Vol. 111, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 16, 1983 Pages 740-749

IN VITRO SYNTHESIS OF PHOTOSYNTHETIC MEMBRANES: I. DEVELOPMENT OF PHOTOSYSTEM I ACTIVITY AND CYCLIC PHOTOPHOSPHORYLATION

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Received February 9, 1983

First successful in vitro synthesis of functional photosynthetic phosphorylating membrane is reported. Etioplasts, highly enriched in cytoplasmic and plastid proteins, isolated from etiolated Cucumber cotyledons pretreated with kinetin and gibberellic acid, and illuminated in a cofactor fortified medium showed commencement of chlorophyll (Chl) synthesis immediately after illumination from exogenous δ-aminolevulinic acid, while photosystem I (PS I) activity commenced 15 min after the onset of illumination. When cotyledons pretreated with kinetin and gibberellic acid were illuminated directly, there was a lag phase of 30 min before the commencement of Chl synthesis and PS I activity developed after 1 h of illumination. In plastids developed both in vivo and in vitro, the electron flow from dichlorophenolindophenol to methyl-viologen was coupled to phosphorylation as observed by an increase in the electron transport rate on the addition of uncouplers. Analysis of polypeptide profiles of the greening plastids in vitro showed the disappearance of many higher molecular weight proteins during greening. Polypeptides of molecular weight 32, 20.5, 19.5 K absent in etioplasts appeared as distinct bands after 4 h of greening in vitro.

Though the potential value of studying the process of greening in vitro in order to understand the process of photosynthesis

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Abbreviations: ALA, δ-aminolevulinic acid; ATP, adenosine 5'-triphosphate; BSA, bovine serum albumin; CF_1, coupling factor I; Chl, chlorophyll; Chlide, chlorophyllide; CPI, P700 chlorophyll a-protein complex; Cyt f, cytochrome f; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCPIP, 2,6-dichlorophenolindophenol; EDTA, ethylenediaminetetraacetic acid; HEPES, N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid; MV, methyl viologen; Pchlide, protochlorcphyllide; PC, plastocyanin; PS I, photosystem I; TES, N-tris(hydroxymethyl) methyl-2-aminoethanesulfonic acid; TMPD, N'N'N'N'-tetramethylphenylenediamine; SDS, sodium dodecyl sulfate.

0006-291X/83 $1.50

was recognized by many scientists since the last decade, see recent reviews (1,2) no significant progress has been made after the demonstration of the ability of the plastids to divide *in vitro* (3) and the introduction of cell-free systems to synthesize Pchlide (4) and Chl (5,6) *in vitro*. Illumination of extracted etioplasts showed signs of disorganization and a rapid loss of their activities (7) and after further studies it has been concluded that there is no evidence that etioplasts grow after isolation (2). Since DNA of the chloroplast contains insufficient information to code for the complete spectrum of the chloroplast proteins and the subunits of many chloroplast proteins are known to be synthesized in different cellular locations, it may be expected that any successful culture system will be a highly complex one including purified macromolecules, cellular membranes and organelles in addition to the plastids (2). However, Daniell and Rebeiz (8,9) recently demonstrated that pretreatment of etiolated tissues with hormones induces excess accumulation of prothylakoid proteins resulting in massive synthesis of Pchlide and other intermediates of the Chl biosynthetic pathway from exogenous ALA. It is known that these enzymes involved in the conversion of ALA to Pchlide are synthesized in the cytoplasm (10). Etioplasts, thus enriched in cytoplasmic proteins and incubated in a cofactor fortified medium, were capable of synthesizing Chl from exogenous ALA at a rate about twice as high as the highest rates observable in greening tissues *in vivo* (11). Complete esterification of chlide *a* to Chl *a* *in vitro* (12) and further progress made in this cell-free system has been reviewed recently (13,14). Electron microscopic studies of these etioplasts showed only prolamellar bodies before illumination. After the onset of illumination, the prolamellar bodies were partially mobilized to form membranes in 2 h of illumination followed by complete mobili-

zation of the prolamellar bodies into huge macrograna in 4 h of illumination (15). The functional characterization of the membranes synthesized *in vitro* presented here is the first report on the development of the photosynthetic apparatus *in vitro*.

