BULG. J. PLANT PHYSIOL., 1996, 22(1–2), 40–52

MEMBRANE PROTEINS LOCALIZATION IN NON-IRRADIATED AND FLASH IRRADIATED PROLAMELLAR BODIES AND PROTHILAKOIDES, ISOLATED FROM WHEAT, MEASURED BY FLUORESCENCE PROBES 1-ANILINE-8-NAPHTHALENE SULFONATE AND PYRENE

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Received July 1, 1996

Summary. The enzyme, responsible for phototransformation of protochlorophyllide (Pchlide) to chlorophyllide (Chlide) in etiolated plants is NADPH-protochlorophyllide oxidoreductase (EC 1.3.1.33, POR). The membrane localisation of the POR and its changes after irradiation are not entirely investigated. In the present work the energy transfer from tryptophan residues of membrane proteins to the fluorescence probes 1-aniline-8-naphthalene sulfonate (ANS) and pyrene was used to study the aggregaton state of the POR, its localization in the lipid phase of the membranes and the enzyme conformational changes after irradiation. Prolamellar bodies (PLBs) and prothylakoids (PTs) were isolated from wheat (*Triticum aestivum* L. cv. Kosack) and incubated with the fluorescence probes. Changes in protein – probe interactions of the PLBs after irradiation and dark incubation showed that in non-irradiated PLBs, POR molecules together with certain amounts of Pchlide and NADPH are organized in large aggregates. The used brief irradiation induces disconnection of the POR molecule and its lateral movement within the lipid phase. Quenching of tryptophan fluorescence and its change after irradiation showed that a major part of POR is localized close to the membrane surface, most probably on the level of lipid polar heads. This supports the idea of other authors, based on the hydrophobicity plot, and the hydropathy of protein, that the enzyme is not an integral membrane protein and is most probably localized on the membrane surface.

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***Key words*:** etioplast, fluorescence probes, fluorescence spectra, NADPH-protochlorophyllide oxidoreductase, protochlorophyllide, *Triticum aestivum* L., wheat

***Abbreviations*:** ANS – 1-aniline-8-naphthalene sulfonate; Chlide – chlorophyllide; Pchlide – protochlorophyllide; POR – NADPH-protochlorophyllide oxidoreductase; PLB – prolamellar body; PT – prothylakoid; Trp –tryptophan

Introduction

The enzyme, responsible for phototransformation of protochlorophyllide (Pchlide) to chlorophyllide (Chlide) in etiolated plants is NADPH-protochlorophyllide oxidoreductase (EC 1.3.1.33, POR). Its amount is about 90% of the total proteins of prolamellar bodies (PLBs) (Griffiths et al., 1984; Ryberg and Dehesh, 1986). PLBs also contain small amounts of other proteins (Lindsten et al., 1988). A minor amount of POR has also been found in the prothylakoids (PTs) (Lindsten et al., 1988). Some other peptides, like the α and β-subunits of ATP-ase (Selstam and Sandelius, 1984), cytochrome *f,* cytochrome b_6, plastocyanin, ferredoxin and ferredoxin-dependent reductase (Savchenko and Chayka, 1988) have also been found in the PTs.

The localisation of POR in the lipid bilayer is not clearly known. Some investigations with washing-out of the enzyme (Grevby at al., 1989; Selstam and Widell-Wigge, 1989; Widell-Wigge and Selstam, 1990) and the high level of hydrophobic amino acids – about 30% (Griffiths, 1991), suggest that the protein is mostly integral. Hydropathy profiles (Spano et al., 1992) and the secondary structure prediction, based on the amino acid composition, reveal no obvious membrane spanning region of the POR (Darrah et al., 1990; Benly et al., 1991) which might indicate a peripheral association of the enzyme. Böddi et al. (1991) and Wiktorsson et al. (1992) suggest that in the PLBs POR is organized in large aggregates which dissociate into smaller units after irradiation and the enzyme is then relocated from PLBs to the PTs and redistributed in the etioplast (Ryberg and Dehesh, 1986; Artus et al., 1992).

