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Use of fluorescence probes 1-aniline-8-naphthalene sulfonate and pyrene for studying the localisation of proteins in inner membranes from wheat etioplasts

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Abstract

The fluorescence probes 1-aniline-8-naphthalene sulfonate (ANS) and pyrene were applied for characterisation of the light-induced changes in etioplast inner membranes (EPIMs) from 7 d-old dark-grown wheat seedlings (*Triticum aestivum* L. cv. Pobeda). The major aim was to obtain information about the localisation of membrane proteins in the EPIMs, using probes situated in different regions of the membranes. The quenching of tryptophan fluorescence showed that the main parts of proteins were accessible to the pyrene buried in the lipid bilayer which suggests that most of the proteins also enter the lipid bilayer. The substantial quenching of the tryptophan fluorescence by the surface-situated ANS demonstrated that a part of the tryptophan residues was probably localised close to the membrane surface. The registered changes after irradiation could be explained by the presence of large aggregates of NADPH-protochlorophyllide oxidoreductase (POR), protochlorophyllide (PChlide) and NADPH in membranes that start to disconnect and redistribute along the prothylakoids.

Additional key words: chlorophyllide; lipids; NADPH-protochlorophyllide oxidoreductase; prothylakoids; protochlorophyllide; *Triticum aestivum* L.; tryptophan.

Introduction

The sequence of chlorophyll (Chl) synthesis reactions in dark grown higher plants is blocked at the level of the light-dependent reduction of protochlorophyllide (PChlide) to chlorophyllide (Chlide). Two different types of connected to each other inner membranes (usually referred to as etioplast inner membranes, EPIM, Ryberg

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Abbreviations: ANS - 1-aniline-8-naphthalene sulfonate; Chlide - chlorophyllide; EPIMs - etioplast inner membranes; PChlide - protochlorophyllide; POR - NADPH-protochlorophyllide oxidoreductase; PT - prothylakoids; PLB - prolamellar body.

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and Sundqvist 1982) appear in the plastids of etiolated plants, the prolamellar bodies (PLBs) and the prothylakoids (PT).

The enzyme, responsible for transformation of PChlide to Chlide, NADPH-protochlorophyllide oxidoreductase (POR; EC 1.3.1.33) is mainly localised in PLBs. It amounts to about 90 % of the total proteins (Griffiths and Oliver 1984, Dehesh and Ryberg 1985, Ryberg and Dehesh 1986) and is thus the dominating polypeptide (Oliver and Griffiths 1980, Dehesh and Ryberg 1985). The PLBs also contain small amounts of other proteins (Lindsten *et al.* 1988). A minor amount of POR has also been found in the PTs (Ryberg and Sundqvist 1982, Lindsten *et al.* 1988). Some other peptides, like the α- and ß-subunits of ATPase, have been also found in the PT (Selstam and Sandelius 1984) together with cytochrome *f*, cytochrome b_6, plastocyanin, ferredoxin and ferredoxin-dependent reductase (Savchenko and Chaïka 1988). The exact localisation in the lipid bilayer of the POR and other membrane proteins is not known; it may be loosely attached to the PLB membranes (Griffiths and Oliver 1984). Investigations of the hydrophobicity of the POR show that the enzyme is an amphiphilic protein tightly associated with the membrane (Selstam and Widell-Wigge 1989, Widell-Wigge and Selstam 1990) and is resistant even to harsh washing procedures (Grevby *et al.* 1989). Hydropathy profiles do not indicate any clear membrane-spanning or thylakoid transfer domains (Spano *et al.* 1992) which might indicate a peripheral association of the enzyme with the membrane on the stromal side. However, the high level of hydrophobic amino acids (about 30 %) (Griffiths 1991) suggests that the protein is mostly integral. The secondary structure prediction based on the amino acid composition revealed no obvious membrane spanning region of the POR from oat (Darrah *et al.* 1990) and *Arabidopsis* (Benli *et al.* 1991).

