PHOTOSYNTHETICA 41 (4): 635-639, 2003

BRIEF COMMUNICATION

Surface charge densities and membrane fluidities in thylakoids with different degrees of thylakoid appression after Norflurazon treatment

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Abstract

Wheat seedlings (*Triticum aestivum* L.) develop plastids (etioplasts and chloroplasts) which exhibit alterations in inner membrane organisation after treatment with Norflurazon (NF), an inhibitor of carotenoid biosynthesis. In dark-grown plants, it results in a decreased amount of partitions (contact zones) between prothylakoids. Under weak red radiation (WRR), plants contain chloroplasts devoid of grana. Using the fluorescent probe 9-amino acridine (9-AA), the average surface charge density of isolated prothylakoids (PTs) was –21.8±3.2 mC m^{-2} and –27.4±2.6 mC m^{-2} in the control and after treatment, respectively. Thylakoid membranes isolated from plants grown under WRR exhibited slightly more negative values, –23.5±2.9 mC m^{-2} and –29.0±2.1 mC m^{-2}, in control and after NF treatment, respectively. The surface charge density of de-stacked thylakoids from greenhouse-grown untreated plants, containing extensive grana stacking, was –34.3±2.5 mC m^{-2}. Assays using the fluorescent probe of DPH (1,6-diphenyl-1,3,5-hexatriene) showed a higher polarisation value when incorporated into thylakoids from NF-treated plants compared to untreated plants grown under WRR. The highest polarisation value was found in untreated plants grown in the greenhouse. This indicates a lower rotation transition of the probe in the lipid environment of thylakoids after NF treatment, which can be interpreted as more rigid membranes. Hence the surface charge density and the mobility of membrane components may play a major role for the formation of partitions in dark-grown plants and in the formation of grana in plants grown under WRR.

Additional key words: 9-amino acridine**;** chloroplast; etioplast; (pro-)thylakoids; stacking; *Triticum*; wheat.

Thylakoids consist of a lipid bilayer to which proteins and protein-complexes are associated. The formation of thylakoid appressions occurs between regions of the membrane where the Coulombic repulsive forces between two adjacent membranes are decreased. This occurs through lateral segregation of thylakoid components, such as displacement of negative membrane surface charges to membrane regions not involved in stacking and/or through cations, screening negative membrane surface charges in areas involved in stacking (Barber 1982). Plants grown in the presence of the herbicide Norflurazon (NF) contain approximately 0.5 and 2.0 % of the normal content of carotenoids in etioplasts and chloroplasts of wheat seedlings, respectively (Dahlin 1989). In etioplasts, the NF treatment leads to low amount of partitions between the prothylakoids. Chloroplasts in NF-treated plants, grown under weak red radiation (WRR) to avoid photodestruction, lack the ability to form normal grana. The inability to form grana after NF treatment is correlated to the deficiency of LHCP, the light-harvesting protein of photosystem 2 (Dahlin 1988). The absence of LHCP, and consequently the excess of stroma-exposed positive charges of lysine and arginine (cf. Bűrgi *et al*. 1987) as well as charged acyl lipids (Sandelius and Dahlin 1990) suggest that the unability to form grana after NF treatment in light may be related to

Received 1 September 2003, *accepted* 2 January 2004.
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Abbreviations: 9-AA – 9-amino acridine; DPH – 1,6-diphenyl-1,3,5-hexatriene; EPIM – etioplast inner membrane; F/F_{max} – relative 9-AA fluorescence in relation to maximum fluorescence recorded after addition of 20 mM M_gCl_2; LHCP – major light-harvesting protein of photosystem 2; NF – 4-chloro-5-(methyl amino)-2-(α,α,α-trifluoro-*m*-tolyl)-3(2H)-pyridazinone or Norflurazon; PLB – prolamellar body; PT – prothylakoid.
Acknowledgements: The financial support from the Carl Trygger Foundation for Scientific Research is gratefully acknowledged. Prof. I.M. Møller is greatly acknowledged for his help with the surface charge measurements. The provision of Norflurazon by *Sandoz Ltd.*, Basel, Switzerland is kindly acknowledged.

differences in membrane surface charges.

In the present paper, the possible correlation between the surface charge densities and the fluidity of membranes are related to the formation of prothylakoid overlap and grana of plastid inner membranes from NF-treated plants grown in darkness or in non-photooxidising WRR, respectively.

