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CHLOROPLAST CULTURE VIII
A NEW EFFECT OF KINETIN IN ENHANCING THE SYNTHESIS AND ACCUMULATION OF PROTOCHLOROPHYLLIDE IN VITRO*

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SUMMARY: By pretreating etiolated cucumber cotyledons with kinetin in the dark, it was observed that the plastids isolated from such tissues were 400% more active in the conversion of δ-aminolevulinic acid into protochlorophyllide, than plastids prepared from water-treated controls. The experimental evidence is consistent with the hypothesis that (a) the kinetin dark-pretreatment of the etiolated tissue, uncouples the joint biosynthesis of prothylakoids and protochlorophyll and results in the accumulation of excess prothylakoid membranes poorly supplied with protochlorophyllide (b) upon isolation of the plastids and incubation with δ-aminolevulinic acid, the latter is very rapidly converted into membrane-bound protochlorophyllide.

INTRODUCTION

Since the introduction of cell-free systems capable of Pchl and Chl biosynthesis (1-6), we have been interested in inducing total chloroplast differentiation in vitro. Such a goal requires among other things (a) a potent cell-free system capable of the massive net synthesis of Pchl, Chl, and thylakoid lipoproteins and (b) the coupling of plastidic and extraplastidic thylakoid protein biosynthesis to pigment and lipid biosynthesis, in vitro. As part of a research program aimed at achieving such a goal, we have just described a cell-free system capable of the net synthesis and accumulation of Pchlide at a rate twice as high

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Abbreviations: ALA: δ-aminolevulinic acid; ATP: adenosine 5'-triphosphate; BSA: bovine serum albumin; Chl: Chlorophyll; Chl(ide): Chlorophyll(ide) which in turn refers to a mixture of Chl and Chlide; EDTA: ethylenediaminetetraacetatic acid; Hepes: N-2-hyroxyethylpiperazine-N'2-ethane sulfonic acid; MP(E): a mixture of Mg-protoporphyrin and Mg-protoporphyrin IX monomethyl ester or monoester; Pchlide: Protochlorophyllide; Pchl: protochlorophyll which in turn refers to a mixture of Pchlide and Pchlide ester; Proto: protoporphyrin IX; Tes: N-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid.

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as that of the tissue from which the plastids were prepared (7). In this communication we report that by pretreating cucumber cotyledons with kinetin, which is a potent protein syntheis promotor *in vivo* (8), the plastids isolated from the kinetin-pretreated tissues were about four fold more active in Pchlide net synthesis than similar plastids prepared from H_2O-pretreated tissues. On the other hand kinetin had no effect on the Pchlide biosynthetic pathway *in vitro*. These results suggest that kinetin pretreatment induces the excess accumulation of prothylakoid proteins which can accept and bind the Pchlide synthesized by the isolated plastids from added ALA. This in turn implies that in plants thylakoid lipoprotein biosynthesis and pigment biosynthesis are not obligatorily tightly coupled and their synthesis , to a degree, may be manipulated independently *in vitro*.

