Protochlorophyllide Photoconversion Initiates the Transformation of Reaggregated Prolamellar Body Tubules *in vitro*

M. Müller and A. Nisius

Fakultät Biologie (Abteilung Professor Dr. H. G. Ruppel) der Universität Bielefeld, Postfach 86 40, D-4800 Bielefeld 1, Bundesrepublik Deutschland

Z. Naturforsch. **37 c**, 476–480 (1982); received January 13, 1982

Prolamellar Body, Photoconversion, NADPH: Protochlorophyllide Oxidoreductase, Saponin

Completely dissolved components of native prolamellar body/primary thylakoids PLB/PT were induced to reaggregate into tubular structures *in vitro*. Incubation of these pchlide containing PLB-like tubules under red light with NADPH : pchlide oxidoreductase led to a specific association of the enzyme protein to the tubules. Photoconversion of the pigment initiates the dispersal of reaggregated PLB-tubules as indicated by changes in the content of their main constituent saponin.

Introduction

Etioplasts, a type of plastids representing a somehow "frozen picture" of the normal development of proplastids into chloroplasts, are characterized by a large paracrystalline array of tubules called PLB.

When etiolated tissue is exposed to light the paracrystallinity of PLB is disrupted: photoconversions of pchlide to chlide occurs at the same time as PLB tubules lose their crystalline configuration.

In vivo there is a good evidence for a correlation between photoconversion and tubules-transformation: by fluorescence microscopy Boardman and Wildman [1] demonstrated that pchlide in etioplasts may be localized in PLB-tubules. According to Kahn [2], pchlide holochrome is a substantial building part of PLB. Henningsen and Boynton [3] proposed that a conformational change in the pchlide holochrome protein is related to the dispersal of PLB tubules. In old leaves of dark grown plants which lack photoconvertible pchlide the tubular complex persits even in the light (Prof. Ruppel, pers. communication). Barley mutants not showing the spectral shift of chlide normally following the photoconversion also are unable to disperse the PLB [4].

Abbreviations: CHLIDE, chlorophyllide; EDTA, ethylenediamin-tetraacetate-tetrasodium salt; HEPES, 4-(2-hydroxymethyl)-1-piperazine-ethane sulfonic acid, PAA, polyacrylamide; PCHLIDE, protochlorophyllide; BLB, prolamellar body; PT, primary thylakoids; SDS, sodiumdodecylsulfate; SDS-PAGE, sodiumdodecylsulfate polyacrylamide gel electrophoresis; TRICINE, N-tris(hydroxymethyl)methylglycine; TRIS, tris(hydroxymethyl)aminomethane.

Reprint requests to Dr. M. Müller.

0341-0382/82/0500-0476 $ 01.30/0

To correlate disruption of PLB tubules with changes in its pigment content exact localization of pchlide within the etioplast is necessary. Recent efforts [5] to separate membranes of the etioplasts into a PLB fraction and a fraction of membraneous vesicles (PT) showed that pchlide might be associated with the PT-rich fraction. The authors suggested that pchlide phototransformation achieved by pchlide holochrome should be associated with the PT-fraction.

All *in vivo* experiments to localize pchlide within the etioplasts [5] are limited by the difficulties to separate PT completely from PLB tubules normally attached to them. To overcome these technical difficulties we used an *in vitro* reconstitution of PLB tubules [6] reaggregated from components of an acetone extract of native PLB/PT. Pchlide was found to be an integral component of these reaggregated tubules. If pchlide is responsible for the maintenance of PLB structure, reaggregated tubules containing pchlide should be dispersed after attack of NADPH: pchlide oxidoreductase, the enzyme catalysing the photoconversion of pchlide to chlide.

As pchlide photoconversion only can be achieved when pigment molecules are attached to specific protein [7] the formation of this photoactive complex with PLB tubules has to be analysed.

As saponins and sterols are known to be the main building units of the PLB [6] and as moreover – parallel to light induced dispersal of PLB – changes of its saponin content were observed [8, 9] *in vivo*, it is of obvious interest to investigate the saponin content of the reconstituted system of PLB tubules (containing pchlide) after photoconversion.

