Evidence for Photoreduction of $NADP^+$ in a Suspension of Lysed Plastids from Etiolated Bean Leaves

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It has been investigated if photoreduction of $NADP^+$ (detected by absorption spectroscopy) may take place in lysed plastids within minutes of exposure to light. This process was made possible by removal of oxidants from the reaction mixture. Lysed plastids were suspended in a medium containing $NADP^+$, catalase, glucose oxidase and glucose; exposure to light simultaneously to addition of ferredoxin and ferredoxin-$NADP^+$ reductase, resulted in increased absorbance in the band around 340 nm, while addition of 3-phosphoglycerate resulted in the disappearance of this band. Ferredoxin and ferredoxin-$NADP^+$ reductase are only effective when photoactivation takes place before their addition to the medium.

Introduction

In the plastids of green leaves, photoreduction of $NADP^+$ occurs by transfer of electrons from water along the photosynthetic transport chain, a highly integrated structure which includes the two active centres of photosystem I and II (PS I and PS II).

In order to set up an experimental system enabling us to check whether $NADP^+$ photoreduction can occur *in vitro* in material which is undergoing the greening process, the transport of electrons related to PS I must be ensured. Many authors agree that it is possible to detect PS I activity before that of PS II [1–3]. Partial but significant transport of electrons typical of PS I has been detected by Wellburn and Hampp [4] within 15 min of initial illumination. Photophosphorylation thought to be typical of PS I has been reported after 15 min lighting of etiolated French beans [5]. A much more extensive and complex system than the one invoked by these authors must, however, be functional in order for $NADP^+$ reduction to effectively take place; it requires the obligatory presence of ferredoxin and ferredoxin-$NADP^+$ reductase. Anderson and Boardman [6] found it was necessary to green the etiolated leaves of French beans for 16 h before being able to detect an increase in the absorbance at 340 nm during the illumination of a suspension of plastids extracted from these leaves. It was only after 2 h illumination of the etiolated leaves of barley that Phung Nhu Hung *et al.* [7] observed an increase in absorbance at 340 nm in the supernatant from plastids (which were removed by centrifugation at the end of the experiment): they attributed this increase to a rise in concentration of NADPH.

The question to be answered now is whether it is possible to detect photoreduction of $NADP^+$ at an earlier stage in the process of greening. If so, the inference will be that the etiolated material already contains all the essential elements for this process. Moreover, all the elements of the transport system must be in a functional state, in the appropriate conformation, and must be integrated into a structure which enables them to fulfill this function correctly. In fact, cytochrome *f*, plastocyanin, ferredoxin (Fd) and ferredoxin-$NADP^+$ reductase (FNR), the protein of P-700 as well as the coupling factor CF_1 have all been detected in the etioplasts of several plant species examined by different authors using various methods [8–11]. Moreover, the FNR and the $NADP^+$-linked glyceraldehyde-3-phosphate dehydrogenase, which are activated by light, are also present in the etiolated material [12]. And finally the ferredoxin-thioredoxin reductase, thioredoxin

Abbreviations: ATP, adenosine triphosphate; Fd, ferredoxin; FNR, ferredoxin-$NADP^+$ reductase; NADP(H), nicotinamide adenine dinucleotide phosphate; PGA, 3-phosphoglycerate; PS, photosystem; P-700, reaction centre of photosystem I.

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system – which allows photoactivation – was found in dark-grown seedlings [13].

Our previous work aimed at detecting photoreduction of $NADP^+$ during the first minutes of illumination of a suspension of etioplasts or of etioplast membranes from French beans used the classical method developed by Vernon and Shaw [15] for the chloroplasts of spinach. A decrease in absorbance at 340 nm, observed during the period of exposure to light of the membrane fraction, was attributed to photooxidation of pigments.

The existence in our etiolated material of all the components, required for the photoreduction of $NADP^+$, led us to perform the present investigation in order to show that the accumulation of NADPH produced by photoreduction is already possible in French bean etioplasts in the minutes which follow exposure to light. In the experiments presented here, we have used the catalase + glucose oxidase system with the aim of eliminating any disturbing oxidations which occur in the illuminated plant extracts. We have used membranes, while keeping the whole etioplast lysate, to prevent any loss of components which might be only weakly bound to membranes. The basic medium used is the one proposed by Anderson and Boardman [6] which contains no exogenous electron donors.

