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LIGHT-DEPENDENT PHOSPHORYLATION OF THYLAKOID MEMBRANE POLYPEPTIDES

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SUMMARY: The phosphorylation of thylakoid membrane proteins was studied using isolated chloroplasts from Euglena gracilis. We have found, using [^{32}P] labelling, that this phenomenon was light-driven, reversible in the dark, and completely inhibited by Carbonyl cyanide m-chlorophenyl-hydrazone (CCCP). Polyacrylamide gel electrophoresis containing SDS has revealed five main bands which have been found to be proteins. Amino acid analysis of the bands has shown that [^{32}P] is incorporated into phosphothreonine.

Many photochemical, molecular and ultrastructural events have been reported in the study of photosynthetic membrane activation: electron transfer, conformational change in the coupling factor, ion flux, electrical potential change. Membrane protein phosphorylation seems to be a new phenomenon in this activation. Protein kinase activity has been reported from Acetabularia chloroplasts (1), from a cellular fraction of Brassica (2), and more recently, from pea chloroplasts (3). The exact function of this phosphorylation is still unknown, but it might play a role in membrane activation and coupling of photochemical events with the ATP synthesis. The present study, using isolated chloroplasts shows a light-driven thylakoid membrane protein phosphorylation.

MATERIALS AND METHODS

Chloroplasts isolation: Euglena gracilis (strain Z) was grown photoheterotrophically, in the medium described by Price (4). Cells were harvested and washed twice with distilled water, followed by centrifugation at 1000 x g for 2 minutes, then washed again with grinding buffer (TB) modified from Salisbury (5). KCl replaced NaCl. The cells were resuspended in this buffer at 0.5 g/ml, broken in a French pressure cell at 1500 psi, and collected in 4 volumes of grinding buffer. The broken cells were centrifuged at 100 x g for 1 minute in a small volume of TR buffer (40mM Tricine-KOH pH 8.4; 0.33M Sorbitol), and

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centrifuged successively at 100 x g for 5 minutes and then one minute, the pellet being discarded each time. Thylakoid membranes were isolated according to Vasconcelos (6).

Incorporation assays: Isolated chloroplasts containing 50 µg of chlorophyll in 300 µl of TR, were incubated with carrier-free [^{32}P]-orthophosphate (100 µCi) from New England Nuclear, Boston. Incubation was at 20°C. The mixture was illuminated (10,000 lux) with two tungsten 150W bulbs filtered to eliminate all wavelengths shorter than 580 nm. Aliquots of 50 µl were withdrawn at regular intervals, and plated on Whatman 3MM paper disks, which were treated according to Mans and Novelli (7). The filters were then counted by measuring Cerenkov radiation in 10 ml of water in a Beckman model LS 355 liquid scintillation spectrometer with a counting efficiency of 51%.

Electrophoresis: The chloroplasts, after a 10 minute incubation, were centrifuged at 12000 x g for 10 sec in a Eppendorf 3200 Microcentrifuge and immediately solubilized in a mixture of 50mM Na_2CO_3, 50mM dithiothreitol (DTT), 2% (wt/vol) SDS, 12% sucrose and 0.04% bromphenol blue, the final chlorophyll concentration of the preparation being 1 mg/ml. SDS gel electrophoresis was done according to Chua (8) using 7.5 to 15% polyacrylamide gels gradient; each slot contained 20 µl.

Isolation of labelled amino acids: Regions of gels corresponding to the phosphorylated proteins were excised and trypsin-treated according to Clegg (9). After digestion and lyophilisation, the peptides were acid-hydrolised in 6N HCL under N_2, at 105°C for two hours. The resulting amino acid mixture was again lyophilized, dissolved in water to remove trace of acid and lyophilized a third

