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# Xanthophyll cycle-dependent quenching of photosystem II chlorophyll a fluorescence: Formation of a quenching complex with a short fluorescence lifetime

**(picosecond time-resolved fluorescence/antheraxanthin/zeaxanthin/nonphotochemical fluorescence quenching)**

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**ABSTRACT** **Excess light triggers protective nonradiative dissipation of excitation energy in photosystem II through the formation of a trans-thylakoid pH gradient that in turn stimulates formation of zeaxanthin and antheraxanthin. These xanthophylls when combined with protonation of antenna pigment–protein complexes may increase nonradiative dissipation and, thus, quench chlorophyll a fluorescence. Here we measured, in parallel, the chlorophyll a fluorescence lifetime and intensity to understand the mechanism of this process. Increasing the xanthophyll concentration in the presence of a pH gradient (quenched conditions) decreases the fractional intensity of a fluorescence lifetime component centered at ≈2 ns and increases a component at ≈0.4 ns. Uncoupling the pH gradient (unquenched conditions) eliminates the 0.4-ns component. Changes in the xanthophyll concentration do not significantly affect the fluorescence lifetimes in either the quenched or unquenched sample conditions. However, there are differences in fluorescence lifetimes between the quenched and unquenched states that are due to pH-related, but nonxanthophyll-related, processes. Quenching of the maximal fluorescence intensity correlates with both the xanthophyll concentration and the fractional intensity of the 0.4-ns component. The unchanged fluorescence lifetimes and the proportional quenching of the maximal and dark-level fluorescence intensities indicate that the xanthophylls act on antenna, not reaction center processes. Further, the fluorescence quenching is interpreted as the combined effect of the pH gradient and xanthophyll concentration, resulting in the formation of a quenching complex with a short (≈0.4 ns) fluorescence lifetime.**

A conserved mechanism protects photosystem II (PSII) in higher plants against damage by excess absorbed excitation energy (1, 2). It has been suggested that excess excitation of the PSII antenna is nonradiatively dissipated, causing nonphotochemical quenching (NPQ) of PSII chlorophyll (Chl) a fluorescence (Fig. 1) (1–4). Excess light triggers NPQ by first causing chloroplast lumen acidification. Proton pumping from either light-driven electron flow or the chloroplast ATPase can induce NPQ (3). Lumen acidity serves at least two roles in the NPQ mechanism. First it activates the xanthophyll cycle deepoxidase (5) that catalyzes violaxanthin (Vx) deepoxidation to zeaxanthin (Zx) via antheraxanthin (Ax) (6). Second, protonation apparently alters the conformation of PSII antenna and/or reaction center pigment–protein complexes, promoting interaction with the xanthophylls and somehow causing NPQ (3, 4, 7). In isolated chloroplasts, NPQ depends on both lumen acidification and Zx plus Ax (4). Uncouplers of the proton gradient reverse NPQ and inhibit deepoxidation (3–5).

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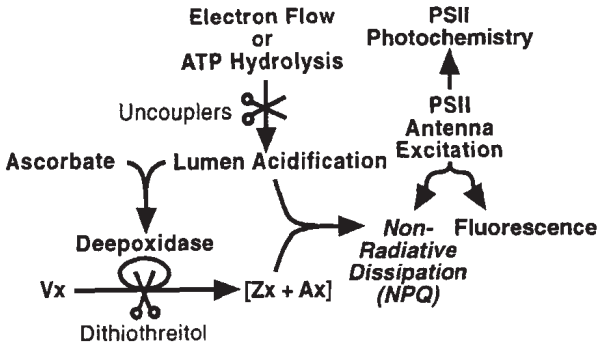


FIG. 1. Schematic summary of the light- and ATP-induced xanthophyll-dependent NPQ mechanism in isolated chloroplasts. Vx, violaxanthin; Zx, zeaxanthin; Ax, antheraxanthin

Dithiothreitol (DTT) inhibits deepoxidation but not lumen acidification (8) or NPQ caused by preformed Zx plus Ax (3, 4).

