the presence of NaF inhibited the exchange. In contrast, the phosphorylation of the 37 kDa band was reduced in the presence of NaF.

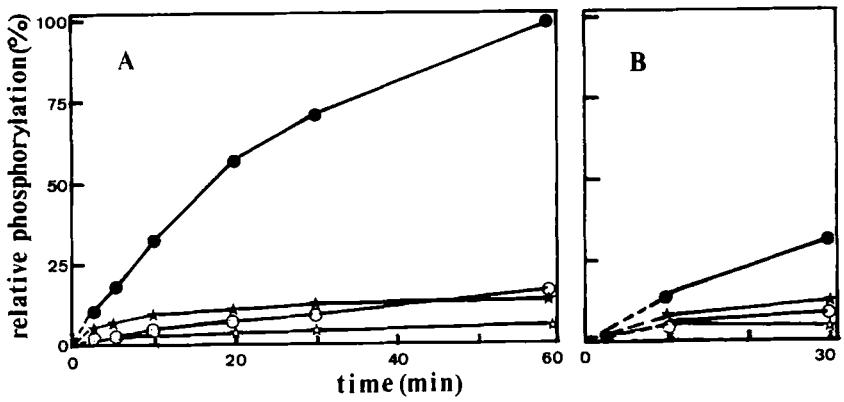

Fig. 6. Area scan of the peaks of the autoradiogram displayed in Fig. 5, 24 kDa (☆), 26 kDa (★), 29 kDa (●) and 37 kDa (○). A: conditions as in Fig. 5, lanes 1–6; B: conditions as in Fig. 5, lanes 7 and 8.

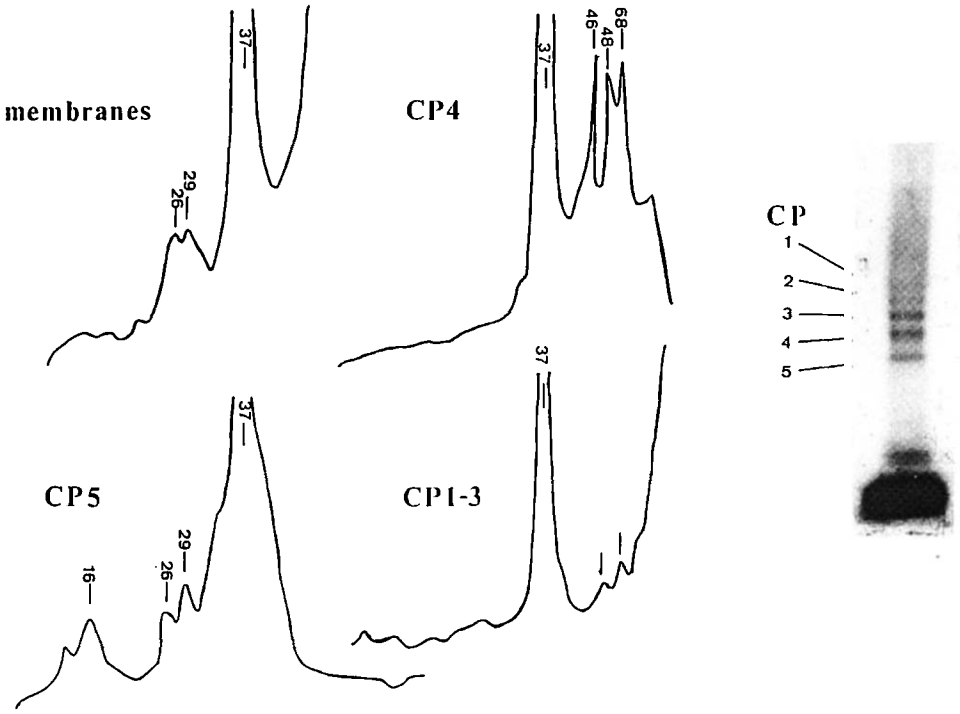

Fig. 7. Phosphorylation of polypeptides in the different chlorophyll-protein complexes of *P. hollandica*. Membrane phosphorylation was achieved as in Fig. 3, lane 4. The complexes were obtained by separation on non-denaturing 'green' gels [29]. The polypeptides in these complexes were resolved on denaturing LiDS gels (cf. Materials and Methods). Lanes from this latter gel were autoradiographed and scanned (left, relative absorbance units).

A first approach to identify the 29 kDa polypeptide included use of non-denaturing green gels. The phosphorylated 29 kDa and 26 kDa band appeared to run in parallel with the lowest band on these gels (Fig. 7). All bands contained the 37 kDa phosphorylated band, band CP4 also contained labeled bands at 46 and 48 kDa.

Discussion

Regulation of light energy distribution in chloroplasts and cyanobacteria may be rather different, i.e., protein phosphorylation and dephosphorylation have been directly linked to fluorescence changes which report on state transitions in the case of chloroplasts [8] but a contribution of this type of regulation in cyanobacteria has not been unequivocally proven. The more rapid fluorescence transients in cyanobacteria have also been explained in terms of photooxidation of chlorophyll bound to PS II [15,16]. Studies on the role of reversible protein phosphorylation in state transitions in cyanobacteria revealed contradictory results. Biggins and Bruce with *Synechococcus* [15] and Schuster et al. with *Fremyella* [17] reported unchanged labelling profiles for thylakoids incubated in the light or in the dark. On the other hand, Sanders and Allen with *Synechococcus* [16] showed an inactivation of the membrane-bound kinase in the dark. Rates of (de)phosphorylation in cyanobacterial membrane preparation have not been studied in the presence of NaF. A marked effect on the rate of (de)phosphorylation could have been indicative for a regulatory function for protein phosphorylation [3–5,10]. Also contributing to a less well-resolved answer on the meaning of phosphorylation as a regulatory mechanism in cyanobacteria is that to our knowledge no defined redox conditions have been set in protein phosphorylation studies in cyanobacteria. The common use of the plastoquinone pool by photosynthesis and respiration [30,32,33] introduces uncertainties in the predicted changes in the redox state by just using light, dark or PS I, PS II light conditions.

