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The relation between changes in non-photochemical quenching, low temperature fluorescence emission, and membrane ultrastructure upon binding of polyionic compounds and fragments of light-harvesting complex 2

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Abstract

Experiments were performed to distinguish some of the proposed mechanisms by which thylakoid membranes regulate the performance of photosynthetic apparatus in relation to non-photochemical quenching, q_N. Aliphatic diamines were used as uncouplers of transmembrane H^+ gradient as they can be transported across the membrane at the expense of hydrogen cations. Diamines did not induce changes in low-temperature fluorescence emission but induced different changes in membrane ultrastructure. Positively charged peptides did not affect membrane ultrastructure but blocked q_N. In addition, they caused an increase of low temperature fluorescence emission between 710 and 720 nm. For control peptide, the maximal fluorescence increase was found at 715 nm. Fragments of light-harvesting complex 2 in their phosphorylated and non-phosphorylated form shifted the position of this increase. We believe that peptides bind to membrane surface and reduce the mobility of membrane components whose migration is needed for observation of q_N. Phosphorylated and non-phosphophorylated LHC2 fragments bind to different binding sites for corresponding forms of the protein.

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Abbreviations: Chl - chlorophyll; LHC2 - light-harvesting complex 2; PS - photosystem; q_N - non-photochemical quenching of chlorophyll fluorescence.
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Additional key words: decyldiamine; ethylenediamine; peptides; membrane fragments; phosphorylation; *Pisum sativum*.

Introduction

Non-photochemical quenching of chlorophyll (Chl) fluorescence (q_N) is a relatively easy-to-measure parameter related to the state of photosynthetic apparatus. Because it may be measured non-invasively (Schreiber 1983), many plant physiologists and biologists routinely use this parameter in experiments. Most of the fluorescence emission of photosynthetic apparatus at room temperature originates from photosystem (PS) 2 (Krause and Weis 1991) and its transients probably reflect changes in the efficiency of utilisation of energy captured by PS2 antennae.

Several mechanisms were proposed for q_N. Each of them was based on different lines of biochemical evidences. Quenching related to xantophyll cycle is supposed to be caused by excitation energy flow from Chl to zeaxanthin, a pigment whose concentration in the membrane increases with rising irradiance (Hager 1966, Gilmore 1997). Another line of evidence points out that proton gradient across the thylakoid membrane is necessary for q_N (Briantais *et al.* 1979). Lowering of solution pH in suspension of isolated PS2 membranes leads to a decrease in fluorescence yield (Krieger and Weis 1992). The analogy between the reduction of fluorescence emission in precipitated isolated LHC2 (Burke *et al.* 1978) and the decrease in fluorescence emission of leaves and thylakoid membranes has suggested the hypothesis (Ruban *et al.* 1992) relating the decrease in fluorescence yield with LHC2 aggregation. This hypothesis was recently modified and nowadays the minor antennae are considered to play crucial role in q_N (Crofts and Yerkes 1994, Ruban *et al.* 1996).

Another mechanism by which plants regulate the energy flow from antennae to reaction centres is phosphorylation of membrane proteins (first observed by Bennett 1977; reviewed by Allen 1992). This mechanism is probably directly related to state 1-state 2 transitions (reviewed by Allen 1992), *i.e.*, increase of absorption cross-section of PS1 at the expense of that of PS2 and *vice-versa*. According to Rintamäkki *et al.* (1997) the phosphorylation pattern of thylakoid membrane proteins *in vivo* varies with irradiance and length of irradiation. Similar observation was reported earlier (Stys *et al.* 1995) on isolated thylakoids. Main evidence between the low-irradiance and high-irradiance phosphorylation lies in the phosphorylation of LHC2 which is high at low irradiance and low at high irradiance—clearly, the role of LHC2 is different in each of these states and the original mechanism, suggesting that phospho-LHC2 leaves PS2 and binds to PS1, can hardly be employed (reviewed by Allen 1992).

In this article, we describe experiments aimed at distinguishing some of the proposed mechanisms for q_N. The low-pH induced quenching was blocked by diamines which are supposed to be transported across the membrane at the expense of protons (Portis and McCarty 1976). Similar changes in q_N were observed when positively charged peptides, which bind to charged surface but are not transported across the membrane, were added to the reaction medium in the absence of diamines. Changes in membrane ultrastructure were observed only in the presence of diamines and differed between ethylenediamine and decyldiamine. The phospho- and non-phospho-

LHC2 fragments induce a red or blue shift in emission of PS1, respectively, which indicates that the main influence of LHC2 may be sought in PS1 and its antennae.