Materials and Methods

Plant material: Avena sativa L. germinated at 25 °C and 75% humidity in complete darkness.

Isolation of PLB/PT: 500 g of 6 days old leaves of Avena sativa were homogenized in 1.5 l of 0.05 M TRIS/HCl pH 8.0 containing 0.25 M sucrose. The filtrat (passed through 8 layers of cheese cloth) was centrifuged for 20 min at $1500 \times g$. The etioplasts were shocked osmotically by resuspending them in 50% Percoll (Percoll/TRIS/HCl 0.05 M, pH 8.0). After formation of the self-generating gradient (15 min at $10000 \times g$) tubules banding at a density of 1.06 g/ml could be isolated.

Reaggregation of tubules in vitro: native PLB/PT were extracted with 80% acetone. The completely dissolved components of the PLB/PT acetone extract were brought to reaggregate by evaporation of the solvent (40–50 °C). Products of reaggregation (tubules) banding at a density of 1.04 g/ml could be isolated on a Percollgradient (25% Percoll in 0.05 M Tris/HCl pH 8.0, 30 min, $20000 \times g$).

NADPH:pchlide oxidoreductase (E.C. 1.6.99): was isolated and purified according to Apel *et al.* [10].

Incubation: reaggregated tubules were incubated with the DEAE-cellulose purified enzyme in the following medium: 510 mM TRICINE/KOH pH 7.2, 20% glycerol, 10 mM HEPES, 1 mM $MgCl_2$, 1 mM EDTA, 0.5 mM Triton-X-100, 1.2 mM NADPH. Incubation was done at 4 °C under red light of high intensity ($30000 \text{ erg} \times \text{cm}^{-2} \times \text{s}^{-1}$, cold light source KL 150 B, Schott & Co, cut-off filter RG 630). After incubation the reaggregated tubules were collected in a further gradient (25% Percoll).

Lipid and pigment measurements: pigments and lipids were extracted into acetone by adding absolute acetone to the fraction to obtain a final concentration of 80%.

Pchlide and chlide concentration was calculated according to Klein and Schiff [11]. Thin-layer chromatography of lipids was done according to Müller [9].

Protein analysis: protein precipitate of the acetone extract of the fractions was solubilized in 10 mM TRIS/HCl pH 8.0, 1 mM EDTA, 2.5% SDS, 5% mercaptoethanol and treated 5 min at 100 °C. Separation was done on a Pharmacia PAA 4/30 gradient gel using the following electrophoretic buffer: 40 mM TRIS/HCl pH 7.4, 20 mM Na-acetate, 2 mM EDTA, 0.2% SDS. After staining proteins with 0.02% Coomassie Blue quantitative measurements were carried out using a densitometer (Quick Scan, Desaga) at 578 nm.

Electron microscopy: isolated fractions were monitored on a Hitachi H-500 electron microscope after negative staining with 1% phosphotungstic acid (pH 7.0)

Results and Discussion

Tubules reaggregated *in vitro* were compared with native PLB tubules by means of electron microscopy. Fig. 1 reveals that the induced formation of PLB-like structures resulted in tubules of similar appearance (*i.e.* diameter) to those reported from intact etiolated leaves and they are considered to be identical.

