Biochimica et Biophysica Acta, 767 (1984) 138–144
Elsevier

BBA 41634

MECHANISM OF THE LIGHT STATE TRANSITION IN PHOTOSYNTHESIS

II. ANALYSIS OF PHOSPHORYLATED POLYPEPTIDES IN THE RED ALGA, *PORPHYRIDIUM CRUENTUM*

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(Received May 21st, 1984)

Key words: Protein phosphorylation; Chlorophyll fluorescence; Phycobilisome; Excitation energy distribution; State transition; (Red alga)

The possible involvement of a reversible protein phosphorylation event in the regulation of excitation energy distribution was studied in the red alga *Porphyridium cruentum*. Whole cells were incubated in phosphate-depleted growth medium containing carrier-free [^{32}P]orthophosphate for several hours to label the intracellular phosphate pools, and they were then converted to State 1 or State 2 by illumination using blue or green light, respectively. The successful transition to State 1 or State 2 was verified by 77 K fluorescence spectroscopy of the chlorophyll emission and the cells were then denatured using either acetone, trichloroacetic acid or boiling detergent. The whole cell lysates were solubilized, treated with RNAase, and analyzed for phosphoproteins by SDS-polyacrylamide gel electrophoresis. At least twelve polypeptides were found to be phosphorylated but no changes in specific radioactivity of the polypeptides were detected when samples from cells in State 1 and State 2 were compared. We conclude that a reversible protein phosphorylation event is not implicated in the state transition in *P. cruentum*. A model is presented for the mechanism of the light state transition in organisms that contain phycobilisomes which is different from the mechanism of energy distribution proposed for higher plants.

Introduction

Regulation of energy distribution between the two photosystems in photosynthesis by illumination of cells in vivo has been shown to occur in higher plants [1,2], green algae [3–5], red algae [6–9] and cyanobacteria [10]. Following terminology suggested by Bonaventura and Myers [3] and Murata [6], excitation absorbed primarily by PS II (light 2) results in the conversion of cells to State 2 and, conversely excitation absorbed primarily by PS I (light 1) converts the cells to State 1. State 2 is characterized by an increase in fluorescence emanating from PS I, whereas State 1 results in an increase in PS II fluorescence. Corresponding changes in the rates of PS I and PS II electron transport reactions accompanying the state transition have been demonstrated [3,9–12].

In higher plants, it has been established that the transition to State 2 occurs following phosphorylation of the LHC by a membrane kinase [13,14] that is controlled by the redox state of plastoquinone [15,16]. The conversion back to State 1 is mediated by dephosphorylation of the phosphorylated LHC by a membrane phosphatase [14].

Abbreviations: PS, Photosystem; LHC, light-harvesting chlorophyll *a*/*b* protein; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; SDS, sodium dodecyl sulfate; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone.

As suggested by Bonaventura and Myers [3], it has been shown that the transition to State 2 is accompanied by an increase in the absorption cross-section of PS I [17,18]. Larsson et al. [19] and Kyle et al. [20] have indicated that this occurs as a result of a lateral migration of the phosphorylated LHC from the stacked region of the thylakoids to the stroma regions that are enriched in PS I. (For review see Ref. 21.)

Red algae and cyanobacteria differ substantially from green algae and higher plants in that their major apparatus for light harvesting is not an intramembrane pigment-protein LHC. Instead, they have phycobilisomes [22,23] which are attached by dense packing to the stromal surface of the thylakoid [24] and their absorbed excitation is transferred almost exclusively into PS II [8]. We have recently reported that proton ionophores such as CCCP, prevent the conversion of the red alga *Porphyridium cruentum* to State 1, suggesting that coupled proton transport is required for the transition [25]. However, it is not known whether this energetic requirement is a prerequisite for a protein phosphorylation event that might be analogous to the phosphorylation of the LHC in higher plants.

The purpose of this investigation was to determine the possible involvement of a reversible protein phosphorylation event in organisms that contain phycobilisomes. The data reveal the existence of at least twelve phosphoproteins in whole cell lysates of the red alga *P. cruentum*, but no changes in protein phosphorylation were detected during the reversible conversion of cells between State 1, State 2 and darkness. We conclude that the mechanism for the regulation of excitation energy distribution in red algae is quite different from that which has been observed in higher plants.