Materials and Methods

Seeds of French bean (*Phaseolus vulgaris* L. cv Commodore) were soaked with tap water and sown in a dark room at 296 K on a vermiculite-perlite mixture 1:1; they were moistened daily with tap water. A dim green safelight was always used when manipulating the etiolated material.

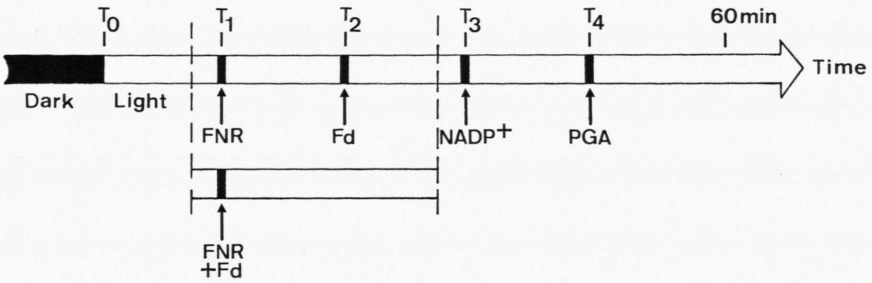
About 30 g of primary etiolated leaves were harvested after 13 to 15 days. Etioplasts were extracted at 275 K as quickly as possible in about 120 ml of 0.1 M Tricine buffer pH 8.0 containing 4 mM $MgCl_2$, 0.1% bovine serum-albumin (w/v), 10% glycerin (v/v), 0.6 M sucrose. The homogenate was filtered through 4 layers of muslin and a nylon cloth. The etioplasts were sedimented by centrifuging once at $2000 \times g$ for 15 min. The pellet of etioplasts was suspended in 0.5 ml of the same Tricine buffer to which 1.7 ml of distilled water was added. The mixture was gently homogenized for 5 min at 273 K and used as such for the experiments. It represented the membranes and stroma from the etioplasts.

To remove the atmospheric oxygen from the suspension, glucose-oxidase (EC 1.1.3.4) and its substrate β-D-glucose were added. The addition of catalase (EC 1.11.1.6) to the etioplast suspension ensured also the elimination of peroxides. The efficiency of this anti-oxidizing system was checked using an oxygen Clark electrode: in its absence, 93–98% of oxygen saturation was measured in the suspension, whereas the oxygen level dropped to 0% in its presence.

The basic reaction medium was the one used by Anderson and Boardman [6] for $NADP^+$ photoreduction by green and greening bean plastids, with the addition of the anti-oxidizing system and the lysed etioplasts. 5 ml of the reaction medium finally contained: 27 mM Tris buffer pH 7.8, 0.26 mM $NADP^+$, 3.3 mM $MgCl_2$, 50 mM glucose, glucose oxidase (600 μg), catalase (200 μg), etioplasts extracted from about 30 g of fresh leaves. The final pH was 7.9 ± 0.1. The suspension was divided into two quartz cells (light path of 1 cm) which were put into a double beam spectrophotometer Varian model Cary 17. The reference cell was kept at all times in the dark without any additions. The sample cell was the only one treated by light irradiance and addition of agents. The sample was irradiated at various points with light of 1000 $\mu E \cdot m^{-2} \cdot s^{-1}$ delivered by a 500 W xenon lamp in a Sylvania projector. Absorbance difference spectra (sample minus reference cell) were recorded throughout the duration of the experiment. FNR (EC 1.18.1.2) and Fd were isolated and purified according to the methods of Shin [17] and Buchanan and Arnon [18], respectively. FNR and Fd solutions contained 1 mg protein $\cdot ml^{-1}$ and were dialyzed against a 5 mM Tris buffer pH 7.4 before use. The specific activities tested with spinach chloroplast membranes were respectively 0.20 μmol NADPH $\cdot$ mg $FNR^{-1} \cdot min^{-1}$ and 0.36 μmol NADPH $\cdot$ mg $Fd^{-1} \cdot min^{-1}$. 10 μl of FNR were first added during the assay in the preilluminated sample in order to stimulate both $NADP^+$ photoreduction and NADPH accumulation. Then 10 μl of Fd were also added. We have verified that such an addition of FNR and Fd in the dark did not cause a significant change in absorbance. A further supply of $NADP^+$ (0.26 mM more) was given and after a further period of illumination, 24 μM PGA was finally introduced.