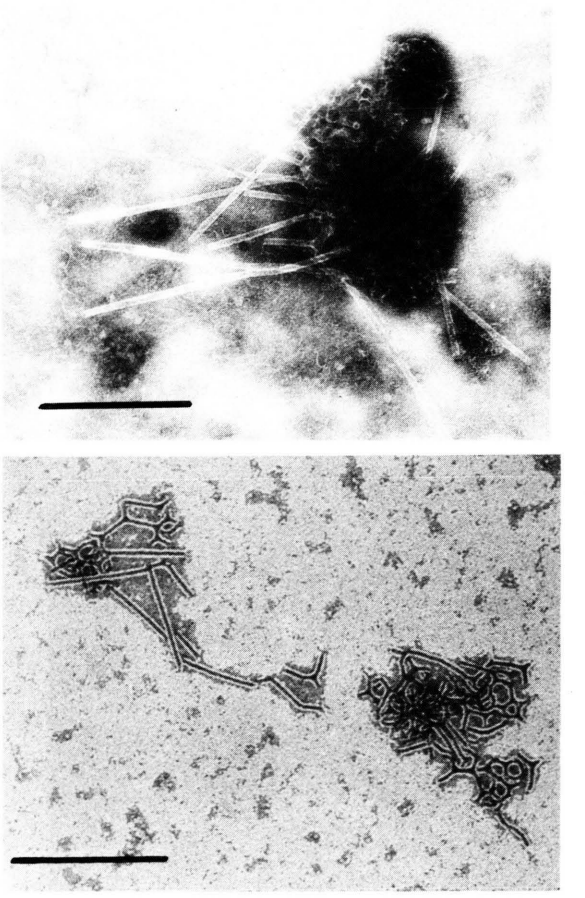

Fig. 1. Electron micrograph of negatively stained PLB tubules a) isolated from 6-days old etiolated *Avena sativa* plants; b) reaggregated *in vitro* from completely dissolved components of an PLB/PT acetone extract (bar: 0.4 µm).

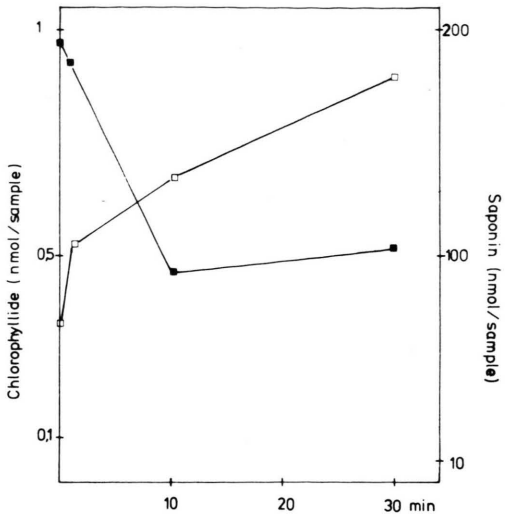

Fig. 2. Light dependent changes in the pigment content (□) and in the saponin content (■) of reaggregated PLB tubules after incubation with NADPH:pchlideoxidoreductase.

Lipid analysis of reaggregated tubules revealed that qualitatively the lipid composition resembles that of native PLB tubules [6].

The aggregates being derived from an acetone extract of PLB/PT were essentially devoid of protein as controlled by electrophoresis. These results fit into earlier investigations trying to reaggregate all SDS-soluble components of the PLB/PT into PLB-like structures which resulted in protein-free tubules [12].

For incubating tubules containing pchlide together with the enzyme catalysing the photoconversion we prepared NADPH:pchlideoxidoreductase. The purity of the enzyme preparation was controlled by gel electrophoresis (Fig. 3b, D). There was a *pair* of closely related polypeptides of a m. wt. of 35 and 37 kD. Recently Oliver and Griffiths [13] presented evidence that both peptides show properties expected to the pchlide reductase, investigating the same material as we did. After incubation with the enzyme under red light of high intensity the tubules were separated from their incubation medium using a Percollgradient and their content of pigment and saponin – before and after photoconversion – was determined.

As a result of pchlide photoconversion the saponin content of the tubular fraction decreases with a temporary retardation while the ratio pchlide/saponin (Table I) remains unaltered. Apparently the saponin content of the tubular fraction diminished to the same extend as pchlide was reduced.