Wheat seeds (*Triticum aestivum* L.) were soaked in tap water with (10^{-4} M) or without NF. The seedlings were grown in darkness or in a greenhouse (untreated only; 16 : 8 h light : dark regime) or in continuous WRR (16 mW m^{-2}; *Philips TL 20W/15*, Ryberg *et al*. 1980) for 6 d at 20±1 °C. Dark-grown plants were used for the preparation of prolamellar bodies (PLBs) and prothylakoids (PTs) (cf. Lindsten *et al.* 1988). Preparation of etioplast inner membranes (not separated into PLBs and PTs, *i.e*. EPIMs) and thylakoids from plants grown in WRR or in the greenhouse was performed as described by Engdahl *et al*. (2001).

Lipids were extracted from suspended membranes according to Sandelius and Sommarin (1986) and analysed according to Sandelius and Dahlin (1990). The total amount of proteins was measured with Coomassie Brilliant Blue according to Lindsten *et al*. (1988). The content of chlorophylls (Chls) was determined spectrophotometrically according to Lichtenthaler and Wellburn (1983).

The fluidity of membrane fractions was measured with fluorescence polarisation of DPH (1,6-diphenyl-1,3,5-hexatriene, cf. Hernando *et al.* 2001). The membranes were suspended at 100 g m^{-3} Chl with 75 μM DPH and incubated for 40 min at room temperature. The membranes were then washed by centrifugation, and DPH polarisation was performed with an excitation wavelength of 365 nm and an emission wavelength of 450 nm using a *Baltzer* filter *B-40 450 9*. Fluorescence polarisation values were estimated generally as described by Ford and Barber (1980).

To measure membrane surface charge density, the fluorescent dye 9-amino acridine (9-AA) was used. This dye carries a net positive charge at neutral pH. When suspended in a solution with low cation content together with membranes, it will be attracted to negatively charged membrane surfaces. The dye will then be concentrated to such an extent that its fluorescence is quenched (Searle and Barber 1978). By titrating the solution with salts (functionally cations), the surface potential is reduced. The dye then exchanges to the bulk solution and its fluorescence is restored. Certain 9-AA fluorescence reflects the same average surface potential for both mono- and di-valent cations. The salts used here were KCl and (DM)Br_2 (N,N,N,N′,N′,N′-hexamethyldecane-1,10-diamine bromide). The measurements were performed with 20 μM 9-AA on 35 g m^{-3} membrane protein in 0.3 M sucrose, 3 mM MOPS, and 50 μM Na-EDTA at pH 7.1. The excitation wavelength was 355 nm. Reabsorption of the 9-AA fluorescence by Chls and carotenoids was kept at a minimum by measuring small volumes (250 mm^3) at an emission wavelength of 498 nm. The addition of the uncoupler FCCP (carbonyl cyanide *p*-trifluoro-methoxy-phenylhydrazone; 10^{-6} M) did not give any change in 9-AA fluorescence, indicating that the dye did not accumulate inside the membrane vesicles (Møller and Lundborg 1985).

The surface charge density (σ) was calculated according to A. Bérczi and I.M. Møller (personal communications), at F/F_{max} = 0.80. This calculation takes into account the monovalent cation concentration of the buffer and also the 2 : 1 salt. This is a modification of the method used by Chow and Barber (1980) which stems back to the theories of Gouy (1910) and Chapman (1913) on colloidal surfaces. The exact equations used are given in Dahlin (1989). The Mann-Whitney U test was used to evaluate differences between paired observations in two groups (Sokal and Rohlf 1981).

Ultrastructural studies of etioplasts in dark-grown plants have revealed a significantly lower amount of partitions between the PTs after NF treatment (Dahlin *et al*. 1983). The intention here was to investigate possible differences in membrane surface charges between etioplast inner membranes from NF and control seedlings. First, increasing amounts of membranes (on a protein basis) were added to a low-salt buffer containing 9-AA. This showed that EPIMs from NF-treated plants were able to quench the 9-AA fluorescence stronger than did EPIMs from untreated plants (Fig. 1). This quenching could partly be restored by adding $MgCl_2$ to the solution, indicating that the quenching actually was due to the