The fluorescence probe 1-aniline-8-naphthalene sulfonate (ANS) is able to bind the membrane proteins and to integrate at the level of the polar heads of the membrane lipids (Slavik, 1982). The non-polar, hydrophobic probe pyrene, is localized in the fatty acid region of the membranes (Podo and Blasie, 1977). An energy transfer from tryptophan (Trp) residues of the membrane proteins to ANS and pyrene can give information about localization of the proteins in the membranes (Dobretsov, 1989).

In previous papers we have reported some light-induced changes of ANS binding (Denev and Minkov, 1992), the microviscosity, measured by pyrene excimerization (Minkov and Denev, 1992), and protein localization in isolated total inner

etioplast membranes (Denev and Minkov, 1996). Due to the complex structure and composition of the object it was not possible to make conclusions about processes localized in PLB and PT membranes. In the present paper we used isolated PLBs and PTs. PLB membrane composition is more simple from a biochemical point of view in comparison with other etioplast membranes, due to the prevailing presence of POR. It also has an unique structure, which undergoes dynamic light-induced changes, the latter being performed mostly by the changes of the aggregational state of POR protein. Since POR is the major enzyme of the PLBs, it is possible to examine the changes in the aggregational state and the localization of POR which appear after a brief illumination, and which is consider to be the main reason of PLBs breaking down after irradiation of etioplasts.

Material and Methods

Wheat plants (*Triticum aestivum* L., cv. Kosack) were grown in darkness in a mixture of peat and sand at 25°C as described earlier (Denev and Minkov, 1992). PLBs and PTs membranes were isolated according to Ryberg and Sundqvist (1982). Before the measurements PLBs and PTs were pelleted by centrifugation and resuspended in sucrose-free TES-HEPES buffer (1 mM $MgCl_2$, 1 mM EDTA, 20 mM TES, 10 mM HEPES, adjusted to pH 7.2 with KOH). The total protein content of the membranes was measured with Coomassie Brilliant Blue G250 as described by Bearden (1978). Samples from the PLBs and PTs suspensions with and without probes were taken for a fluorescence pigment quantitative analysis before and after flash irradiation. Fluorescence emission spectra of the pigments were recorded at 77 K. The excitation wavelength was 440 nm. The changes of PLBs and PTs were examined via the energy transfer and binding of probes, especially of ANS to the membranes. The investigations were performed using suspensions containing PLBs (100 µg/ml protein) and ANS (Mg-salt, Reachim) 10, 15 or 20 µM, or pyrene (Serva) 4, 6, 8, or 10 µM. Before the measurements, samples were incubated with the probes on ice for 1 h. Phototransformation of the Pchlide was performed by giving 3 flashes of "white light" from a photographic flash (Braun F 800), at a distance of 0.1 m (impulse energy 120 J). The energy transfer between Trp of membrane proteins and the probes was measured by quenching of Trp fluorescence after excitation at 286 nm. The calculations were performed as it was described by Dobretsov (1989). Means of 5 measurements are given for all experiments. The results were statistically processed and the values of σ and P were calculated. Changes in probe–membrane interactions were discussed if the differences had been with P less than 0.01. All the fluorescence measurements were performed with a SLM 8000C spectrofluorimeter (SLM Aminco, Urbana, IL USA) and accompanying software. All spectra were corrected for variations in the sensitivity of the photomultiplier.

Results

Initially the effect of the fluorescence probes ANS and pyrene on the activity of the Pchlide:POR:NADPH complex was studied. The low temperature fluorescence spectra (77 K) of samples containing fluorescence probes and isolated PLBs and PTs were examined. PLBs showed a main fluorescence peak at 657 nm originating from the phototransformable Pchlide and a lower peak at 633 nm coming from non-phototransformable Pchlide (Fig. 1A). The addition of ANS to a PLB sample caused no changes