The specificity of the reaction was controlled by further experiments: the saponin content of the tubules incubated under the same conditions but *without* enzyme (Table I) did not change. Also incubation of tubules with enzyme in the dark of with enzyme under red light but without NADPH, induced no dispersal to tubules (Table I). This is

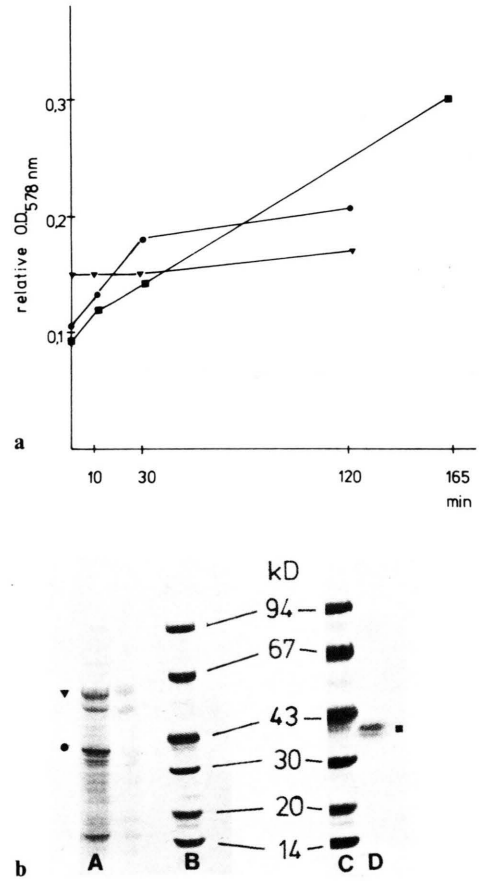

Fig. 3. a) association of proteins with reaggregated tubules exposed to red light dependent on incubation time. The amount of proteins was determined by measuring the relative $O.D._{578}$ after gel electrophoresis and staining of the proteins. Measurements are done after association of reaggregated tubules with ■ purified NADPH:pchlide oxidoreductase; ● NADPH:pchlide oxidoreductase from crude extract; ▼ 59000 dalton protein from crude extract. b) polypeptide composition of A: the triton-X-100 soluble fraction of the PLB suspension; B, C: marker proteins; D: NADPH:pchlide oxidoreductase purified on DEAE cellulose. Separation of the polypeptides was carried out by SDS-PAGE on a 4%–30% gradient gel.

Table I. Changes occuring during incubation of reaggregated tubules with/without NADPH:pchlideoxidoreductase (under red light, 30000 erg × cm^{-2} × s^{-1}, at 4 °C).

Essay:	Light exposure [min]	
	0	30
chlide content of the enzyme preparation	not detectable	not detectable
pigment content of tubules after incubation with the enzyme and isolation on a Percoll-gradient pchlide + chlide at the beginning of the essay = 100%	pchlide: 84% chlide: 16%	pchlide: 46% chlide: 38%
pchlide/saponin (molar ratio)	1.1×10^{-2}	1.0×10^{-2}
saponin content of the tubules incubated *without* enzyme after isolation on a Percollgradient	100%	94%
saponin content of the tubules after incubation *with* enzyme and isolation on a Percollgradient		
without NADPH, in the light	100%	92%
with NADPH, in the dark	100%	81%
with NADPH, in the light	100%	33%
total of saponin (tubular-bound and free) *after* incubation with the enzyme, *before* gradient centrifugation	100%	97%

evidence that tubules containing pchlide need enzyme as well as NADPH and light to be dispersed after photoconversion.

Table I indicated that the total of saponin (that is tubular bound plus free saponin after incubation but before gradient centrifugation) remains more or less constant during incubation. Apparently saponin is not degraded enzymatically. After illumination, a smaller amount of saponin is found in the tubular band of the second gradient, while an increased amount of saponin is found within the rest of the gradient. We therefore postulate that – similar to the situation in the etioplast *in vivo* [9] – there is a balance between tubular bound and the apparently dissolved state of saponin. Like in the experiments reported herein, etioplasts *in vivo* also display a rapid decrease of the tubular saponin under illumination without immediate decrease of the overall saponin in the etioplast.

As indicated by the saponin content of the tubular fraction, number and/or size of the tubules is reduced after photoconversion *in vitro* and one is tempted to suppose that tubules disrupt just in those areas where the conformational change of the pigment-protein complex upon photoconversion induces the loss of tubular configuration of saponin.

