Optical Multichannel Analysis of Protochlorophyllide Phototransformation in Detergent-Solubilized Etioplast Membranes of Wheat

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Extracts of wheat etioplast membranes obtained after treatment with 7 mM *n*-octyl-β-D-glucopyranoside (OG), *n*-dodecyl-β-D-maltoside (DM) or Triton X-100 contained the three spectral forms of Pchlide (the photoactive $Pchlide_{638}$ and $Pchlide_{650}$ and the inactive $Pchlide_{630}$) in various relative amounts . The OG extract had a Pchlide composition close to that of the intact membranes whereas the DM extract was enriched in $Pchlide_{638}$ and the Triton extract was enriched in $Pchlide_{630}$. Measurements of the kinetics of phototransformation and of time-resolved absorbance spectra during phototransformation in continuous light shows that the inactive $Pchlide_{630}$ is in fact slowly transformed to Chlide, especially in the Triton extract where this form is more abundant. Addition of NADPH favours the phototransformation of $Pchlide_{630}$ and the slow regeneration of $Pchlide_{638}$ and $Pchlide_{650}$ from $Pchlide_{630}$ in darkness after illumination. No such regeneration was however observed in the Triton extract. NADPH had only slight effects on the Chlide shift towards shorter wavelengths after phototransformation in solubilized membranes.

Introduction

The enzyme NADPH-protochlorophyllide reductase (EC 1.3.1.33) is a major membrane protein in etioplasts of dark-grown angiosperms [1]. It catalyzes the photoreduction of Pchlide *a* to Chlide *a*, an important light-requiring step in plant greening (see [2, 3] for reviews). In the etioplast membrane, the enzyme form stable complexes with substrates Pchlide and NADPH [4]. These complexes are generally termed photoactive complexes because the Pchlide they contain is reduced to Chlide as soon as light is turned on. Two spectrally distinct photoactive complexes, $Pchlide_{650}$ and $Pchlide_{638}$, are normally found *in vivo* [2–5]. Their molecular architecture is still unknown. They have been suggested to consist of large size aggregates containing several enzyme subunits and several pigment molecules, with $Pchlide_{650}$ being more aggregated than $Pchlide_{638}$ [5–8]. A minor spectral form, $Pchlide_{630}$ or inactive Pchlide, is not transformed in the light and is only present in low amounts *in vivo* [3, 5].

In early studies Pchlide-protein complexes have been isolated from homogenates of dark-grown leaves, using Triton X-100 or saponin as solubilizing agent [9–11]. These solubilized complexes (holochromes) contained photoactive Pchlide mainly under the $Pchlide_{638}$ form. Purified prolamellar bodies were recently solubilized with *n*-octyl-β-D-glucoside [12]. After detergent treatment $Pchlide_{638}$ was also the main active form. In the present work we have used *n*-octyl-β-D-glucoside, *n*-dodecyl-β-D-maltoside or Triton X-100 for the solubilization of Pchlide complexes from wheat etioplast membranes. Extracts with different proportions of the three Pchlide spectral forms were obtained. The spectroscopic and kinetic properties of the phototransformation with or without exogenous NADPH were studied in these extracts with the aim of further characterizing the three different Pchlide complexes.

Abbreviations: $Pchlide_x$, protochlorophyllide with an absorbance maximum at x nm; $Chlide_x$ chlorophyllide with an absorbance maximum at x nm; OG, *n*-octyl-β-D-glucopyranoside; DM, *n*-dodecyl-β-D-maltoside; T, Triton X-100; M, etioplast membranes.

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Materials and Methods

Plant material

Wheat (*Triticum aestivum cv.* Minaret) seedlings were grown on vermiculite and tap water during 6–7 days in darkness at 22 °C.