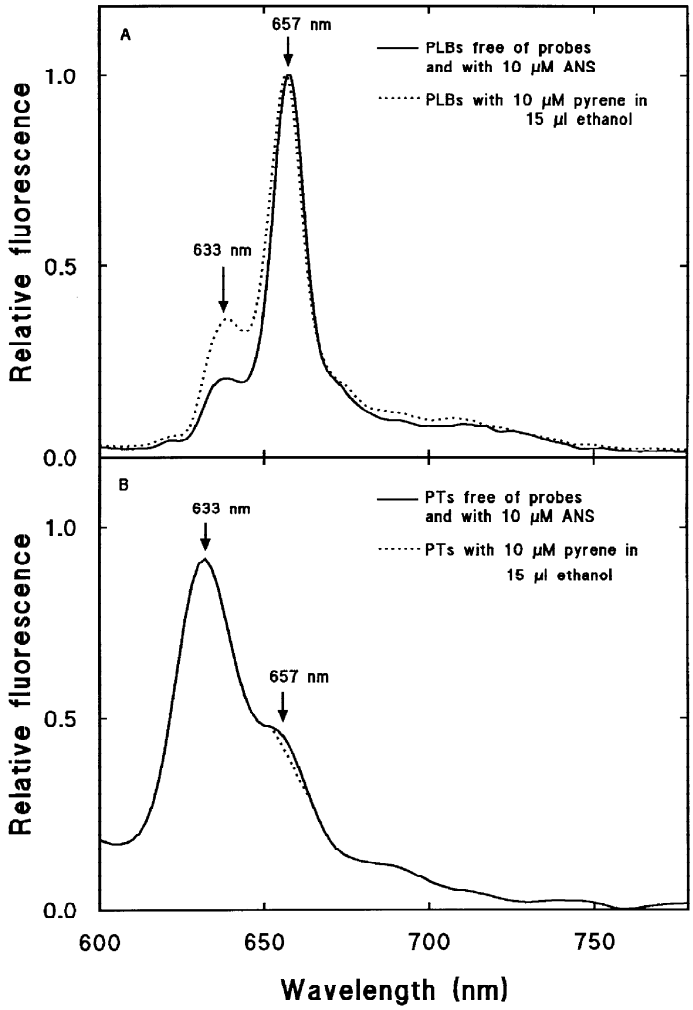

Fig. 1. Fluorescence emission spectra at 77 K of isolated PLBs (A) and PTs (B). The samples were incubated in darkness, on ice for 1h with or without fluorescence probes. Before the measurements, samples were frozen in liquid nitrogen. The excitation wavelength was 440 nm

