

The Role of NADPH in the Reversible Phototransformation of Chlorophyllide P_{682} into Chlorophyllide P_{678} in Etioplasts of Oat

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Absorption spectra and room temperature fluorescence variations upon light excitation were studied in etioplasts in which the protochlorophyllide had been reduced to chlorophyllide prior to the experiment. In the presence of 1 mM NADPH, the absorption band was located around 682 nm and the fluorescence variations reflected the occurrence of the chlorophyllide microcycle (reversible phototransformation of chlorophyllide P_{682} into chlorophyllide P_{678}), whereas, in the absence of NADPH, the absorption band was located around 676 nm and the fluorescence exhibited only a slow decrease with illumination time. After washing, NADPH-treated etioplasts still exhibited an absorption maximum of 682 nm, which shifted irreversibly to 678 nm upon illumination. Re-addition of only 10 μ M NADPH restored the reversibility of the reaction. Plastids were also prepared after a brief illumination of the intact leaves. These plastids contained the P_{682} chlorophyllide. In this preparation, essentially the same phenomena were observed as in NADPH-treated etioplasts after washing. Moreover, enzymic oxidation of NADPH resulted in the transformation of P_{682} into P_{678} in darkness. Addition of the Hill electron acceptors 2,6-dimethylbenzoquinone on FeCN caused an acceleration of the phototransformation of P_{682} into P_{678} . We interpret these results as being due to the oxidation of NADPH during the phototransformation of P_{682} into P_{678} . The nature of the electron acceptor and the physiological role of this reaction is discussed.

Introduction

A chlorophyllide microcycle in intact etiolated bean leaves has been recently characterized by Franck and Inoue [1] and by Sironval *et al.* [2]: room temperature illumination by red light of a leaf containing the P_{682} chlorophyllide results in a large variation of the 690 nm fluorescence intensity such as in Fig. 1A. This fluorescence transient is concomitant with an absorbance (and fluorescence) shift of the chlorophyllide. At the F_0 level, the chlorophyllide absorbance is located at 682 nm; it shifts to 678 nm during the $F_0 \rightarrow F_M$ increase and then goes back to 682 nm during the $F_M \rightarrow F_S$ decrease. In etiolated oat leaves, similar phenomena are observed in the same conditions, although with slightly different kinetics (Fig. 1B). The chlorophyllide microcycle

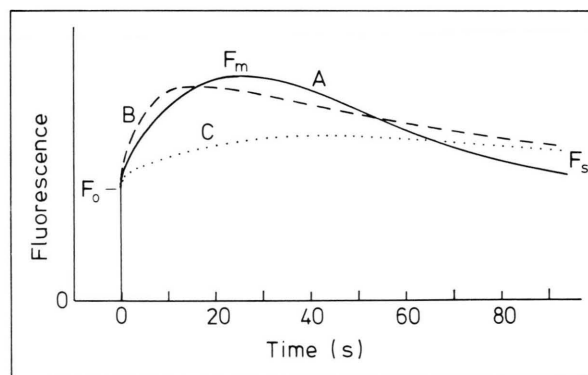


Fig. 1. Room temperature fluorescence variations at 690 nm of etiolated leaves during an illumination given 2 min after a short saturating flash. A: 14-day old bean leaf, red light excitation (633 nm); B: 7-day old oat leaf, red light excitation; C: 7-day old oat leaf, blue light excitation. The curves were adjusted to the initial F_0 fluorescence intensity.

* P_X stands for the pigments whose absorption maximum in the red is located at X nm at 77 K.

Abbreviations: NADPH, nicotinamide adenine dinucleotide phosphate; TES, 2-[(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)-amino] ethane sulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid; DMBQ, 2,6-dimethylbenzoquinone; ADP, adenosin-5'-diphosphate; FeCN, $K_3[Fe(CN)_6]$.

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is also observed under blue light excitation but the amplitude of the fluorescence changes is then smaller than in red light (Fig. 1C), may be due to screening effects by carotenoids.

