

# On the Correlation between Oxygen Uptake in Plastids of Greening Etiolated Oat Leaves and Pigment Photooxidation

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Z. Naturforsch. **40c**, 699–704 (1985); received May 20, 1985

Dedicated to Prof. Dr. Wilhelm Menke on the Occasion of His 75th Birthday

Oxygen Uptake, Etiolated Leaves, Photooxidation

Changes in oxygen evolution or uptake in illuminated plastids were studied during the early stages of greening in etiolated leaves. An oxygen uptake was observed in etioplasts, which persisted even after the appearance of oxygen evolution. This oxygen uptake was most pronounced in etioplasts or in plastids prepared after redarkening the plants. Incubation of pre-illuminated etioplasts in the presence of NADPH resulted in an inhibition of the uptake in a subsequent illumination. Addition of NADPH had three further consequences: a shift of the chlorophyllide absorbance band to 682 nm, an inhibition of pigment photodestruction and the appearance of a light-induced fluorescence transient at room temperature. Inhibition of pigment photodestruction by NADPH was maximal when the experimental conditions favoured the formation of the  $P_{682}$  chlorophyllide-protein.

It is inferred that the oxygen uptake seen under these conditions is due to pigment photooxidation. Inhibition of both phenomena by NADPH is ascribed to the specific interaction of the nucleotide with the pigment-protein complexes and to the ability of these complexes to undergo the light-induced chlorophyllide microcycle, described by Franck and Inoue (Photobiochem. Photobiophys. **8**, 85–96, 1984). In the leaf, NADPH is thought to play the same role.

## Introduction

Illumination of etiolated leaves from higher plants results within a few seconds in the photoreduction of protochlorophyllide. Under prolonged illumination, chlorophyll accumulates and the photosynthetic apparatus starts to develop. The appearance of the ability to evolve oxygen in the light is a multi-step process including light and dark reactions operating in a defined sequence. We were recently able to show that preillumination of leaves by two 5 min white light periods, spaced apart by a 90 min dark interval, enables isolated plastids to evolve oxygen in the light. A typical Kok-Joliot pattern is obtained when oxygen is measured as a consequence of short saturating light flashes [1].

Another light reaction which also occurs in such slightly greened organelles is a light-induced oxygen uptake. It was first described in etiolated leaves by Gabrielsen *et al.* [2] and by Madsen [3]. Further ob-

servations on this oxygen uptake in etioplasts were carried out by Redlinger and McDaniel [4] who ascribed this phenomenon to pigment photooxidation, although no direct proof was presented. Their assumption seems, however, plausible since it is known that native chlorophyll(ide) molecules are easily bleached [5] and that the triplet state of chlorophyllide is easily populated in illuminated etioplasts [6].

The fact that oxygen evolution is now detected very soon in the course of development raises the question whether oxygen uptake observed under such conditions is to be associated with pigment bleaching or with some precursor activity of photosystem II, since an oxygen uptake has recently been observed in photosystem II of blue-green algae [7].

In the present paper, we describe the modifications of the oxygen exchanges of plastids isolated from leaves at progressive stages in the beginning of the greening process. We provide experimental evidence that the oxygen uptake which takes part in these exchanges is due to pigment photodestruction. We show that NADPH, which is known to be the reductant in protochlorophyllide reduction [8], plays an important role in preserving the pigments from photooxidation. This result is discussed in terms of the specific effect of the nucleotide on the pigment-protein complexes.

**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.

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Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen  
0341-0382/85/0009-0699 \$ 01.30/0



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## Material 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.

Plastid preparations were obtained from cut-off leaf sections of the plants. The entire preparation was carried out in green safety light according to the procedure described earlier [1]. Bleaching experiments were carried out with etioplasts prepared in the same way but resuspended in a buffer pH 7.5 containing 20 mM TES, 20 mM Hepes, 1 mM MgCl<sub>2</sub> and 5% glycerol, causing lysis of the plastids. Broken etioplasts were then precipitated by centrifugation at 6000 × g for 10 min and resuspended in the same buffer with 0.6 M sucrose added. They were either used directly for measurements or stored at -20 °C.

Oxygen measurements were carried out by polarography with the three-electrode-system described by Schmid and Thibault [9]. During the measurements, osmotic integrity of the plastids was maintained by suspension in buffer pH 7.5 containing 20 mM TES, 20 mM Hepes, 1 mM MgCl<sub>2</sub> and 0.5 M sucrose. Electrolyte (KCl) concentration was adjusted to 0.1 M.