Irradiation induces a series of changes of the etioplast membranes, especially breaking-down of the PLBs ultrastructure. Böddi *et al.* (1991) and Wiktorsson *et al.* (1992, 1993) suggest that in the PLBs, POR is organised in large aggregates which dissociate into smaller units after irradiation. The POR is then relocated from PLBs to the PTs and redistributed in the etioplast (Dehesh *et al.* 1986, Ryberg and Dehesh 1986), especially along the thylakoids (Artus *et al.* 1992).

In our previous papers (Denev and Minkov 1992, Minkov and Denev 1992) we reported some light-induced changes of the EPIMs conformation, using changes of the 1-aniline-8-naphthalene sulfonate (ANS) binding to the isolated etioplast inner membranes and changes of the microviscosity, measured by pyrene excimerization.

In the present paper, we used the energy transfer from tryptophan residues of the membrane proteins to both ANS and pyrene to obtain some information about the localisation of the proteins in the EPIMs.

Materials and methods

Wheat plants (*Triticum aestivum* L. cv. Pobeda) were grown in darkness in a mixture of peat and sand at 25 °C. Etioplast inner membranes (EPIMs) were isolated from 3 cm leaf sections taken 1 cm from the top of the leaves according to the method

described by Ryberg and Sundqvist (1982) with the difference that the osmotic shock was performed with TES-HEPES buffer without NADPH. The EPIMs were pelleted by centrifugation at 7700 × *g* (15 min), and the pellet was resuspended in the same buffer and used for investigation without any sonication.

The total protein content of the EPIMs was measured with *Coomassie Brilliant Blue G250* as described by Bearden (1978). The absorbance was measured at two wavelengths, 595 and 750 nm, and bovine serum albumin was used as a standard. The PChlide and Chl contents were calculated by the fluorescence emission at 626 and 670 nm in 80 % acetone, using the molar absorption coefficients given by Kahn (1983).

Membrane suspensions containing 100 kg m^{-3} protein were titrated with different amounts of the probes. ANS (*Reachim*) was taken from a 1 mM stock solution (in bidistilled water) and added to the membranes until the final concentrations of the probe was 10, 15 or 20 μM. The pyrene (*Serva*) was added from a 1 mM stock solution (in ethanol) in small portions (1 μmol per min) as described by Dobretsov (1989) to a final concentration of 4, 6, 8, or 10 μM. In order to reach good integration with the membranes, the latter were incubated with the probes on ice for 1 h.

Samples from the EPIMs suspensions with and without probes were taken for a low temperature fluorescence analysis before and after flash irradiation. Fluorescence emission spectra were recorded at 77 K. The excitation wavelength was 440 nm.

Phototransformation of the PChlide was performed by giving 5 flashes of "white light" from a photographic flash, at a distance of 10 cm (impulse energy 120 J).

The nonradiative energy transfer between the tryptophan of membrane proteins and the probes was measured by quenching of tryptophan fluorescence at 345 nm after excitation at 286 nm. The presence of other fluorescent molecules in the EPIMs ($NADPH/NADP^+$, PChlide/Chlide) made it impossible to prove that all registered changes of the fluorescence were only due to the singlet-singlet transfer of the energy from tryptophan to the fluorescence probes. Because of that we restricted our investigations to calculating the accessible part of the tryptophan in the protein(s) of the EPIMs for the quenching, caused by the fluorescence probes. The energy transfer occurs if the distance between tryptophan and the probe is less than 1.2 times the Förster radius (R_0), which is equal to 2.7-2.8 nm, both for the pyrene and ANS (Dobretsov 1989). Tryptophan residues according to this condition could be divided into two parts: quenched ß and nonquenched (1 - ß) from the probe. A method for calculating ß was described in Dobretsov (1989):

$$ß = a/[F_0/(F_0\text{-}F)]\text{min} \quad (1)$$

where *a* is a constant either equal to 0.75, if the acceptors are in the volume of the lipid phase (pyrene), or equal to 0.65, if they are on the surface (ANS) (Dobretsov 1989). $[F_0/(F_0\text{-}F)]$min is the maximum possible quenching of tryptophan fluorescence in the largest concentration (infinite) of the acceptor (the probe).