MATERIALS AND METHODS

Cucumber seeds (*Cucumis sativus* L.) were germinated in moist vermiculite at 28° C for 3 days in the dark. Cotyledons excised with hypocotyl hooks were pretreated in the dark with 0.5 mM kinetin and 2 mM gibberellic acid for 20 h. After removing the hooks each 5 g tissue was gently hand homogenized, with 10 pestle strokes, in the dark in 12.5 ml of the isolation medium that consisted of 0.5 M sucrose, 15 mM HEPES, 30 mM TES, 1 mM $MgCl_2$, 1 mM EDTA, 5 mM Cysteine and 2% BSA with the final pH of 7.7. The homogenate was passed through 4 layers of cheese cloth and the plastids were pelleted by centrifuging the homogenate at 200 g for 3 min and by centrifuging the resultant supernatant for 7 min at 1500 g. The pelleted plastids were suspended in a medium that consisted of 0.5 M sucrose, 15 mM HEPES, 30 mM TES, 20 mM $MgCl_2$, 5 mM $MnCl_2$, 2.5 mM EDTA, 20 mM ATP, 40 mM NAD, 8 mM methionine, 15 nM phytol (dissolved in absolute methanol, final methanol conc. < 1%) and 1% BSA, with a final pH of 7.7. Each incubation consisted of 1 ml of plastid suspension (10-15 mg plastid protein), one additional ml of the resuspension medium, 0.1 ml of 10 mM ALA and 0.9 ml of H_2O. The plastids were irradiated with cool white fluorescent light of 30 $\mu E\ m^{-2}s^{-1}$ intensity at 25° C for different durations on a reciprocating water bath operated at 60 oscillations per min. Samples were collected at different durations of illumination and assayed for their photosynthetic capacity.

For studying the process of greening *in vivo*, cotyledons pretreated with hormones were exposed to white light of 70 $\mu E\ m^{-2}s^{-1}$ intensity for different durations and plastids were isolated as mentioned earlier. Photosystem I electron transport was measured by continuous recording of O_2 uptake with a Clark type electrode (Yellow Springs Instrument Co., USA). The reaction mixture was magnetically stirred and thermoregulated by circulating water at 20° C. White actinic light of intensity $10^6 erg.cm^{-2}s^{-1}$ was passed through 20 cm water before illuminating the sample (16). SDS-polyacrylamide gel electrophoresis was carried out as described in (17) with the following modifications. The plastid proteins were precipitated with TCA (final conc. 5%) and the pellets were obtained by centrifugation at 3000 g for 5 min. The pellets were washed twice with ether and dissolved in SDS containing sample buffer and the ratio of protein to SDS was 1:20. The samples were boiled for 2 min after adding 0.5% B-mercaptoethanol, 10% glycerol and a trace of bromophenol blue. Each sample containing 200 ug protein was loaded in a 1 mm thick slab gel and electrophoresis was carried out for 12 h at 150 V (17 mA). The gels were stained with 0.2% coomassie brilliant blue R-250 in 50% ethanol, 7% acetic acid for 4 h and destained by successive washings in 20% ethanol and 7% acetic acid. The gels were calibrated using the

following maker proteins: Phosphorylase A (94000), BSA (68000), r-globulin heavy chain (50000) ovalbumin (44000), r-globulin light chain (23000) and cytochrome C (11500).

RESULTS AND DISCUSSION

When plastids were isolated in the dark from hormone pretreated cotyledons and incubated in a cofactor fortified medium in the presence of ALA, chlorophyll synthesis commenced immediately after the onset of illumination and the rate of synthesis was linear upto 1 h of illumination beyond which there was no addi-