Results

As Anderson and Boardman [6], we noted that the isolated plastids of French beans rapidly lose their capacity to photoreduce $NADP^+$. Consequently the etioplasts were extracted very rapidly and submitted to the test immediately after lysis. At the beginning both the experimental suspension and the control suspension contained 0.26 mM of $NADP^+$ as well as the anti-oxidant system. We systematically recorded at 298 K the difference of absorbance between experimental and control samples. The results presented here correspond to the following time scale:



Scheme 1.

Fig. 1 illustrates the effect of illumination during the period T_0T_1. In the sample suspension after 1 min in the light, a slight decrease in absorbance is observed at about 340 nm (spectrum a, Fig. 1); the drop is greater after 3 and 7 min (spectra b and c, Fig. 1).

When the absorbance at about 340 nm reached its minimal value, at point T_1 on Scheme 1 (a time which varied between 2 and 7 min, according to the experiment), FNR was added in the dark to the preilluminated cell; the spectrum obtained thereafter was taken as the base line in Fig. 2. After 2 min illumination, an increase of 0.02 units in the absorbance between 330 and 340 nm was observed (spectrum a, Fig. 2). The subsequent addition of Fd to the cell was immediately followed by 30 sec of light which brought about a new increase in absorbance of the same amplitude. When light was given for up to 4 min, the total increase in absorbance reached 0.07 units (spectrum c, Fig. 2).

When, after the period of preillumination T_0T_1 (Scheme 1) FNR and Fd were added together in the

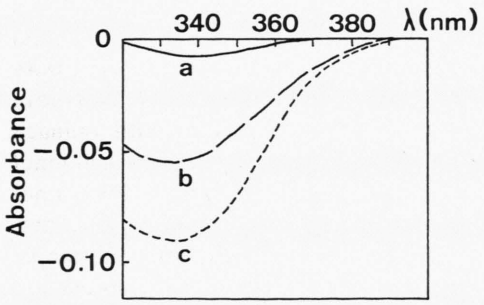


Fig. 1. Control: absorbance difference spectra of a lysed etioplast suspension illuminated for 1, 3 and 7 min (spectra a, b and c, respectively) with a continuous polychromatic light at room temperature during the T_0T_1 period (see Scheme 1). At time T_0, 1 ml contained 27 mM Tris-HCl buffer pH 7.8, 3.3 mM $MgCl_2$, 0.26 mM $NADP^+$, 50 mM glucose, glucose oxidase (600 μg), catalase (200 μg), etioplasts from 6 g of fresh bean leaves.

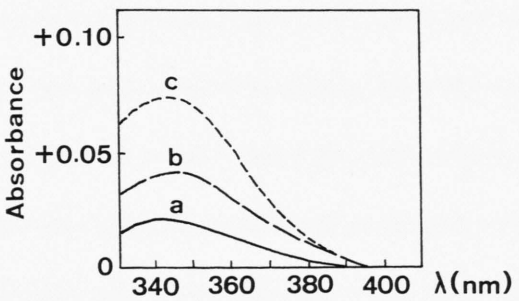


Fig. 2. Effect of successive additions of FNR and Fd on the absorbance level around 340 nm in a lysed etioplast suspension. FNR was added at time T_1 (see Scheme 1); the suspension was then illuminated for 2 min (spectrum a). At time T_2, Fd was added, then light was applied for 30 s and 4 min (spectra b and c, respectively). At time T_0, the conditions were the same as in Fig. 1.

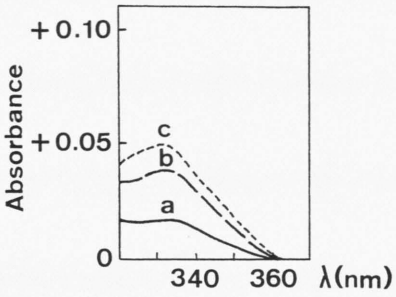


Fig. 3. Effect of a simultaneous addition of FNR and Fd on the absorbance level around 340 nm in a lysed etioplast suspension. FNR and Fd were added at time T_1 (see Scheme 1); the suspension was illuminated for 2, 4 and 8 min (spectra a, b and c, respectively). At time T_0, the conditions were the same as in Fig. 1.

dark at point T_1, an increase in absorption between 330 and 335 nm was also observed during the minutes which follow illumination (Fig. 3).