The investigations were performed as follows: first the quenching of tryptophan fluorescence by probes in nonirradiated samples was measured, then the samples were irradiated with 5 flashes from a photographic flash at a distance of 10 cm (impulse energy 120 J) and the quenching of tryptophan fluorescence was measured

immediately. The samples were later kept in darkness and measurements were performed at the 10th, 20th and 30th min after the brief irradiation. The described scheme was chosen by two reasons: (*1*) The Shibata shift and most of the light-induced changes appear during the first 30 min. (*2*) The limited number of measuring points prevents samples of non-specific destruction, due to the UV radiation used to excite the tryptophane fluorescence.

To measure the fluorescence excitation spectra of pyrene, large unilamellar vesicles were prepared using extrusion through a double 200 nm polycarbonate filter and Liposofast Basic Equipment (*AVESTINI*, Ottawa, Canada) (MacDonald *et al.* 1991). The lipid composition of the vesicles was MGDG : DGDG : PG (40 : 40 : 20, m/m/m) (Selstam and Sandelius 1984).

Means of 5 measurements are given for all experiments. The results were statistically processed. All the fluorescence measurements were performed with a *SLM 8000C* fluorescent spectrophotometer upgraded with *SLM 8100* motherboard (*SLM Aminco*, Urbana, U.S.A.) and accompanying software. Excitation and emission bandwidths were 8 nm, and the integration time was 0.1 s. All spectra were smoothed 10 times (using a fixed bandwidth, sharp cut-off, three point and low pass digital filter) and corrected for variations in the sensitivity of the photomultiplier.

Results

In order to see if the system of PChlide reduction is affected by the probes, we initially investigated the influence of ANS and pyrene on the activity of the PChlide:POR:NADPH complex. The addition of ANS to an EPIMs sample caused no changes in the PChlide and Chlide spectral forms (Fig. 1*A,B*). When pyrene was added to EPIMs in a concentration of 10 μM, a small decrease of the fluorescence at 657 nm was found, corresponding to a decrease of the phototransformable PChlide (Fig. 1*C*). This effect was mainly due to the presence of about 1 % ethanol as there was no substantial difference between samples with pyrene and those with the same amount of ethanol and no probes (results not shown). However, the ethanol of pyrene-containing samples did not influence significantly the rate of photoreduction since the changes of PChlide and Chlide amounts before and after irradiation (Fig. 2) were similar in the probe-free EPIMs and in the EPIMs-samples incubated with both probes.

Tryptophan fluorescence in EPIMs samples free of probes did increase after irradiation (Fig. 3*A*). Probes-containing samples did not show the same pattern of changes in the tryptophan fluorescence as the control (Fig. 3*B,C*). Since all the other conditions had not been changed, we assumed that it was due to an effective quenching of tryptophan fluorescence by probes. We used the obtained values to calculate the value of quenched tryptophan residues (ß). Because of the difference between the constants *a* (see Materials and methods; Eq. 1), the largest values of ß for ANS and pyrene were also different. To compare the results, the values were recalculated as a percent of the maximal possible value of ß, and showed that in the non-irradiated EPIMs 38 % of the tryptophan was available for quenching by ANS.

The accessible part of tryptophan residues for quenching by pyrene was 48 and 70 % by both probes (Fig. 4). Within the first minute after flash irradiation, the accessibility of the tryptophan residues by pyrene rapidly increased to 54 %. Later only a slight increase was seen. The ANS-containing samples did not show statistically verified changes of tryptophan quenching during the first minute. Only after the 10th min a slow decrease in quenching was registered. There were no significant variations of tryptophan quenching in samples which contained both probes.

