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$NADP^+$/NADPH CONTROL OF THE PROTOCHLOROPHYLLIDE-, CHLOROPHYLLIDE-PROTEINS IN CUCUMBER ETIOPLASTS

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SUMMARY

$NADP^+$ favours the formation of some quantity of the photoinactive $P_{649-642}$ protochlorophyllide-protein during the inactivation of the $P_{657-650}/P_{(645)-637}$ protochlorophyllide-proteins in isolated cucumber etioplasts. The reduction of $NADP^+$ by the (glucose-6-phosphate + glucose dehydrogenase) system changes back all inactive $P_{649-642}$ and part of inactive $P_{633-628}$ into active $P_{657-650}/P_{(645)-637}$. Light then changes the restored active complexes into the $P_{696-682}$ chlorophyllide-protein.

INTRODUCTION

We have previously found [1] that a certain amount of the inactive protochlorophyllide-protein complex $P_{649-642}$ is formed besides inactive $P_{633-628}$ while the photoactive $P_{657-650}/P_{(645)-637}$ ** complexes disappear during the incubation of a suspension of cucumber etioplasts in the dark at 283 K. $P_{649-642}$ is transformed back into photoactive $P_{657-650}/P_{(645)-637}$ when NADPH is added to the incubate.

The present paper deals with the effects of the addition of $NADP^+$ and then of the enzymatic reduction of $NADP^+$ into NADPH on the pigment-proteins found in an incubation of isolated cucumber etioplasts.

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** P_{x-y} = forms of protochlorophyllide- or chlorophyllide-proteins with 77 K temperature emission at x and absorption at y nm. (x) means that there is no fluorescence emission in the etioplast from the $P_{(x)-y}$ pigment-protein.

MATERIAL AND METHODS

Cucumber seedlings (*Cucumis sativus* L, cv "long vert de Chine") were grown in darkness at 293 K ± 1 on a moist mixture of vermiculite (50%)—perlite (50%). Etiolated cotyledons (30 g) from day 7 seedlings were ground at 277 K in 60 ml ice-cold medium (0.33 M Tris—HCl, 0.6 M sucrose, 4 mM $MgCl_2$, 0.2% bovine serum albumin, 10% glycerol, pH 7.6), using an homogenizer (Braun, type M × 322; 2 × 5 s at high speed). The homogenate was filtered through 8 layers of cheese-cloth and 1 layer of 30 μm mesh nylon tissue, then centrifuged at 200 *g* for 7 min at 277 K. The supernatant was centrifuged at 1500 *g* for 7 min at 277 K. The pellet was resuspended in ice-cold isolation medium (5 ml) and centrifuged at 1500 *g* for 7 min at 277 K. The final pellet was resuspended in 5 ml isolation medium (= fresh suspension). All manipulations were carried out in dim green light.

Fresh suspensions were incubated during 110 min in the dark at 288 K on a shaker operated at 4 r.p.m. Three types of suspensions (7.8 mg etioplast protein · ml^{-1}) were examined: (1) control suspensions without any addition; (2) suspensions to which $NADP^+$ (0.5 mM) was added at the start of the incubation, i.e. at time $t = 0$; (3) suspensions with $NADP^+$ added as in series 2, to which the NADPH generating enzyme system (glucose-6-phosphate 1 mM + glucose-6-phosphate dehydrogenase 0.4 U/mg protein) was added at $t = 70$ min.

Two samples were taken simultaneously from suspension types 1, 2 and 3 at the desired time. One of them was frozen at 77 K in darkness; the other was illuminated twice at 5 s interval using a photographic 1 ms flash light (light energy 125 J; Multiblitz Report Porba. 50 E) and frozen at 77 K.

Absorption spectra of the dark and the illuminated samples were recorded at 77 K using a Cary 17 spectrophotometer. Fluorescence emission spectra were recorded at 77 K using the appartus described by Sironval et al. [2]; they were not corrected. The etioplast protein content was estimated by the Lowry method [3].

Chemicals: $NADP^+$, glucose-6-phosphate and glucose-6-phosphate dehydrogenase (EC 1.1.1.49), were purchased from Boehringer.

RESULTS

Fig. 1 reproduces the 77 K absorption and emission spectra of the etioplasts prepared as described in Material and Methods. These spectra show the following results.