One proposed NPQ mechanism includes a role for Vx in preventing or reversing pH-dependent aggregation of PSII light-harvesting antenna complexes (LHCIIs) (9). Here, decreasing the Vx concentration, which is equivalent to increasing the sum of the Zx and Ax concentrations, [Zx + Ax] (Fig. 1), enhances LHCII aggregation and hence NPQ by creating weakly fluorescent long-wavelength-absorbing chlorophylls. However, Vx deepoxidation apparently does not induce long-wavelength-absorbing chlorophylls in leaves (10), and there is no indication of long-wavelength chlorophylls in other chloroplast studies (11, 12). Another suggested NPQ mechanism involves the trans-thylakoid pH gradient (ΔpH) causing $Ca^{2+}$ release from and inhibition of the PSII donor side (13); thus, due to PSII donor side inhibition, the quinone acceptor of PSII ($Q_A$) remains oxidized and photochemically quenches Chl a fluorescence.

The processes competing for the de-excitation of the excited state determine the quantum yield of fluorescence—i.e., $\phi_F = k_f/(k_f + k_p + k_t + k_n)$, where $k_f$ is the rate constant of Chl a fluorescence, $k_p$ is the rate constant of photochemical quenching by oxidized $Q_A$, $k_t$ is the rate constant of energy transfer

Abbreviations: Ax, antheraxanthin; *c*, center of fluorescence lifetime distribution; Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DTT, dithiothreitol; *f*, fractional intensity of fluorescence lifetime or distribution; $F_m$ ($F'_m$), maximal fluorescence intensity in the absence (presence) of NPQ; $F_o$ ($F'_o$), minimal fluorescence intensity in the absence (presence) of NPQ; LHCP (LHCII), light-harvesting pigment–protein complex (of PSII); NPQ, nonphotochemical quenching of PSII Chl a fluorescence; PAM, pulse-amplitude modulation fluorimeter; PSII, photosystem II; $Q_A$, primary quinone electron acceptor of PSII; Vx, violaxanthin; Zx, zeaxanthin; ΔpH, trans-thylakoid proton gradient; $\langle\tau\rangle$($\langle\tau\rangle'$), average lifetime of Chl a fluorescence in the absence (presence) of NPQ; *w*, width at half maximum of fluorescence lifetime distribution.

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from fluorescent PSII to weakly fluorescent photosystem I, and $k_n$ is the rate constant of nonradiative dissipation. NPQ is thus presumably determined by $k_n$, which is apparently proportional to the lumen proton concentration and [Zx + Ax] (4). However, current kinetic models cannot be used to interpret available data and determine whether NPQ occurs in the PSII antenna or reaction center complexes (for a review, see ref. 14). On the one hand, the kinetic model of Butler and Kitajima (15) predicts that increasing $k_n$ in the PSII antenna should quench both the minimal ($F_o$; maximum $k_p$) and maximal ($F_m$; $k_p = 0$) fluorescence intensity; quenching that affects charge separation in the reaction center should not affect $F_o$. On the other hand, the exciton radical pair equilibrium model (16) for PSII suggests that the reaction center P680 Chl a is a shallow trap and that charge recombination of the radical pair can allow an exciton to diffuse back to the antenna. Therefore, the exciton radical pair model, which assumes PSII photochemistry is trap-limited, contrasts with Butler and Kitajima's model (15), which assumes that exciton diffusion from the antenna to the reaction center limits charge separation. Thus, in the exciton radical pair model, $F_m$ and $F_o$ quenching by NPQ could be proportional to each other in either the reaction center or antenna complexes, but quenching of $F_m$ and *not* $F_o$ indicates an effect on charge separation (14). Several reports show that NPQ of $F_o$ is proportional to that of $F_m$ (3, 9, 17), in contrast to a report by Krieger *et al.* (13), which shows no $F_o$ quenching.