Etioplast membranes preparation and solubilization

Lysed etioplasts, prepared as in [13], were washed in 25 mM Hepes-NaOH buffer, pH 7.6, with 25 mM sucrose and 1 mM $MgCl_2$ by a 10 min centrifugation at $4300 \times g$. The pellet was resuspended in 25 mM Hepes-NaOH buffer, pH 7.6, with 25% (v/v) glycerol and 1 mM $MgCl_2$. This preparation is referred to as intact membranes. For solubilization, an incubation of 15 min on ice with the indicated concentration of *n*-octyl-β-D-glucoside, *n*-dodecyl-β-D-maltoside or Triton X-100 was followed by a 10 min centrifugation at $7000 \times g$. The clear supernatants ("extracts") were stored in liquid nitrogen until used. All operations were done at 0–4 °C under dim green light.

Fluorescence and absorbance spectroscopy

Fluorescence spectra were measured at 77 K under 436 nm exciting light (half bandwidth: 30 nm) in the device described in [14] but with the detector being replaced by an EMI 92558 B photomultiplier. Spectra were not corrected for the detector sensitivity.

Absorbance spectra were measured in a 5 mm cuvette with an optical spectrometric multichannel analyzer (OSMA, Princeton Instruments) equipped with a 1024 channels PDA diode array detector and a Spex Model 1681 monochromator. The low intensity measuring light ($\lambda > 580$ nm, $9\ W \cdot m^{-2}$) was provided by a stabilized Oriel light source. A quartz halogen QH-300 fiberoptic illuminator (Labsphere) was used for actinic illumination at right angle to the path of measuring light. The light was filtered through a broad blue filter with maximum transmission at 440 nm (bandwidth: 55 nm, intensity: $115\ W \cdot m^{-2}$). In this combination absorbance spectra could be measured during actinic illumination without significant interference from actinic light or fluorescence of the sample.

Results

Pchlide spectral forms and phototransformability in detergent extracts of etioplast membranes

n-Octyl-β-D-glucoside (OG), *n*-dodecyl-β-D-maltoside (DM) and Triton X-100 (T) were tested for their ability to solubilize phototransformable Pchlide-protein complexes from intact etioplast membranes (M). Fig. 1 shows the effect of different concentrations of these detergents on the 77 K fluorescence intensities measured in the membrane extracts at 632 nm (fluorescence of inactive $Pchlide_{630}$) and 655 nm (fluorescence of active $Pchlide_{650}$). Solubilization with Triton was accompanied by an almost complete disappearance of the 655 nm fluorescence. A high F_{655}/F_{632} ratio could only be obtained at low concentrations of this detergent (1 mM or less), when solubilization was only partial. OG and DM gave fractions with relatively intense 655 nm fluorescence at concentrations lower than 10 mM. Triton X-100 and DM had to be used at concentrations higher than their critical micellar concentration (CMC) for efficient solubilization whereas OG was most efficient at a concentration about half CMC.

We have analyzed in more detail the properties of the membrane extracts obtained with 7 mM OG, DM or Triton since, as shown in Fig. 2, they contained different proportions of the three Pchlide forms. Intact membranes showed a main band of photoactive Pchlide at 657 nm, a weak band of

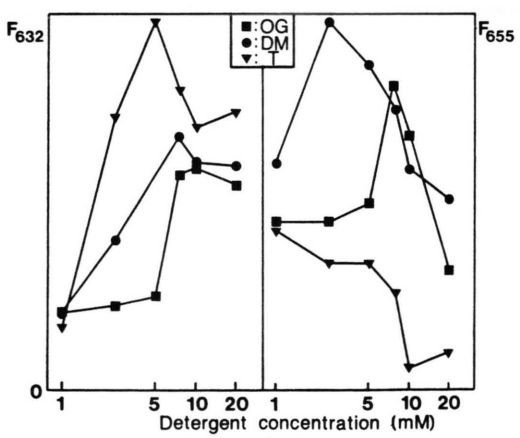

Fig. 1. Relative 77 K fluorescence intensities at 655 and 632 nm as a function of the concentration of octyl-glucoside (OG), dodecyl-maltoside (DM) or Triton X-100 (T) used for membrane solubilization.