Materials and methods

Isolation of thylakoids: Thylakoids were isolated from three-weeks-old pea (*Pisum sativum* L. cv. Tyrkys) seedlings grown on a perlite substrate in a heated greenhouse under supplementary irradiation with halide lamps (200 μmol m^{-2} s^{-1}). 3 g of leaves were crushed in 25 cm^3 of isolation medium (0.4 M sorbitol, 2.5 mM $MgCl_2$, 10 mM KCl, 1 mM $MnCl_2$, 50 mM Tricine, 1 % bovine serum albumine, pH 7.5) on ice and filtered through eight layers of cotton gaze. Chloroplasts were sedimented at 5000×*g* for 4 min at 4 °C. The pellet was resuspended in a shock medium (15 mM $MgCl_2$, 10 mM KCl, 1 mM $MnCl_2$, 50 mM tricine, pH 7.1) and stirred for 1 min. Thylakoids were sedimented at 5000×*g* for 4 min, resuspended in the shock medium with 0.33 M sorbitol, and stored on ice.

In all cases and for all experiments described except electron microscopy, the dependence of the observed effect on concentration of either diamine or peptide was tested to exclude a possible qualitative change at certain concentration. Finally, the concentration of 10 mM was chosen for diamines and 0:83 mM for peptides. The concentrations above these values either did not change the extent of observed effect (diamines) or caused precipitation (control peptide).

Fluorescence spectra: Thylakoids were diluted in resuspension medium supplemented with the desired concentration of polypeptides or polyamines to give final Chl concentration of 25 g m^{-3} and mixed with 10 cm^3 of 2 mM rhodamine B solution (internal fluorescence standard). The mixture was kept on ice for 5 min, pipetted into a shallow (0.4 mm) groove of a metal holder, and frozen in liquid nitrogen. Chl concentration in the sample was 1 g m^{-3}. Emission spectra were measured with a *Fluorolog* fluorimeter (*SPEX*) equipped with halogen lamp and double monochromators. Excitation wavelength was 480 nm. Spectral emission and excitation bandwiths were 2 and 4 nm, respectively.

Fluorescence yield: Influence of polypeptides and polyamines on the Chl fluorescence yield and its light-induced changes were assayed with a PAM fluorimeter (*Walz*, Germany). Thylakoids were diluted to 20 g(Chl) m^{-3} with the resuspension medium with 3 mM of sodium ascorbate in a cuvette, and tested compounds (reagents) of desired concentration were added. Changes in the fluorescence yield upon red actinic irradiation (λ >650 nm, 300 μmol m^{-2} s^{-1}) and Chl relaxation in the dark were recorded. The maximal fluorescence yield was determined using "white-light" saturation pulses (3000 μmol m^{-2} s^{-1} for 1 s). The fluorescence yield was measured at room temperature.

Peptide synthesis was performed as described earlier (Cheng *et al.* 1994, Stys *et al.* 1995) . In general, the t-Boc strategy was used (Barany and Merrifield 1980). In the place of phophothreonine, Thr[$OPO(OPh)_2$] was incorporated and deprotection was carried out as described by Grehn *et al.* (1987). Peptides were synthesized in the

laboratory of Dr. Ivo Bláha in the Institute of Organic Chemistry and Biochemistry of the Academy of Science of the Czech Republic in Prague (peptides SRPLSDQEKRKQISVRGLAGVENV, RKSATTKKVASSGSP) and in the laboratory of Dr. Henry Franzén at the Biomedical Unit at Lund University (RKSAT(PO_4)TKKVASSGSP).

Fixation and preparation of samples for electron microscopy: Samples for electron microscopy were prepared essentially as described in Wollenberger *et al.* (1995). Samples were fixed overnight with 1 % osmium tetroxide, dehydrated with acetone, and embedded in Spurr resin (*Polysciences*, USA). Thin sections were stained with uranyl acetate at room temperature and examined with a *Philips EM 420* electron microscope.