In this paper we compared, in parallel, the xanthophyll concentration, the Chl fluorescence lifetimes, and the quenching of $F_m$ and $F_o$ to gain insight into the NPQ mechanism. Analyses of the fluorescence lifetimes clearly show that NPQ is due to a xanthophyll-dependent increase in the fractional intensity of a specific Chl a component with a short fluorescence lifetime. The lifetime data suggest that NPQ is due to the formation of a xanthophyll-dependent quenching complex. The lifetime and intensity ($F_m$ and $F_o$) data together indicate that NPQ differs mechanistically from $Q_A$ quenching. The results are discussed in terms of the current biophysical and structural models of PSII fluorescence and xanthophyll-induced NPQ.

## MATERIALS AND METHODS

**Time-Resolved Chl a Fluorescence Measurements.** Fluorescence lifetimes were measured with a multifrequency cross-correlation fluorimeter (model K2; ISS Instruments, Urbana, IL) (18–20). Sample excitation at 610 nm was provided by a cavity-dumped rhodamine 6G dye laser (Coherent, Palo Alto, CA) pumped by a mode-locked Nd:YAG laser (Coherent). The sample was excited under "magic-angle" (54.7°) conditions. Data were collected at 16 separate sinusoidally modulated excitation frequencies ranging from 7 to 300 MHz. The frequencies were mixed randomly during acquisition to minimize systematic errors from sample bleaching. Emission at wavelengths >620 nm was monitored through a red Hoya R-64 cutoff filter. The shift in phase angles (phase) and demodulation ratios (modulation) of the sample fluorescence emission were analyzed for fluorescence lifetime information. Further details and explanation of the multifrequency cross-correlation technique can be found elsewhere (18–20). The fluorescence lifetime data are presented as the lifetimes ($\tau$) and fractional intensities ($f_i = \alpha_i\tau_i/\Sigma\alpha_i\tau_i$, where $\alpha_i$ is the preexponential amplitude factor representing the fractional contribution of the component with the lifetime $\tau_i$). Plots of Lorentzian lifetime distributions were normalized to a value of 1 for the major component.

**Chloroplast Isolation.** Fresh spinach (*Spinacia oleracea*) and lettuce (*Lactuca sativa* L. cv. Romaine) leaves were obtained locally and stored at least 12 h (dark, 4°C) before chloroplast isolation (3). The chloroplasts were osmotically shocked (10–15 s) in 1 ml of distilled water and then brought to 5 ml and 30 $\mu$M Chl a + b in a reaction medium containing 50 $\mu$M methylviologen, 0.3 mM ATP, 0.1 M sucrose, 10 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$, 10 mM Tricine, 1 mM $KH_2PO_4$, and 0.2% (wt/vol) defatted bovine serum albumin adjusted to pH 8.0 with NaOH. This procedure yielded the thylakoid membranes used in this study.