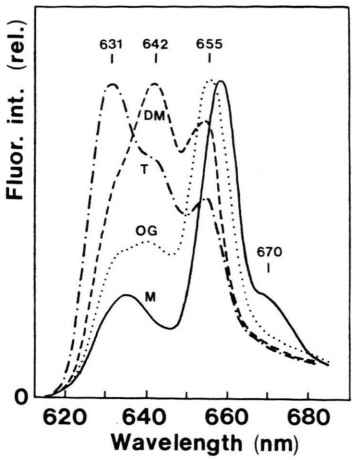

Fig. 2. 77 K fluorescence spectra of etioplast membranes (M) and of the extracts obtained after solubilization with 7 mM octyl-glucoside (OG), dodecyl-maltoside (DM) or Triton X-100 (T). The spectra were normalized at their maximum.

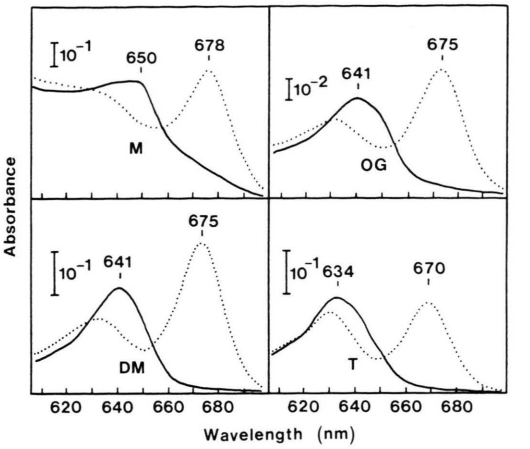

Fig. 3. Room temperature absorbance spectra before (——) or immediately after (– – –) a 10 s actinic light pulse in intact etioplast membranes (M) and in extracts of membranes solubilized with 7 mM octyl-glucoside (OG), dodecyl-maltoside (DM) or Triton X-100 (T).

inactive Pchlide at 633 nm and a shoulder around 670 nm. Solubilized membranes showed always three emission components of varying amplitudes with maxima around 632, 642 and 655 nm (Fig. 2). These three maxima are slightly blue-shifted when compared to the known maxima of the three main Pchlide forms at 633, 645 and 657 nm *in vivo* or in membrane fractions [15, 16]. The main fluorescence band was at 655 nm in the OG extract, 642 nm in the DM extract and 631 nm in the Triton extract. The 670 nm component (a minor form of Pchlide according to [15]) was no longer detected after solubilization.

Room temperature absorbance spectra of the same preparations before and after illumination are shown in Fig. 3. The Pchlide maximum (at 650 nm in intact membranes) was shifted to 641 – 642 nm in OG- and DM-solubilized membranes and to 635 nm in Triton-solubilized membranes. A 10 s illumination by white light showed that the photoconvertibility was higher in DM- and OG- than in Triton-solubilized membranes as revealed by the lower relative amplitude of the Chlide band after illumination in the Triton extract. By comparing the amplitudes of Pchlide absorbance in Fig. 3 it appears that DM and Triton at 7 mM could solubilize almost entirely the Pchlide-protein complexes whereas OG at the same concentration was less efficient in that respect.

The three Pchlide components found in 77 K fluorescence spectra could not be distinguished clearly in room temperature absorbance spectra due to the broadness of the bands but it is reasonable to assume that the differences in absorbance spectra correspond to different proportions of the three Pchlide absorbance bands at around 628 – 630, 636 – 638 and 650 nm which correspond to the three fluorescence bands [3, 5]. The comparison of the fluorescence ratio F_{655}/F_{642} with the absorbance ratio A_{650}/A_{638} in intact membranes and in the three detergent extracts indicates efficient energy transfer from $Pchlide_{638}$ to $Pchlide_{650}$ in intact membranes and in the OG extract (Table I).