Illumination for pigment bleaching measurement was provided by a LEITZ projector equipped with a broad red filter and a heat absorbing filter. Light intensity was about  $3.5 \times 10^5$  ergs · cm<sup>-2</sup> · sec<sup>-1</sup>.

Absorption spectra were measured at +5 °C in a Perkin-Elmer spectrophotometer.

Fluorescence measurements were performed under blue light in the device described in [10].

## Results

When etiolated leaves were slowly greened under a regime of successive 5 min illumination periods, separated by 2 hours dark periods, oxygen evolution rapidly developed, together with chlorophyll accumulation. Fig. 1 shows the oxygen patterns obtained under continuous light and under short light flashes of plastids prepared of etiolated leaves either at the end of the dark period or within a short time after each of the three preillumination periods. If one takes out the first preillumination, which does not lead to any ability to evolve oxygen, the effect of each preillumination period seems to be an enhance-

ment of the oxygen evolution at the expense of an oxygen uptake which can already be observed in etioplasts. The flash sequence recorded after the second preillumination showed clearly that oxygen

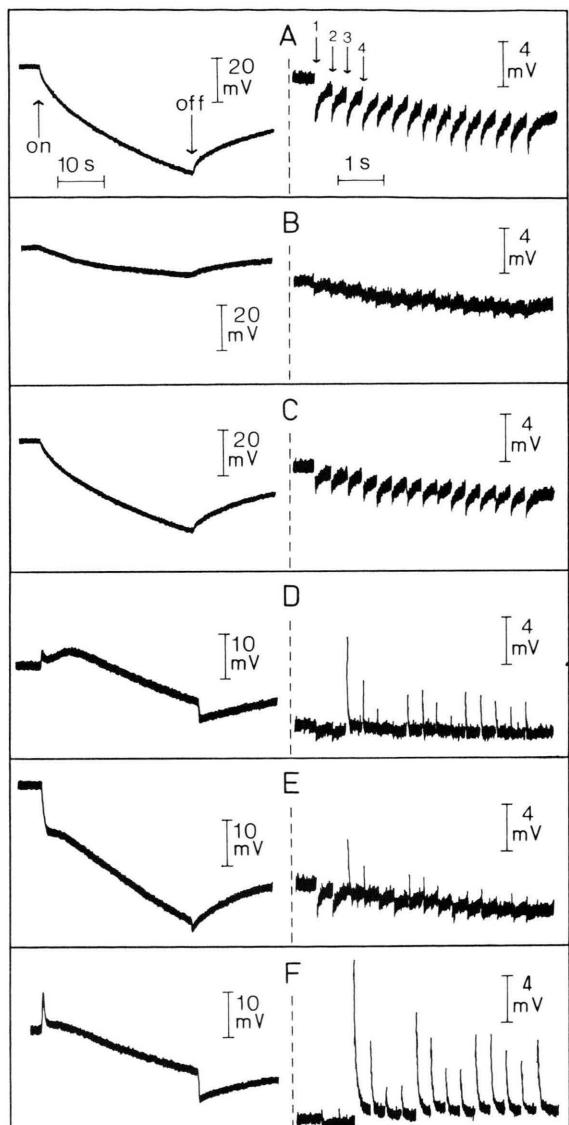


Fig. 1. Patterns of oxygen exchanges induced by continuous red light (left) or by short xenon flashes (right) in plastids prepared after various pre-treatments of etiolated leaves: A: etiolated; B: 5 min light; C: 5 min light + 2 h darkness; D: 5 min light + 2 h darkness + 5 min light; E: 5 min light + 2 h darkness + 5 min light + 2 h darkness; F: 5 min light + 2 h darkness + 5 min light + 2 h darkness + 5 min light. The amount of plastids on the electrode was kept constant throughout from A to F by adjusting the amount of protein to a constant value of 0.5 mg.

evolution proceeds according to Kok's mechanism as soon as it appears (if one disregards the abnormally long life-time of the  $S_3$  state [1]).