In the intact leaf, the P_{682} chlorophyllide is the product of a dark shift which follows the formation of the P_{678} chlorophyllide upon illumination of the



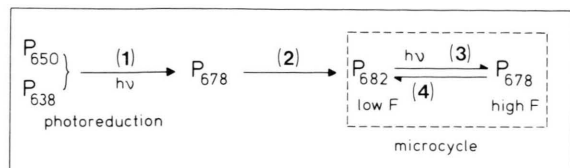
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active protochlorophyllides P_{650} and P_{638} (reactions (1) and (2) in Scheme I).



Scheme I

The chlorophyllide microcycle that occurs upon illumination of leaves in which P_{682} has just appeared, can be conveniently described by the interplay of the light reaction (3) and the dark reaction (4), where P_{682} exhibits a lower fluorescence yield than P_{678} at room temperature.

The physico-chemical nature of the reactions involved in the microcycle is unknown. It has been suggested by Oliver and Griffith [3] that the product P_{678} of protochlorophyllide reduction (reaction (1)) is a ternary complex between chlorophyllide, the enzyme protochlorophyllide reductase and $NADP^+$. In P_{682} , $NADP^+$ would be replaced by NADPH (reaction (2)). Since illumination of P_{682} apparently restores the P_{678} species, one would predict that NADPH is oxidized or replaced by $NADP^+$ in the light during reaction (3). In order to check the validity of this hypothesis, we have studied the influence of the nucleotide on reactions (2), (3) and (4) in isolated plastids. The effect of some redox mediators on this system is also reported.

Materials and Methods

Etiolated oat plants Avena sativa were grown on soil for seven days in complete darkness at 24 °C. The seedlings were either used directly in the experiment or transferred to white light (800 lux) for the indicated length of time.

Broken etioplasts were obtained from cut-off leaf sections of the etiolated plants. 80–100 g of leaves were cut to small pieces with scissors and suspended in 300 ml of cold buffer, pH 7.5, containing 20 mM Tes, 20 mM Hepes, 0.5 M sucrose, 1 mM $MgCl_2$ and 1 mM EDTA (buffer 1). The suspension was blended in a Braun Typ MX 32 blender under cooling, 3 times for 1 s at high speed following by 2 times 10 s at low speed. The brei was filtered through four layers of cheesecloth. Large cell fragments were sepa-

rated by a quick centrifugation of 2 min at $500 \times g$. The plastids were sedimented by centrifugation at $1500 \times g$ for 10 min and resuspended in a buffer, pH 7.5, containing 20 mM Tes, 20 mM Hepes, 1 mM $MgCl_2$ and 5% w/v glycerol (buffer 2). Broken etioplasts were then sedimented by centrifugation at $6000 \times g$ for 10 min and stored at -20 °C in buffer 1, with EDTA omitted. Measurements were performed with broken etioplasts resuspended in buffer 2.

P_{682} containing intact plastids (PL 682) were obtained from leaves first illuminated for 5 min. 80–100 g of leaves were quickly cut after the illumination and suspended in 300 ml of cold buffer, pH 8.6, containing 50 mM Tricine, 50 mM KOH, 1 mM $MgCl_2$, 1 mM EDTA and 25% w/v glycerol (buffer 3).

Plastid isolation was then carried out as described above, except that buffer 3 was used. After the second centrifugation, the intact plastids were resuspended in the same buffer, with EDTA omitted, and used directly in the experiment.

All experiments were performed under a green safety light. *Fluorescence measurements* were performed under blue light excitation in the device described in [4].

Absorption spectra were measured around +4 °C using a Perkin-Elmer (Lambda 3) spectrophotometer. The blank consisted of a milk suspension in water.

NADPH enzymic oxidation: NADPH was oxidized chemically by addition of 10 μ l of a glutamate dehydrogenase solution (GDH) purchased from Boehringer, 1 mM ADP, 10 mM α -Ketoglutaric acid and 1 mM NH_4Cl . GDH was omitted in the control sample. The incubation time was 5 min at room temperature.