**Experimental Protocol.** Fig. 2 shows an example, using spinach thylakoids, of the Chl fluorescence intensity determinations with the pulse-amplitude modulation fluorimeter (PAM) (model 103; Heinz Walz, Effeltrich, F.R.G.) (21). We measured $F_o$ (minimum fluorescence) with a <0.15 $\mu$mol of photons per $m^2$ per s (1.6 kHz) beam and $F_m$ (maximum fluorescence) with a 2-s light pulse (5000 $\mu$mol of photons per $m^2$ per s after passing through a Heinz Walz model DT-cyan filter). The white continuous actinic light (450–500 $\mu$mol of photons per $m^2$ per s; Corning CS1-75 infrared filter) was turned on as the PAM measuring beam was switched to 100 kHz. After a 15-min light exposure, 3 mM DTT was added to stop deepoxidation (Fig. 1) and to assure thiol activation of the ATPase (22). The minimal fluorescence intensity in the presence of NPQ ($F'_o$) was monitored 1 min after turning off the continuous light, and then the sample was transferred to dark at 0°C. The sample, now in a "quenched" condition as far as the Chl a fluorescence is concerned, was prepared for the lifetime measurements by diluting a portion to a final volume of 3 ml and 7.5 $\mu$M Chl a + b; all other reagents were the same as in the reaction mixture except that 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU; 10 $\mu$M) was added. The quenched sample was analyzed in the lifetime instrument at 5°C within ≈15 min. Then, as shown in Fig. 2*B*, the sample was rapidly placed in the PAM where the quenched maximal fluorescence intensity in the presence of NPQ ($F'_m$) was measured, the $\Delta$pH was uncoupled with nigericin, and the "unquenched" $F_m$ was determined. The unquenched fluorescence lifetime data were either immediately taken, or the samples were stored (dark, 0°C) until the quenched samples were finished; neither the lifetimes nor $F_m$ changed during ice storage (<4 h).

The [Zx + Ax] was controlled by the DTT concentration added before illumination [i.e., 3 mM for complete inhibition and usually <0.25 mM for subsaturating inhibition (4)]. The high [Zx + Ax] was obtained by omitting DTT during the first 15 min of actinic illumination, and the low [Zx + Ax] samples had 3 mM DTT before illumination. We restricted changes in fluorescence during the lifetime measurements to changes in nonradiative dissipation by adding DCMU, which eliminates photochemical quenching by inhibiting $Q_A^-$ oxidation, and methylviologen, which inhibits energy transfer changes (23). Under these conditions, the ATPase $\Delta$pH remained very constant for >0.5 h.

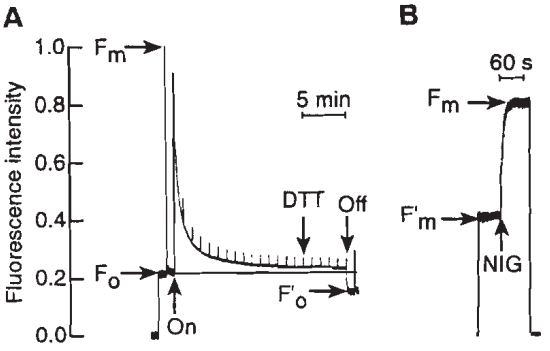


FIG. 2. Chl a fluorescence intensity determinations in spinach thylakoids for $F_m$, $F'_m$, $F_o$, and $F'_o$. The final unquenched $F_m$ was measured 60 s after adding 2 $\mu$M nigericin (NIG). The sample cuvette for the PAM was at 15°C. Similar results were obtained with lettuce thylakoids. "On" and "Off" refer to turning the continuous actinic light on and off, respectively.

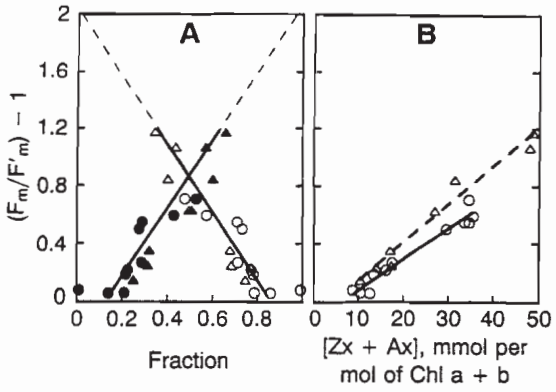


FIG. 5. Relationship between $(F_m/F'_m) - 1$ and the Lorentzian fractional intensities (*A*) and the [Zx + Ax] (*B*). (*A*) Solid symbols indicate the quenched $f_2$ values in Table 1 of spinach (▲) and lettuce (●) thylakoids, respectively. Open symbols indicate the quenched $f_1$ values in Table 1. (*B*) The plots were fit by linear regression, and the $r^2$ values for spinach (△) and lettuce (○) were 0.969 and 0.956, respectively. For spinach and lettuce the [Vx + Ax + Zx] were 83 ± 6 and 82 ± 3 mmol per mol of Chl a + b, respectively.

