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Zeaxanthin Formation and Energy-Dependent Fluorescence Quenching in Pea Chloroplasts under Artificially Mediated Linear and Cyclic Electron Transport[1]

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ABSTRACT

Artificially mediated linear (methylviologen) and cyclic (phenazine methosulfate) electron transport induced zeaxanthin-dependent and independent (constitutive) nonphotochemical quenching in osmotically shocked chloroplasts of pea (*Pisum sativum* L. cv Oregon). Nonphotochemical quenching was quantitated as Stern-Volmer quenching (SV_N) calculated as (F_m/F'_m)-1 where F_m is the fluorescence intensity with all PSII reaction centers closed in a nonenergized, dark-adapted state and F'_m is the fluorescence intensity with all PSII reaction centers closed in an energized state. Reversal of quenching by nigericin and electron-transport inhibitors showed that both quenching types were energy-dependent SV_N. Under light-induced saturating ΔpH, constitutive-SV_N reached steady-state in about 1 minute whereas zeaxanthin-SV_N continued to develop for several minutes in parallel with the slow kinetics of violaxanthin deepoxidation. SV_N above the constitutive level and relative zeaxanthin concentration showed high linear correlations at steady-state and during induction. Furthermore, F_o quenching, also treated as Stern-Volmer quenching (SV_O) and calculated as (F_o/F'_o)-1, showed high correlation with zeaxanthin and consequently with SV_N (F_o and F'_o are fluorescence intensities with all PSII reaction centers in nonenergized and energized states, respectively). These results support the view that zeaxanthin increases SV_N above the constitutive level in a concentration-dependent manner and that zeaxanthin-dependent SV_N occurs in the pigment bed. Preforming zeaxanthin increased the rate and extent of SV_N, indicating that slow events other than the amount of zeaxanthin also affect final zeaxanthin-SV_N expression. The redox state of the primary electron acceptor of photosystem II did not appear to determine SV_N. Antimycin, when added while chloroplasts were in a dark-adapted or nonenergized state, inhibited both zeaxanthin-SV_N and constitutive-SV_N induced by linear and cyclic electron transport. These similarities, including possible constitutive F_o quenching, suggest that zeaxanthin-dependent and constitutive SV_N are mechanistically related.

Light energy that is not used photosynthetically is dissipated radiatively as fluorescence or nonradiatively as heat. Nonradiative energy dissipation at PSII is thought to serve a protective function against the potentially damaging effects of excess light and appears to be under photosynthetic control (3, 14, 32). Nonradiative dissipation of excitation energy at PSII is seen experimentally as a component of q_N[2] (23). The light-doubling technique (6) and advances in instrumentation (23) have enabled resolution of various q_N components (see ref. 13 for a review). The major component is ΔpH-dependent quenching, q_E (15), which also appears to depend on the redox state of a membrane component (20). Recently, q_E has been related to "down regulation" of photochemistry at PSII (32).

The mechanism for q_E is unclear. Exchange of protons for Mg^{2+} (16), conversion of PSII from fluorescent to nonfluorescent forms (32), and zeaxanthin formation have been implicated (9). Zeaxanthin is formed from violaxanthin (34) by action of violaxanthin deepoxidase whose activity requires an acidified lumen (11). Depending on treatment, zeaxanthin formation results in increased irreversible or reversible q_N (8, 9). Irreversible or slowly reversible zeaxanthin-dependent nonphotochemical quenching may be related to photoinhibition (8). Rapidly reversible zeaxanthin-dependent q_N is concluded to be q_E (9, 10). Zeaxanthin-dependent q_N appears to have a photoprotective function (3).

Whether q_E comprises more than one component is controversial. The results of several laboratories support the view that zeaxanthin-dependent q_E adds to an underlying zeaxanthin-independent q_N (3, 9, 10). Other studies conclude instead that zeaxanthin sensitizes q_E to ΔpH and that, at saturating ΔpH, zeaxanthin does not increase total q_N (18). Both q_N (5) and zeaxanthin-dependent q_E (3, 9) have been correlated with F_o quenching (q_O) which, according to the Butler-Kitajima model (7), suggests quenching occurs in the pigment bed.

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[2] Abbreviations: q_N, coefficient for nonphotochemical quenching; q_E, coefficient for energy-dependent nonphotochemical quenching; Q_A, primary electron acceptor of PSII; MV, methylviologen (1,1′-dimethyl-4,4′-bipyridinium dichloride); DBMIB, dibromothymoquinone (2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone); PQ, plastoquinone; q_P, coefficient for photochemical quenching; FQR, ferredoxin-quinone reductase; VAZ, the sum of the xanthophyll cycle pigments violaxanthin, antheraxanthin, and zeaxanthin; ASC, ascorbate; F_m, fluorescence intensity with all PSII reaction centers closed in nonenergized, dark adapted state; F'_m, fluorescence intensity with all PSII reaction centers closed in an energized state; F_o, fluorescence intensity with all PSII reaction centers open in nonenergized state; F'_o, fluorescence intensity with all PSII reaction centers open in energized state; q_o, coefficient for nonphotochemical quenching of F_o.

However, where zeaxanthin dependent and constitutive q_N are thought to be separate activities, zeaxanthin-independent quenching is proposed to quench in the reaction center (1, 9).

Here the relationships between nonphotochemical quenching and zeaxanthin formation in osmotically shocked pea (*Pisum sativum* L. cv Oregon) chloroplasts under artificial electron transport and saturating light are reported. We used Stern-Volmer data treatment of nonphotochemical quenching, previously used by Bilger and Björkman (3), because it allowed quantitation of concentration-dependent quenching. The results suggest that at saturating ΔpH, zeaxanthin quantitatively increases nonphotochemical quenching above an underlying zeaxanthin-independent quenching. Both types of quenching are energy-dependent and have other similar properties that suggest a mechanistic relationship. Zeaxanthin-dependent quenching and possibly constitutive quenching appear to quench in the pigment bed.