Applying an analogous experimental approach as used in the studies with cyanobacteria, Schuster et al. in a study with *Prochloron* on light/dark transitions observed neither differences in phosphorylation, nor changes in the yield of modulated fluorescence [17]. Similar observations were made in the present study with *P. hollandica* (Fig. 2). The lack of changes in the phosphorylation pattern after changes in the light conditions (also including use of 710 nm PS I light) are possibly due to the complicating interplay between photosynthesis and respiration in *P. hollandica* (Matthijs, H.C.P. et al., unpublished observations) just like in cyanobacteria [33]. Under defined redox conditions, however, we were able to demonstrate phosphorylation to be less in relatively membrane-oxidizing conditions and to be enhanced in reducing conditions (Fig. 5). The data represented in Fig. 6 would suggest that gradual

TABLE I

Phosphate incorporation in membrane polypeptides from Prochlorothrix hollandica in the absence or presence of the phosphatase inhibitor NaF.

Membranes (prereduced with the NADPH-generating system) were labeled during 30 min in the presence of 5 mM Mg ion and with or without 10 mM NaF. The final ATP concentration in this experiment was 50 μM (10 μCi [γ-^{32}P]-ATP). Phosphorylation was continued for another 60 min, either without any addition (upper two lines) or after dilution of the label with a 20-fold excess of cold ATP (1 mM final). The area of the peaks from the 29 and 37 kDa bands were estimated from scans. The numbers indicated under 29 kDa are inclusive of the 26 kDa band.

Condition	Incorporation (% of maximum)	
	29 kDa	37 kDa
Without NaF	46	80
With NaF	100	46
Without NaF plus chase	23	100
With NaF plus chase	81	53

incorporation of label especially occurs in the 29 kDa band. These data could be misleading in that no information on the relative amount in the membranes of the phosphorylated polypeptides is available [13].

From the relative staining density on gels, we have observed that the 29 kDa band is always very weak, the 26 kDa band is much stronger staining, the possible presence of various polypeptides of 26 kDa contributing to this cannot be excluded at this moment. In addition, we have observed that the phosphorylation of the 29 kDa band is much more favoured by reducing conditions than the other bands considered in this study (Fig. 6). Studies to identify the 29 kDa band further are in progress. The observed kinase activity in the bands of Fig. 5 (autoradiogram) may not necessarily have a role in the regulation of light energy distribution analogous to the one in chloroplasts. Relatively fast dephosphorylation would be a prerequisite for such a regulatory role.

Experiments aiming at detection of those phosphorylated polypeptides being susceptible to fast dephosphorylation by a phosphatase revealed that in particular phosphorylation and dephosphorylation of a 29 kDa polypeptide was affected by NaF (Table I). The difference in phosphorylation in the absence or presence of NaF of about 50% is very much like data found for LHC II in chloroplasts [10]. The regulation of the (de)phosphorylation of the 29 kDa polypeptide thus shows similarities with the conditions of LHC II (de) phosphorylation in chloroplasts. However, the 29 kDa polypeptide has not yet been proven to be a part of the chlorophyll *a/b*-protein complex in *P. hollandica* [29]. Some analogy with the data documented on *Prochloron* interestingly exists. Schuster et al. [17] demonstrated that besides the 34 kDa polypeptide some additional proteins in the 27–30 kDa range were relative intensely labeled and belonged to the fastest running complex on green gels (identified as $CPII_L$ for the lower CPII band by these authors [17]). A similar observation was made for *P. hollandica*, the 29 kDa band appeared to run in parallel with the complex with the highest mobility on green gels. This complex CP 5, has earlier been identified as a chlorophyll *a/b*-protein complex [29]. Resolution of these complexes in experiments like the one in Fig. 7 is hampered by a need to expose the membranes to conditions such as lengthy incubation at ambient temperature in contrast to the normally favoured fast procedure at low temperature used in running green gels. All green complexes contained the 37 kDa polypeptide as a major phosphorylated band. Such a 37 kDa polypeptide is commonly found in the phosphorylated polypeptide pattern of chloroplasts [34]. The effect of NaF on its (37 kDa band) phosphorylation has not been discussed in literature. A suggestion for the identity of the 37 kDa band could be a phosphatase on basis of its strong (auto)phosphorylation and the effect of NaF. In contrast to the results shown here for *P. hollandica* this heavily labeled band appeared to be absent in the experiments with *Prochloron* [17]. In conclusion, the results of this preliminary study indicate similarities in the molecular mechanism and type of regulation in the (de)phosphorylation of LHC II and the 29 kDa polypeptide of *P. hollandica*. Our present attempts to identify the 29 kDa polypeptide could help in assigning a role for membrane protein phosphorylation in light energy distribution in prochlorophytes.

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