Table I. Ratio of the 77 K fluorescence intensities at 655 and 642 nm and of the room temperature absorbances at 650 and 638 nm in intact membranes (M) and in membrane extracts obtained with 7 mM octyl-glucoside (OG), dodecyl-maltoside (DM) or Triton X-100 (T) respectively.

	F_{655}/F_{642}	A_{650}/A_{638}
M	4.15	1.43
OG	2.12	0.81
DM	0.89	0.71
T	0.79	0.52

Kinetics of the phototransformation and changes in the difference spectra during the phototransformation

The kinetics of phototransformation were studied by recording a series of successive absorbance spectra during an illumination with actinic blue light at room temperature. A spectrum was first measured before actinic illumination. Then 10 recordings of 0.5 s duration each were done consecutively, the beginning of the first recording being synchronized with the onset of actinic illumination. An example of the kind of data obtained by this procedure with DM-solubilized membranes is shown in Fig. 4. During the illumination the Chlide absorbance maximum shifted progressively towards shorter wavelengths. This rapid shift was observed with the three types of solubilized material, but was most pronounced in the case of Triton.

Kinetics of Chlide formation could be reconstituted by calculating the absorbance differences at maximum Chlide absorbance between each spectrum of the series and the spectrum of the non-illuminated sample. Fig. 5 shows the kinetics obtained for the DM-, OG- and Triton-solubilized membranes which were or were not incubated with 10 mM NADPH for 15 min on ice before measurement. The kinetics of various samples were normalized on the maximum Chlide absorbance measured after 10 s without NADPH.

The rate of Chlide formation decreased in the order OG > DM > Triton, which may be related to

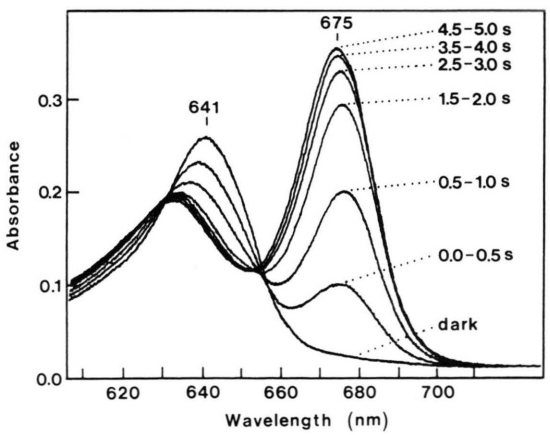

Fig. 4. Selected absorbance spectra of a series of 10 recordings (0.5 s duration each) during actinic illumination of the 7 mM dodecyl-maltoside extract. Recording periods as indicated.

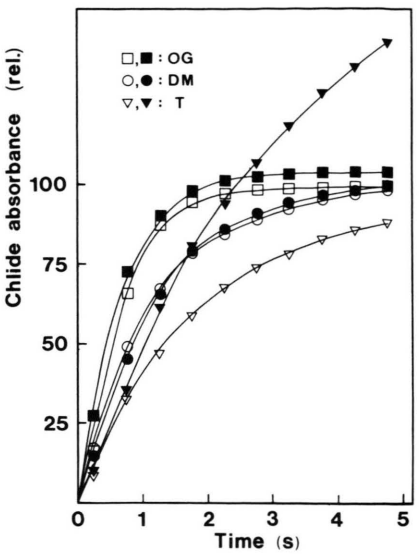

Fig. 5. Reconstituted kinetics of Chlide accumulation during actinic illumination of the 7 mM octyl-glucoside (OG), dodecyl-maltoside (DM) or Triton X-100 (T) extract. Open symbols: no addition. Closed symbols: 10 mM NADPH was added 15 min before illumination. The kinetics were normalized on the maximum Chlide absorbance measured after 10 s illumination in the absence of NADPH.