MATERIALS AND METHODS

Chloroplast Isolation

Pisum sativum L. cv Oregon, pea, was grown at 20°C in a growth cabinet at 650 μE m^{-2} s^{-1} PAR and a 16-h photoperiod. Plants were dark-adapted for 12 to 16 h before harvesting to eliminate background levels of zeaxanthin, then chilled in the dark at 4°C for 1 h before chloroplast isolation. Chloroplasts were isolated according to Horton and Black (12) with slight modifications. The resuspension medium contained 0.1% BSA and the reaction medium contained 10 mM KCl. Immediately prior to each treatment, chloroplasts in resuspension buffer were osmotically shocked for 15 to 20 s in 1:10 dilution with distilled water then brought to 3 mL with reaction medium. Chloroplast concentration was equivalent to 30 μg total Chl/mL for all reactions.

Simultaneous Measurement of Absorbance and Room-Temperature Chl Fluorescence

Room-temperature Chl-fluorescence induction and the 505 minus 540 nm absorbance change associated with zeaxanthin formation were measured simultaneously with a PAM 101 Chlorophyll Fluorometer (Heinz Walz, Effeltrich, FRG) and a DW-2000 UV-VIS spectrophotometer (SLM-Aminco), respectively. Chloroplast suspensions in spectrofluorometric cuvettes were illuminated at right angles to the spectrophotomer beam with actinic light from a Unitron microscope lamp and saturating light from a KL-1500 flash unit (Heinz Walz) through the PAM fiber-optic unit. Both light sources were filtered through Corning CS2-58 (red) and CS1-75 (IR) filters. Actinic light and saturating flash intensities at the cuvette were 425 μE m^{-2} s^{-1} and 2960 μE m^{-2} s^{-1}, respectively, unless otherwise stated. This actinic light intensity was saturating for ΔpH as indicated by neutral red uptake (26). A Corning CS4-96 filter protected the photomultiplier tube from the actinic light and saturating flashes. Opal glass, a quartz diffuser plate, and a beam scrambler between the cuvette and the photomultiplier tube were used to reduce light-scattering effects. All reactions were at 20°C and stirred continuously.

Stern-Volmer Fluorescence-Quenching Parameters

In the absence of standard nomenclature for Stern-Volmer (SV) treatments of Chl fluorescence, we chose the expressions SV_N to equal $(F_m/F'_m) - 1$ and SV_O to equal $(F_o/F'_o) - 1$ for nonphotochemical quenching of F_m and F_o, respectively. The SV_N and SV_O nomenclature parallel the quenching coefficient expressions q_N and q_O. Similarly, nonphotochemical quenching that is reversible by uncouplers or electron transport inhibitors is energy-dependent SV_N or SV_E. The nomenclature used for fluorescence-intensity indicators follows the recommendations of van Kooten and Snel (30).

In a conventional SV plot, absolute quencher concentration is plotted against the SV expression, the resulting slope being the SV-quenching constant. Here, the plots are SV-type rather than classical SV plots. Zeaxanthin concentrations are expressed relative to Chl *a* and not as absolute concentrations since the latter in the membrane cannot be readily determined. Unless otherwise stated, zeaxanthin-dependent SV_N was resolved as ΔSV_N, calculated as SV_N with zeaxanthin formed minus SV_N with zeaxanthin formation completely inhibited with $\geq$1 mM DTT (33). ΔSV_O was resolved in a similar way as ΔSV_N. q_P (23) was estimated using the initial F_o instead of F'_o.

Pigment Analysis

Zeaxanthin concentrations relative to Chl *a* were determined with a new HPLC method that resolves zeaxanthin, lutein, and most of the chloroplast pigments at or near baseline in about 13 min. Acetone extracts (20 μL containing about 90 μg mL^{-1} Chl *a*) were chromatographed on an ODS-1 column (Alltech Associates, Inc.) at 2 mL min^{-1} starting with an aqueous mixture of acetonitrile/methanol/0.1 M Tris-HCl buffer (pH 8.0) (72:8:3) for 4 min followed by a 2.5 min linear gradient to methanol hexane (4:1). Further details of this method will be published elsewhere. The detectable limit of zeaxanthin for the concentration and volume of extract analyzed was about 2 mmol zeaxanthin mol^{-1} Chl *a*.

RESULTS

Effects of Methylviologen on Photochemical and Nonphotochemical Fluorescence Quenching

MV used to support linear electron transport quenched F_m in dark-adapted, nonenergized chloroplasts (Fig. 1). Accordingly, even saturating flashes of over 20,000 μE m^{-2} s^{-1} did not restore F_m to levels before MV addition (data not shown). Vernotte *et al.* (31) reported a similar effect which they attributed to nonphotochemical quenching of PSII by oxidized PQ. In the presence of ascorbate, inhibiting electron flow with DBMIB restored the original F_m (Fig. 1A and B). DBMIB also fully restored the F_m that was further decreased by uncoupling with nigericin (Fig. 1B). In contrast, DCMU did not restore MV-mediated F_m quenching completely (data not shown). These effects are consistent with quenching of F_m by oxidized PQ and highlight the potential of MV and uncouplers to confound F_m.