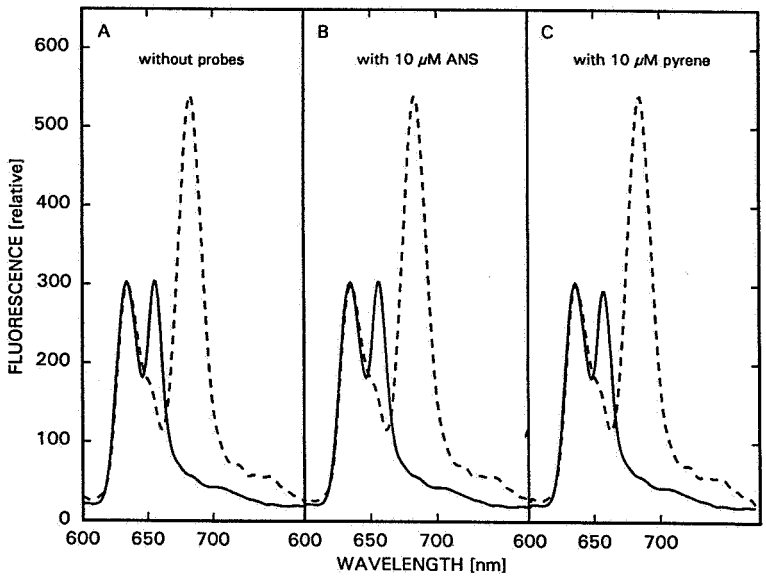

Fig. 1. Fluorescence emission spectra at 77 K of isolated EPIMs. The samples *B* and *C* were incubated on ice in darkness for 1 h with 10 μM ANS and pyrene, respectively. Before the measurements, chlorophyllide-containing samples were irradiated with 5 flashes of "white light" and then immediately frozen in liquid nitrogen. The excitation wavelength was 440 nm.

To make conclusions about processes in the inner etioplast membranes, the probable probe localisation must be defined. The fluorescence probe ANS is able to bind to the membrane proteins and to integrate at the level of the polar heads of the membrane lipids (Slavik 1982). According to NMR studies, the nonpolar, hydrophobic probe pyrene is localised in the fatty acyl region of the membranes (Pownall and Smith 1973, Podo and Blasie 1977). We assume that probably the main part of the pyrene molecules can be found in the lipid phase of the EPIMs, because there is no quenching of the pyrene fluorescence after incorporation of an equimolar amount of iodine ions (results not shown).

The probable binding of pyrene to the EPIMs-membrane proteins was also investigated. The pyrene molecules, localised in lipid bilayer, are able to form excited dimers (excimers) (Pownall and Smith 1973). Binding of pyrene to proteins prevents formation of excimers (Dobretsov 1989). To examine pyrene-protein interactions, we recorded pyrene emission spectra in EPIMs using excitation wavelength of 286 nm.

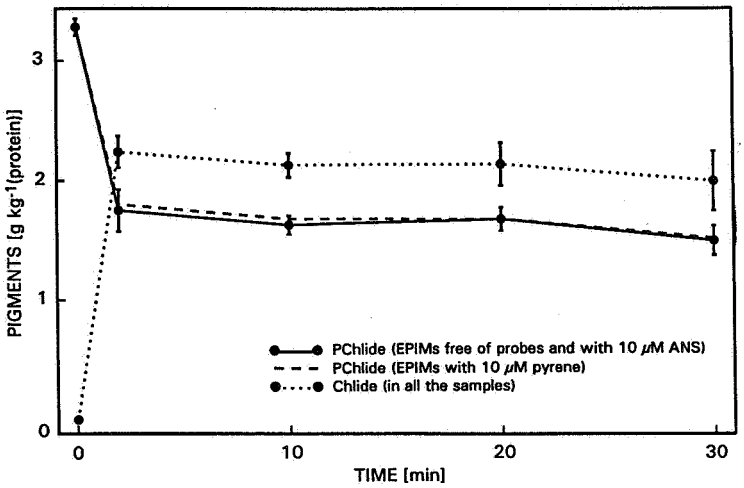

Fig. 2. Light-induced changes of pigment amounts in EPIMs. The membranes were incubated on ice for 1 h with and without ANS and pyrene in darkness. The samples (except the control) were irradiated with 5 flashes of "white light" and during the following dark period (30 min) certain volumes of samples were extracted with acetone for measuring protochlorophyllide and chlorophyllide.