Methods

P. cruentum (UTEX 161) was grown autotrophically on the enriched sea-water medium F/2 of Guillard and Ryther [26] at a light intensity of 25 $\mu E \cdot m^{-2} \cdot s^{-1}$. The state transition was induced as described previously [9] by illumination of the cells with either light 1 (Corning glass No. 5.60 plus an Optics Technology 600 nm short pass filter) at an intensity of 150 $\mu E \cdot m^{-2} \cdot s^{-1}$, or light 2 (Optics Technology 500 nm long pass plus 600 nm short pass filters) at an intensity of 500 $\mu E \cdot m^{-2} \cdot s^{-1}$. The state transition was verified by measuring the fluorescence emission of the cells at liquid nitrogen temperature and the spectra were similar to those of Ley and Butler [8] and those presented in our previous report [9].

Preparation of the algae for the investigation of phosphoproteins was as follows: exponential phase cells were harvested by centrifugation, washed once and suspended in phosphate-depleted medium (F/2 minus supplemental phosphate) at a cell concentration of 50 μg chlorophyll *a* per ml. 500 μCi carrier-free [^{32}P]orthophosphate (New England Nuclear, Boston, MA) was added and the 2 ml sample was incubated in light for 2–4 h. Longer incubations of 12–16 h gave identical results, but the protein fraction was of much higher specific radioactivity. The cells were then diluted with 5 vol. F/2 medium to a chlorophyll concentration of approx. 10 μg/ml and incubated for a further hour in the light. The sample was then divided into two, and one sample was converted to State 1 by illumination of the cells with light 1, and the other was converted to State 2 by illumination with light 2. The incubations for this stage were conducted in a glass chamber of 2.5 cm pathlength and the cells were stirred to ensure equilibration with the air and to promote rapid killing of the cells by added denaturant at the end of the incubation period. Illumination times were occasionally varied between 15 s and 5 min with the same results. Immediately prior to cell denaturation, the reactions were always sampled by rapidly withdrawing and freezing small aliquots for 77 K fluorescence analysis to ensure that the cells in question were in the required light state. Routinely, the cells were then killed by addition of 4 vol. cold acetone. The insoluble fractions were collected by centrifugation, washed twice with 95% acetone and twice with 100% acetone. Residual acetone was removed from the powders by evacuation. Alternate procedures in experimental protocol gave identical results. These included denaturing the cells with 15% trichloroacetic acid or boiling 2% SDS. Occasionally, a single reaction vessel containing radioactive cells was alternated between light 2, light 1 or darkness, and samples were rapidly withdrawn and denatured for protein analysis.

The washed residues were prepared for protein analysis by boiling in 2% SDS, 50 mM dithiothreitol, 0.1 M Tris-HCl (pH 6.8) for 5 min. The solubilized fractions were then microfuged for 10 min and the supernatant was treated with RNAase (100 μg/ml) overnight at 30°C. The fractions were then analyzed by SDS-polyacrylamide gel electrophoresis using the discontinuous system of Laemmli [27] with 12% polyacrylamide in the resolving slab gel of 1.5 mm thickness. The slab gels were stained with Coomassie brilliant blue R-250, destained, dried and radioautographed on X-ray film using standard procedures. All electrophoretic reagents and protein markers were obtained from Bio-Rad (Richmond, CA) and the enzymes RNAase, proteinase K and pronase were obtained from Sigma (St. Louis, MO).

Results

Fig. 1 shows representative 77 K fluorescence emission spectra of radioactive cells in State 1 and State 2 that were subsequently analyzed for phosphoproteins. The spectra confirm that the cells were in State 1 and State 2 prior to denaturation of the samples and protein analysis. Fig. 2 shows data from the denaturing slab gels of samples that were analyzed by SDS-polyacrylamide gel electrophoresis. Fig. 2A is of a lane stained with Coomassie R-250 to show the distribution of the major species of polypeptides in the whole cell lysates. Considerable difficulty was encountered in the resolution of polypeptides from such whole cell lysates of *P. cruentum* at protein concentrations required for detection by staining. Most of the problems were due to extensive streaking and were most probably caused by mucopolysaccharide cell wall components interfering with entry of the polypeptides into the stacking gel. Much better resolution in the autoradiographs was obtained by performing the SDS-polyacrylamide gel electrophoresis at very low concentrations of protein of high specific radioactivity.