To account for MV quenching, a saturating pulse was delivered before and after MV addition. When PQ oxidation

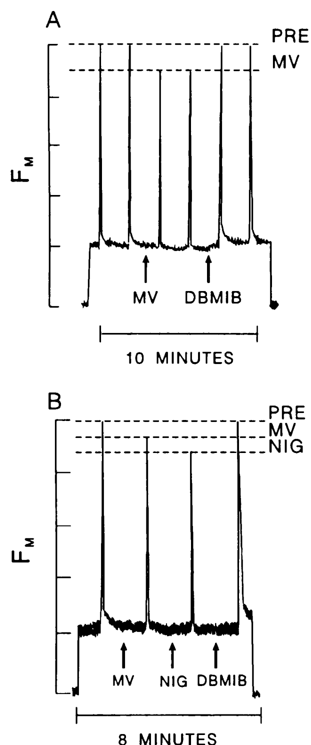

Figure 1. The effects of MV on F_m in osmotically shocked pea chloroplasts. Saturating flashes were delivered to dark-adapted chloroplasts exposed only to the low level 1.6 kHz PAM illumination. MV-induced quenching of F_m (A) was further enhanced by nigericin (NIG, B). DBMIB restored F_m in both cases. Final concentrations were 60 mM ascorbate (present from the beginning of the experiment), 0.1 mM MV, 2 μM NIG, and 2 μM DBMIB.

was not inhibited with DBMIB or when linear flow was uninterrupted by DCMU, the latter F_m was used; when DBMIB or DCMU was present, the former F_m was used for calculations. Since DCMU and reduced DBMIB abolish ΔpH, these inhibitors also enabled resolution of SV_E from ΔpH-independent SV_N. However, only data employing DBMIB are strictly quantitative because, as previously mentioned, DCMU does not fully restore F_m.

Ascorbate is required if DBMIB is used to restore maximum fluorescence because oxidized DBMIB quenches fluorescence. Ascorbate is also necessary for zeaxanthin formation in broken chloroplasts (24). Preliminary experiments showed that ascorbate affected the dynamic relationship between photochemical electron transport, zeaxanthin formation, and SV_N. We therefore characterized these effects. Figure 2 shows the effects of ascorbate and MV on 1-q_P (panel A), SV_N (panel B), and zeaxanthin formation (panel C) in osmotically shocked pea chloroplasts. The zero time for A and B represents the time from beginning of actinic illumination in C (ON). In the absence of MV or ascorbate, 1-q_P was high, SV_N was low, little if any SV_E was observed, and no detectable zeaxanthin was formed (confirmed by HPLC analysis). The addition of MV reduced 1-q_P markedly and stimulated a low level of constitutive SV_E, but again no detectable zeaxanthin was formed. Ascorbate and ascorbate + MV stimulated SV_E

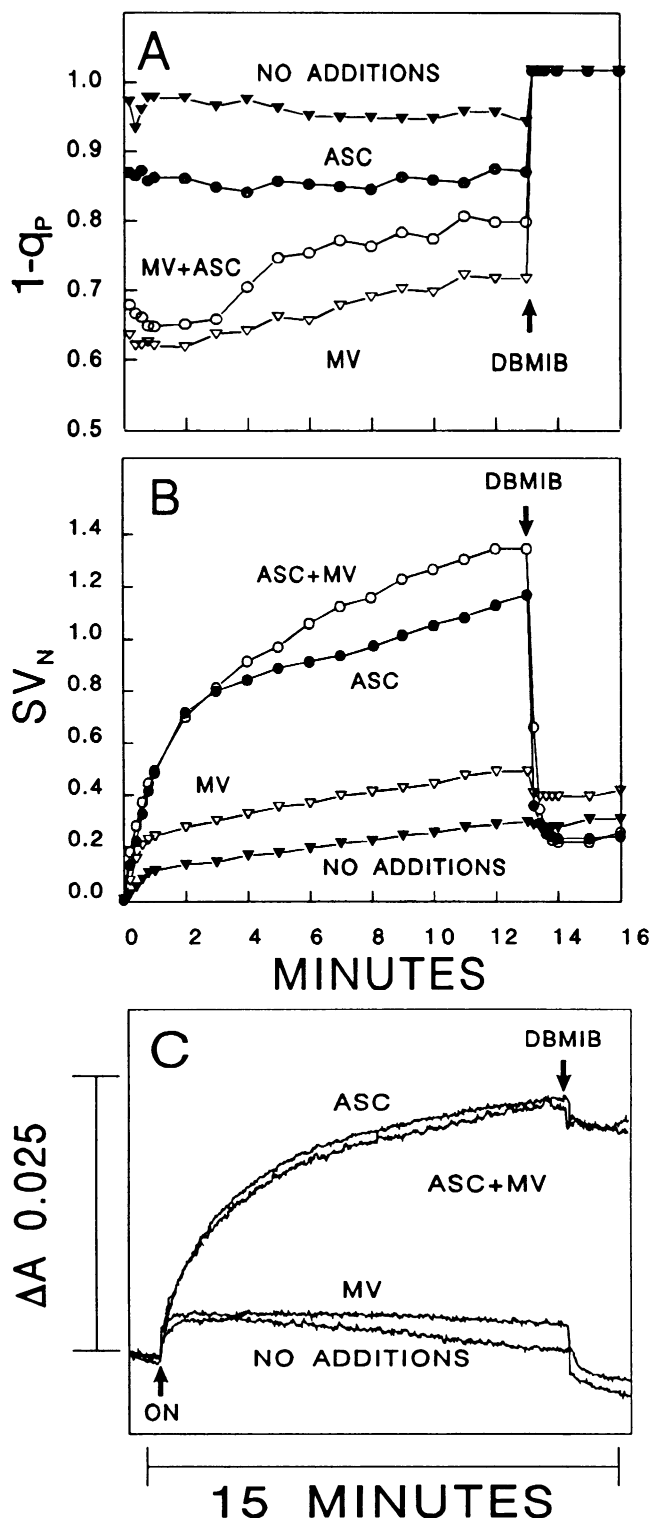

Figure 2. Effects of ascorbate and MV on (A) 1-q_P, (B) SV_N, and (C) 505 nm kinetics of zeaxanthin formation. Final zeaxanthin levels determined by HPLC were 37.1 and 36.7 mmol mol^{-1} Chl *a* for the ASC + MV and ASC reactions, respectively. No detectable zeaxanthin was formed in the MV only or "no additions" reactions. Final DBMIB concentration was 2 μM.