MATERIALS AND METHODS

Cucumber seeds (*Cucumis sativus* L. cv. Beit Alpha MR) were germinated in moist vermiculite at 28°C for 3 days in the dark (9). The cotyledons were excised with hypocotyl hooks in the dark or under subdued laboratory light (~5 ftc) and were pretreated in the dark with H_2O or with a 0.5 mM aqueous solution of kinetin for various periods of time. The pretreatment was carried out in deep petri dishes, 10 cm in diameter, which contained 5 g of excised cotyledons (with hooks) and 9.0 ml of H_2O or of a 0.5 mM kinetin solution. After removing the hooks in the dark each 5 g of tissue was gently hand homogenized, with 10 pestle strokes, in the dark or under subdued laboratory light in 12.5 ml of an isolation medium that consisted of: 0.5 M sucrose, 20 mM Hepes, 30 mM Tes, 1 mM $MgCl_2$, 1 mM EDTA, 5 mM cysteine and 2% BSA at a pH adjusted to 7.7 at room temperature (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 resulting supernatant for 7 min at 1500 g. The pelleted plastids were suspended in a medium that consisted of 0.5 M sucrose, 0.2 M Tris-HCl pH 7.7, 1 mM $MgCl_2$, 2.5 mM EDTA, 1.25 mM methanol, 20 mM ATP, and 1% BSA. Each incubation consisted of 1 ml of plastid suspension (about 4-5 mg of plastid proteins), one additional ml of the suspension medium, 0.1 ml of 10 mM ALA and 0.9 ml of H_2O. The plastids were irradiated with white fluorescent light (320 uw cm^{-2}) for 30 sec before incubation. The latter was carried out in the dark at 28°C for 2 h on a metabolic shaker operated at 50 strokes per min. The reaction was stopped by precipitation with 15 ml of acetone: 0.1N NH_4OH (9:1 v/v). The Proto, MP(E)and Pchlide pools were monitored by spectrofluorometry at room temperature in hexane-extracted acetone as described elsewhere (6). Fluorescence spectra were recorded on an SLM spectrofluorometer model 8000 DS, interfaced with a Hewlett Packard microcomputer system model 9825 (10) The conversion of the digital spectral data into quantitative values was performed automatically by the microcomputer just following the recording of pertinent spectra. The software for the post-run calculations was recently developed in our laboratory. Total proteins were determined by biuret (11).

RESULTS AND DISCUSSION

We have previously proposed that etiochloroplasts diffentiated less satisfactorily *in vitro* than *in vivo* partly because Pchl and prothylakoid membranes

accumulation appeared to be limited in vitro, by a structural protein synthesized in the cytoplasm and in the absence of which the massive formation of prothylakoids and grana was not possible (4, 12). It was therefore conjectured that should a method be found for obtaining etiochloroplasts containing excess Pchl-binding prothylakoids but lacking stochiometric amounts of membrane-bound Pchl then these plastids may be able to synthesize Pchlide, in vitro, at very high rates in order to saturate the Pchlide binding sites. We have indeed demonstrated earlier that in etiochloroplasts all the Pchl is membrane bound (13).

Several independent observations suggested that the forementioned goal may be experimentally feasible. First we noticed that when excised etiolated cucumber cotyledons were incubated overnight in the dark with an aqueous solution of kinetin, they underwent a 370% increase in size. However, their Pchlide content increased only by about 128%. On the other hand, cytokinins are known to (a) promote the differentiation of plastids in vivo (14, 15) (b) to increase the size and number of chloroplasts per cell (11, 17) and (c) to increase the rate of RNA (18), DNA (19) and protein biosynthesis in higher plants (20-22). Furthermore it is well known that in vivo, Pchl accumulation rapidly ceases in the dark due to a feed back inhibition of ALA biosynthesis which may be relieved however by the addition of exogenous ALA (23, 24). Altogether these observations raised the possibility that the forementioned kinetin treatment may have uncoupled the etioplast prothylakoid biosynthesis from Pchl biosynthesis which in turn resulted in the accumulation of excess kinetin-induced prothylakoid membranes devoid of stochiometric amounts of membrane-bound Pchl.

In order to test the above hypothesis 3-day old etiolated cucumber cotyledons were excised, with hypocotyl hooks, then were incubated either with distilled H_2O or with a 0.5 mM aqueous kinetin solution, for 20 h in the dark at 28°C as described in Methods. The plastids were then isolated and their tetrapyrrole biosynthetic capability was determined by monitoring the conversion of exogenous ALA into Proto, MP(E) and Pchlide (see Methods). As shown in Table I (exp A) the Pchlide Net synthesis and accumulation capabilities of the plastids prepared from kinetin-pretreated tissues was about 160% higher than those of the plastids

Table I. Effect of Kinetin-Pretreatment of Etiolated Cotyledons on the Tetrapyrrole Biosynthetic Capacity of Isolated Plastids.
Cotyledons were harvested with hypocotyl hooks from 3-day old etiolated cucumber seedlings either in the dark or under subdued laboratory light (6 uw cm^{-2}). They were preincubated either with distilled water or with a 0.5 mM aqueous solution of kinetin for 20 h in the dark at 28°C. The plastids were isolated either in the dark or under subdued laboratory light, were given a 30 sec phototransforming light treatment (320 uw cm^{-2} of white fluorescent light) then were incubated with ALA, in the dark as described in Methods. The Δ change refers to the pigment pool contents of the plastids at the end of the incubation period minus the pigment content before incubation.