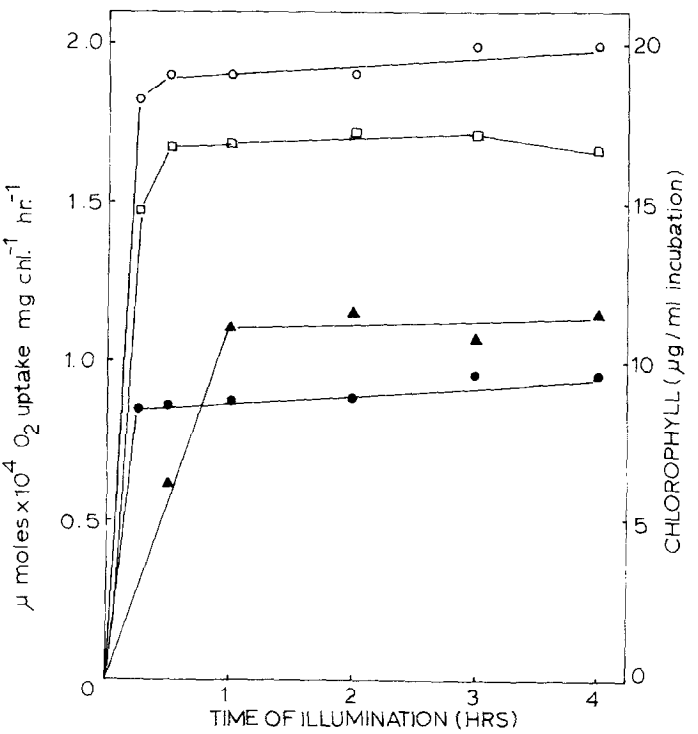

Fig.1: Rates of chlorophyll synthesis and PS I electron transport in greening plastids in vitro. Etioplasts were isolated from 3 day old etiolated Cucumber cotyledons that were pre-incubated in 0.5 mM kinetin and 2 mM gibberellic acid in the dark and illuminated in a cofactor fortified medium as described in methods. The rate of electron flow from exogenous electron donors to MV was measured as O_2 uptake in the reaction mixture containing 0.1 M sucrose, 40 mM HEPES-NaOH buffer, pH 7.5, 0.1 mM TMPD or 0.2 mM DCPIP, 1 mM ascorbate, 2 μM DCMU, 0.1 mM methyl viologen and chloroplasts equivalent to 1-2 μg Chl/ml. For uncoupling 10 μM gramicidin plus 5 mM NH_4Cl were added. White light of intensity 10^6 erg. $cm^{-2}s^{-1}$ was used and the values reported were obtained by averaging five experiments. ▲——▲ Chlorophyll; ●——● DCPIP/ascorbate to MV coupled to photophosphorylation; O——O DCPIP/ascorbate to MV uncoupled with gramicidin plus NH_4Cl; □——□ TMPD/ascorbate to MV.

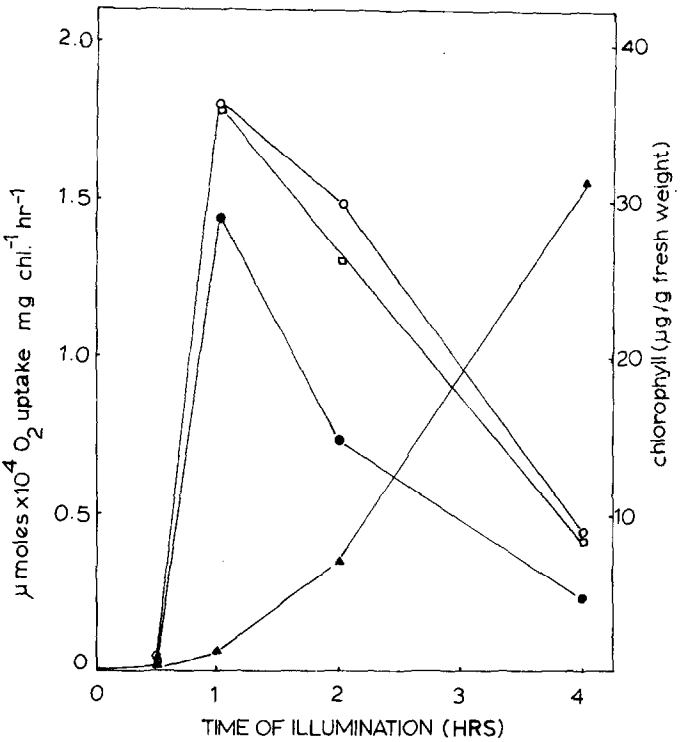

Fig.2: Rates of chlorophyll synthesis and PS I electron transport in greening cotyledons in vivo. Experimental conditions were the same as in Fig.1. ▲——▲ chlorophyll; ●——● DCPIP/ascorbate to MV coupled to phosphorylation; ○——○ DCPIP/ascorbate to MV uncoupled with gramicidin + NH_4Cl; □——□ TMPD/ascorbate to MV.