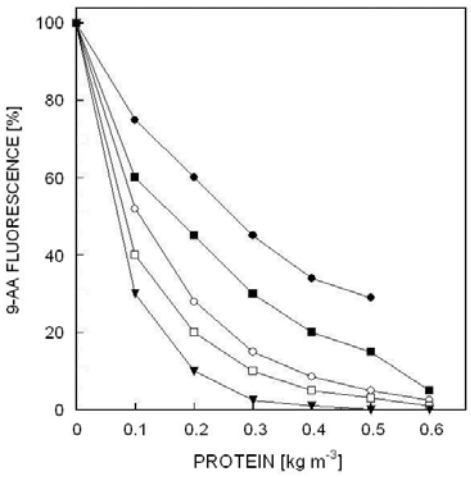

Fig. 1. Representative data of changes in 9-AA fluorescence following addition of thylakoids and EPIMs isolated from control and NF-treated wheat seedlings. *Circles* denote EPIMs isolated from dark-grown wheat seedlings; *squares* denote thylakoid membranes from wheat seedlings grown in weak red radiation, WRR (16 mW m^{-2}). *Triangles* denote thylakoids from untreated plants grown in the greenhouse. *Open and filled symbols* denote membranes from seedlings grown in the presence or absence of NF, respectively.

accumulation of 9-AA to the membrane surface, and not to the lumen.

Isolated PLB and PT membranes from control and NF-treated dark-grown plants were then used for surface charge density measurements using 9-AA. No major differences in 9-AA quenching or surface charge densities were observed between PLBs isolated from untreated and NF-treated seedlings (not shown). In PTs on the other hand, calculations of the surface charge density (σ) confirmed a difference in the concentrations of membrane negative charges between PTs from untreated and NF-treated plants. The latter ones exhibited higher surface charge density, –27.4±2.6 mC m^{-2}, as compared to –21.8±3.2 mC m^{-2} in PTs from untreated plants ($p < 0.03$). These membranes were then used for steady-state polarisation (*p*) measurements using the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH). No major difference could be observed between the control membranes and the membranes from NF-treated etioplasts. This suggests that migration of membrane components (fluidity) is fairly similar in membranes from control and NF-treated plants.

The low amount of partitions between plastid inner membranes after NF treatment is not only confined to dark-grown plants. Herbicide treated plants grown in non-photo-oxidising WRR contain chloroplasts lacking grana and LHCP (Axelsson *et al*. 1982, Dahlin 1988). Careful 9-AA fluorescence measurements and surface charge density (σ) calculations showed that also the σ of isolated thylakoids from NF-treated plants was higher (more negative) than that of corresponding thylakoids from untreated plants (Table 1). Surprisingly, the average surface charge density of de-stacked thylakoids isolated from untreated greenhouse-grown plants was *ca*. –34 mC m^{-2} (Table 1). That is, plants containing normal grana exhibited the highest σ of all membranes assayed (Table 1). This value is by far more negative than the –25 mC m^{-2} which Barber (1982) reported as the highest σ value allowing thylakoid appression. Obviously, other parameters than average surface charge density are involved in regulating the formation of thylakoid appression and stacking. DPH measurements showed that this probe exhibits a higher polarisation value (*p*) when incorporated into the thylakoids of NF-treated plants as compared to untreated seedlings grown in WRR (Table 1). This indicates a lower rotation transition of the probe (cf. Hernando *et al.* 2001), and consequently a more rigid lipid phase after NF treatment. In thylakoids of greenhouse-grown plants, the *p* value was approximately 0.215, which is significantly the lowest value of all membranes assayed ($p<0.03$, cf. Table 1), indicating a relatively high fluidity. An inversed relationship was observed between polarisation values and fatty acid to protein ratios. Thylakoids from NF-treated plants exhibited the highest *p* value. These membranes also contain the highest amounts of fatty acids on a protein basis, whereas thylakoids from W-plants exhibit lowest fatty acid to protein ratios as well as lowest *p* values (Table 1).

Table 1. Lipid/protein ratios, surface charge densities (σ), and fluidity of thylakoid membranes isolated from leaves of wheat grown with or without NF during seed imbibition. The fluidity is expressed as a steady-state DPH fluorescence polarisation (*p*). C – untreated seedlings grown in the greenhouse, WRR – untreated seedlings grown in weak red radiation (WRR), WRR/NF – NF-treated seedlings grown in WRR. Means followed by standard deviation ($p<0.03$); there were five experiments.