Results

It was shown previously [3, 4] that illumination of etioplast membranes in the presence of NADPH results in the production of the P_{682} chlorophyllide, normally observed in the intact leaf. We have repeated this experiment, using various NADPH concentrations. After the addition of NADPH, broken etioplasts were illuminated by a 15 sec red light pulse at room temperature and were then incubated on ice and in darkness for 40 min. Their absorbance spectra at 4 °C are shown in Fig. 2A. Non-illuminated etioplasts exhibited a main peak at 650 nm and a shoul-

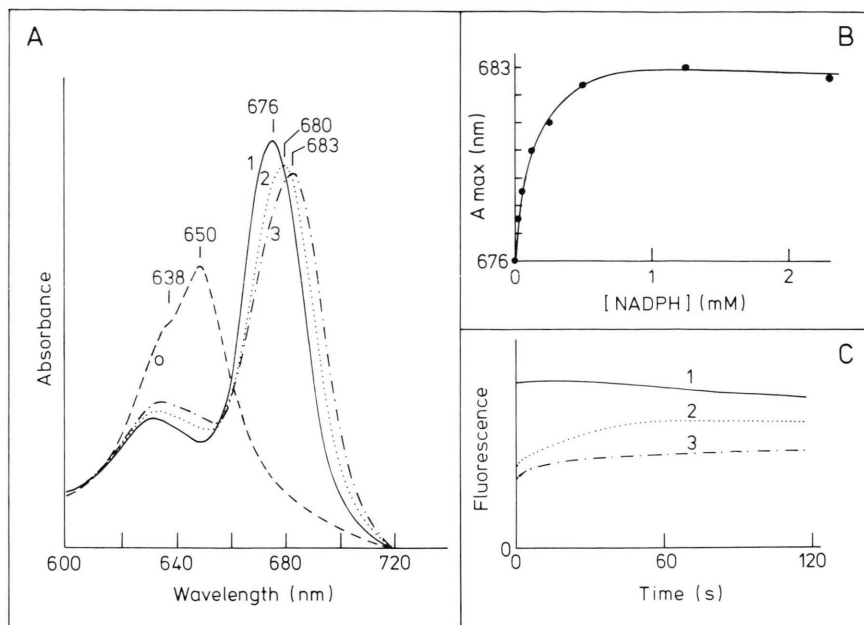


Fig. 2. Effect of NADPH in broken etioplasts. NADPH was added before a 15 sec red light illumination; the latter was followed by a 40 min incubation in darkness prior measurements. A: absorbance spectra without (1) and with 0.1 mM (2) or 0.5 mM (3) NADPH; (0): dark control with or without NADPH; B: position of the chlorophyllide absorbance maximum as a function of NADPH concentration; C: 690 nm fluorescence variations of the samples incubated without (1) and with 0.1 mM (2) or 0.5 mM (3) NADPH.

der around 638 nm (Fig. 2A, trace 0) which characterize the two active protochlorophyllide species normally found in the intact leaf. Illuminated control etioplasts (without NADPH) contained a chlorophyllide form absorbing at 676 nm (Fig. 2A, trace 1). In the presence of increasing concentrations of NADPH, the chlorophyllide absorbance maximum was shifted towards longer wavelengths (Fig. 2A, traces 2 and 3). Fig. 2B shows the dependance of the chlorophyllide absorbance maximum on the NADPH concentration. The curve reached a plateau at 682–683 nm at concentrations between 1 and 2 mM. We recorded the room temperature fluorescence kinetics of these samples after a 20 fold dilution in buffer 2 (it was checked that this dilution did not affect the spectral characteristics of chlorophyllide). The sample which was not supplemented with NADPH exhibited a high fluorescence yield which slowly decreased with the illumination time (Fig. 2C, trace 1). The samples which had been incubated with NADPH had a lower fluorescence yield at the beginning of the illumination (Fig. 2C, traces 2 and 3); their fluorescence emission slowly increased and reached a steady-state maximum whose amplitude decreased when higher NADPH concentrations were used during the incubation period.