Calculation of fluorescence parameters was done generally as defined in Krause and Weis (1991). Non-photochemical quenching q_N was calculated according to the formula

$$q_N = \frac{F_m - F_m'}{F_m'}$$

Results and discussion

Influence of charged compounds on membrane ultrastructure: The influence of charged compounds on membrane ultrastructure was examined by electron microscopy of isolated thylakoids. Polyionic compounds such as ethylenediamine or nigericin are frequently used as uncouplers of the transmembrane H^+ gradient across thylakoid membranes (Portis and McCarty 1976). These compounds are probably transported across the thylakoid membrane in uncharged state while they lose H^+ ion on the side where the environment is deficient in H^+ (stroma) and accept it on opposite side (lumen). In this way the ion balance remains unaffected but the identity of ions is changed. To our knowledge, the influence of these positively charged ion compounds on membrane ultrastructure was not systematically examined although it is known that polyamines modify membrane surfaces and adhesion of membrane lamellae (see Gulbrand *et al.* 1984 for summary including discussion of mechanisms which cause this effect). The compounds used in our study are indicated in Table 1. Despite the fact that for both diamines the mechanism of membrane transport should be the same, the influence on membrane ultrastructure (Fig. 1*A* - free membranes in medium) was different—ethylenediamine induced formation of tight membrane stacks (Fig. 1*B*) while decyldiamine induced formation of elongated stacked regions (Fig. 1*C*) similar to that observed in presence of polylysine (Berg *et al.* 1974). Hence both compounds, apart from possible transport across the membrane, modify also the surface of the membrane and do it in different way. We believe that ethylenediamine, due to its shorter aliphatic chain, can link only membranes with flat surfaces, while decyldiamine is able to link even regions from which extend bulky and space occupying domains such as subunits on stromal side of PSI or ATP-synthase.

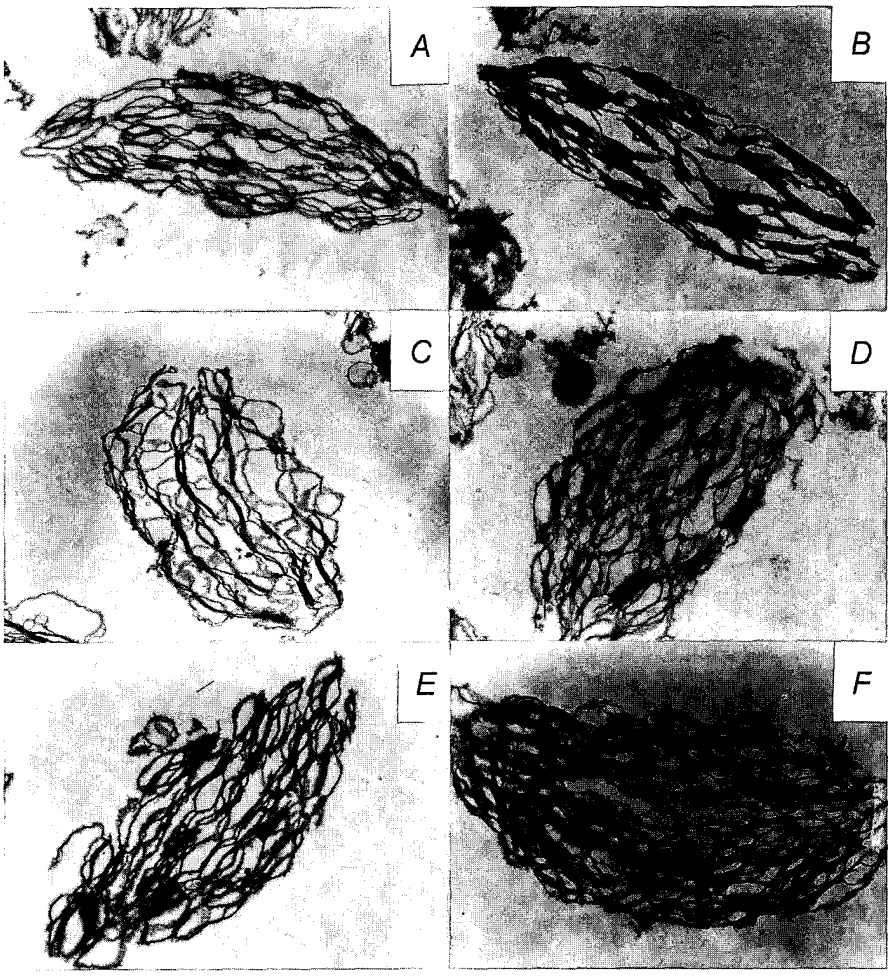

Fig. 1. Electron microscopic pictures of thin layer section of thylakoid membrane in the absence (*A*) and in presence of (*B*) ethylendiamine or (*C*) decyldiamine in the medium. The ultrastructural changes observed in the presence of diamines differ from each other (see text). Peptides caused irregular minor ultrastructural changes which were difficult to evaluate and were not statistically significant (*D* - P1, SRPLSDQEKRKQISVRGLAGVENV, *E* - P2, RKSAT(PO4)TKKVASSGSP, *F* - P3, RKSATTKKVASSGSP).