and zeaxanthin formation. Ascorbate, however, lowered 1-q_P relative to the no-additions treatment and increased 1-q_P relative to the MV-only treatment. Hence, in the former case, addition of ascorbate stimulated electron flow, whereas in the latter case electron flow through Q_A was seemingly reduced. The reason for these apparently opposite effects of ascorbate on 1-q_P is not clear. Perhaps in the absence of a more suitable electron acceptor, the small amount of dehydroascorbate that is unavoidably present in our ascorbate solutions served as an electron acceptor, mediating Q_A oxidation. Siefermann and Yamamoto (24) reported that dehydroascorbate could be reduced photosynthetically and thus supports de-epoxidation of zeaxanthin in light, but not if an alternative (competitive) electron acceptor such as MV is present. In the present study when MV was present, ascorbate somehow either reduced electron flow or served as an alternative electron donor. Regardless of mechanism, MV kept Q_A relatively oxidized, as indicated by the 1-q_P values even with ascorbate present. These results show that maximal SV_E development required zeaxanthin formation but neither SV_E nor zeaxanthin formation required complete reduction of Q_A.

Correlation of Zeaxanthin and SV_N at Steady-State and during Induction

Earlier, we (10) reported that the kinetics and extent of zeaxanthin formation qualitatively correlated with q_E under both linear and PSI-cyclic electron transport. Here we quantitated the steady-state and kinetic relationships between zeaxanthin formation and SV_N under artificially mediated electron transport. In Figure 3, steady-state SV_N and zeaxanthin formation were varied with increasing DTT concentrations. DTT inhibited deepoxidase activity (33) but had no effect on the quantum yield of photosynthesis, electron transport rates (4), or ΔpH (28). Figure 3A shows SV_N development at various DTT concentrations under phenazine methosulfate-mediated cyclic electron transport. Similar experiments were also done under MV-mediated electron transport (development data not shown). Figure 3B shows zeaxanthin concentration at 13 min, plotted against the corresponding ΔSV_N. According to classic SV quenching, the relationship between fluorescence-quencher concentration and quenching is directly proportional to the expression $(F_m/F'_m) - 1$. The SV-type quenching for the two types of electron transport closely fit the same line ($r^2 = 0.992$), indicating that induction of zeaxanthin-SV_N by both electron transport systems was similar if not identical. The linear relationship indicates a concentration-dependent quenching mechanism. The SV-type quenching constant was 0.031 SV_N units/mmol zeaxanthin mol^{-1} Chl *a*.

The quantitative relationship of SV_N and zeaxanthin during induction was examined using 505 nm absorbance changes to follow zeaxanthin formation continuously. Figure 4 shows SV_N kinetics (A) and corresponding 505 nm kinetics (B) under MV-mediated electron transport. The zero time in Figure 4A corresponds to the beginning of actinic illumination (ON) in Figure 4B. Without DTT, the development of SV_N and 505 nm change were high. Zeaxanthin forms irreversibly under these conditions and the irreversible part of the 505 nm change (Fig. 4B) corresponds to zeaxanthin formation. Small