The nature of the oxygen uptake, seen after each dark period, is puzzling. In etioplasts, such an oxygen uptake was tentatively ascribed by Redlinger and McDaniel [4] to a photooxidation of both protochlorophyllide and chlorophyllide. This assumption was made on the basis that the rate of uptake decreased together with increased pigment bleaching. However, this correlation could have been purely coincidental since it would have occurred in the case of any pigment-sensitized photoreaction. Furthermore, the uptake was not observed when the leaves were irradiated for a short time directly before etioplast isolation. This fact, which is confirmed by our data in Fig. 1, remained unexplained. It shows that the occurrence of an uptake depends on whatever protochlorophyllide reduction takes place in the intact leaf or in the isolated plastids.

In the leaf, protochlorophyllide reduction is followed by two successive dark spectral shifts of the product chlorophyllide: a rapid shift from  $P_{678}^*$  to  $P_{682}$  is followed by a slow shift from  $P_{682}$  to  $P_{673}$  [11, 12]. In isolated plastids the rapid shift does not occur and  $P_{678}$  is directly transformed to  $P_{673}$  within some 30 min. It has been shown, however, that the formation of  $P_{682}$  also occurs in isolated plastids provided NADPH is added to the medium [8, 13]. In this case,  $P_{682}$  is stable for at least 2 hours at 0 °C. Since addition of NADPH to isolated plastids thus restores the ability to produce the  $P_{682}$  chlorophyllide form, normally found in the intact leaf, we have measured the rate of oxygen uptake and the extent of pigment photodestruction in plastids in the presence and absence of NADPH.

Broken etioplasts with a high active/non-active protochlorophyllide ratio were first irradiated by a 15 sec red light pulse with or without NADPH (1 mM) in order to transform all the active protochlorophyllide into chlorophyllide. They were then incubated in darkness for 20 min on ice. The absorption maximum of chlorophyllide was found at 673 nm in the non-treated sample while it was located at 682 nm in the sample supplemented with NADPH (Fig. 2A). Upon illumination with red light, the non-treated sample exhibited a large oxy-

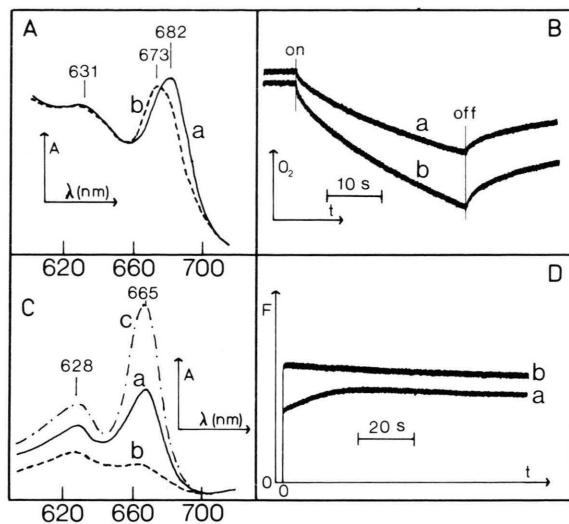


Fig. 2. Effect of NADPH on A: the absorption spectra of an etioplast suspension; B: the oxygen uptake in continuous red light; C: the pigment content after a 10 min red light illumination as measured by the absorption spectra of methanol extracts (c = not-illuminated control sample); D: the room temperature fluorescence variations at 685 nm. Aliquots were first illuminated by a 15 sec red light pulse in the presence (a) or the absence (b) of NADPH (1 mM) and then incubated in ice for 20 min.

gen uptake. After 10 min illumination its pigment content decreased to about 8% of its initial value, as shown by comparison of the absorption spectra of methanol extracts of plastids which were illuminated and not-illuminated. In the sample supplemented with NADPH, the oxygen uptake was reduced by 40% while about 55% of the pigments still remained (Fig. 2B and C).

Another light-induced reaction which depends on the presence of NADPH is the chlorophyllide "microcycle" described by Franck and Inoue [14] and by Sironval *et al.* [15]. This microcycle consists in a light-induced, dark-reversible spectral shift on the  $P_{682}$  chlorophyllide, associated with a room temperature fluorescence transient. In plastids, it occurs only upon addition of NADPH since the presence of the reduced nucleotide is required for the formation of the  $P_{682}$  chlorophyllide and sensitizer of the reaction. Fig. 2D shows that the fluorescence variation which reflects this microcycle is indeed observed in the sample treated with NADPH, while the control sample showed a higher fluorescence yield slowly decreasing with the illumination time.