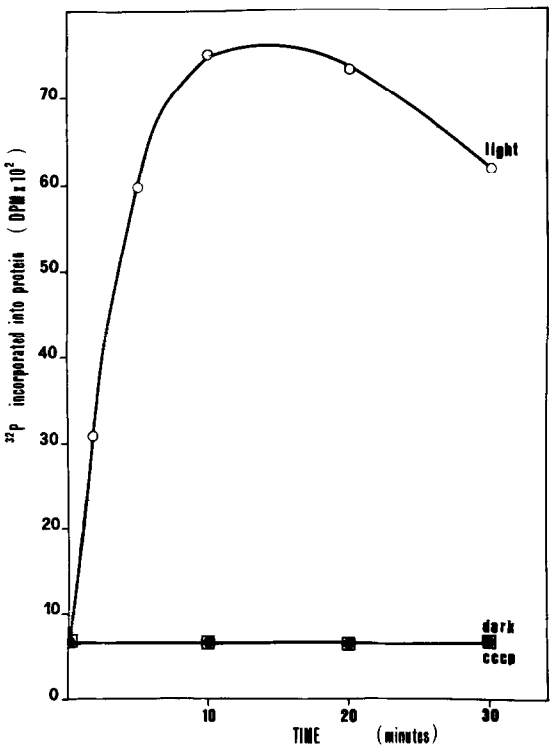


Fig. 1 Incorporation kinetics of [^{32}P] orthophosphate into proteins by isolated Euglena chloroplasts. Incorporation in the light ○ and incorporation in the light with CCCP □ , incorporation in the dark ● .

time, the amino acid mixture was then dissolved in propan-2-ol (10% v/v) and chromatographed on Whatman 3MM paper, according to Jones (10). The paper was dried and put in a folder with one X Ray film (RPR2X-Omat) for radioautography.

RESULTS: The incorporation of [^{32}P] in proteins (Fig. 1) increases during the first 10 minutes of illumination, then it reaches a plateau for an additional 10 minutes before a slow decrease in observed (even with continuous illumination). The control in the dark shows no incorporation, as does an other control in the light, but with addition of CCCP.

The pattern given by the radioautograph (Fig. 2b) revealed up to 10 phosphorylated bands, among which six are prominent as seen in figure. Bands I to

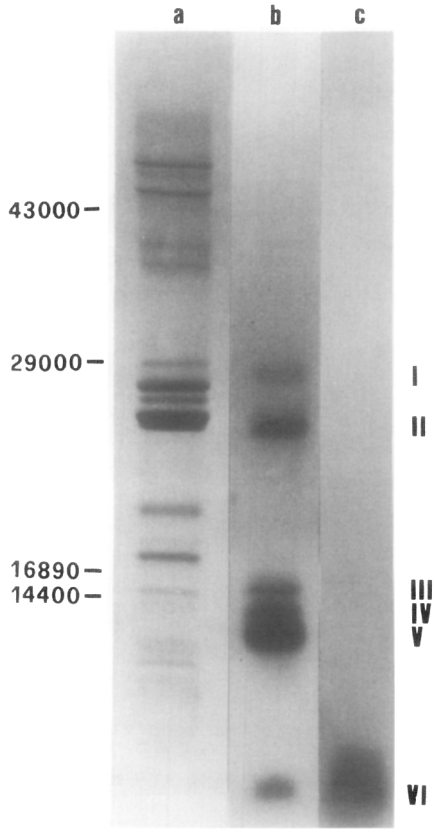


Fig. 2 In vitro incorporation of [^{32}P] orthophosphate into chloroplast proteins. After electrophoresis, the gel is fixed with 50% methanol, 7% acetic acid and put in a folder with one X Ray film (RPR2X-Omat) for radioautography.
a) stained gel showing chloroplast proteins,
b) radioautography showing chloroplast phosphoproteins,
c) effect of proteinase K on sample.
Proteinase K is used at 50 µg/ml, directly in the solubilisation mixture and incubation is done for 2 hours at 37°C.