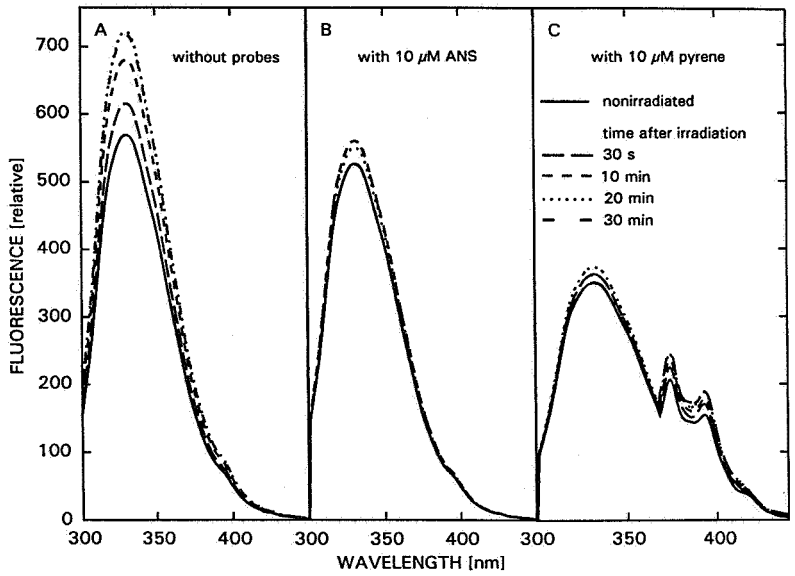

Fig. 3. Changes in tryptophan fluorescence of EPIMs and its quenching by the probes. Samples were taken from EPIMs incubated in darkness on ice for 1 h. The membranes in *B* and *C* were incubated with 10 μM ANS or 10 μM pyrene, respectively. The samples (without the control) were irradiated with 5 flashes of "white light" and the fluorescence was recorded during the following dark period (30 min). The excitation wavelength was 286 nm.

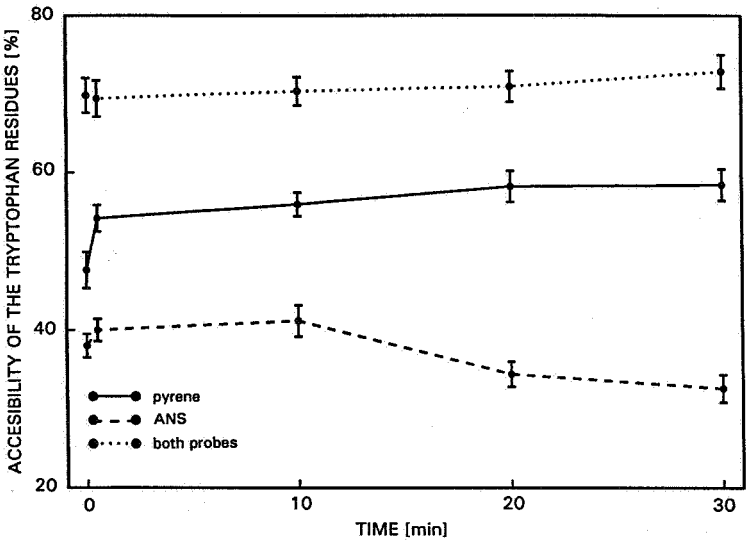

Fig. 4. Changes of the accessibility of tryptophan residues for quenching by the probes. The samples (without the control) were irradiated with 5 flashes of "white light" and the fluorescence at 345 nm was measured during the following dark period (30 min). The excitation wavelength was 286 nm.