\*  $P_X$  stands for the pigment-protein complex whose absorption maximum is located at  $X$  nm.

These results confirm that oxygen uptake in etioplasts is related to pigment photooxidation, since an external factor such as NADPH affects both phenomena in the same way. It shows furthermore that in such a preparation the  $P_{682}$  form is much less subject to photooxidation than the  $P_{673}$  form and suggests that the use of light energy in the chlorophyllide microcycle (whatever the fate of the absorbed light is in this reaction) avoids photooxidation.

The coincidence of the two effects of NADPH — namely formation of  $P_{682}$  and inhibition of photooxidation — could however be fortuitous, since it would be conceivable that the inhibition of pigment photooxidation is the consequence of increasing the redox potential by NADPH addition without any specificity of this molecule. Further experiments clearly showed that a large effect of NADPH on photooxidation was only seen in the case where the experimental conditions favoured also the formation of  $P_{682}$ . In this sense we took advantage of the fact that:

- the formation of  $P_{682}$  hardly occurs if NADPH is added after completion of the shift to  $P_{673}$ ;
- heating the sample for 5 min at 50 °C after or prior to the addition of NADPH inhibited the formation of  $P_{682}$ .

The absorption maximum of the chlorophyllide band before the 10 min of bleaching irradiation and the relative concentration of the remaining chlorophyllide after irradiation are given in Table I, for each of the indicated pretreatments. Relative chlorophyllide concentrations were calculated on the basis of the absorption spectra of methanol extracts, by comparison with a control sample which was submitted to the same treatments except that the 10 min bleaching irradiation was replaced by a second 15 sec

light pulse (this light pulse was given in order to reduce any active protochlorophyllide eventually regenerated during the dark incubation time).

The data clearly demonstrates that the largest inhibition of pigment bleaching in the presence of NADPH corresponds to the occurrence of a chlorophyllide absorption maximum at 682 nm. It seems, however, that in the sample heated in the presence of NADPH, chlorophyllide is also some-

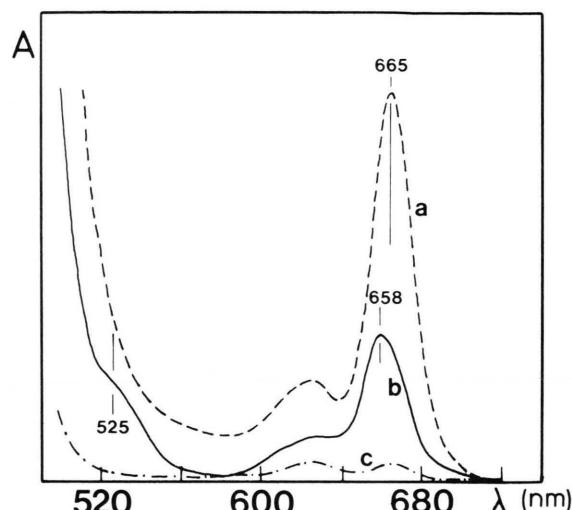


Fig. 3. Effect of sodium dithionite on the absorption spectrum of methanol extracts of etioplasts which were first illuminated by a 15 sec red light pulse and then incubated on ice in darkness for 20 min. The plastids were then either kept in darkness for further 10 min (a) or illuminated for 10 min by red light in the presence of sodium dithionite (2 mg/ml added immediately before the illumination (b) or without addition (c)).

Table I. Effect of NADPH on the position of the chlorophyllide absorption band and on the photostability of chlorophyllide.

Treatment after the initial red light pulse	NADPH added at time 0		NADPH added at time 40'		No addition	
	Chlorophyllide Absorption band [λnm]	Relative concentr.	Chlorophyllide Absorption band [λnm]	Relative concentr.	Chlorophyllide Absorption band [λnm]	Relative concentr.
40 min incubation at 0 °C	682	0.66	674	0.08	673	0.06
heat treatment (A)	672	0.06	672	0.03	672	0.03
heat treatment (B)	674	0.27	672	0.03	672	0.04

The etioplast suspensions were first illuminated by a 15 sec red light pulse (time 0 = before the red pulse) and then incubated in darkness for 40 min. Heat treatment (5 min at + 55 °C): the sample was first heated and then kept in ice for 35 min (A) or was first kept in ice for 35 min and then heated (B).

what protected from photodestruction, although to a much lesser extent than in the not-heated sample.