Experiment	Treatment	Δ Change after 2 hr of incubation Pchlide	MP(E)	Proto
		nmoles/100 mg plastid protein		
A	Cotyledons were harvested in the dark and were pretreated either with water or with kinetin; the plastids were isolated in the dark, then were incubated with ALA			
	a. Water pretreatment	21.58	71.74	91.17
	b. Kinetin pretreatment	56.40	8.86	451.40
B	Cotyledons were harvested under 5 ftc (6 μ2 cm^{-2}) of white light and were pretreated either with water or with kinetin; the plastids were isolated under subdued laboratory light, and were incubated with ALA			
	a. Water pretreatment	18.24	73.86	267.36
	b. Kinetin pretreatment	73.88	85.46	172.02

prepared from the H_2O-pretreated control. When the lag-phase of Chl biosynthesis was first eliminated (25) by exposing the cotyledons to laboratory light before the kinetin or H_2O dark-pretreatment, then the biosynthetic capabilities of the plastids were assayed, those plastids isolated from the kinetin-pretreated tissues were about 400% more active in Pchlide net synthesis and accumulation than the H_2O controls (Table I, exp B). It is also apparent that the plastids prepared from kinetin-pretreated tissues were more potent in converting the nascent Proto into MP(E) and Pchlide than the water controls as evidenced by the lower amounts of Proto that they accumulated (Table I exp Ba, vs Bb). Altogether these results suggested that we may have succeeded with the fore-discussed treatment in uncoupling prothylakoid membrane biosynthesis from Pchl biosynthesis and in preparing etiochloroplasts containing excess Pchlide-binding prothylakoid proteins

If the above hypothesis is correct and if the Pchlide biosynthesis-enhancing effect of the kinetin pretreatment is due to the pigment-uncoupled accumulation of

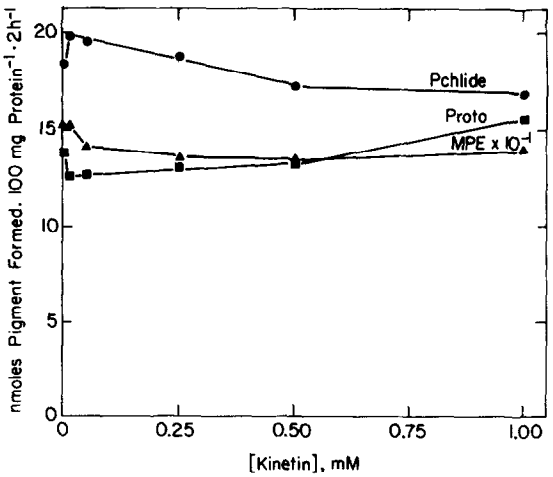

Figure 1. Effect of different concentrations of kinetin on the tetrapyrrole biosynthetic activity of isolated plastids. Etiochloroplasts were isolated from 4-day old etiolated cucumber cotyledons that had been irradiated for 4 h with 320 uw cm^{-2} of cool white fluorescent light in order to eliminate the lag-phase of Chl biosynthesis (25). The plastids were isolated as described in Methods and were incubated for 2h, in the dark at 28° with or without the different concentrations of kinetin indicated on the abscissa.
●——● Pchlide; ▲——▲ MP(E); ■——■ Proto

