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PATHWAY FROM PHOTOINACTIVE $P_{633-628}$ PROTOCHLOROPHYLLIDE TO THE $P_{696-682}$ CHLOROPHYLLIDE IN CUCUMBER ETIOPLAST SUSPENSIONS

B. EL HAMOURI, M. BROUERS and C. SIRONVAL

Photobiology Laboratory, University of Liège, Sart Tilman, 4000 Liège (Belgium)

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SUMMARY

A pathway from the $P_{633-628}$ inactive protochlorophyllide to the $P_{696-682}$ chlorophyllide is demonstrated in Cucumber etioplast suspensions. Following the addition of $NADP^+$ to a dark incubated suspension the photoinactive protochlorophyllide $P_{633-628}$ is transformed into another photoinactive protochlorophyllide $P_{649-642}$. The reduction of the added $NADP^+$ in the dark transforms $P_{649-642}$ into photoactive $P_{657-650,637}$. Light transforms the $P_{657-650,637}$ protochlorophyllide into a chlorophyllide protein complex which is $P_{690-676}$ in the absence of NADPH and becomes $P_{696-682}$ in the presence of NADPH.

INTRODUCTION

We have previously shown that a new photoinactive protochlorophyllide-protein complex, $P_{649-642}$, was formed during the dark incubation of an isolated Cucumber etioplast suspension [1] and that the addition of $NADP^+$ favours the formation of $P_{649-642}$ [2]. $P_{649-642}$ is transformed into the photoactive $P_{657-650,637}$ protochlorophyllide-protein complex by adding NADPH [1] or by reducing previously added $NADP^+$ [2]. The photoactive $P_{657-650,637}$ complex reconstituted in this way is transformed by light into a chlorophyllide-protein complex $P_{696-682}$ [2]. A spectrally similar chlorophyllide-protein is formed in bean leaves as the result of the dark, rapid shift which occurs within the seconds following a strong light flash [3,4].

This paper deals with the effect of $NADP^+$ and NADPH on the state of the protochlorophyllide and chlorophyllide-protein complexes during the incubation of Cucumber etioplasts in the dark. It is shown that the formation of the photoactive $P_{657-650,637}$ occurs along a pathway which first utilizes the photoinactive $P_{633-628}$ commonly found in etioplasts to produce $P_{649-642}$. It is also shown that $P_{696-682}$ is produced, either by illuminating a fresh etiolated suspension in the presence of NADPH, or by reducing $NADP^+$ into NADPH in a suspension which already contains other chlorophyllide-proteins.

MATERIAL AND METHODS

The materials and the methods have been described elsewhere [1,2]. Incubations were performed in the dark at 288 K. Illuminations consisted of two successive flashes with 10-s dark interval. Each flash was sufficient to saturate protochlorophyllide reduction. Absorption and emission spectra were recorded at 77 K. NADPH or $NADP^+$ were added to a final concentration of 0.5 mM. The reduction of added $NADP^+$ into NADPH was performed by the NADPH regenerating enzyme system: glucose 6-phosphate and glucose-6-phosphate dehydrogenase in the concentrations reported elsewhere [2].

RESULTS

The etioplast suspension was first incubated in darkness at 288 K during 30 min in order to partly inactivate the native protochlorophyllide-protein $P_{657-650,637}$. This incubation led to the formation of a mixture of inactive $P_{633-628}$ (main pigment) and $P_{649-642}$ (minor pigment) [1,2]. The suspension was then illuminated in order to convert the remaining, active $P_{657-650,637}$ into the chlorophyllide-protein $P_{690-676}$ (Fig. 1, t=0).