The effect of non-transportable polyions was examined using multiply charged peptides containing carboxylic and other acidic groups. Such compounds, although positively charged in neutral medium, cannot become completely uncharged at any conditions and thus cannot be transported across the membrane by the same mechanism as diamines. As control peptide we used peptide P1 (positively charged peptide with sequence SRPLSDQEKRKQISVRGLAGVENV). To examine specific binding of LHC2 we used phosphorylated (RKSAT(PO4)TKKVASSGSP) and non-phosphorylated (RKSATTKKVASSGSP) fragments of LHC2, denoted P2 and P3.

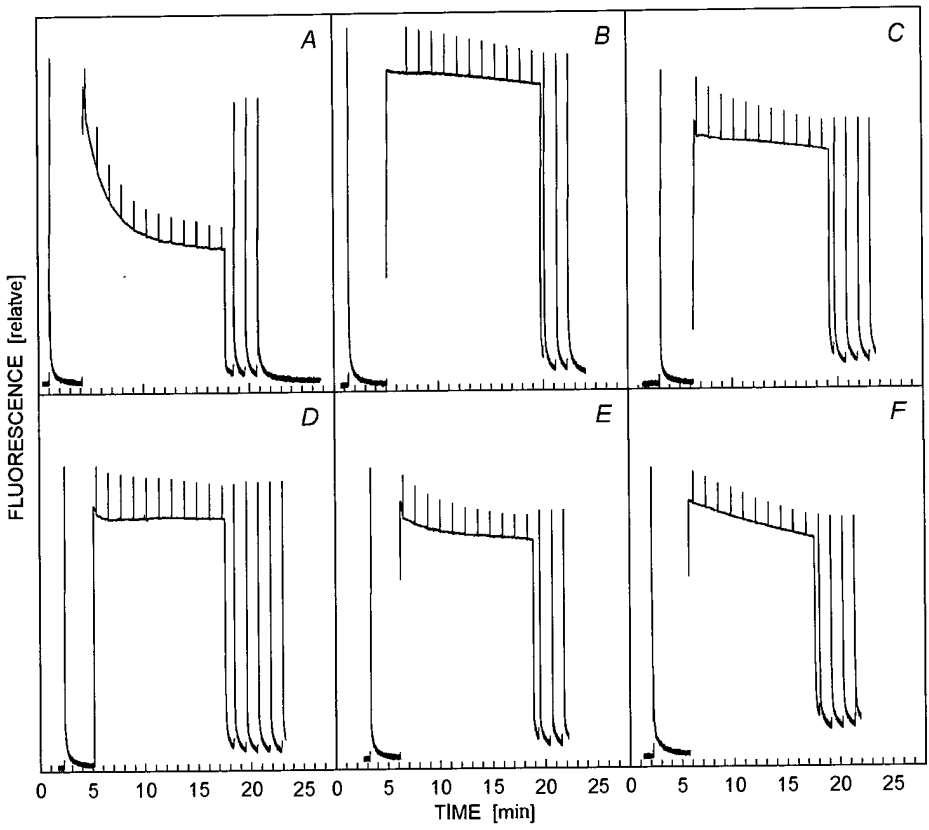

Fig. 2. Course of variable fluorescence of isolated thylakoids in suspension medium (*A*), in suspension medium with added 10 mM ethylenediamine (*B*), 10 mM decyldiamine, (*C*) 0.83 mM peptide fragment of phosphorylation site of glycogen dehydrogenase P1, SRPLSDQEKRKQISVRGLAGVENV (*D*), 0.83 mM phosphorylated fragment of LHC2 P2, RKSAT(PO4)TKKVASSGSP (*E*), and 0.83 mM unphosphorylated fragment of LHC2 P3, RKSATTKKVASSGSP. Both the presence of diamines (*B* and *C*), of the peptide from glycogen phosphorylase (*D*) and of the phophorylated LHC2 fragment (*E*) the non-photochemical quenching is inhibited. In the presence of non-phosphorylated LHC2 fragment (*F*) a steady linear decrease of fluorescence was observed.