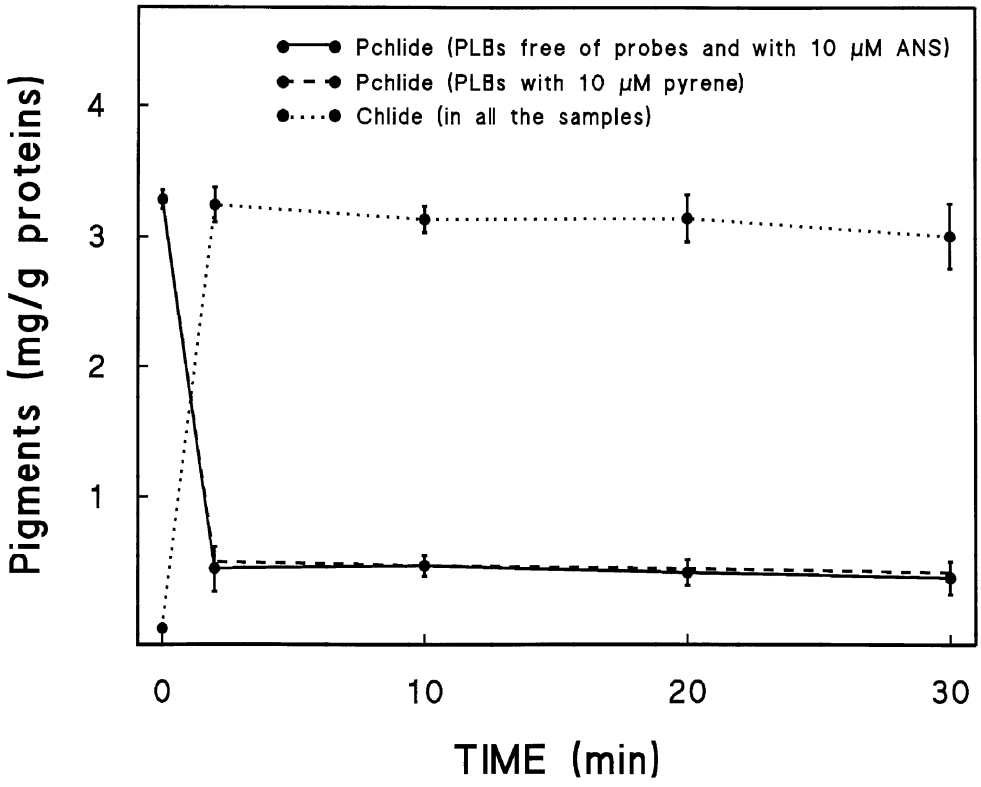

Fig. 2. Light-induced changes of pigment amounts in PLBs. The membranes were incubated on ice for 1h with or without ANS and pyrene in darkness. The samples (except the control) were irradiated with 3 flashes of white light and during the following dark period (30 min) certain volumes of samples were extracted with acetone for measuring Pchlide and Chlide

in the Pchlide spectral forms. When pyrene was added to PLBs in a concentration of 10 μM, a small decrease in the ratio between fluorescences at 657 nm and at 633 nm was found, corresponding to a decrease of the phototransformable Pchlide. This effect was mainly due to the presence of about 1% ethanol (pyrene was added from 1 mM ethanol stock solution) as there was no substantial difference between samples with pyrene and those with the same amount of ethanol without 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 PLBs and in the PLBs-samples incubated with both probes. The probes did not influence the PTs pigment spectra (Fig 1B).

Fluorescence spectra of Trp in PLBs and PTs samples with and without probes had maximum at 330 nm (Figs. 3, 4). The intensity of Trp fluorescence in PLBs-containing samples increased after irradiation (Fig 3 A, B, C). There were no significant