Fig. 2B and C are autoradiographs of the actual samples shown in Fig. 1, and are of cells in State 1 and State 2, respectively. The amount of protein applied to the electrophoretic sample slots in B and C was 30-fold less than that used in A. The two radioautograms shown in Fig. 2 reveal the

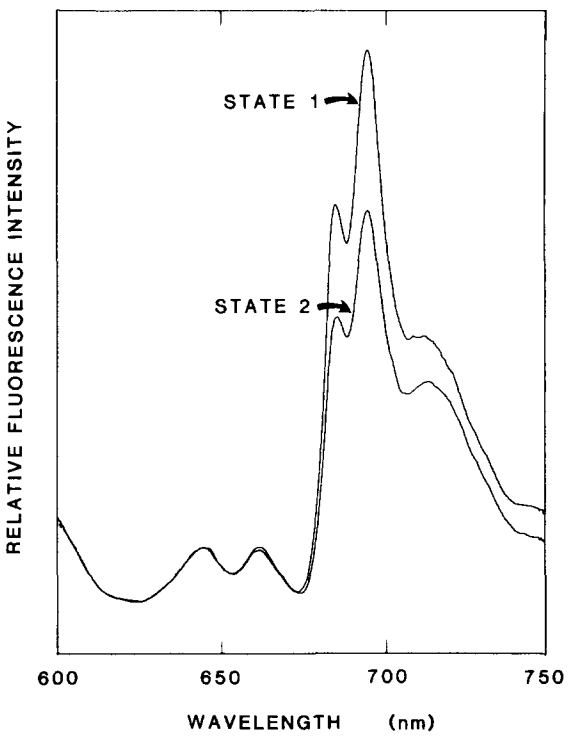

Fig. 1. 77 K fluorescence emission spectra of *P. cruentum* in State 1 and State 2. The excitation wavelength was 560 nm and the spectra were resolved at a band-pass of 0.9 nm. The spectra were normalized at 645 nm. These spectra were of radioactive cells that were analyzed for phosphoproteins as shown in Fig. 2B and C.

existence of at least twelve bands labelled with [^{32}P]P_i. The apparent M_r of the twelve bands labelled with radioactive phosphate were approx. 120, 105, 90, 69, 63, 48, 43, 41, 21, 17, 14 and 13 kDa. Although none of the radioactive bands in Fig. 2B and C or the Coomassie-stained bands in Fig. 2A were identified, it is pertinent to note that the major phosphorylated polypeptides in *P. cruentum* were of minor abundance.

Fig. 3 shows densitometric traces of the autoradiographs displayed in Fig. 2A and B to quantitate the radioactivity in the labelled bands. The profiles of phosphoproteins derived from the cells in State 1 and State 2 are superimposable, except for some very minor differences that we do not consider significant. Additionally, no differences were detected between samples derived from cells in either States 1 and 2, or from cells that had been equilibrated in darkness for 10 min (data not

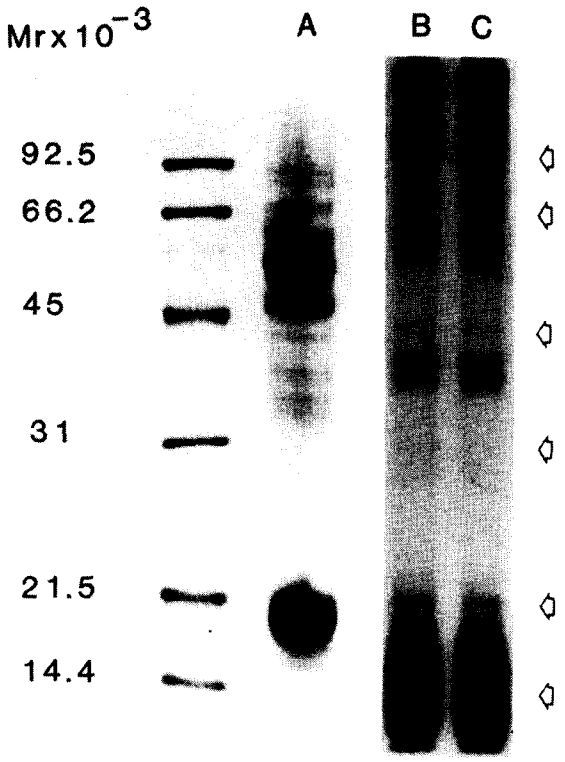

Fig. 2. SDS-polyacrylamide gel electrophoresis of the solubilized protein fraction of *P. cruentum*. (A) A representative example of a whole cell lysate stained with Coomassie brilliant blue. The amount of protein applied was 120 μg. (B and C) Radioautograms of samples derived from cells that had been denatured in State 1 and State 2, respectively. 4 μg protein in 25 μl was applied per sample slot and the radioactivity in lane B = $5.02 \cdot 10^6$ cpm, and lane C = $4.93 \cdot 10^6$ cpm. The fluorescence spectra of the cells prior to denaturation are shown in Fig. 1.