In this experiment, it was not possible to decide whether the effect of NADPH on the fluorescence

kinetics operates during the fluorescence measurement or during the incubation period, or both. To resolve this problem, we first prepared P_{682} containing broken etioplasts as described above, using a 1 mM NADPH concentration; we then removed the excess NADPH by washing. For this purpose, we diluted the sample by 100 times in buffer 3 (which proved to be more suitable during such treatment) and precipitated the etioplasts by centrifugation at $8000 \times g$ for 10 min. The pellet was then resuspended in buffer 2. After such treatment, the etioplasts still exhibited an absorbance maximum at 682 nm (Fig. 3A, trace 1a), indicating that the presence of the P_{682} chlorophyllide was no more dependent on the presence of NADPH in the medium. Upon illumination by blue light at room temperature, the fluorescence intensity started from a low level and reached a maximum within some 40 sec. (Fig. 3B, trace 1a). When the absorbance spectrum was recorded after such blue or red illumination the absorbance maximum was shifted irreversibly to 678 nm (Fig. 3A, trace 1b). At a second illumination, the fluorescence increase could not be repeated (Fig. 3B, trace 1b) nor did the chlorophyllide absorbance band shift further.

In the presence of only $10 \mu\text{M}$ NADPH, the amplitude of the fluorescence increase was strongly reduced and an identical fluorescence kinetics was

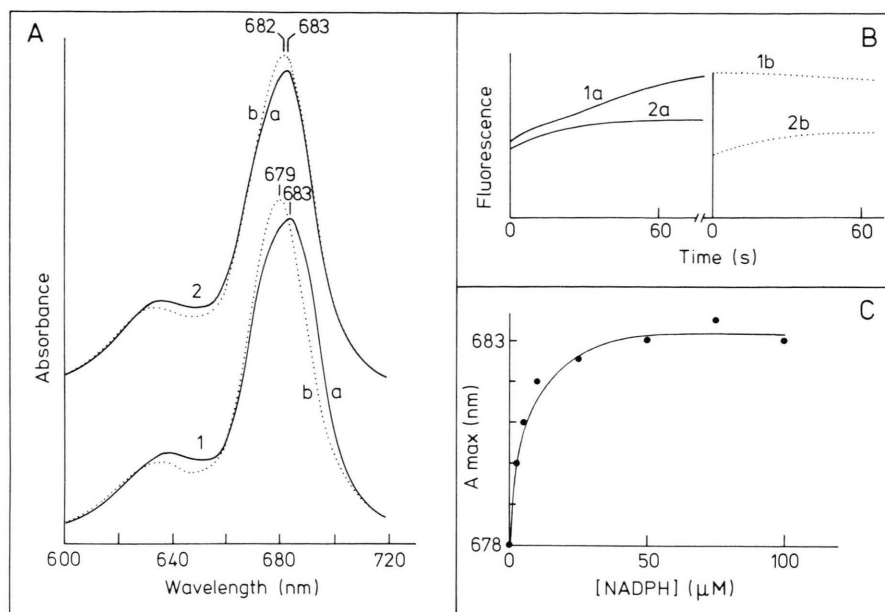


Fig. 3. Effect of NADPH in washed, P_{682} containing broken etioplasts. A: absorbance spectra before (a) or 3 min after (b) a 60 sec red light illumination without (1) or with $10 \mu\text{M}$ (2) NADPH; B: 690 nm fluorescence variations at a first (1) or second (b) illumination separated by a 3 min dark interval under the conditions 1 and 2 used in A; C: position of the chlorophyllide absorbance maximum after a first illumination as a function of NADPH concentration.

observed at each successive illumination (Fig. 3B, traces 2a and 2b). The absorbance spectra recorded before or after each illumination had a maximum located at 682 nm (Fig. 3A, traces 2a and 2b).