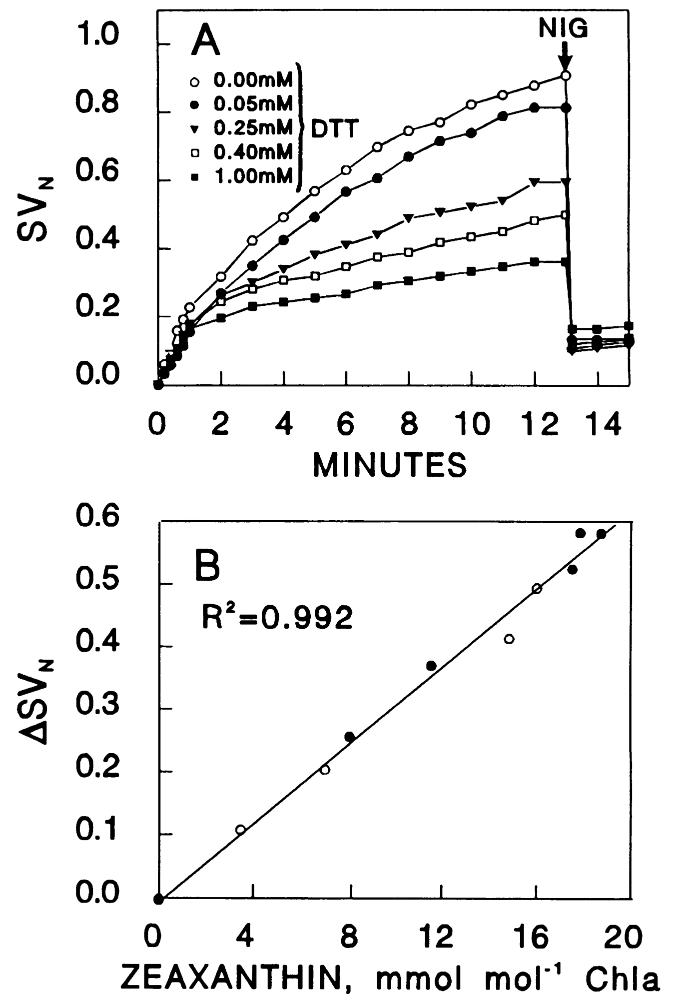

Figure 3. Relationship between zeaxanthin concentration and ΔSV_N at steady-state under linear and cyclic electron transport. Zeaxanthin levels were varied with DTT. Panel A shows SV_N development under cyclic conditions with 2 μM DBMIB, 1 μM phenazine methosulfate and 60 mM ascorbate. Panel B shows the SV-type plot of zeaxanthin concentration against ΔSV_N at steady-state (13 min) using data from experiment in panel A for cyclic conditions (hollow symbols) and a similar experiment for linear conditions (solid symbols) with 60 mM ascorbate and 0.1 mM MV (development data not shown). The final zeaxanthin levels were determined by HPLC. ΔSV_N is the difference between SV_N when zeaxanthin was formed (<1 mM DTT) minus SV_N when it was totally inhibited (1 mM DTT). The equation for the regression line is $y = 0.031x + 0.001$.

absorbance changes at 505 nm observed with DTT present were apparently unrelated to zeaxanthin formation; HPLC pigment analyses of samples at the end of the treatments confirmed that no detectable zeaxanthin had formed. In the absence of DTT, the final pigment concentration was 37.1 mmol zeaxanthin mol^{-1} Chl *a*. DBMIB-reversible SV_N that developed in the presence of DTT is constitutive SV_E (Fig. 4A). Likewise, the DBMIB-reversible SV_N that was DTT sensitive (DTT absent − present) is zeaxanthin SV_E. Constitutive SV_E reached steady-state in about 1 min, whereas

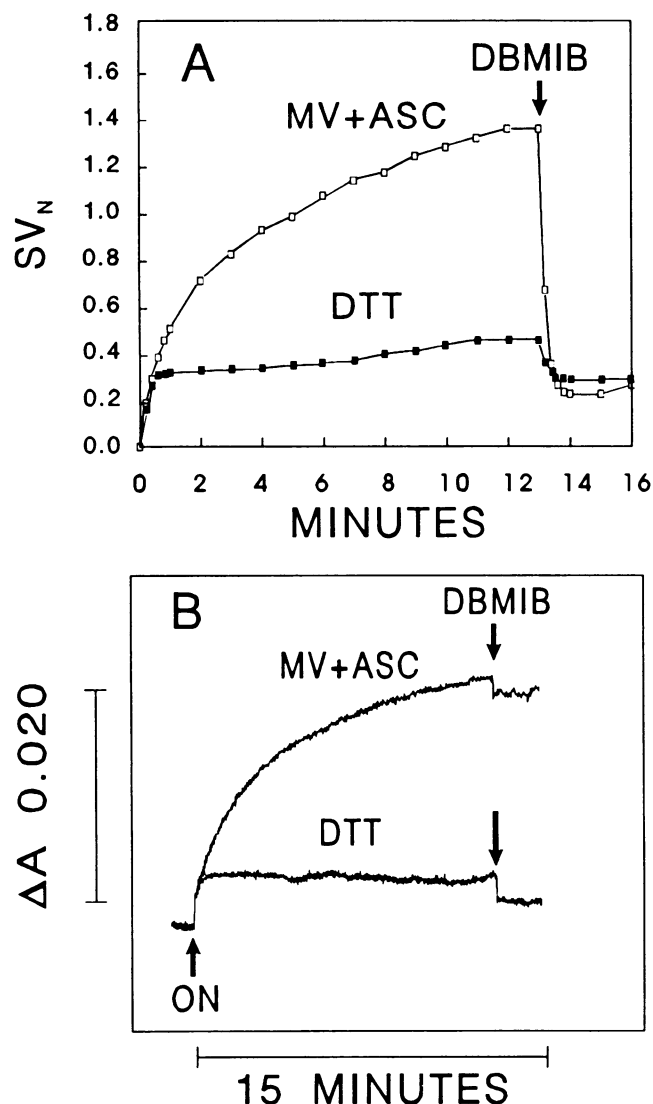

Figure 4. Kinetics of (A) SV_N and (B) 505 nm changes under linear electron transport. The final amounts of zeaxanthin formed were 37.1 and 0.0 mmol zeaxanthin mol^{-1} Chl *a* for the MV + ASC and DTT reactions, respectively. Final DBMIB and DTT concentrations were 2 μM and 2 mM, respectively. Linear conditions were as described in Figure 3.

zeaxanthin SV_E developed more slowly and appeared to correlate with zeaxanthin.

Figure 5 shows an SV-type plot of the zeaxanthin-dependent part of nonphotochemical quenching (ΔSV_N) in Figure 4. The quantity of zeaxanthin at each point of fluorescence quenching was estimated from the corresponding 505 nm absorbance change (Fig. 4B) calibrated for zeaxanthin concentration by HPLC analysis. The SV-type treatment shows a linear relationship ($r^2 = 0.997$) between relative zeaxanthin concentration and ΔSV_N during the course of zeaxanthin formation. The SV-type quenching constant was 0.026 ΔSV_N units/mmol zeaxanthin mol^{-1} Chl *a*.