At the following stage (at point T_3 on Scheme 1), a supplement of $NADP^+$ was added, resulting in an increase in concentration of 0.26 mM. The increase in absorbance around 330 nm attained 0.1 units after only one minute's illumination (spectrum a, Fig. 4). The absorption increased even more (to 0.15 units) after a total period of 5 min exposure to light (spectrum b, Fig. 4).

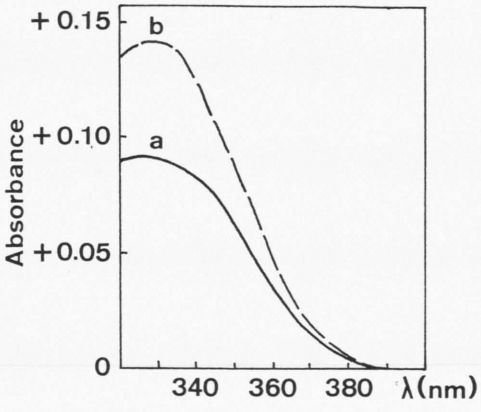


Fig. 4. Effect of an addition of $NADP^+$ on the absorbance level around 340 nm in a lysed etioplast suspension. 0.26 mM $NADP^+$ was added at time T_3 (see Scheme 1); the suspension was then illuminated for 1 and 4 min (spectra a and b, respectively). At time T_0, the conditions were the same as in Fig. 1.

In order to find out whether this increase in absorbance at around 340 nm was due to NADPH, PGA was added (at point T_4 on Scheme 1) to induce the rapid and specific oxidation of the nucleotide by the $NADP^+$-linked glyceraldehyde-3-phosphate dehydrogenase. The addition of PGA indeed brought about a strong decrease in absorbance in the same band (Fig. 5). The increased absorbance reappeared upon readdition of $NADP^+$ followed by immediate exposure to light (result not shown).

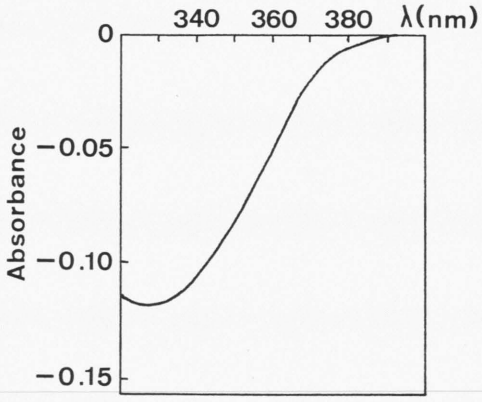


Fig. 5. Effect of an addition of PGA on the absorbance level around 340 nm in a lysed etioplast suspension. 24 μM PGA was added at time T_4 (see Scheme 1). At time T_0, the conditions were the same as in Fig. 1.

When FNR and Fd were present in the reaction mixture from the beginning in darkness at time T_0, prolonged lighting of up to 5 to 10 min did not provoke any change in absorbance in the band between 310 and 400 nm (result not shown). If, later, at stage T_1, a new quantity of FNR and Fd was added, an increase in absorbance was observed as illustrated in Fig. 2, 3 and 4. If on the other hand no FNR and Fd were added at point T_1, the absorbance at 340 nm failed to increase.

The experimental system we have described thus makes it possible to modulate at will the level of absorbance in the UV. However, we were only able to record such changes during the very first hour of spectrophotometric observation. After that delay irreversible modifications affected the variations in absorbance; they seem to be due to a degradation of the system under investigation. This was confirmed by the marked distortions observed in the absorption spectra of the chlorophyll pigments themselves.

Discussion

Our results clearly show that it is possible to observe a substantial increase in absorbance around 340 nm in the first minutes of exposure to light of a suspension of lysed etioplasts from French beans. The following procedures must be respected if such an increase is to be observed: 1) the etioplasts must be extracted as quickly as possible in order to avoid degradation of the system; 2) it is essential to work in the absence of measurable oxygen, to prevent the oxidation of NADPH, as was anticipated by our working hypothesis; 3) FNR, some Fd and some $NADP^+$ must be added to compensate the inevitable dilution of the components in the suspension of lysed etioplasts; 4) in order for the addition of FNR and Fd to produce its effect, the suspension must be preilluminated for 1 to 5 min before their introduction. The shortest time sequence tested and found to lead to an increase in absorbance at 340 nm involved 1 min of preliminary photoactivation followed by addition of FNR and Fd in darkness, then 2 min of illumination.