Treatment	Fatty acid/protein [mol kg^{-1}]	σ [mC m^{-2}]	*P*
C	910±30	–34.3±2.5	0.215±0.004
WRR	950±40	–23.5±2.9	0.230±0.005
WRR/NF	1030±40	–29.0±2.1	0.241±0.006

Surface-exposed membrane charges create an environment of electrostatic forces acting within the plane of the membrane as well as between different membranes. At physiological pH, the main membrane charges facing the stroma arise from oxidised carboxyl groups of proteins (Prochaska and Gross 1977). The contribution of charged lipids to the membrane surface charges is more obscure. Exposed charges of membrane lipids (Murphy and Woodrow 1983) as well as individual fatty acids (Doltchinkova and Nikolov 1997) may contribute to the surface charges. On the other hand, Barber and Gounaris (1986) argued that the bulk of charged lipids (*e.g*. PG and SQDG) are associated with proteins in such a way that the charges are not exposed to the stroma. The method of 9-AA fluorescence has been used to measure surface charge densities on a variety of biological membranes, such as plasmalemma vesicles from roots (Møller *et al*. 1984), mitochondrial membranes (Møller *et al*. 1981), and thylakoids as well as sub-fractionated thylakoids (cf. Barber 1982). Brauer *et al.* (2000) tested the method on artificial liposomes. They concluded that change in 9-AA fluorescence is a reliable method for the calculation of surface charge density of membranes. As shown in this study, there is a general correlation between the average surface charge density, thylakoid appression, and membrane fluidity, except for thylakoids from greenhouse-grown plants, as discussed later. The differences in fluidity of the membranes studied here are likely to depend of at least two things. Firstly, the altered fluidities after NF-treatment could be due to difference in protein-lipid interactions (cf. Páli *et al*. 2003) since the lipid/protein ratio is largely unaffected by the herbicide treatment

(Table 1). Secondly, the subsequent relative increase in content of zeaxanthin (cf. Dahlin 1988) may increase the rigidification of the membranes (Latowski *et al.* 2002).

The main protein of LHC2 (LHCP) is normally concentrated to the appressed regions of the thylakoids. This has been shown with fractionation studies (Andersson and Anderson 1980) and immunogold localisation (Dahlin 1989). This correlates well with the apparent inability to form grana in plants lacking LHC2, thereby exposing the more negatively charged PS2 (Dobrikova *et al.* 2000). On the other hand, partitions between plastid inner membranes are not entirely regulated by LHCP since PTs of NF-treated dark-grown plants exhibit lower degree of partition as compared to untreated plants (Dahlin *et al*. 1983). Thylakoids in greenhouse-grown control plants contain normal grana. Surprisingly, these membranes also exhibit the highest negative average surface charge density (Table 1). This value may be explained in terms of lateral segregation of charges in order to allow appression. Considering the apparent higher amount of LHC1 in thylakoids of greenhouse-grown plants, and in turn negative surface charges (Barber 1980), a displacement of excess negative charges to membrane regions not partaking in the appressions is likely. That is, the excess of exposed negative charges of PS1 and its light-harvesting antennae (LHC1) have to be displaced to membrane regions not partaking in the appressions (Chow *et al.* 1991). This will lead to that certain areas will have a less negative value of –25 mC m^{-2} and allow appression. However, this requires a membrane with relatively high fluidity which allows such displacement, as seen in the *p* value from untreated greenhouse-grown seedlings (Table 1). The *p* values after NF treatment indicate a lower rotation transition. The reason for this is unclear since the contents of uncoloured lipids are largely unaffected by the treatment (Sandelius and Dahlin 1990).

Finally, in light-grown plants the negative effects of NF on the accumulation of membrane and stromal proteins were found (Gray *et al*. 2002). This is due to the photodestruction of one or several plastid signal(s) which affect nuclear gene expression. However, in darkness or under non-photooxidising conditions, no major effects on the transcripts have been found (Sullivan and Gray 2002). The only obvious effect in NF-treated wheat grown under non-photooxidising conditions is the lack of LHCP (Dahlin 1989). It therefore may be assumed that the NF-induced increase in the surface charge density originates from the inability of LHCP to anchor to the thylakoids in the absence of carotenoids. This in turn increases the relative proportion of negative membrane charges.

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