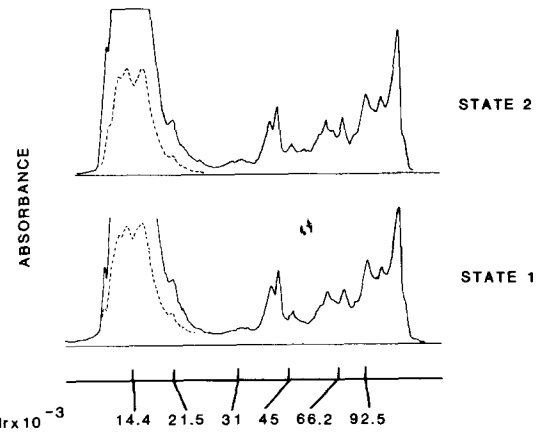

Fig. 3. Densitometric traces of the autoradiographs of the protein gels shown in Fig. 2B and C corresponding to cells in State 1 and State 2. The dashed lines indicate a scale reduction of 2.4.

shown). We conclude that the state transition in *P. cruentum* is not a result of, or accompanied by a protein phosphorylation-dephosphorylation event.

Two important control experiments were performed. Fig. 4 shows data of a control experiment which confirms the radioactive bands shown in Figs. 2 and 3 to be polypeptides due to their proteinase sensitivity. Short-term treatment of the radioactive samples with either proteinase K or pronase gave essentially identical results. Second, we considered the possibility of loss of [^{32}P]P_i from some phosphoproteins by dephosphorylation following solubilization and preparation of the samples for the electrophoresis step. To check on possible protein phosphatase activity in the cell lysates, [^{32}P]P_i-labelled phosphohistone was included as an internal control in a sample of *P. cruentum*, denatured and prepared under identical conditions. No loss in specific activity of the radioactive phosphohistone was observed, indicating the absence of unspecific phosphoprotein phosphatase activity in the solubilized *P. cruentum* lysates (data not shown).

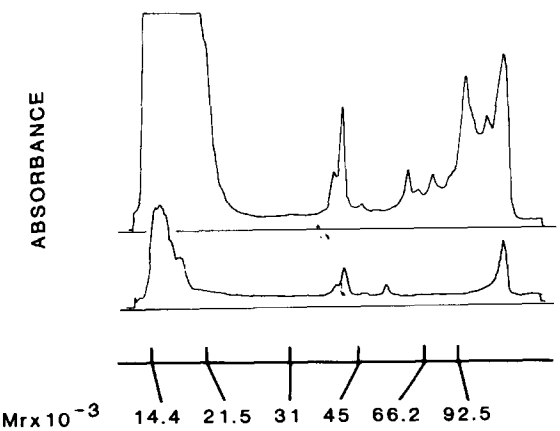

Fig. 4. Densitometric traces of autoradiographs showing the effect of proteinase treatment on the radioactive bands in *P. cruentum*. The upper (control) trace shows a typical labelling pattern and the lower trace shows the sample following treatment with proteinase K (100 μg/ml) for 6 h at 30°C.

Discussion

The data presented show that at least twelve polypeptides in *P. cruentum* can exist in phosphorylated form. It is not known whether any of these phosphoproteins are functional in metabolic regulation. Although there were some major differences in experimental protocol during the investigation, the profile of the radioactive bands was reasonably consistent. Noteworthy is the fact that none of the most abundant polypeptides appear to be phosphorylated such as those of apparent M_r approx. 50, 48 and 18 kDa. The latter group most likely contain the bulk of the phycobilisome subunits [24,28].

The present study was initiated to specifically investigate the possibility of the participation of a reversible protein phosphorylation event in the state transition in *P. cruentum*. At the level of resolution employed, our data indicate that no major changes in the level of phosphorylation of the polypeptides occur accompanying the state transition. We conclude, therefore, that the regulation of energy distribution in *P. cruentum* does not involve a reversible protein phosphorylation event. Similar data, presently in preliminary form, using the cyanobacterium *Anacystis nidulans* have been obtained, indicating that the mechanism of the state transition in organisms containing phycobilisomes may be general and radically different from that of the green algae and higher plants.

Fig. 5 shows a general model for the light state transition in cyanobacteria and red algae which we now present to summarize the data from various lines of investigation and upon which to base future experiments. Diagram A shows the cells in State 2. In this state, excitation absorbed by the phycobilisomes is transferred almost exclusively into PS II [8] with a high probability of excitation transfer from PS II to PS I. This is depicted schematically in A by virtue of the close physical proximity of the two photosystem pigment protein complexes in the thylakoid membrane. The linear electron transport in State 2 is therefore driven by excitation primarily absorbed by the phycobilisome, and the extent of the spillover from PS II adjusts the overall rate of electron transport. We would also note here that in field conditions, marine algae that contain phycobilisomes would