These results show that in NADPH washed, P_{682} containing broken etioplast, reaction (3) of Scheme I still occurs, while the reverse reaction (reaction (4) in Scheme I) only occurs upon re-addition of NADPH. In Fig. 3C the dependance of the ability to regenerate the P_{682} chlorophyllide after illumination upon readdition of various NADPH concentrations is shown. Comparison of Fig. 3C and Fig. 2B clearly shows that much smaller NADPH concentrations are required to produce P_{682} by reaction (4) than by reaction (2) of Scheme 1.

The specific role of NADPH in the chlorophyllide microcycle could be well demonstrated by an experiment in which plastids were isolated from leaves in which the formation of the P_{682} chlorophyllide had already occurred. Thus, we subjected etiolated leaves to a 5 min illumination by white light and then rapidly prepared intact plastids following the procedure described in methods. Their absorbance spectrum showed a main absorbance peak around 682 nm (Fig. 4A, trace 1a), indicating that these plastids contained the P_{682} chlorophyllide species as it is produced in the leaf. We thus labelled this preparation PL (682). Upon illumination of PL (682) by

blue or red light, the same processes as those reported upon illumination of washed, P_{682} containing broken etioplast were observed: a slow, irreversible fluorescence increase and an irreversible absorbance shift to 678 nm, corresponding to reaction (3) in Scheme I (Fig. 4A and B, traces 1a and 1b). Again, addition of small concentrations of NADPH allowed reaction (4) to occur, resulting in the reversibility of these changes (Fig. 4A and B, traces 2a and 2b).

In PL (682) preparations in which no exogenous NADPH was used, we found that a short, dark incubation (5 min) with the enzyme glutamate dehydrogenase (see methods) resulted in a dark absorbance shift from 682 to 678 nm (Fig. 4A, trace 3). After that treatment, the sample exhibited a high fluorescence yield at the beginning of the illumination followed by a slow decrease (Fig. 4B, trace 3). Since under our conditions GDH catalyses the formation of glutamate, using NADPH as reductant, these results establish the specificity of NADPH in the occurrence of the P_{682} chlorophyllide formed *in vivo*. Moreover, it can be demonstrated that oxidation of the endogenous NADPH in darkness has the same effect as a first illumination of non-treated PL (682): namely an increase of the fluorescence yield and an absorbance shift to 678 nm.

We have repeated the same experiment using NADPH-washed, P_{682} containing broken etioplasts