prothylakoid proteins which are devoid of stochiometric amounts of bound Pchlide, then the addition of kinetin to incubated plastids should have no enhancing effect on the reactions of the Pchl biosynthetic pathway *per se*. This was precisely found to be the case as illustrated in Fig. 1. The lag-phase of the excised etiolated cotyledons was eliminated by illumination for 4 h with 320 uw cm^{-2} of cool white fluorescent light (4,26) then the plastids were isolated and their tetrapyrrole biosynthetic capabilities were determined in the presence of different concentrations of kinetin . It is obvious that exogenous kinetin did neither inhibit nor enhance the reactions between ALA and Pchlide *per se* as evidenced by the constant rates of Proto, MP(E) and Pchlide biosynthesis depicted in Fig. 1.

Finally we investigated the effect that the length of the *in vivo* kinetin-pretreatment may have on the tetrapyrrole biosynthetic activity of the isolated plastids. As shown in Figure 2 pretreatment of the tissue for 8 h resulted in the highest rates of MP(E) net synthesis and accumulation, while a pretreatment of 20 h was needed to achieve the highest rates of Pchlide net synthesis and

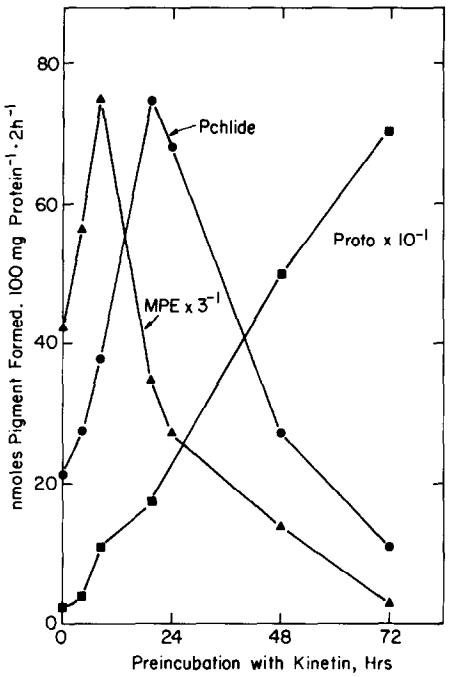

Figure 2. Effect of the length of kinetin pretreatment in vivo on the tetrapyrrole biosynthetic activity of the isolated plastids. Four-day old etiolated cotyledons were harvested and pretreated with kinetin as described in Table I. The plastids were isolated as described in Methods and were incubated for 2 h in the dark with a 10 mM solution of ALA as described in Table I (exp B).
●——● Pchlide; ▲——▲ MP(E); ■——■ Proto

accumulation. The above results suggest that it takes about 20 h of kinetin-pretreatment in vivo, for the plastids to accumulate the full complement of Pchlide-poor prothylakoids. When this is accomplished the isolated plastids no longer accumulate massive amounts of MP(E) but instead accumulate large amounts of membrane-bound Pchlide (Fig. 2). Prolonged preincubation with kinetin, however, for more than 20 h , appears to damage the plastids as evidenced by a significant decrease in the rates of Pchlide and MP(E) net synthesis and an enhanced rate of Proto accumulation (Fig. 2).

The high rate of Pchlide net synthesis and accumulation (73.88 nmoles Pchlides per 100 mg plastid protein 2 h^{-1}) which was reported for the plastids isolated from kinetin-pretreated cotyledons (Table I, exp Bb) is 14 fold higher than the highest Pchlide biosynthetic rate that we reported in 1975 (6). It is about 3 fold higher than the highest rate that we just reported for newly optimized

isolation and incubation media (7). The obvious advantages of such in vitro high rates of Pchlide net synthesis from added ALA are two fold (a) they are likely to be very useful for the study of the stepwise enzymology of the newly proposed 4-branced Chl a biosynthetic pathway (27) and (b) preliminary evidence indicates that the kinetin-pretreatment described in this work also results in very high rates of Chl net synthesis and accumulation in the light, in vitro, which may be further manipulated for the induction of enhanced rates of grana formation in vitro.

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