The influence of peptides on membrane ultrastructure is depicted on Fig. 1*D-F*. No significant deviation of membrane morphology from the control sample (Fig. 1*A*) was found. This observation is rather surprising since generally peptides bind to membrane surface and this was supported also by our observations described below.

Table 1. Structures of compounds used in the study.

ethylenediamine	$NH_3(CH_2)_2\ NH_3$
decyldiamine	$NH_3(CH_2)_{10}\ NH_3$
control peptide (P1)	SRPLSDQEKRKQISVRGLAGVENV
phosphorylated LHC2 fragment (P2)	RKSAT(PO_4)TKKVASSGSP
non-phosphorylated LHC2 fragment (P3)	RKSATTKKVASSGSP

Table 2. Parameters of non-photochemical quenching as defined in Krause and Weiss (1991).

Sample	F_0/F_m		F_v/F_m		q_N
	dark adapted	light adapted	dark adapted	light adapted	
suspension medium	0.19	0.21	0.81	0.79	0.74
ethylenediamine	0.19	0.20	0.81	0.82	0.06
decyldiamine	0.27	0.29	0.80	0.79	0.05
control peptide (P1)	0.21	0.24	0.79	0.76	0.12
phospho-LHC2 (P2)	0.21	0.23	0.81	0.77	0.15
non-phospho-LHC2 (P3)	0.22	0.26	0.78	0.75	0.15

Influence on fluorescence parameters: Each of the compounds used in the study, *i.e.*, ethylenediamine, decyldiamine, and peptides P1, P2, and P3, was able to block non-photochemical quenching (Fig. 2*A-F* and Table 2). Upon addition of various compounds used in this study, we observed both quantitative differences in the extent of q_N and differences in the course of fluorescence decrease. Thus our experiments point out that q_N may be blocked not only by depletion of the trans-membrane proton gradient, but also by modification of membrane surface. Positively charged peptides bind to membrane surface and islands of membrane components are formed below them (Sackmann 1990). If nothing else, this leads to restriction in diffusion of membrane components. In principle, none of the experiments reported so far demonstrated that q_N was inhibited in the presence of diamines by elimination of H^+ gradient and not by binding of diamines to membrane surface. We believe, however, that the fact that diamines change membrane ultrastructure, while the peptides do not, is caused by transport of diamines to lumenal side of the membrane. Peptides most probably mainly restrict the lateral migration of some membranes components. This points out that lateral migration of membrane components (not necessarily proteins) is a second factor required for the formation of q_N.

Differences between effects of individual compounds were found in low-temperature fluorescence spectra. Since we assumed that majority of changes involve Chl *b*-containing antenna systems we used excitation wavelength 480 nm. Incubation of membranes in the dark did not lead to any spectral change in presence of any

compounds used. When the membranes were irradiated for 5 min at 300 μmol m^{-2} s^{-1} in the respective medium prior to low-temperature measurements, some changes appeared. The presence of peptides in the medium lead to increase in fluorescence emission between 710 and 720 nm (Fig. 3*B*). This effect was not observed in the presence of diamines (Fig. 3*A*). However, there were systematic differences between the influence of individual peptides. Control peptide induced maximal fluorescence increase at 715 nm. Phosphorylated fragment of LHC2, P2, induced maximal increase at 720 nm and the non-phosphorylated fragment, P3, induced maximal increase at 712 nm (Fig. 3*B*). Similar changes were observed earlier in experiments in the presence of ATP, *i.e.*, when phosphorylation of membrane proteins was observed (Stys *et al.* 1995).