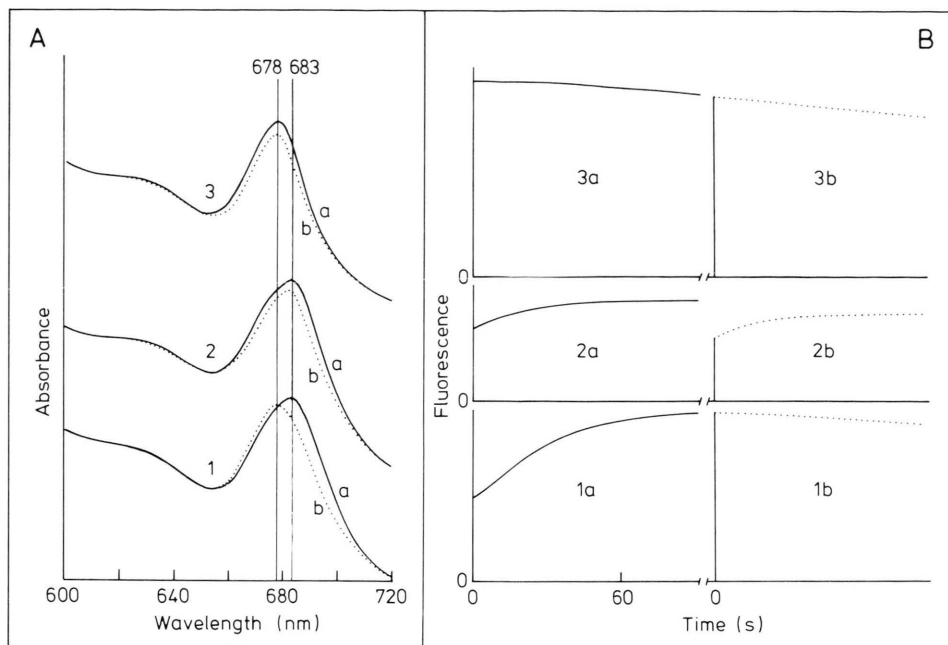


Fig. 4. Effect of NADPH and of the NADPH oxidizing system (GDH) in PL₆₈₂ (PL) plastids extracted after a 5 min illumination of the leaves. A: Absorbance spectra before (a) or after (b) a 30 sec red light illumination in the control (1), with 10 μ M NADPH (2) and with the NADPH oxidizing system (GDH, see methods (3)). B: 690 nm fluorescence variations at a first (a) or second (b) illumination under the conditions 1, 2, and 3 used in A.

instead of intact PL (682) and found the same results. However, addition of GDH to non-illuminated etioplast did not alter the spectral properties of the active protochlorophyllide nor did it affect its ability to be converted into chlorophyllide upon illumination (not shown).

The effect of the electron acceptors DMBQ and FeCN on reaction (3) has been investigated in PL (682). Addition of 0.2 mM DMBQ caused a strong acceleration of the fluorescence increase upon illumination, when compared to the control (Fig. 5, traces 1 and 2). An effect of the same kind was observed when 1 mM FeCN was added, although in a less pronounced way than with DMBQ (Fig. 5, trace 3). FeCN did not have any effect on the absorption spectrum of the sample before the illumination, while DMBQ induced a slight shift towards shorter wavelengths (not shown). Addition of these compounds did not modify the effect of NADPH to allow the reversibility of the fluorescence increase. Therefore, a rapid fluorescence increase could be repeated upon successive illumination of PL (682) in the presence of both DMBQ and NADPH (Fig. 5, traces 4a and b).

It was checked that DMBQ was not reduced chemically by NADPH during such an experiment.

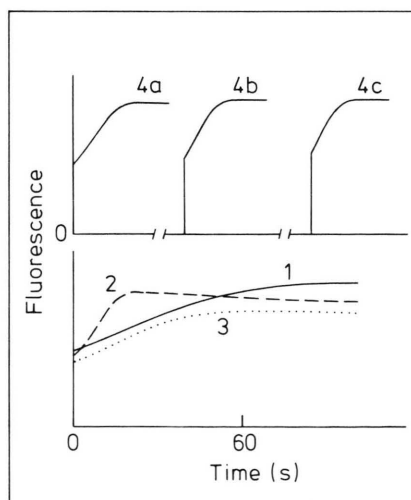


Fig. 5. Effect of DMBQ and FeCN on the 690 nm fluorescence variations in PL (682). (1): control; (2): 0.2 mM DMBQ, (3): 1 mM FeCN. (4): 0.2 mM DMBQ + 0.5 mM NADPH at a first (a), second (b) and third (c) illumination.

Discussion

The first comments on the data presented here concern the formation of P_{682} directly after the photoreduction of protochlorophyllide (reaction (2)). Our results fully confirm previous experiments (3 and 5) showing that NADPH restores the ability to transform P_{678} into P_{682} after the illumination in an etioplast suspension. Since enzymic oxidation of NADPH in plastids isolated after the *in vivo* formation of P_{682} leads to the regeneration of the P_{678} species, it may be concluded that NADPH plays the same role *in vivo* and *in vitro*. The hypothesis of Oliver and Griffiths [3] following which P_{678} and P_{682} are ascribed to ternary complexes between chlorophyllide, the reductase and $NADP^+$ or NADPH respectively thus provides a satisfactory model for all observations.