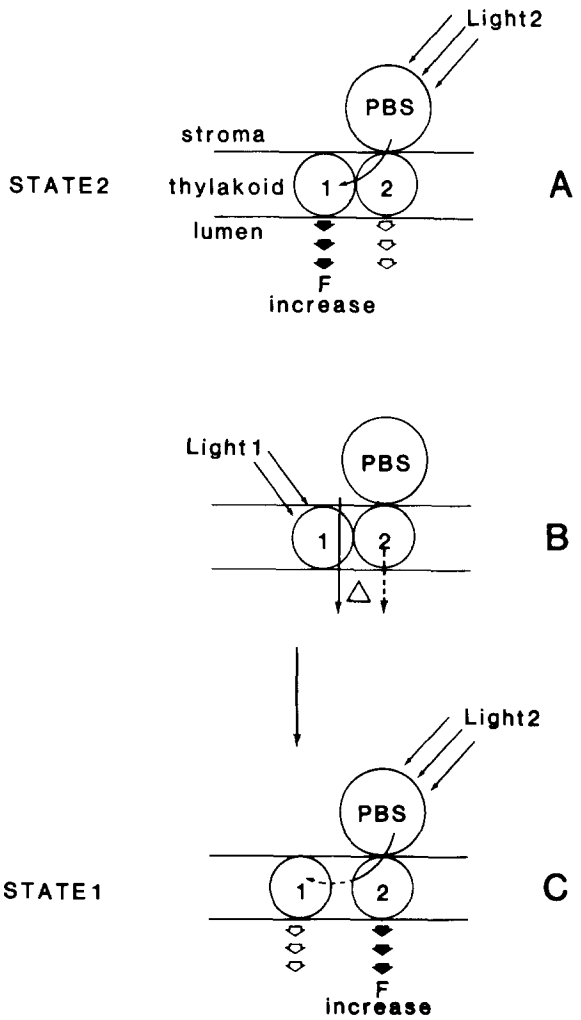

Fig. 5. (A) Model of the mechanism of excitation energy redistribution in cyanobacteria and red algae. In B, Δ represents a change in charge distribution in the local environment of the two photosystems. PBS, phycobilisome.

be expected to be predominantly in State 2 when present in the upper photic zone of the water column.

The transition to State 1 is shown in Fig. 5B and C and occurs as follows. Preferential excitation of PS I results in turnover of the in vivo cyclic electron transport pathway. This must include electron transfer through the cytochrome b_6-f complex, because the transition of cells to State 1 is inhibited by DBMIB [5,9], which inhibits the oxidation site of PQH_2. There is also the require-

ment that the PS I electron transport be energetically coupled as the transition to State 1 is prevented by proton ionophores [5,9]. As our results above strongly suggest that no phosphorylation event is required for the transition to State 1, then the proton gradient per se, rather than ATP and protein kinase activity, must trigger the state transition. As proton gradients are certainly generated by cells in State 2, we propose that the PS I-generated gradient in light 1 must be to some extent localized. This is indicated conceptually in diagram B, where Δ is intended to indicate a difference in charge distribution between the two intramembrane complexes PS I and PS II. Precedence for the consideration of differences in local transmembrane electrochemical potential in this mechanism derive from the work and insight of Prochaska and Dilley [29,30] and Theg and Homann [31]. They showed the existence of a localized domain of H^+ and Cl^- in an intramembranous compartment associated with PS II. Theg and Junge [32] have also suggested the possibility of two H^+ sequestering domains in thylakoids, each dominated by different photosystems.

We have shown previously [9] that algal cells can be chemically cross-linked following the state transition using glutaraldehyde, and that the fixed cells retain the fluorescence and electron transport properties characteristic of the light states. This was taken to indicate the existence of a conformational change in the mechanism of the state transition which is now shown schematically in Fig. 5C as a change in physical proximity between the two photosystems. Only a very short distance would be necessary to account for the change in spillover between PS II and PS I [8], in contrast to the massive lateral migration of antenna complexes envisaged to change the distribution of excitation energy in the thylakoids of higher plants [21]. We suggest that this is brought about by the postulated change in localized charge distribution via electrostatic interactions. Factors contributing to such a charge redistribution may include localized gradients of protons and counterions and, or changes in redox state of components of the electron transport pathways.

Acknowledgements

We wish to thank Professor David L. Brautigan of the Division of Biology and Medicine for providing the [^{32}P]P_i-labelled phosphohistone and for valuable advise throughout the project. The research was supported by the Science and Educational Administration of the United States Department of Agriculture under Grant Number 81-CRCR-1-0767 from the Competitive Research Grants office and PCM-8302983 from the National Science Foundation.

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