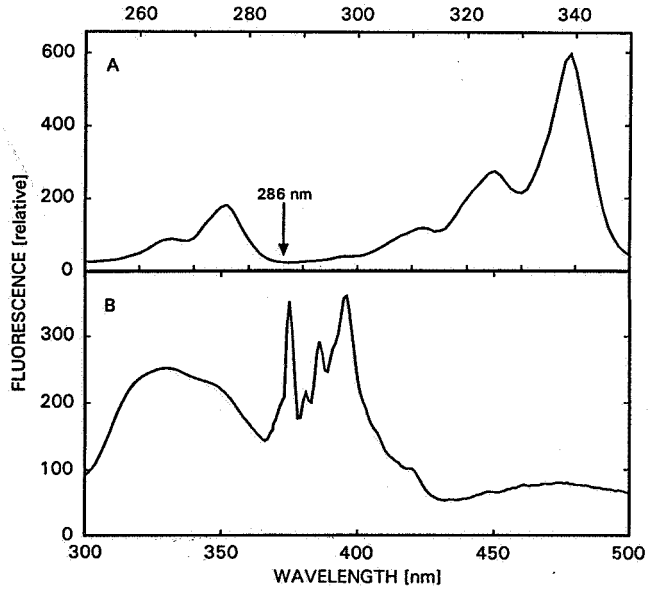

Fig. 5. *A*: Fluorescence excitation spectra of pyrene in model lipid vesicles (MGDG : DGDG : PG = 40 : 40 : 20, m/m/m). The emission wavelength was 375 nm. *B*: Fluorescence emission spectra of isolated EPIMs. The sample was incubated on ice in darkness for 1 h with 10 μM of pyrene. The excitation wavelength was 286 nm.

In model lipid vesicles, the excitation spectra of pyrene did not show the peak at 286 nm (Fig 5*A*), therefore the recorded emission of the pyrene in EPIMs at the excitation wavelength 286 nm (Fig. 5*B*) was due to the transfer of excitation energy from tryptophan residues of membrane proteins. Such type of transfer is possible only if the distance between proteins (tryptophan residues) and pyrene is not longer than 3.1 nm which means that we have registered emission only from pyrene molecules which closely surround the EPIMs proteins. There is still a significant emission of the excimers (Fig. 5*B*), which means that the major part of pyrene molecules involved of energy interaction with proteins is not bound to them.

Discussion

The energy transfer between tryptophan and the used probes is possible at a distance not longer than 1.2 times of the critical Förster radius (R_0), equal either to 2.6 nm for ANS or 2.8 for pyrene (Dobretsov 1989). The quenching of tryptophan fluorescence by probes in the membranes showed that the main part of proteins was accessible to pyrene and thus penetrated into the lipid bilayer. The substantial quenching of the tryptophan fluorescence by ANS suggests that a part of the tryptophan residues is probably localised close to the membrane surface.

The relationship between the photoreduction of PChlide and the changes of quenching accessibility of tryptophan residues of EPIMs-proteins were examined by calculation of linear correlation coefficients. The correlation coefficient between the changes in the accessibility of tryptophan residues for pyrene and the concentration of PChlide was -0.85. The value for ANS was 0.70. The obtained values were higher than 0.5 which meant that the found changes were surely connected to the photoconversion of PChlide.

Our results can be explained using the idea that POR, together with PChlide and NADPH, is organised in large aggregates in the EPIMs and especially in the PLBs (Böddi *et al.* 1991, Wiktorsson *et al.* 1992, 1993). The assembled POR molecules are tightly attached to each other within the aggregates. In that case the probes which interact with tryptophan can be regarded as associated on the aggregates' surface. If a part of tryptophan residues is localised within the aggregates they are not be able to interact with probes *via* an energy transfer.

The increase of tryptophan fluorescence in the EPIMs samples free of probes (Fig. 3*A*) and the changes in quenching by the probes immediately after irradiation (Fig. 4) suggest that the aggregated proteins are disconnected and start to separate. Such separation opens the possibility for interaction between probes and the aggregated tryptophan residues, which are shielded in the inner part of the EPIMs. On the other hand, the complicated dynamics of the quenching suggests that in these conditions not only the aggregational state of membrane proteins is affected, but also their redistribution within the EPIMs, perhaps along the PTs.

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