1. During the incubation of control etioplast suspensions the photoactive protochlorophyll(ide) proteins $P_{657-650}$ and $P_{(645)-637}$, characteristic of the fresh etioplasts (spectra a), were changed into inactive $P_{633-628}$ and traces of inactive $P_{649-642}$. The inactivation process was completed to about 70—80% at $t = 70$ min. At $t = 110$ min the controls still contained a certain amount of photoactive protochlorophyll(ide)-proteins, as attested by the feeble chlorophyll(ide) band seen after flash illumination (spectra b).

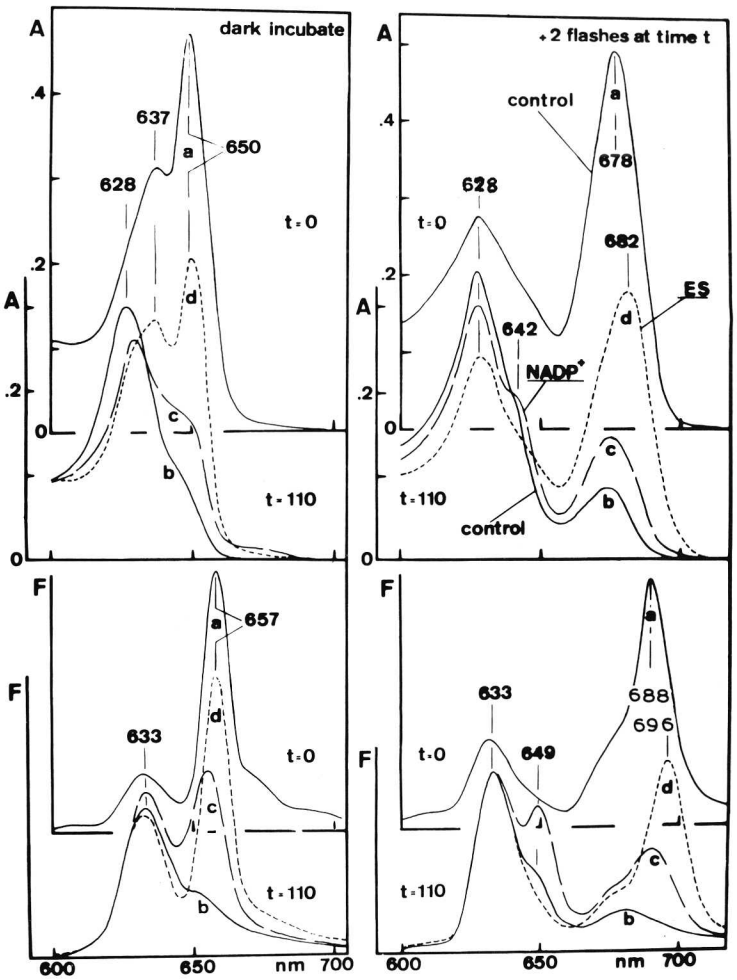

Fig. 1. 77 K absorption (A, above) and fluorescence emission (F, below) spectra of samples of cucumber etioplast suspensions, plunged in liquid nitrogen before (left panel) and after (right panel) their illumination by two intense 1 ms light flashes, at time $t = 0$ or $t = 110$ min of the incubation of the suspensions in darkness at $T = 288$ K. a) Control suspension frozen at $t = 0$. b) Control suspension frozen at $t = 110$ min. c) $NADP^+$ (0.5 mM) containing suspension frozen at $t = 110$ min. d) $NADP^+$ (0.5 mM) containing suspension supplied with NADPH generating enzyme system, ES (glucose-6-phosphate, 1 mM + glucose-6-phosphate dehydrogenase, 0.4 U/mg etioplast protein) at $t = 70$ min, and frozen at $t = 110$ min.

2. $NADP^+$ addition to the fresh suspension at $t = 0$ slowed the inactivation process down. Flash illumination at $t = 110$ min then clearly revealed the 642 nm absorption and the 649 nm emission of inactive $P_{649-642}$, showing that the formation of this particular protochlorophyll(ide)-protein was favoured by the addition of $NADP^+$ (spectra c).

3. The inactivation process was reversed completely when the NADPH

generating enzyme system was added at $t = 70$ min to the incubation already provided with $NADP^+$ since $t = 0$. The restoration of $P_{657-650}$ and $P_{(645)-637}$ was ended at $t = 110$ min (spectra d). Flash illumination changed the restored protochlorophyll(ide)-proteins into chlorophyll(ide)-proteins; the amount of chlorophyll(ide) produced by light was slightly less than that produced in the fresh control (at $t = 0$).