tional synthesis of Chl but Chl synthesized was maintained without any degradation (Fig.1). On the other hand, when hormone pretreated cotyledons were illuminated, there was a lag phase of 30 min during which no Chl could be detected (Fig.2). Though the phototransformation of the Pchlide synthesized in the dark should have resulted in the accumulation of some Chl, no Chl could be detected in pigment extracts. Newly formed Chl molecules, forming the reaction centres are 'bound Chl' not extractable by any solvent system (18) and this was recently pointed out to be a serious defect in spectrophotometric determination of Chl in pigment extracts of tissues under different physiological conditions (19). The lag phase in Chl synthesis is due to the delay in the synthesis of ALA (20) and this lag is eliminated in vitro because of the exogenous supply of ALA. After the

commencement of Chl synthesis in cotyledons the rate of synthesis was slow till 2 h of illumination beyond which synthesis was linear (Fig.2).

Photosystem I activity as measured by the rate of electron flow from exogenous electron donors DCPIP/ascorbate to MV was observed 15 min after the onset of illumination in illuminated plastids *in vitro* (Fig.1) whereas it was observed only after 1 h of illumination in cotyledons (Fig.2). Previous investigators have failed to detect any PS I activity in etiolated leaves or etioplasts (21,22). The commencement of PS I reaction during greening of etiolated seedlings varied from less than 1 h (21,22) to more than 6 hrs (23,24) and this difference could be due to variation in plant material, age of the seedlings and intensity of illumination (1). In both *in vivo* and *in vitro*, the electron flow from DCPIP/ascorbate to MV was coupled to phosphorylation since an increase in the electron transport rate was observed on the addition of uncouplers (Figs.1, 2). It is known that coupling factor I is present in etioplasts and its biosynthesis is completed in the etioplasts, illumination bringing about a modification in its function (25). As seen in Fig.4, the α and β subunits of coupling factor I were major bands in the polypeptide profile of etioplasts and this remained unchanged during 4 h of illumination. Electron flow from TMPD/ascorbate to MV, which is not coupled to ATP formation was similar to the uncoupled reaction of DCPIP/ascorbate to MV in both *in vivo* and *in vitro* (Figs.2, 1). This indicates that even if the PS I electron flow is not coupled to phosphorylation there is still a lag period in the commencement of the PS I reaction which is shorter in incubated plastids (Fig.1) and longer in cotyledons (Fig.2). Recently, PS I electron donors have been classified based on the sensitivity of the reaction to inhibitors, $DCIPH_2$ donating electrons to

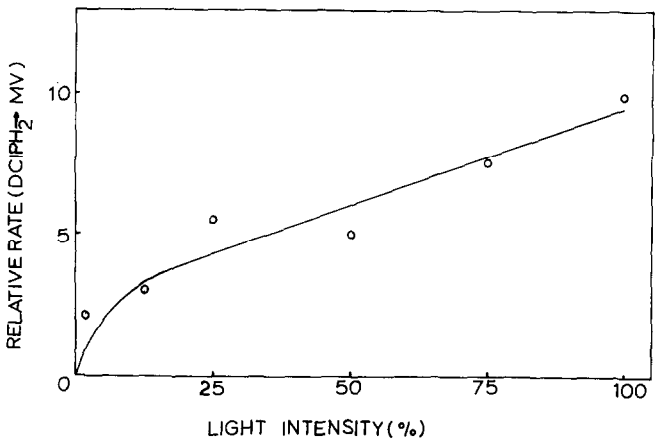

Fig.3: Rate of PS I electron transport from DCPIP/ascorbate to MV as a function of light intensity in plastids after 2 h of illumination in vitro. Experimental conditions were the same as in Fig.1.

P_{700} and TMPD to Cyt f/PC region. The commencement of both these reactions in plastids incubated in vitro may probably suggest the simultaneous induction of synthesis of the electron transport chain components.

An important feature of the photosystem activities detected in the initial stages of greening is that very high light intensities are required for saturation of the reactions. As seen in Fig.3, the rate of electron flow from DCPIP/ascorbate to MV increased with increase in the intensity of light in plastids after 2 h of illumination, suggesting that at the early stages of greening most of the Chl was present in the reaction centres, there being very little light harvesting Chl. However, as greening progresses in vivo, more light harvesting Chls became associated with the reaction centres resulting in a decrease in the electron transport rate when expressed on a Chl basis (Fig.2).