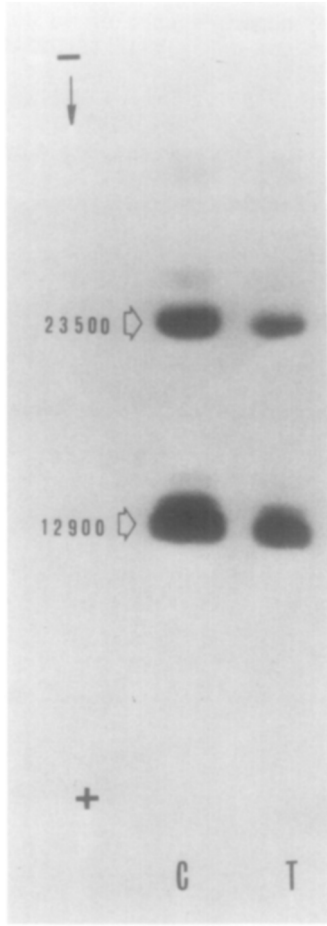
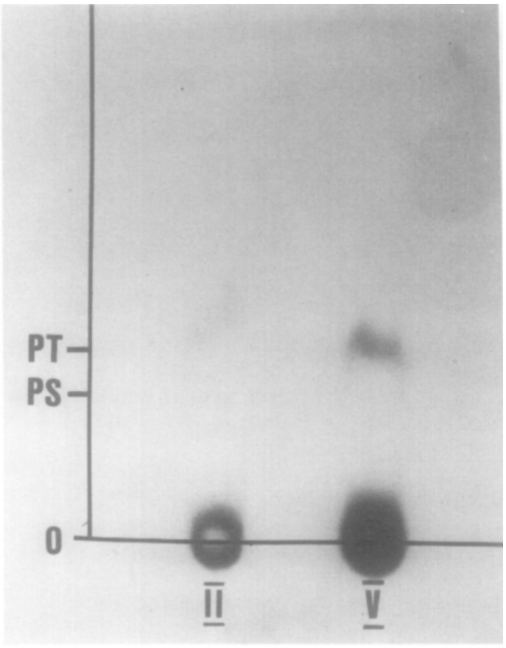


Fig. 3 Location of phosphoproteins. Radioautograph of total chloroplasts proteins (C) and of isolated thylakoids membrane proteins (T).

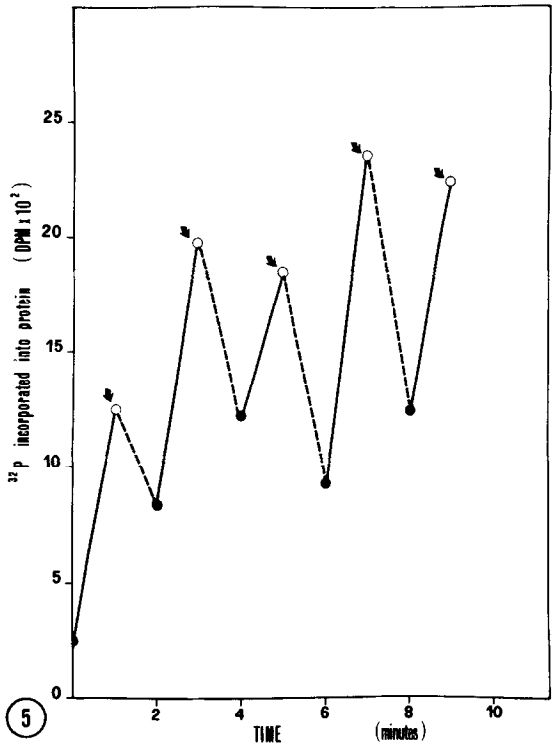
V are lost after treatment with proteinase K, leaving band VI intact (Fig. 2c). RNase treatment has no effect on any of the six bands (data not shown). TCA treatment of the gels at 90°C eliminates band VI (it may be phospholipids).

The isolated thylakoid membranes give the same pattern of incorporation as the total chloroplasts, establishing the membrane nature of these phosphorylated peptides (Fig. 3). Chromatographic analyses of labelled bands II (M_r: 23,500) and V (M_r: 12,900) permits the identification of the phosphorylated amino acids as beeing phosphothreonine and not phosphoserine (Fig. 4).