4. The chlorophyll(ide)-protein $P_{696-682}$ was formed when the restored, photoactive protochlorophyll(ide)-proteins were illuminated (spectra d). $P_{696-682}$ was never found in control samples or in samples with only $NADP^+$ added. In the latter cases, the $P_{688-678}$ and $P_{675-668}$ chlorophyll(ide)-proteins were formed (spectra a, b and c). At 77 K the yield of the 696 nm fluorescence emission of $P_{696-682}$ appeared abnormally weak in comparison to the yield of the 688 nm emission of $P_{688-678}$.

DISCUSSION

The reversible inactivation of $P_{657-650}$ has been reported previously by Gassman [4] and by Dujardin [5]. Gassman submitted etiolated bean leaves to hydrogen sulfide and obtained the conversion of $P_{657-650}$ into $P_{633-628}$. This was reversed by admitting air or nitrogen. Dujardin converted $P_{657-650}$ into $P_{633-628}$ in darkness by incubating etiolated bean leaves at 320 K for 15 min. The reverse conversion was obtained by returning the leaves at room temperature in darkness. Dujardin concluded that the process was a reversible denaturation of the protein moiety of $P_{657-650}$ though she could not exclude the de novo protein synthesis. She observed an abnormally weak 77 K fluorescence emission of the chlorophyllides formed by light after renaturation. We also find an abnormally weak 77 K fluorescence of the $P_{696-682}$ chlorophyllides after reactivation.

On the other hand, Griffiths [6,7] reconstituted photoactive $P_{657-650}/P_{(645)-637}$ by suspending etioplast membranes in a medium containing cholate solubilized protochlorophyllide and NADPH. He concluded from experiments using [4-^{3}H]NADPH that in $P_{657-650}/P_{(645)-637}$ the protein moiety behaves as an enzyme which binds protochlorophyllide and NADPH, with "direct juxtapositioning of NADPH with protochlorophyllide" [8] (see also ref. 9). Fig. 1 shows that the $P_{657-650}/P_{(645)-637}$ complexes are inactivated during the incubation of isolated cucumber etioplasts at 288 K in the dark, and that they became fully reactivated by increasing the level of NADPH in the incubation medium. Thus the inactivation might be due to the release of NADPH from (enzyme—protochlorophyllide—NADPH) complexes, the reactivation being the reverse process.

We have shown previously that a little amount of a hitherto unnoticed, inactive protochlorophyllide, $P_{649-642}$, appears in addition to inactive $P_{633-628}$ when $P_{657-650}$ and $P_{(645)-637}$ become inactivated in a suspension of cucumber etioplasts. The formation of $P_{649-642}$ is also a reversible process, since $P_{649-642}$ disappears while $P_{657-650}$ and $P_{(645)-637}$ are reconstituted; there is no trace left of $P_{649-642}$ in spectra d, Fig. 1, after illumination of the suspensions in which $P_{657-650}$ and $P_{(645)-637}$ have been restored. Thus

we have the reactions:

inactivation reaction $P_{657-650}/P_{(645)-637} \rightarrow P_{649-642}$ (1)

reactivation reaction $P_{649-642} \rightarrow P_{657-650}/P_{(645)-637}$ (2)

The facts reported here indicate also that: 1) the addition of $NADP^+$ to the fresh etioplast suspension slows down the inactivation process and favours $P_{649-642}$ formation against $P_{633-628}$ formation; 2) the chlorophyllide-protein $P_{696-682}$ is produced when restored $P_{657-650}/P_{(645)-637}$ are illuminated in the presence of the NADPH generating enzyme system.

If one accepts that $P_{657-650}/P_{(645)-637}$ are (protein—protochlorophyllide—NADPH) complexes [8,9], $P_{649-642}$ might be a (protein—protochlorophyllide—$NADP^+$) complex, $P_{633-628}$ being then a (protein—protochlorophyllide) complex without NADPH or $NADP^+$ or the free pigment [8]. $P_{696-682}$ would then be tentatively ascribed to a (protein—chlorophyllide—NADPH) complex. Brodersen [9] suggests similarly that $P_{688-678}$ is the (enzyme—chlorophyllide—$NADP^+$) complex. However, by themselves, the facts reported here do not force us to suppose that the control of the inactivation/reactivation of $P_{657-650}$ and $P_{(645)-637}$ by NADPH or $NADP^+$ actually involves the formation of ternary complexes.

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