the decreasing proportion of long-wavelength Pchlides as revealed in fluorescence and absorbance spectra of dark samples (see Fig. 2 and 3). A large increase of the extent of Chlide formation in the presence of NADPH was observed in Triton-solubilized membranes but not in DM- or OG-solubilized ones. In the Triton extract supplemented with NADPH it was even possible to exhaust Pchlide completely if the illumination was prolonged up to 30 s (not shown). We thus suspected that even $Pchlide_{630}$, although usually considered as an inactive form, could be in fact rapidly photoreduced in this case, although maybe more slowly than the two photoactive forms. To verify this, we calculated difference spectra of partial phototransformation at the beginning or after some seconds of illumination. Fig. 6 compares difference spectra reflecting partial phototransformation in the periods 0.5–1.0 s and 4.0–5.0 s in Triton- and DM-solubilized membranes (no valuable data were obtained with OG extracts due to the lower signal-to-noise ratio). The difference spectra of Fig. 6 reveal that long-wavelength Pchlides ($Pchlide_{638}$ and $Pchlide_{650}$) are transformed at the beginning of illumination (spectra a)

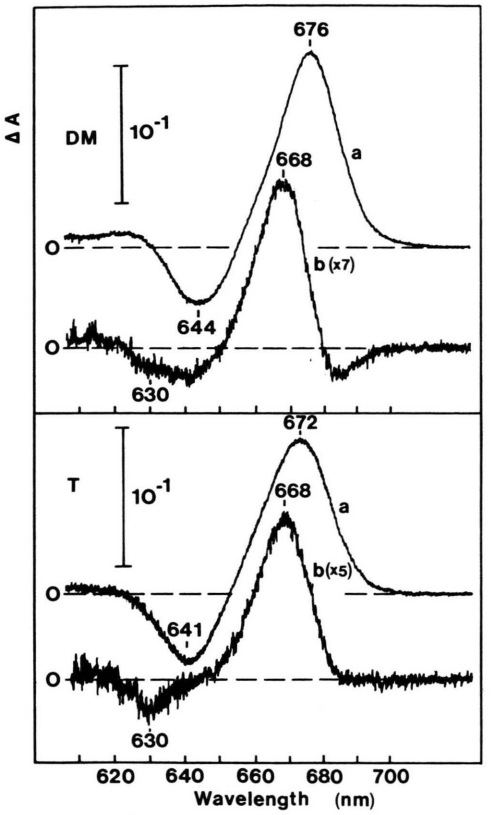

Fig. 6. Difference absorbance spectra calculated at the beginning (a: time 1 s minus time 0.5 s) or at the end (b: time 5 s minus time 4 s) of phototransformation in the 7 mM dodecyl-maltoside (upper part) or the 7 mM Triton X-100 (lower part) extract.

whereas a transformation of short-wavelength Pchlide ($Pchlide_{630}$) occurred later, resulting in the appearance of a negative shoulder at 630 nm in the DM extract and of a single negative band at this wavelength in the Triton extract (spectra b). The difference spectra calculated in the period 4.0 to 5.0 s (spectra b) also show that the Chlide produced from $Pchlide_{630}$ has an absorbance maximum around 668 nm (this value may however be somewhat underestimated due to a slight interference of a blue shift of the already formed Chlide during illumination).

Chlide shift and photoactive Pchlide regeneration after phototransformation

The effect of 10 mM NADPH on the position of the Chlide absorbance maximum and on the extent of regeneration of photoactive Pchlides after illumination is depicted in Table II for intact and solubilized membranes. In intact membranes NADPH induced the red Chlide shift from 678 to 684 nm during the 15 min dark period following illumination. This shift corresponds to the rapid Chlide shift normally found in leaves [17, 18]. It was not observed in solubilized membranes.