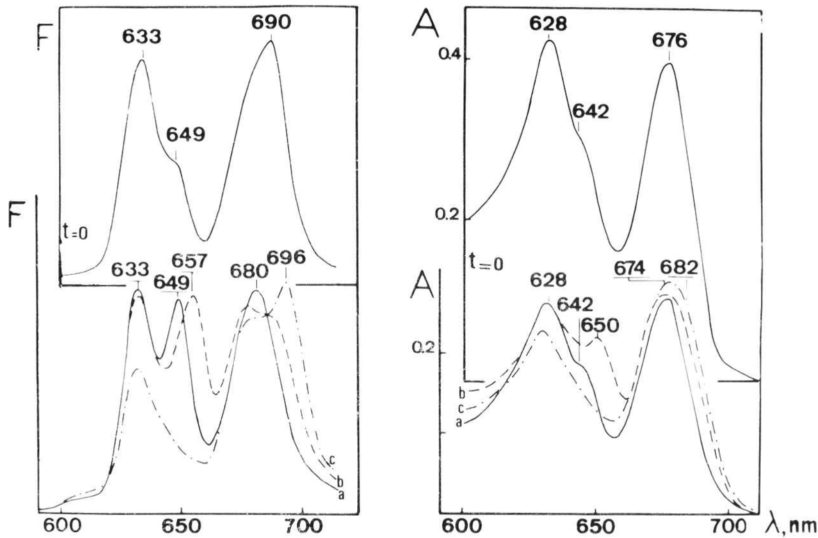


Fig. 1. 77 K absorption (A) and fluorescence emission (F) spectra of a Cucumber etioplast suspension. The suspension was illuminated by two light flashes immediately after a 30-min dark incubation at 288 K and a sample was frozen at 77 K in order to register the spectra at time 0 of the experiment (t = 0). Three other samples were frozen at 77 K 30 min (a) and 60 min (b,c) later. They were taken from a suspension treated as follows: (a) the suspension was supplied at time 0 with $NADP^+$ (0.5 mM) and the incubation was prolonged for 30 min in the dark at 288 K (=time 30 of the experiment); (b) NADPH regenerating enzyme system (see Methods) was added at time 30 and the dark incubation was prolonged for 30 min at 288 K (=time 60 of the experiment); (c) the suspension was illuminated by two light flashes at time 60.

When $NADP^+$ was added and the dark incubation was prolonged, the amount of $P_{649-642}$ was increased while the emission and absorption maxima of the chlorophyllide bands shifted towards shorter wavelengths (680 and 674 nm respectively) (Fig. 1a). The subsequent dark reduction of $NADP^+$ brought about the disappearance of $P_{649-642}$ and the reformation of photoactive $P_{657-650,637}$; the chlorophyllide bands shifted partly towards longer wavelengths (Fig. 1b). Light transformed the reconstituted $P_{657-650,637}$ protochlorophyllide into the $P_{696-682}$ chlorophyllide-protein complex (Fig. 1c).

$P_{696-682}$ was never found in control suspensions or in the presence of $NADP^+$. In these suspensions light transformed $P_{657-650,637}$ into a pigment-protein complex with fluorescence emission at 690 nm and red absorption at 676 nm ($P_{690-676}$) which was later changed in the dark into a pigment-protein complex with emission and absorption at shorter wavelengths. The effect of NADPH on the chlorophyllide-protein was not dependent on the

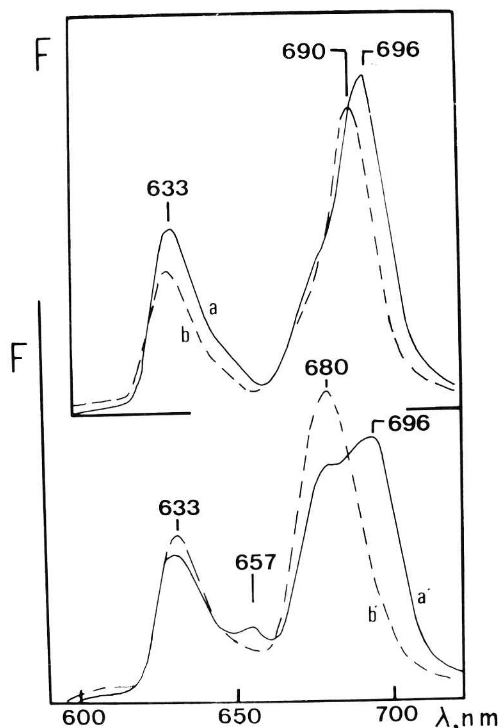
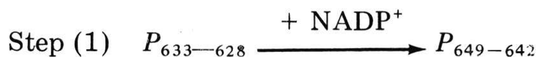