Although the correlation in Figure 5 is high, close examination of the data shows that zeaxanthin SV_E did not correlate with zeaxanthin until after about 5 mmol zeaxanthin mol^{-1} Chl *a* had formed. This amount of zeaxanthin formed during the first min of illumination. While there could be a lag between zeaxanthin formation and expressed quenching, the data during the first min is inconclusive because ΔpH induction, constitutive SV_E development (Fig. 5A), and light-scattering changes obscure the results during this period (Fig. 5B). After 1 min, zeaxanthin SV_E development clearly followed zeaxanthin formation. Importantly, this direct relationship between zeaxanthin and SV_E seen kinetically was in the absence of DTT, in contrast with the previous experiment where steady-state zeaxanthin levels were varied with DTT.

To further investigate the temporal relationship between zeaxanthin formation and SV_N development, we examined the effects of preforming zeaxanthin (Fig. 6). Zeaxanthin was formed by an initial light treatment, and its further development was inhibited with DTT. After a 3-min dark relaxation period, the chloroplasts were given a second light treatment. Chloroplasts were also treated with DTT from the outset to determine the contribution of constitutive SV_E. Figure 6A shows that during the first min of the initial light treatment, the kinetics of SV_N with DTT present and absent were superimposed. In the second light treatment (Fig. 6B), SV_N during the first min developed faster with zeaxanthin already formed than with zeaxanthin formation inhibited. As before, subtracting SV_N with ≥1 mM DTT present from total SV_N gave ΔSV_N or zeaxanthin SV_E (Fig. 6B). The curves clearly show zeaxanthin SV_E developed faster and to a higher extent when zeaxanthin was preformed (Fig. 6B). The higher SV_N extent during the second light treatment, even with no further zeaxanthin formed, suggests that other slow changes in addition to the amount of zeaxanthin present affect total zeaxanthin-SV_E expression. As noted earlier, DTT inhibits zeaxanthin formation without inhibiting ΔpH (28). Because DTT did not inhibit zeaxanthin SV_E after zeaxanthin was formed, DTT apparently had no observable effect on zeaxanthin SV_E other than inhibiting violaxanthin deepoxidase activity.

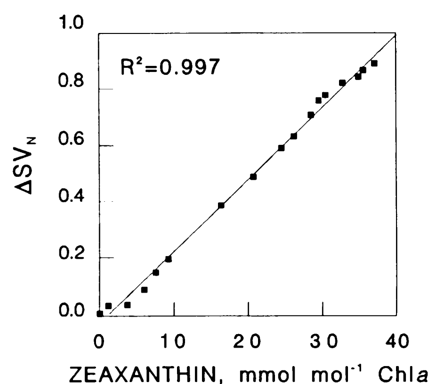

Figure 5. Relationship ΔSV_N and zeaxanthin concentration during induction. ΔSV_N was calculated from the data in Figure 4 as the difference between SV_N in the MV + ASC reaction minus SV_N in the DTT reaction at corresponding times. Zeaxanthin concentrations were determined from the Δ505 nm absorbance calculated as absorbance of the MV + ASC reaction minus absorbance of the DTT reaction at corresponding times and calibrated for zeaxanthin by HPLC. The equation for the regression line is $y = 0.026x - 0.033$.

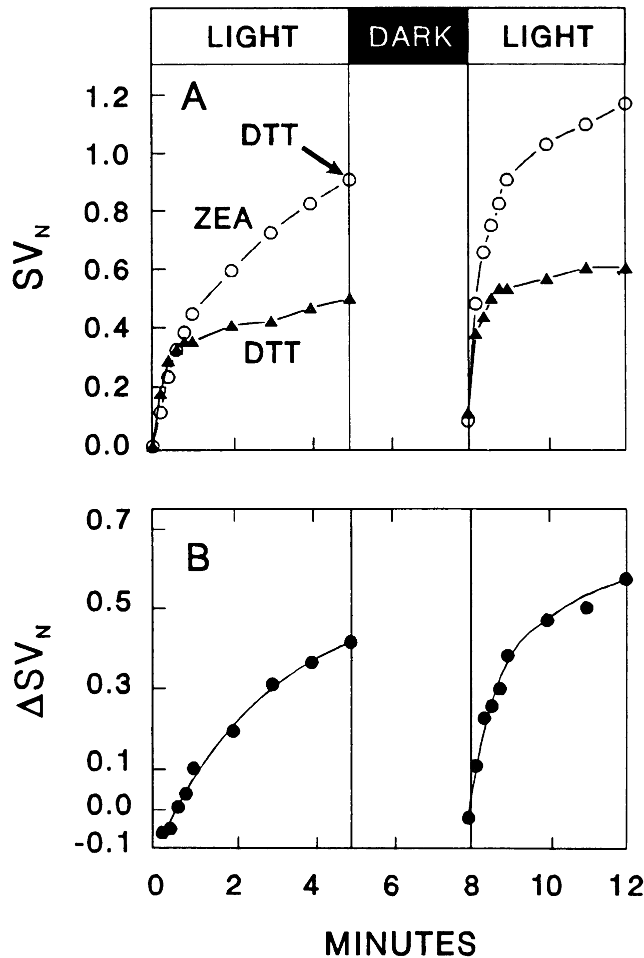

Figure 6. Kinetics of SV_N formation under linear electron transport during sequential light treatments. A, DTT was added at the beginning of the reaction (DTT) or at the end of the first light period (ZEA). B, ΔSV_N representing zeaxanthin-dependent SV_E was obtained by subtracting SV_N in the DTT reaction from SV_N in the ZEA reaction. The final zeaxanthin levels formed were 17.2 and 0.0 mmol mol^{-1} Chl *a* for the ZEA and DTT reactions, respectively. Final DTT concentration when present was 2 mM.