When the illumination is not continuous, but is for alternating periods of one minute in the light and one minute in the dark (Fig. 5), the incorporation



(4)



(5)

Fig. 4 Identification of [^{32}P] labelled amino acid in bands II and V. Localisation of radioactivity in threonine
PT phosphothreonine
PS phosphoserine

Fig. 5 Effect of pulsed illumination on [^{32}P] incorporation into proteins. Conditions are as in Figure 1, but with 25 µl aliquots.
light off
○ bulk incorporation at the end of light period
● bulk incorporation at the end of dark period

follows the light stimulation. The response time of the membrane to irradiation is therefore under one minute. Figure 5 also shows the two-states of the membrane, during illumination, and in the dark. The excited state, in illumination, is represented by white dots, and the ground state, by black dots.

DISCUSSION: As can be seen in Figure 1, the phosphorylation of thylakoid peptides is light-dependent, the maximum incorporation being during the first ten minutes. The control in the dark, or in the light with CCCP, gave no incorporation at all, the coupling of photochemical events to the ATP synthesis seems thus to be essential for phosphorylation to occur.

The molecular weights of the two main phosphorylated peptides (M_r: 23,500, 12,900) is quite similar to those obtained by Bennett (3), working with pea chloroplasts: M_r: 26,000, 9,000, with the difference that band V is definitly more prominent with Euglena chloroplasts, and that five well-defined proteins are phosphorylated instead of two.

Our results exhibit similarities with those of Bennett (3) not only with regard to the molecular weights of these peptides, but also reveal the same pattern of incorporation, (the maximum being around ten minutes) and the same major phosphorylated amino acid: threonine. These similarities are of interest, considering the evolutionery divergences between Euglena and pea.

Three possibilities for the fate (during the dark period), of the [^{32}P] phosphate incorporated during the light period, are illustrated in Figure 6: the first (I) is shown not to be the case, since our results demonstrated no

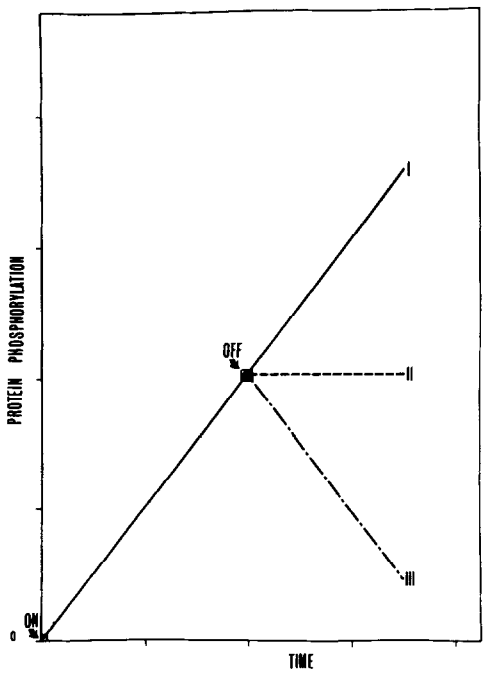


Fig. 6 Illustration of three possibilities in membrane protein phosphorylation. The beginning and end of illumination are indicated respectively by ON and OFF. I, II, III, represent respectively situations where incorporation continues (I), remains constant (II) or decreases (III) during the dark period.

phosphorylation in the dark (Fig. 1); the second (II) would have revealed a light-dependent phosphorylation, but not necessarily related to membrane activation, while the third (III) one would give us a light-dependent phosphorylation of peptides, possibly related to membrane activation during photosynthesis. We would thus observe, using pulse illimination, a transition of the membrane between two states. The kinetics of [^{32}P] phosphate incorporation during pulse irradiation (Fig. 5) shows that the third possibility was the correct one. Since the phosphorylation decreases soon as irradiation is stopped, the phenomenon is probably implied in photosynthetic membrane activation. The possibility that these peptides could be chlorophyll-protein complex is of great interest, considering what is known about the coupling of photochemical events to the ATP synthesis.

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