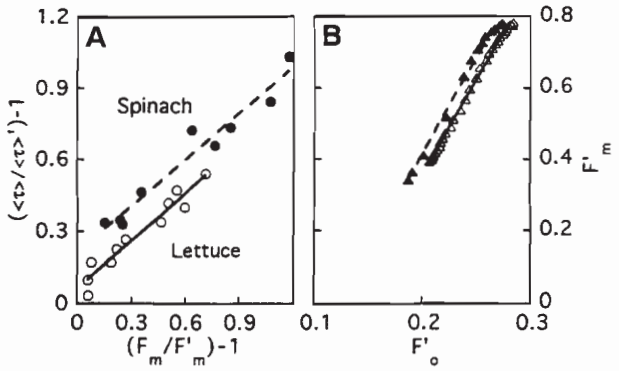


FIG. 6. (*A*) Relationship between $(\langle\tau\rangle/\langle\tau\rangle') - 1$ and $(F_m/F'_m) - 1$ for spinach and lettuce thylakoids. The $\langle\tau\rangle$ and $\langle\tau\rangle'$ values were calculated for each point as in Table 2. The equations of the regression lines for spinach and lettuce were $(\langle\tau\rangle/\langle\tau\rangle') - 1 = 0.64(F_m/F'_m - 1) + 0.21$; $r^2 = 0.96$ and $(\langle\tau\rangle/\langle\tau\rangle') - 1 = 0.67(F_m/F'_m - 1) + 0.06$; $r^2 = 0.94$, respectively. For spinach (and lettuce) ratios of variable fluorescence to maximal fluorescence ($F_v/F_m$) before light treatment were 0.79 ± 0.01 (and 0.80 ± 0.004). (*B*) Plots of $F'_o$ against $F'_m$ in lettuce chloroplasts at 30°C (△) and 25°C (▲). Both $F'_o$ and $F'_m$ are normalized relative to the preillumination $F_m$ (see Fig. 2); the preillumination $F_v/F_m$ was 0.785 for both samples. The [Zx + Ax] was 63 ± 4 mmol per mol of Chl a + b for both samples. The equations of the regression lines were $F'_o = 0.19F'_m + 0.13$; $r^2 = 0.997$ (30°C) and $F'_o = 0.19F'_m + 0.12$; $r^2 = 0.990$ (25°C).

quenched and quenched with low [Zx + Ax]. The low $\chi^2$ values indicated a goodness of fit similar to the Lorentzian models (Fig. 4 legend). Also similar to the Lorentzian model, [Zx + Ax] had little or no significant effect on the fractional intensities and lifetimes in the unquenched samples (Table 2). Furthermore, in the quenched samples the major changes during quenching occurred in the fractional intensities (values in parentheses) and not the lifetimes. In contrast to the Lorentzian model, the discrete analysis showed larger lifetime changes between the quenched and unquenched samples along with fractional shifts in both the plant species. The quenched $\langle\tau\rangle'$ values (i.e., the average lifetime of Chl a fluorescence in the presence of NPQ) are clearly shorter in the high than in the low [Zx + Ax] samples, whereas the unquenched $\langle\tau\rangle$ values are independent of [Zx + Ax]. In Fig. 6*A* the slopes of the linear plots of $(F_m/F'_m) - 1$ against $(\langle\tau\rangle/\langle\tau\rangle') - 1$ were 0.64 and 0.67 for spinach and lettuce, respectively. Importantly, as with the Lorentzian model, these analyses show that the changes in measured $F_m$ that are correlated with changes in $\langle\tau\rangle$ are mainly due to changes in the fractional intensities of the discrete exponential lifetime components.