Investigations by electron microscopy of those tubules which – after photoconversion – banded at the same density within the gradient as before, did not reveal any changes. This result indicates that in the tubular fraction of the gradient only complete PLB tubules of similar appearance are collected and the fraction lacks tubular subunits produced after photoconversion. So the diminished saponin content of the tubular fraction is likely to result from the reduced number of those tubules that consist of the same constituents before and after treatment.

We like to interprete our experiments as follows:

1) the first light induced step in the morphological transformation of tubular PLB is related to pchlide photoconversion into chlide – the only reaction occuring in our *in vitro* system found to decrease the tubular state of PLB saponin;
2) pchlide is an integral component of PLB-tubules being able to form a photoactive complex with NADPH:pchlideoxidoreductase, the enzyme catalysing the photoconversion.

To follow up the formation of this complex we analyzed the protein content of the reaggregated tubules after incubation with the enzyme by aid of gel electrophoresis. Fig. 3a shows the association of the purified NADPH:pchlideoxidoreductase (35/37 kD) with the tubules which occurs in the light as well as in the dark. So the association of the holochrome protein to tubules containing pchlide is independent of photoconversion.

The specificity of this bound was demonstrated in a further experiment when tubules were incubated with a crude enzyme preparation containing additional proteins of the m. wt. of 16, 54, 59 kD. Although these proteins were represented in re-

sembling amounts we discern – concerning the association of proteins to tubules – 3 groups of proteins:

a) There was no association of proteins of the m. wt. of 16 and 54 kD.
b) there was no enhanced association of the protein of 59 kD, during the treatment
c) the amount of the proteins of 35/37 kD m. wt. increased with length of incubation (Fig. 3a).

In conclusion, there are tubules aggregated *in vitro* and built up only by lipids and pigments. These pigment containing tubules should have specific points binding the protein in such a manner that photoconversion takes place. This causes conformational changes of the photoactive complex affecting the dispersal of the PLB tubules.

To apply this model to the greening process *in vivo*, we suppose the light induced PLB destruction to be switched on by pchlide photoconversion of the pigment component of PLB tubules. So photoconversion triggers the dispersal of the reservoir – "PLB" containing most of the components which are needed during the greening process.

Acknowledgements

The authors wish to thank Professor Dr. H. G. Ruppel for helpful discussions and critical reading of the manuscript. The aid of Dr. B. Müller in translating the manuscript is gratefully acknowledged.

[1] N. K. Boardman and S. G. Wildman, Biochim. Biophys. Acta **59,** 222–224 (1962).
[2] A. Kahn, Plant Physiol. **43,** 1781–1785 (1968).
[3] K. W. Henningsen and J. E. Boynton, J. Cell Sci. **5,** 757–793 (1969).
[4] D. von Wettstein, in: Membranes and Disease (C. Bollis, J. F. Hoffman, and A. Leaf, eds.), pp. 123–130, New York 1976.
[5] C. Lütz and U. Männing, in: Photoreceptors and Plant Development (J. De Greef, ed.), pp. 229–236, University Press, Antwerpen 1980.
[6] A. Nisius, M. Müller, and H. G. Ruppel, Z. Naturforsch. **37 c,** 251–255 (1982).
[7] J. H. C. Smith and A. Benitez, Plant Physiol. **29,** 135–143 (1954).
[8] J. Kesselmeier and H. G. Ruppel, Z. Pflanzenphysiol. 93, 171–184 (1979).
[9] M. Müller, Z. Naturforsch. **36 c,** 869–874 (1981).
[10] K. Apel, H. J. Santel, T. E. Redlinger, and H. Falk, Eur. J. Biochem. **111,** 251–258 (1980).
[11] S. Klein and J. A. Schiff, Plant Physiol **49,** 619–626 (1972).
[12] H. G. Ruppel, J. Kesselmeier, and C. Lütz, Z. Pflanzenphysiol. **90,** 101–110 (1978).
[13] R. P. Oliver and W. T. Griffiths, Biochem. J. **195,** 93–101 (1981).
[14] H. C. Lancer, C. E. Cohen, and J. A. Schiff, Plant Physiol. **57,** 369–374 (1976).