Fig. 2. 77 K fluorescence emission spectra (F) of Cucumber etioplast suspensions. *Above:* freshly isolated etioplasts were illuminated by two light flashes (2 × 1 msec) in the presence (a) or in the absence (b) of NADPH (0.5 mM) and immediately frozen at 77 K. *Below:* freshly isolated etioplasts were illuminated by two light flashes (2 × 1 msec) and frozen at 77 K after a 20-min dark incubation at room temperature (a′) in the presence of NADPH (0.5 mM) and (b′) in the absence of NADPH.

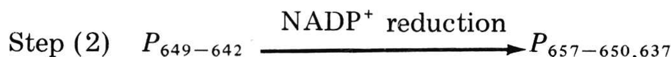
prolonged dark incubation used in the experiments reported in Fig. 1, since in the presence of NADPH the action of light on a fresh etioplast suspension resulted in $P_{696-682}$ formation as well (Fig. 2, above). In that last case any subsequent dark shift towards shorter wavelengths was clearly inhibited by the presence of NADPH (Fig. 2, below).

DISCUSSION

In the absence of precursors, isolated etioplasts did not synthesize any additional amounts of protochlorophyllide during their incubation in darkness. $P_{633-628}$ was the only source for $P_{649-642}$ formation in the suspension which had been deprived of $P_{657-650,637}$ by illumination (Fig. 1, $t = 0$) and to which $NADP^+$ had then been added. As soon as $NADP^+$ was reduced by NADPH regenerating enzyme system the transformation of $P_{649-642}$ into $P_{657-650,637}$ was observed in the dark (Fig. 1, b). These facts demonstrate the following sequence:



Step (1) $P_{633-628} \xrightarrow{+\ NADP^+} P_{649-642}$



Step (2) $P_{649-642} \xrightarrow{NADP^+\ \text{reduction}} P_{657-650,637}$

A similar pathway using exogenous ALA-Protochlorophyllide as a source for $P_{649-642}$ was demonstrated in etioplasts isolated from ALA fed bean leaves [5].

If we adopt Griffiths conclusion following which $P_{657-650,637}$ is a membrane component consisting of a ternary complex: protein-protochlorophyllide-NADPH [6,7], $P_{649-642}$ should be a protein-protochlorophyllide-$NADP^+$ complex and $P_{633-628}$ free protochlorophyllide or a protochlorophyllide-protein [2].

On the other hand, Griffiths claims that NADPH provides the reductant needed for protochlorophyllide photoreduction [7,8]. Thus, the first chlorophyllide-protein complex formed by light should contain $NADP^+$. The fluorescence emission and red absorption maxima of this complex are found in bean leaves at 688 and 678 nm, respectively [3,4]. In our control experiments with Cucumber etioplasts the first product of light had a red absorption centered at 676 nm and an emission band at 690 nm. Thus the light step was:

Step (3) $P_{657-650,637} \xrightarrow{h\nu} P_{690-676}$

In the absence of NADPH the absorption and emission of $P_{690-676}$ shifted towards shorter wavelengths in darkness (Fig. 1a). Otherwise the reduction of $NADP^+$ in the preilluminated, then incubated etioplast suspension resulted in a dark shift towards longer wavelengths; $P_{696-682}$ was formed in

that case (Fig. 1b). $P_{696-682}$ was also found in a fresh suspension within the seconds following the action of light on $P_{657-650,637}$ provided that NADPH was present (Fig. 2). This introduces step (4):

$$\text{Step (4)} \quad P_{690-676} \xrightarrow{\text{NADP}^+ \text{ reduction}} P_{696-682}$$

Step (4) furnishes a clue to the biochemical interpretation of the rapid red shift towards longer wavelengths which produces $P_{696-682}$ in leaves within the seconds which follow a brief illumination with strong light [3,4]. This shift might be due to an increase of the NADPH level.

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