Attempts to inhibit pigment bleaching by removal of oxygen were also made. This was done by adding sodium dithionite at a concentration of 2 mg/ml. Addition of this product had a dramatic effect on the pigment composition of the sample after illumination by 10 min red light. The amplitude of the red absorption bands in methanol extracts was higher than in the sample illuminated without dithionite, but the chlorophyllide band normally located at 665 nm was shifted to 658 nm.

vitro, which might be favoured by the presence of NADPH [21, 22]. The results shown in Table I strongly suggest, however, that the effect of NADPH has to be considered as an interaction with the pigment-protein complexes rather than as a direct interaction with the pigment alone. Indeed, efficient inhibition of photodestruction by NADPH was only observed in the case where its addition resulted in the absorption shift to 682 nm of the plastids suspension, *i.e.* in non-heated samples or in samples which did not yet undergo the Shibata shift to  $P_{673}$ .

This view is supported also by the fact that, during slow greening under 5 min light – 2 h darkness cycles, the oxygen uptake is mainly observed when plastids are prepared after a dark period (Fig. 1). In this case, sample illumination results in the reduction of the regenerated protochlorophyllide without formation of the  $P_{682}$  species. On the other hand, preillumination of the intact leaves allows the normal pathway to occur in the leaves. Therefore, the oxygen uptake is less pronounced in plastids prepared a short time after each preillumination.

Following this interpretation, one would predict that, in whole leaves, where the two chlorophyllide species appear in series, chlorophyllide  $P_{682}$  would be less easily bleached than chlorophyllide  $P_{673}$ . Axelsson [5] reported the opposite result. However, close examination of the experimental procedure used reveals that any light-induced chlorophyllide blue shift would have been computed as pigment photodestruction. We know now that such a blue shift occurs without photodestruction upon illumination of the  $P_{682}$  species [14]. Therefore, the observation of Axelsson should be reinvestigated.

The effect of sodium-dithionite on the pigment composition of illuminated etioplasts is not trivial. Since addition of this compound leads to a decrease of the oxygen concentration in the medium, an inhibition of pigment bleaching is expected. The red absorption band in methanol decreases indeed less in this case than in the control, but it is shifted to shorter wavelengths, indicating that the pigment is no more chlorophyllide. The appearance of a shoulder around 525 nm might indicate the formation of pheophytin or of a reduced species of chlorophyllide [23, 24].

The question remains what function belongs to the chlorophyllide microcycle described by Franck and Inoue [14]. The chemical nature of this particular photoreaction is not understood at the present time.

## Discussion

In green plants, carotenoids have been shown to protect chlorophyll against photooxidative destruction [16, 17]. Although etioplasts contain a large amount of carotenoids, the native chlorophyll(ide) is easily destroyed by light, as well as protochlorophyll(ide) [4, 5]. For some structural reason, carotenoids are thus not able to exert their protective role at this stage.

In etioplasts, protochlorophyll(ide) and chlorophyll(ide) are present in form of pigment-protein complexes located in the prolamellar body and in the prothylakoids [18, 19]. NADPH plays an essential role for the properties of these complexes by acting as a cofactor in the photoreduction of protochlorophyllide and by promoting the chlorophyllide spectral shift to  $P_{682}$  [8, 13]. Apart from these specific effects of the nucleotide, redox potential effects have also been reported. Lowering the redox potential to around –350 mV stabilizes the photoactive protochlorophyllide  $P_{678}$  [20]. However, the formation of chlorophyllide  $P_{682}$  in etioplasts has up to now only been observed in the presence of NADPH.

The effect of NADPH, reported here, namely that of a simultaneous inhibition of the oxygen uptake and of pigment bleaching strengthens the hypothesis that the oxygen uptake, always observable in young, greening plastids is well due to photooxidation. To explain this particular effect of NADPH, one may consider the simple fact that lowering the redox potential of the medium might make oxidation reactions less probable. It is also known that the triplet state of chlorophyll or chlorophyllide can sensitize a large variety of reversible reduction reactions in

Our data suggest that it might compete with pigment photooxidation since it is observed together with the inhibition of pigment bleaching upon addition of NADPH. In the intact etiolated leaf, this microcycle is repeatedly observed upon successive illuminations. One of its possible physiological role could be the

protection of newly synthesized pigments against photodestruction.

#### Acknowledgement

F. Franck thanks EMBO for the award of a fellowship.

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