Zeaxanthin F_o Quenching

According to Butler and Kitajima (7), nonradiative dissipation of energy in the pigment bed of PSII quenches both F_m and F_o proportionally, whereas energy dissipation in the reaction center quenches only F_m. Figure 7A shows the zeaxanthin-concentration dependency of SV_N and SV_O, and Fig. 7B shows the same data replotted, SV_N *versus* SV_O at corresponding zeaxanthin concentrations. MV-mediated electron transport was used and the actinic light intensity was saturating for ΔpH. Zeaxanthin formation and the associated fluorescence quenching were inhibited at various times with DTT. SV_N was determined as in the previous experiments. SV_O was measured after quenching had reached steady-state by switching the PAM illumination from 100 to 1.6 kHz and simultaneously turning off the actinic light. The presence of MV assured rapid and complete opening of traps for accurate measurement of F'_o. F'_o held momentarily then relaxed within 90 s to a steady-state F_o level.

Both SV_N and SV_O increased linearly with zeaxanthin concentration (Fig 7A) and, consequently, proportionally with each other (Fig. 7B). The SV_N and SV_O at zero zeaxanthin represents constitutive F_m and F_o quenching, respectively, at the detectable limit of zeaxanthin. The SV-type constants for SV_N and SV_O were 0.025 and 0.012 units/mmol zeaxanthin mol^{-1} Chl *a*, respectively. The results are consistent with zeaxanthin quenching occurring in the pigment bed. F_o quenching at zero zeaxanthin implies that constitutive quenching also occurs in the pigment bed. The possibility appears remote that constitutive quenching is owing to a low constitutive level of zeaxanthin which is undetectable. For that possibility, the Stern-Volmer relationship would need to change abruptly beginning from the limit of detectability of about 2 mmol zeaxanthin mol^{-1} Chl *a* down to absolute zero zeaxanthin. Nevertheless, the evidence for constitutive F_o quenching is less conclusive than for zeaxanthin F_o quenching, the former being limited to a single point and the latter comprising several measurements. Obtaining further quantitative data for constitutive F_o quenching, however, may prove difficult given that F_o quenching is small and constitutive quenching, by its nature, cannot be easily varied.

The Stern-Volmer type constants for SV_N ranged from

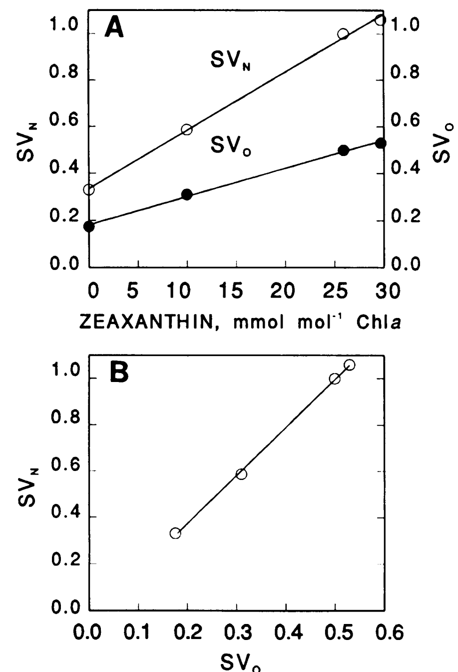

Figure 7. Zeaxanthin-dependent SV_O and SV_N quenching under MV-mediated energized conditions. A, Stern-Volmer type plot for F_m and F_o quenching. The slope of the regression lines (SV-type constants) were 0.025 (r^2 = 0.998) and 0.012 (r^2 = 0.997) for F_m and F_o, respectively. B, Plot of SV_N against SV_O for corresponding zeaxanthin concentrations from panel A. Illumination was 10 min at 350 $\mu E\ m^{-2}\ s^{-1}$ for all reactions. DTT was added either before illumination or at different times during the reaction. Final reagent concentrations were 60 mM ascorbate, 50 μM MV, and 1 mM DTT when added.

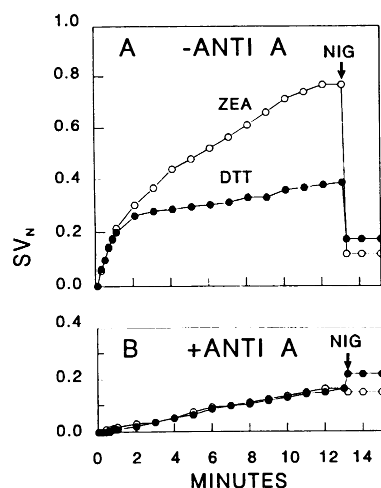

Figure 8. Effects of antimycin (2 μM) on zeaxanthin SV_N and constitutive SV_N induction under cyclic electron transport. A, Zeaxanthin SV_N (ZEA) and constitutive SV_N (DTT) development in the absence of antimycin. B, Effect of antimycin added before illumination on ΔSV_N. Final zeaxanthin levels formed were 13.9 and 14.8 mmol zeaxanthin mol^{-1} Chl *a* for the reactions without DTT in A and B, respectively. No detectable zeaxanthin was formed in the presence of DTT. Final concentrations were 2 μM nigericin and 2 mM DTT. Cyclic electron transport conditions were as described in Figure 3.

0.025 to 0.031 SV_N units/mmole zeaxanthin mol^{-1} Chl *a*. Inasmuch as the xanthophyll concentration is normalized to Chl, the numerical value of the SV constant is in part a function of the xanthophyll to Chl ratio of the sample. The ratio for the samples used in these experiments was 84 ± 1.6 mmol VAZ mol^{-1} Chl *a*. We have grown peas under conditions that yield plants with 104 mmol VAZ mol^{-1} Chl *a*. These peas show proportional F_o and F_m quenching similar to that in Figure 7 but with a lower Stern-Volmer type constant. This effect is currently under further study. The observation of variability in the VAZ to Chl ratio is not new. Even larger differences have been reported between sun-grown and shade-grown leaves of several species (29).