Another feature concerns the question whether the P_{678} spectral species formed after illumination of P_{682} by reaction (3) is equivalent to the P_{678} species produced as a result of protochlorophyllide photoreduction by reaction (1). Until now, only a spectral similarity was reported. Our data here strongly suggest that the two species are equivalent, *i.e.* they both represent chlorophyllide-protein complexes bound to $NADP^+$. We found indeed that in those two conditions under which a 678 nm absorbing chlorophyllide is found, addition of NADPH induces the shift to 682 nm. Therefore, we tend to say that NADPH is oxidized during photoreaction (3) while it is reduced or substituted by NADPH, during dark reaction (4). Thus, the phototransformation of P_{682} into P_{678} seems to be similar to the photoreduction of protochlorophyllide in the sense that, in both cases, NADPH is oxidized. In the case of the photoreduction the photoreceptor protochlorophyllide is also the substrate to be reduced. In the case of the phototransformation of P_{682} into P_{678} , the photoreceptor is chlorophyllide (see the action spectrum in [1]) with the nature of the oxidant being unknown. Further reduction of the pigment seems to be excluded since this would result in a large blue shift not observed in fact. Another possibility would simply be that oxygen is reduced in the reaction. However, we have recently shown that addition of NADPH to an etioplast suspension inhibits chlorophyllide photooxidation as measured by the rate of pigment photodestruction and oxygen uptake upon illumination. Thus, oxygen uptake was inhibited in the presence of

NADPH. This contradicts the hypothesis that oxygen serves as an electron acceptor in a light-triggered oxidation of NADPH. Hence, we have to assume that there exists a non-identified electron acceptor which is reduced when NADPH is oxidized. It was observed by Sironval *et al.* [6] and by Jouy [7] that illumination of leaves containing P_{682} results in the occurrence of complex absorbance changes between 400 and 600 nm. We feel that part of these changes might reflect the reduction of the unknown acceptors, namely to increase the rate of the phototransformation of P_{682} into P_{678} , suggesting that compounds with appropriate redox potentials might serve as electron carriers in the reaction. Some additional comment on the effect of NADPH on the pigment-protein complexes is needed. We found here that a much smaller concentration of the nucleotide — about 20 times less — is required to transform P_{678} into P_{682} via reaction 4 than via reaction (2). This suggests either that the accessibility to NADPH is very much increased during the initial formation of P_{682} or that different binding sites with different affinities for NADPH occur for the two reactions. In this sense, ElHamouri and Sironval [8] have shown that three different NADPH-binding peptides of 65, 36 and 15 Kd occur in etioplast membrane fractions.

The physiological role of the reaction involved in the chlorophyllide microcycle may be questionable, since continuous consumption of NADPH is not expected to present any advantage for the organism. In our previous paper [9] we have suggested that these reactions might compete with pigment photooxidation, therefore protecting to some extent the newly formed chlorophyllide against intense irradiation. Another hypothesis is that these reactions would represent some precursor activities to the normal photosynthetic reactions. This idea is in contrast to the current concept that the pigments belonging to the microcycle are bound to the 36 Kd peptide whose unique function would be the reduction of protochlorophyllide into chlorophyllide. It is known, however, that the product chlorophyllide is not released immediately as a free molecule as would be expected. Our observation here is that plastids containing a stable P_{682} chlorophyllide-protein complex can be obtained from shortly irradiated leaves. Even after the Shibata shift, chlorophyllide is found to be bound to one or several polypeptides [10, 11]. It must be pointed out also that oxygen evolution in etiolated leaves appears after only two short illumi-

nations, spaced apart by a 90 min dark interval [12]. At each of the two illumination, protochlorophyllide is reduced and the chlorophyllide microcycle occurs [13]. A detailed analysis of the changes in the pigment-protein relationships which occur during this

period leading to active photosynthesis is actually in progress.

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