The regeneration of photoactive $Pchlide_{638}$ and $Pchlide_{650}$ after illumination was evaluated by measuring the amplitude of the absorbance decrease at 645 nm during a second 10 s illumination spaced from the first one by 15 min of darkness. It was significantly enhanced by NADPH in all preparations except in Triton-solubilized membranes where it did not occur. The NADPH-induced regeneration of photoactive Pchlide in DM-solubilized membranes is illustrated in Fig. 7, which shows difference spectra reflecting the absorbance changes during the 15 min dark period after a first 10 s illumination, with or without NADPH. Photoactive Pchlides absorbing around 645 nm (probably a mixture of $Pchlide_{638}$ and $Pchlide_{650}$) were accumulated at the expense of $Pchlide_{630}$. Absorbance changes in the Chlide region reflect a slow dark shift of Chlide which was only slightly inhibited by NADPH.

Discussion

Etioplast membranes contain three major spectrally distinct Pchlide-protein complexes. The photoactive $Pchlide_{650}$ and $Pchlide_{638}$ are stable complexes between the reductase, Pchlide and NADPH

Table II. Effect of 10 mM NADPH on the position of the Chlide absorbance maximum after a 15 min dark period following a 10 s light pulse and on the extent of photoactive Pchlide regeneration as measured by the amplitude of the absorbance decrease at 645 nm (ΔA_{645}) at a second 10 s light pulse (expressed in % of the initial 645 nm absorbance of the dark sample). M: intact membranes; OG, DM and T: membrane extracts obtained with 7 mM octyl-glucoside, dodecyl-maltoside or Triton X-100, respectively.

	Position of Chlide maximum [nm]		ΔA_{645} [%]	
	Control	NADPH [10 mM]	Control	NADPH [10 mM]
M	673	684	3.2	14.6
OG	671	673	4.4	11.3
DM	673	674	3.2	10.1
T	670	670	<1.0	<1.0

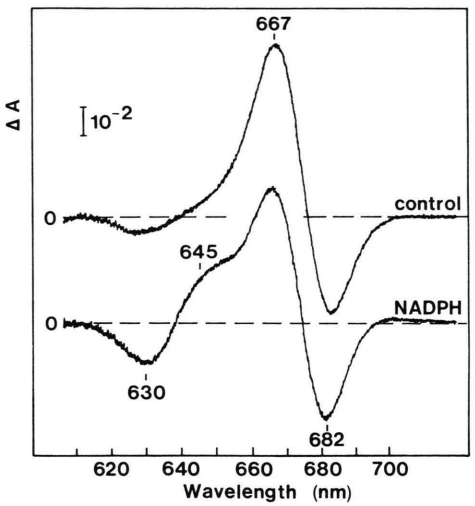

Fig. 7. Difference between the absorbance spectra recorded after a 15 min dark period or immediately after a 10 s actinic illumination in the presence or absence of 10 mM NADPH in the 7 mM dodecyl-maltoside extract.

[4]. They are transformed into Chlide-containing complexes within a few microseconds after light absorption by Pchlide [19]. The difference between $Pchlide_{650}$ and $Pchlide_{638}$ has been suggested to be due to different oligomeric states of the complex [6–8]. The third spectral form $Pchlide_{630}$ is not readily reducible in the light. This inactive Pchlide is either not bound to the enzymatic site for the photoreduction or is a pigment-enzyme complex lacking NADPH [4, 20]. In Pchlide holochrome or in solubilized membranes, the same spectral forms are generally found but the photoactive $Pchlide_{638}$, which is present in low amounts in intact plastids, becomes the major species due to a modification of $Pchlide_{650}$ to $Pchlide_{638}$ which results from partial disaggregation [6].