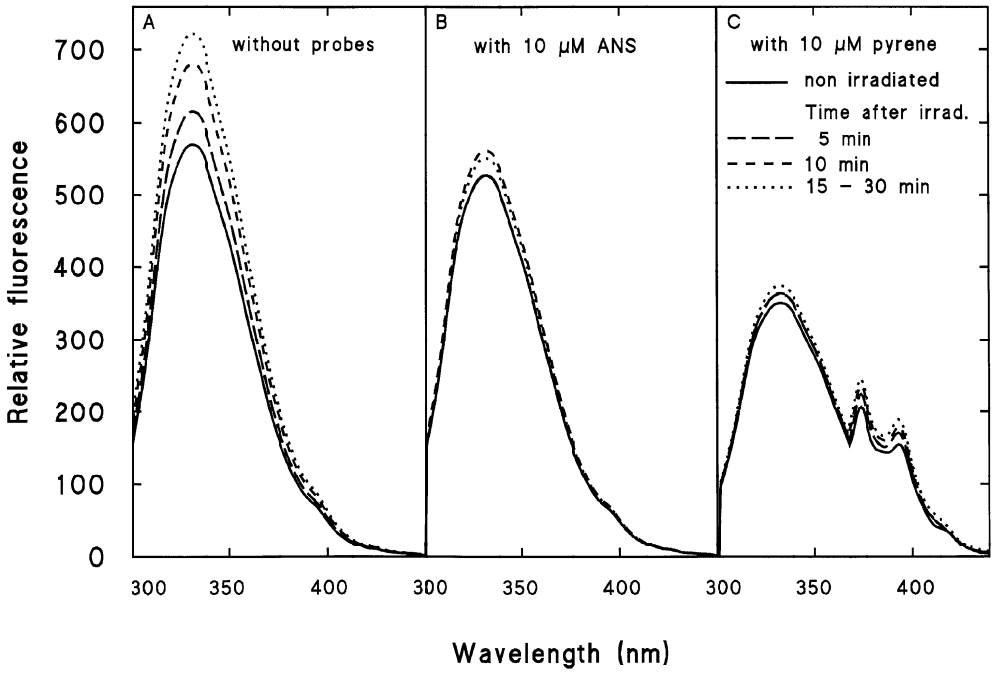

Fig. 3. Changes in tryptophan fluorescence of isolated PLBs and its quenching by the probes. Samples were taken from PLBs incubated in darkness on ice for 1 h. The membranes, presented in parts B and C were incubated with 10 μM ANS or 10 μM pyrene respectively. The samples (without the control) were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

changes of Trp fluorescence in PTs (Fig. 4 A, B, C). Considering the fact that the major part (at least 90%) of PLBs proteins are POR on the basis of fluorescence intensity, using a concentration curve, we calculated the number of emitting Trp residues in probe-free PLBs samples. It was 3.53 per molecule POR before irradiation and the latter increased up to 3.96. It is well known that the wavelength of Trp fluorescence maximum depends very much on the polarity of environment (Eftink and Ghiron, 1981). Solutions of *in vitro* synthesized POR, kindly supplied to us by Dr. Clas Dahlin (Plant Physiol. Dept., University of Göteborg, Sweden), as well as pure (Merck) solutions of Trp in TES-HEPES buffer (pH 7.2) had a fluorescent pick at 350 nm which suggests that the Trp residues in POR molecules and in other proteins (in PTs) are not in a direct contact with the surrounding water. Because of that in the calculations about the Trp quenching we used the concentrations of the probes, bound to the membranes.

The concentrations of the bound and non-bound ANS were calculated in consideration of the fact that the bound form of probe absorbs at 390 nm and has fluorescence emission at 460–480 nm (Slavik, 1982). The concentration of binding sides

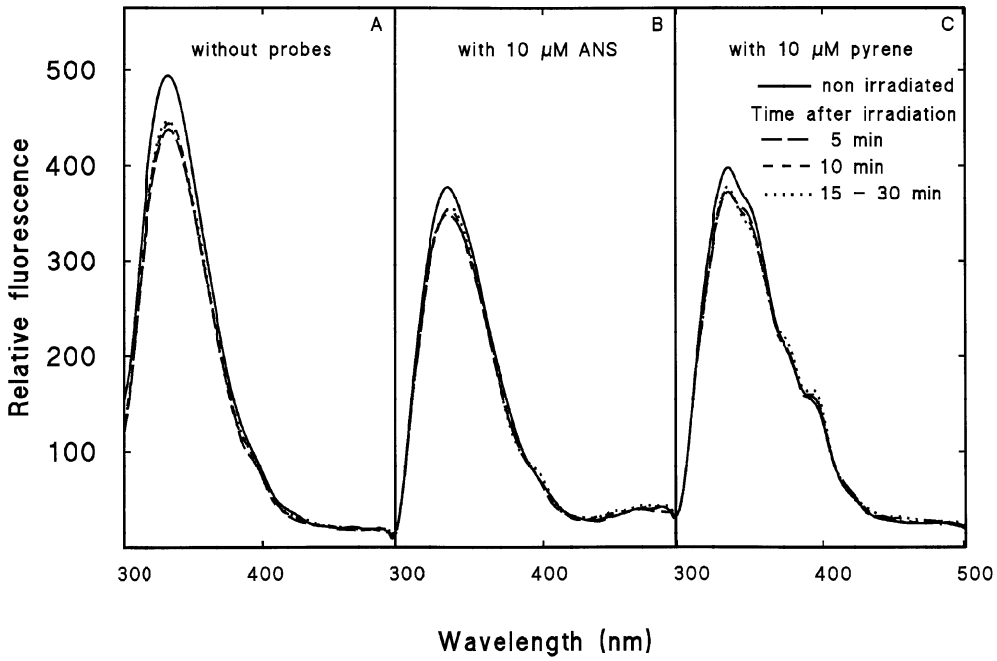

Fig. 4. Changes in tryptophan fluorescence of isolated PTs and its quenching by the probes. Samples were taken from PTs incubated in darkness on ice for 1 h. The membranes, presented in parts B and C were incubated with 10 μM ANS or 10 μM pyrene respectively. The samples (without the control) were irradiated with 3 flashes of white light and the fluorescence was recorded during the following dark period (30 min). The excitation wavelength was 286 nm