On the other hand: 1) an addition of electron donor is not required; 2) the exogenous FNR and Fd added do not need to originate from French beans, since in these experiments FNR and Fd from green spinach were found to be effective. Indeed, Dujardin *et al.* [10] have already shown the immunochemical identity between electron carriers of etioplasts of French bean and those of tobacco chloroplasts. Hence, there is no genus specificity.

During our experiments, the maxima and minima of the variations of absorbance in the near UV were not situated at exactly 340 nm. However, we noted that a mixture of a pure solution of NADPH (absorbing at 340 nm) with Fd and FNR led to a maximum of absorbance at 335 nm.

Several arguments tend to support the idea that the increase in absorbance at 335–340 nm indeed corresponds to the photosynthetic production of NADPH from exogenous and endogenous $NADP^+$: 1) Exogenous FNR, Fd and $NADP^+$ are known to stimulate the photoreduction of $NADP^+$ very strongly in membranes from chloroplasts [19, 20]. We observed similar events in our experiments with etioplasts; 2) light is necessary in order to observe the increase in absorbance around 340 nm. Therefore both our data and those of the literature suggest that light permits the reduction of Fd and the activation of FNR [21], thus leading to an accumulation of NADPH during the first phase of greening of etiolated material. Moreover, light inhibits the action of the glucose-6-phosphate dehydrogenase, which is capable of reducing $NADP^+$ in darkness [21]; 3) the introduction of an excess of PGA resulted in the disappearance of the positive band of absorbance around 340 nm through the oxidation of NADPH in darkness. This implies that the suspensions tested in our experiments could contain the enzymes and substrates of the first phase of the Calvin cycle; Bradbeer *et al.* [22] have indeed demonstrated that dark-grown bean leaves contain all the enzymes of the Calvin cycle; these results have been confirmed by Anderson on the etiolated pea [12]. Moreover, Wallis and Bradbeer [23] have found 2.7 nmol of ATP per leaf in 14 day etiolated French bean leaves, the ratio ATP/NADPH being equal to 3.8, whereas the optimal ratio for the accomplishment of the Calvin cycle is only 2.

We have observed that photoactivation of the suspension of lysed etioplasts is necessary before the addition of FNR and Fd in order to observe the subsequent increase in absorbance around 340 nm. If FNR and Fd are included in the reaction mixture, as soon as it is prepared in darkness, the level of absorbance in the near UV remains constant for at least the first 5 min of light exposure. On the other hand, French bean chloroplasts treated in the same way show a significant increase at about 340 nm after 3 min only, whereas spinach chloroplasts from market spinach already begin to accumulate NADPH during the first minute of receiving light. Thus we must assume that during the photoactivation, one (or more) component(s) of the electron transport chain might take up an appropriate position (in the photosynthetic chain), or could simply change its (their) conformation. Nikolaeva has noted that less than 1 min is needed to increase the activity of the FNR added in a chloroplast suspension (personal communication); this delay could correspond to the time required for accurate positioning of connectin, a protein of 7–8 kDa which binds FNR to the green thylakoids. Moreover, Vallejos *et al.* [24] and Ceccarelli *et al.* [25] have detected a protein, a trimer of 17.5 kDa, which was necessary for the fixation of FNR to the green thylakoids.

Having obtained photoreduction of $NADP^+$ within the first minutes of lighting of lysed etioplasts of French beans, we conclude that P-700 begins to func-

tion very quickly. Perhaps it is already functional in the etiolated membranes. Nechushtai and Nelson [26] have found in etiolated plants of French beans, the subunit I of PS I containing both P-700 and the site of binding of plastocyanin.

What is the electron donor in the system? The medium of Anderson and Boardman [6] did not contain any. Ours is not supposed to include a donor but we have observed that glucose and glucose oxidase added to a solution of oxidized dichlorophenol indophenol were able to reduce it. We concluded that the glucose with glucose oxidase may be a possible electron donor.

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