The comparison of the fluorescence and absorbance spectra of membranes solubilized by OG, DM or Triton shows that the relative amount of $Pchlide_{650}$ to $Pchlide_{638}$ decreased according to the detergent used, in the order OG > DM > Triton in our experimental conditions (7 mM during 15 min), suggesting an increasing disaggregation of the complexes. This is also reflected in the decreasing ratio of A_{650}/A_{638} and F_{655}/F_{642} (Table I). The differences between the two types of measurements (the fluorescence ratio being always higher than the absorbance ratio) is explained by excitation energy transfer from $Pchlide_{638}$ to $Pchlide_{650}$ [21]. This transfer is particularly efficient in OG extracts, suggesting also a large size of the complex obtained when using this detergent. Due to its shorter hydrophobic tail, OG preserves large size oligomers but has a low solubilizing efficiency as shown by the much lower Pchlide absorbance in OG extracts than in intact membranes or in the DM and Triton extracts (Fig. 3).

The kinetics of phototransformation obtained with the different extracts used show that increasing disaggregation of the complex decreases the rate of Chlide formation during illumination. Kinetic differences can be explained by the successive photoreduction of photoactive Pchlides ($Pchlide_{638}$ and $Pchlide_{650}$) and inactive $Pchlide_{630}$ as shown by the absorbance difference spectra recorded at the beginning or at the end of the phototransformation. The amount of $Pchlide_{630}$ is particularly high in the Triton extract and therefore its transformation contributes to a large extent of the kinetics. It was shown earlier [22] that addition of exogenous Pchlide to solubilized membranes results in its slow reduction to Chlide in the light if NADPH is added. Our results show that endogenous $Pchlide_{630}$ behaves in the same way even without NADPH addition. Since most Pchlide can be precipitated by high-speed centrifugation after Triton solubilization ([6] + data not shown) one must admit that $Pchlide_{630}$ is bound to the solubilized complex. It may be suggested that after detergent treatment $Pchlide_{630}$ represents a state of the pigment loosely bound to the reductase. In this state Pchlide is reduced into Chlide at a lower rate. The short-wavelength (668 nm) of the Chlide produced indicates that the pigment is under a monomeric state. It must be mentioned that earlier studies on chlorophyll formation *in vivo* showed that etiolated leaves fed with the Pchlide precursor δ-aminolevulinic acid accumulate a short-wavelength Pchlide that is slowly transformed to Chlide during a series of brief light flashes [23–25]. This transformation occurred through regeneration of $Pchlide_{650}$ in the min time scale between flashes and is not comparable to the much faster transformation of $Pchlide_{630}$ (in the s time scale) found here for the Triton extract, although this transformation may involve a rapid, unresolved step of $Pchlide_{650/638}$ regeneration.

In DM- and OG-solubilized membranes NADPH had only slight effects on the extent of phototransformation but induced the subsequent

regeneration of photoactive $Pchlide_{650}$ and $Pchlide_{638}$ from the remaining $Pchlide_{630}$. Such NADPH-induced regeneration of photoactive Pchlides from inactive Pchlide after a short irradiation is well documented in intact membranes [20, 26]. In the Triton extract a large enhancement of the phototransformation was found in the presence of NADPH but no dark regeneration of photoactive Pchlides was observed after irradiation. These results suggest that the regeneration of stable, photoactive $Pchlide_{650}$ and $Pchlide_{638}$ is dependent on a high degree of organization of protein subunits which was not preserved after Triton treatment.

The rapid shift from $Chlide_{678}$ to $Chlide_{684}$, which normally occurs in leaves [17, 18], was only found in intact membranes supplemented with NADPH. In solubilized membranes $Chlide_{678}$ rapidly shifted to $Chlide_{670}$ which most probably reflects Chlide dissociation from the protein [4] or aggregate dissociation [8]. This process was only slightly slowed down by NADPH. The formation of $Chlide_{684}$, which has been ascribed to a NADPH-Chlide reductase complex [4] is therefore particularly sensitive to modifications of the membrane environment. Previous experiments [13] have led to the proposal that the action of NADPH on the formation of $Chlide_{684}$ might involve another binding site than the one where the nucleotide acts as reductant in the phototransformation. It is possible that this site is inactivated or not retained during solubilization. Alternatively $Chlide_{684}$ formation could be related to an aggregation process (as proposed in [17]) that is prevented by the detergent.

Acknowledgements

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