"N" was also calculated (Denev and Minkov, 1992). We found that in non-irradiated PLBs samples containing 10 μM ANS (as a total concentration) only 1.84 μM of the probe was bound to PLBs, in 20 μM ANS containing samples the amount of bound probe was 2.09 μM. An increase to 2.27 (10 μM ANS) and to 2.38 μM (20 μM ANS) after irradiation of the bound probe amouns was found (Fig. 5A). The calculated parameters for PTs were 4.26 μM (10 μM ANS) and 5.01 μM (20 μM ANS). There were not significant changes after flash irradiation up to the end of dark incubation of the membranes (Fig. 5B). The value of N in non-irradiated PLB was 7.9 mmol/g membrane protein. After the irradiation N increased and 10 min later it was about 16% higher than that in non-irradiated membranes. During further incubation, N decreased to about 5% lower than in the dark membranes and then it was followed by a gradual increase (Fig. 5A). In the PTs during all the experiment N was about 12 mmol/g (Fig. 5B). Probably the main part of pyrene molecules could be found in the lipid phase of the membranes, because there was not a quenching of pyrene fluorescence (results not shown) in the presence of an equimolar amount of iodine ions, added as a fresh-prepared KJ solution in TES-HEPES buffer (Dobretsov, 1989).

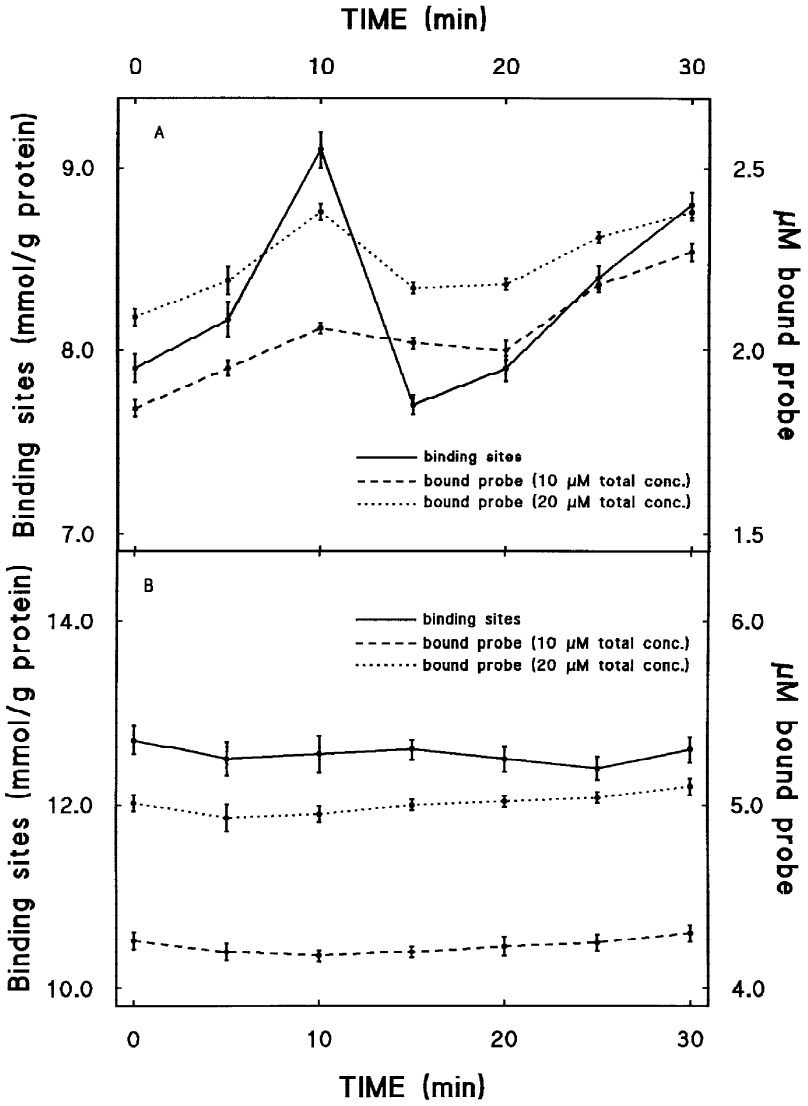

Fig. 5. Binding of ANS to isolated PLBs (A) and PTs (B) before and after irradiation with 3 flashes of white light. Samples were incubated on ice in darkness for 1 h with 10 μM or 20 μM ANS. The samples (without the control) were irradiated with 3 flashes of white light and kept for different time in darkness. The fluorescence was measured at 470 nm with the excitation wavelength set to 390 nm

The non-radiative energy transfer between the Trp of membrane proteins and the probes was measured by quenching of Trp fluorescence at 330 nm after excitation at 286 nm. The presence of other fluorescent molecules in the membranes (NADPH, Pchlide, Chlide) made it impossible to prove that all registered changes of the fluores-

cence were only due to the singlet-singlet transfer of the energy from Trp to the fluorescence probes. Because of that we restricted our investigations to calculating the accessible part of the Trp in the protein(s) of the membranes for the quenching, caused by the fluorescence probes. The energy transfer between Trp and the probes used is possible at a distance not greater than 1.2 times the Förster radius (R_0), equal to 2.6 nm for ANS and to 2.8 nm for pyrene (Dobretsov, 1989). Trp