**Effects of NPQ on $F_m$ and $F_o$.** Fig. 6*B* shows $F'_o$ plotted against $F'_m$ for experiments with lettuce thylakoids similar to the experimental conditions used in Fig. 2*A*. Here, after a preillumination period where NPQ was induced, we recorded the values over a 1-h dark period during which the ATP-induced NPQ slowly and completely relaxed. DCMU was omitted from these samples to allow for complete $Q_A^-$ oxidation between the high-intensity light flashes used for the $F'_m$ measurements. The $F'_o$ and $F'_m$ showed a linear correlation with only a slight deviation at the high range of $F'_m$ and $F'_o$. In short, these data established that $F'_o$ and $F'_m$ are quenched in a linear proportion to each other and confirmed that NPQ does not affect charge recombination in the reaction center.

## DISCUSSION

**Xanthophyll Cycle-Induced Quenching of Chl a Fluorescence.** Increasing the Vx deepoxidation to Zx + Ax during NPQ correlates with the conversion of a 2-ns Lorentzian fluorescence lifetime component to a 0.4-ns component (Fig. 4 and Table 1); this conversion decreases the fluorescence yield and quenches $F_m$ (Fig. 5). Because the fluorescence lifetimes of the Lorentzian distributions were independent of xanthophyll concentration during NPQ and also because the extent of NPQ is not inhibited at low temperatures (ref. 26; this study), we conclude that NPQ is not controlled by rates of diffusive collisions. We instead suggest that NPQ is due to the formation of quenching complexes. The shorter fluorescence lifetime of the $c_2$ compared to the $c_1$ component is consistent with a higher rate constant of nonradiative dissipation ($k_n$) for the $c_2$ component. The differences between the fluorescence lifetime parameters of the two components during the quenching process suggest that they arise from antenna complexes in either an unquenched ($c_1$) or quenched ($c_2$) conformation, respectively. Interestingly, the apparent changes in the con-

Table 2. Discrete lifetimes, given in ns, and fractional intensities (in parentheses) under conditions of quenched and unquenched Chl fluorescence in spinach and lettuce thylakoids with either high or low [Zx + Ax]

| | Spinach | | | | Lettuce | | | |
|---|---|---|---|---|---|---|---|---|
| | Quenched | | Unquenched | | Quenched | | Unquenched | |
| | High* | Low* | High* | Low* | High† | Low† | High† | Low† |
| $\tau_1$ | 4.35 (0.08) | 3.05 (0.31) | 4.55 (0.10) | 6.49 (0.04) | 3.21 (0.27) | 2.72 (0.70) | 2.99 (0.68) | 2.99 (0.65) |
| $\tau_2$ | 1.73 (0.53) | 1.54 (0.58) | 2.37 (0.85) | 2.39 (0.86) | 1.41 (0.56) | 1.09 (0.26) | 1.46 (0.28) | 1.53 (0.30) |
| $\tau_3$ | 0.40 (0.39) | 0.29 (0.10) | 0.31 (0.05) | 0.41 (0.10) | 0.17 (0.17) | 0.00 (0.03) | 0.00 (0.04) | 0.04 (0.05) |
| $\langle\tau\rangle'$ or $\langle\tau\rangle$‡ | 1.41 | 1.88 | 2.50 | 2.35 | 1.67 | 2.20 | 2.45 | 2.41 |
| $\chi^2$ | 1.891 | 0.888 | 0.892 | 1.480 | 2.90 | 2.36 | 1.58 | 1.08 |

The analysis was made using a three-exponential decay model.
*The high and low [Zx + Ax] for spinach were 31 and 10 nmol per mol of Chl a + b, respectively.
†The high and low [Zx + Ax] for lettuce were 34 and 13 nmol per mol of Chl a + b, respectively.
‡$\langle\tau\rangle$ (unquenched) and $\langle\tau\rangle'$ (quenched) = $\Sigma f_i\tau_i$.