Though the development of PS I activity could be detected after 15 min of illumination, CP I, the P_{700} Chl a protein complex and associated polypeptide could not be detected in the polypeptide

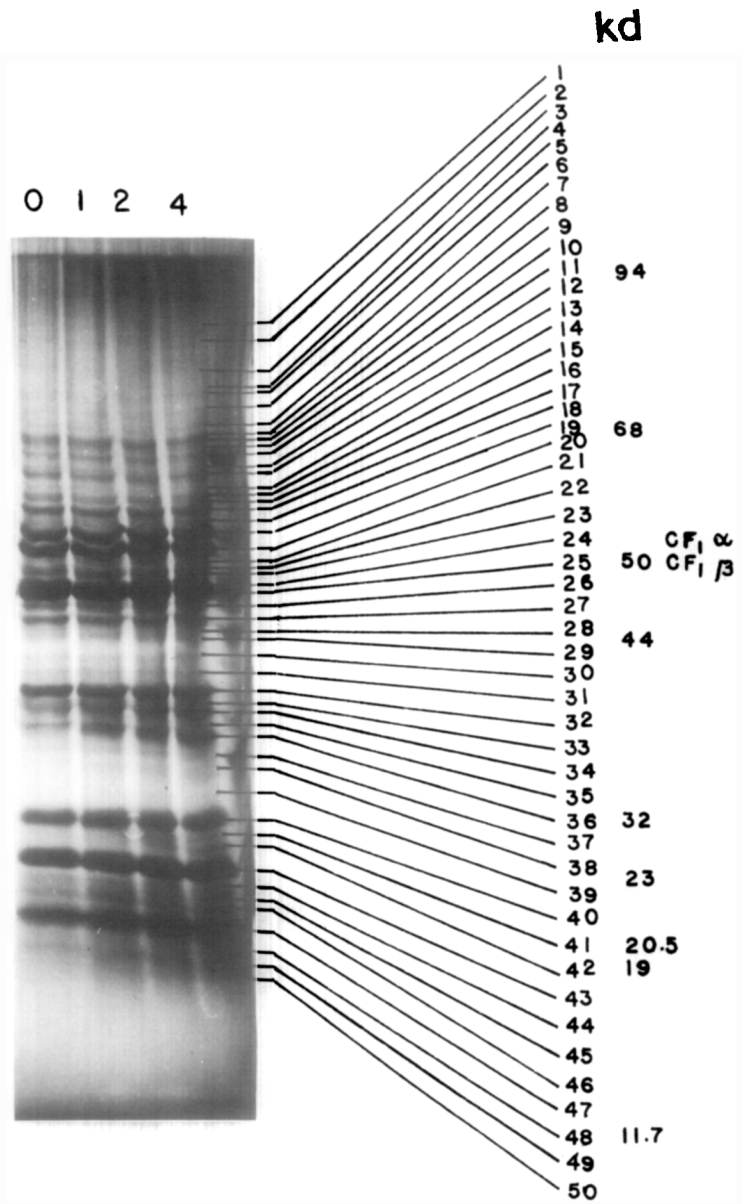

Fig.4: Comparison of the polypeptide patterns of etioplasts from Cucumber cotyledons pretreated with hormones (Oh) and etiochloroplasts greened in vitro for 1, 2 and 4 h. The polypeptides readily visible in the gels have been numbered in the order of increasing electrophoretic mobility. The α and β subunits of coupling factor I and the polypeptides appearing as distinct bands after 4 h of illumination but absent in etioplasts have been marked along with the molecular weight of standard proteins.

profile of etiochloroplasts even after 4 h of illumination (Fig.4) Similar observation that CP 1 is not essential for PS I activity in greening tissues has been reported earlier (26) and CP I deve-

lops only after 24 h of illumination in etiolated barley seedlings (27). During greening some polypeptides remained unchanged, some disappeared and some were newly synthesized (Fig.4). Many higher molecular weight proteins (between 1 and 17 bands) either disappeared or were reduced in intensity during 4 h of greening while polypeptides of molecular weight 20.5 and 19.5 K (bands 41, 42) absent in the etioplasts increased in intensity during greening to form distinct bands after 4 h of illumination (Fig.4). The appearance of the 32 KD polypeptide, probably derived from a higher molecular weight protein and the development of H_2O splitting activity in plastids *in vitro* will be discussed in a subsequent communication.

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