residues according to this condition could be divided into two parts – quenched **b** and non-quenched **(1–b)** from the probe. A way for calculating **b** was described by Chekrigin and Dobretsov (according to Dobretsov 1989): $\mathbf{b = a/[F_0/(F_0 - F)]min,}$ 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), $\mathbf{[F_0/(F_0 - F)]min}$ is the maximum possible quenching of Trp fluorescence in the largest concentration (infinite) of the acceptor (the probe). Because there is a difference between the constants **a**, the largest values of **b** for ANS and pyrene are different too. In order to compare the results, the data were recalculated as a percent of a maximal possible value of **b**. The data show that in the non-irradiated PLBs, 58 % of the fluorescent Trp residues were accessible for quenching by pyrene, 52% – by ANS, and 86% – when both probes were added together. After the irradiation, the quenching by pyrene increased to 82%, and the quenching by ANS increased to about 72%. The quenching by both probes increased up to 92% after irradiation (Fig. 6A). In the non-irradiated PTs 55% of Trp residues were available for quenching by ANS and 45% were quenched by pyrene. The results show that in the presence of both probes all the Trp residues were accessible for quenching. There were no significant changes after the irradiation.

Discussion

Our results indicate that the light-induced changes of probe–membrane interactions appear generally in the PLBs. Since POR is the major protein in PLBs, it is possible to use the results and make conclusions about its localization. The results can be best explained accepting the idea that POR, together with Pchlide and NADPH, are organized in large aggregates in the PLB membranes (Böddi et al., 1991; Wiktorsson et al., 1992). Within the complexes the assembled POR molecules are tightly attached to each other. In such a case the Trp residues which have been in the inner part of the complexes are located beyond the distance which enables energy transfer between them and the probes. It is possible that a small part of Trp residues but not more than one per two POR molecules, are overshadowed within the aggregates. This model gives a good explanation why about 48% and 42% of Trp emitting in non-irradiated PLBs are not accessible for quenching by ANS and respectively by pyrene. The increase of Trp fluorescence after irradiation suggests that the aggregated proteins were disconnected and started to separate, which eliminated the shadowing of Trp. Such