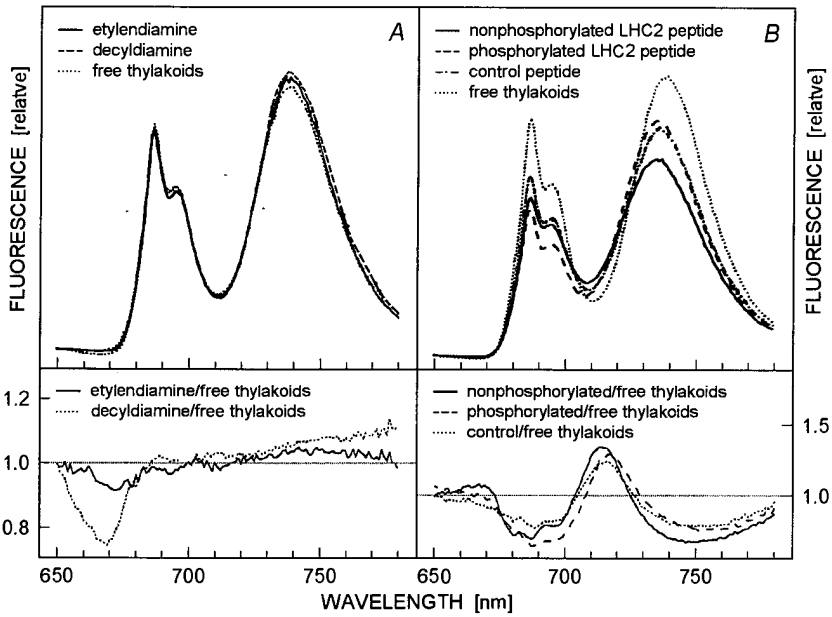

Fig. 3. 77 K fluorescence emission spectra of thylakoids frozen at high energy state after 10 min irradiation with 1000 μmol m^{-2} s^{-1} of "white light", at conditions when the non-photochemical quenching occurred in free membranes. *Upper panels* represents emission spectrum of free thylakoids (*dotted line*), thylakoids in the presence of (*A*) 10 mM ethylenediamine (*solid line*) and 10 mM decyldiamine (*dashed line*), or (*B*) in the presence of 0.83 mM peptide P1, SRPLSDQEKRKQISVRGLAGVENV (*short dashed line*), 0.83 mM peptide P2, RKSAT(PO4)TKKVASSGSP (*long dashed line*) and 0.83 mM peptide P3, RKSATTKKVASSGSP (*solid line*). *Lower panels*: ratio of the spectra recorded with and without (*A*) 10 mM ethylenediamine or (*B*) added peptides in the medium.

We believe that the fluorescence increase between 710 and 720 nm is an indication of restriction of lateral migration (perhaps it reflects inappropriate aggregation of photosystems or antenna systems). The LHC2 fragment occupied specific and different binding sites on the membrane which had became accessible

upon membrane irradiation. These observations indicate that LHC2 may be involved in fine tuning of PS1 antenna and that phosphorylated and non-phosphorylated LHC2 influence different sites.

In conclusion, we demonstrated that diamines known as ΔpH uncouplers have a paralell effect on membrane ultrastructure. The effect on ultrastructure depends on the length of the aliphatic chain. The differences in membrane ultrastructure cannot be put in relation to non-photochemical quenching. The mechanism of non-photochemical quenching involves probably also migration in the membrane plane. The low-temperature experiments indicate that there are specific binding sites for both phosphorylated and non-phosphorylated LHC2. It supports the results of Rintamäkki *et al.* (1997) and Stys *et al.* (1995) which indicate that there are two stages in the adaptation of thylakoid membranes to light—an early stage, in which LHC2 is the prominent phosphoprotein, and the late stage, when LHC2 is functional in non-phosphorylated form. It might be hypothesised that both non-phosphorylated and phosphorylated LHC2, in response to irradiation, physically migrate and bind to a specific binding site on outer antennae of PS1.

Question to be solved is to which extent the observation on isolated thylakoids may be applied to observations on intact leaves. The similarity of results on isolated thylakoids (Stys *et al.* 1995) and on intact leaves (Rintamäkki *et al.* 1997) shows that at least in the case of phosphorylation, results on isolated thylakoids and intact leaves are comparable. Isolated thylakoids inavoidably miss many enzyme components, products, and intermediates such as ribulose-1,5-bisphosphate carboxylase/-oxygenase, the terminal electron acceptor, the electron carriers such as $NADP^+$ and ferredoxin, substrates and products of the ATP synthase reaction to list just the components certainly related to the thylakoid membrane. In a combination of mild isolation methods, optimisation of isolation media, and addition of protective compounds such as ascorbate, similar behaviour of the signal of variable fluorescence to that observed in intact leaves may be preserved. We thus believe that the observations presented in this article reflect at least a part of the mechanisms which contribute to q_N quenching in intact leaves.

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