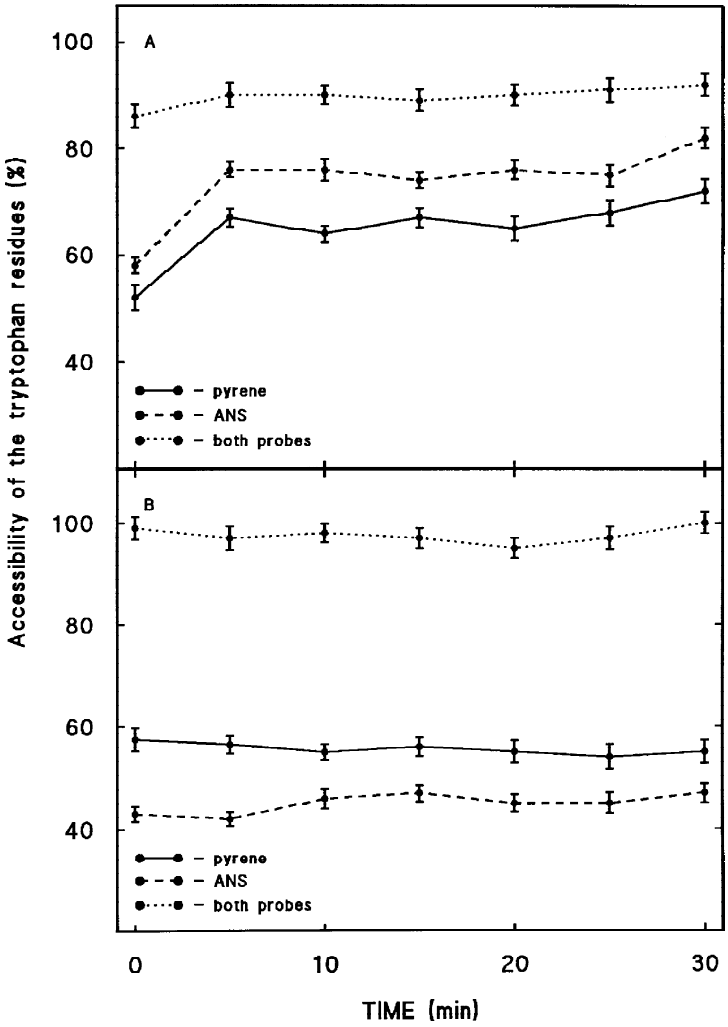

Fig. 6. Changes of the accessibility of tryptophan residues in PLBs (A) and PTs (B) for quenching by the probes. The samples (without the control) were irradiated with 3 flashes of white light and the fluorescence at 333 nm was measured during the following dark period (30 min). The excitation wavelength was 286 nm

a separation opens a possibility for binding more ANS molecules (see above). After separation of the complexes, Trp becomes more accessible for quenching by probes (Fig. 6A). No photoactive complexes of POR, NADPH and Pchlide, or their aggregates have been reported in the PTs. The absence of significant light-induced changes of the quenching accessibility of Trp residues in PTs (Fig. 6B) supports this explanation.

Another questionable moment is the localization of proteins in the lipid phase. According to our results 58% of Trp residues in non-irradiated PLBs and 82% in the irradiated ones are accessible to the deep buried in lipids pyrene. This fact suggests their possible localization within the lipids. On the other hand, 72% of Trp residues are accessible to the surface-localized ANS (concerning the lipid phase). One acceptable explanation is that the predominant part of Trp residues (not less than 60%) are situated at the level of polar heads in the lipids, which could explain the high accessibility of Trp by both probes. Such a distribution of Trp suggests that at least a part of POR molecules are situated close to the lipid surface most probably also on the level of lipids polar heads. Our data do not give enough information about the distribution of the rest of the enzyme. It is possible that after the irradiation part of the enzyme molecules start leaving the lipid phase. Most probably within the first thirty minutes the main part of POR remains within the membranes. This supports the hypothesis that after photoreduction, POR migrates laterally on the membrane surface (Ryberg and Dehesh, 1986; Artus et al., 1992).

The accessibility of Trp residues in PTs for quenching by probes suggests that most of the proteins are situated close to the membrane surface.

***Acknowledgements*:** The work was partly supported by Grants K 414 and MU-HP-3 from the National Science Fund, Bulgaria,.

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