Antimycin Inhibition of Zeaxanthin SV_E and Constitutive SV_E

We reported previously (10) that antimycin inhibited q_E development without affecting zeaxanthin formation under either linear or cyclic electron flow. The sensitivity of each of the two types of quenching to antimycin, however, was not determined. Figure 8 shows that antimycin does indeed inhibit both zeaxanthin and constitutive SV_E. Under cyclic electron transport, SV_N that developed in the absence of antimycin (Fig. 8A) were completely inhibited when antimycin was added prior to illumination (Fig. 8B). As in our previous report (10), antimycin did not inhibit zeaxanthin formation (see legend in Fig. 8 for HPLC data). Since zeaxanthin formation requires a ΔpH (11), the inhibition of SV_N under these conditions (2 μM antimycin) cannot be ascribed to uncoupling. Furthermore, in preliminary experiments neither zeaxanthin formation nor ΔpH measured as neutral-red uptake was affected by 2 μM antimycin. Zeaxanthin formation was 50% inhibited at 50 μM antimycin, which is similar to the 9-aminoacridine quenching data of Oxborough and Horton for uncoupling (19).

In isolated chloroplasts, a low level of SV_N that is independent of zeaxanthin and ΔpH is usually present. This component is clearly evident in Figure 8B; nigericin did not reverse this low-level, antimycin-resistant SV_N. The rate and extent of this SV_N were nearly identical for reactions with and without zeaxanthin formation. The slow induction kinetics of this ΔpH-independent SV_N suggest that it may be the same component Oxborough and Horton (19) observed after the reversal of ΔpH with DCMU. They termed this component qR and suggested it was probably photoinhibition, or the coefficient for irreversible quenching. The possibility, however, that this type of SV_N is an artifact, reflecting an inherent sensitivity of isolated chloroplasts to damage, is not excluded.

Antimycin added prior to illumination completely inhibits SV_N, but its addition during illumination either has no effect or only partly reverses the previously formed SV_N (10, 19). Accordingly, Figure 9 shows that antimycin added prior to illumination completely inhibited SV_N; antimycin added during illumination only slightly reversed the partly formed SV_N. However, after an intervening dark period, SV_N in both antimycin-treated reactions was completely inhibited. Apparently, the antimycin site was changed to its sensitive form in the dark. These results and those in Figure 8 indicate antimycin inhibits both zeaxanthin SV_E and constitutive SV_E by a common mechanism.

DISCUSSION

Zeaxanthin Formation and SV_N in Osmotically Shocked Chloroplasts

Use of osmotically shocked chloroplasts under artificially mediated electron transport coupled with Stern-Volmer treatment of quenching proved highly satisfactory for the quanti-

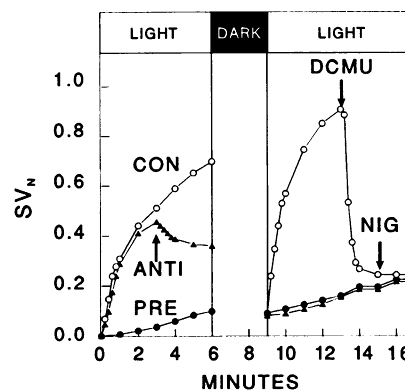

Figure 9. Effects of dark relaxation on the inhibitory effects of antimycin on SV_N under linear electron transport. Antimycin (2 μM) was added at 3 min in the "anti" reaction, added at the beginning in the "pre" reaction, and absent in the control reaction. All three reactions were interrupted with a 3-min dark period. Final DCMU and nigericin concentrations were 0.1 mM and 2 μM, respectively. Linear electron transport conditions were as described in Figure 3.

tation of nonphotochemical quenching, SV_N. Both zeaxanthin-dependent and independent forms of nonphotochemical quenching seen in leaves and intact chloroplasts (1, 3, 9) were also seen in these preparations. The use of various photosynthetic mediators and inhibitors showed unambiguously that both zeaxanthin and constitutive nonphotochemical quenching were energy-dependent quenching, SV_E, and that both could be supported by artificial linear and cyclic electron transport. A few precautions were necessary for accurate quantitation of SV_N. When MV was used as the electron transport mediator, DBMIB was better than DCMU for the measurement of F_m. The latter gave incomplete recovery of the fluorescence quenching presumably due to nonphotochemical quenching by oxidized PQ. Uncoupling MV-linear electron transport further increased the confounding effect of PQ quenching. Use of DTT to vary zeaxanthin formation was especially advantageous; we observed no effect of DTT on the SV_E mechanism under these conditions other than inhibition of the deepoxidase enzyme. DTT inhibits ascorbic acid peroxidase in whole chloroplasts (22), but this activity is rapidly lost in broken chloroplast systems (2).

Correlations between zeaxanthin and nonphotochemical quenching have now been observed in leaves (1, 4, 8) and whole chloroplasts (9) under a variety of experimental conditions. Here the correlations at steady-state and during induction between zeaxanthin and SV_N were seen under light-saturating conditions with both artificially mediated linear and cyclic electron transport. Furthermore, the zeaxanthin-dependent quenching was in addition to an underlying zeaxanthin-independent SV_N under these saturating conditions. We conclude, in agreement with Demmig-Adams *et al.* (9), that zeaxanthin quantitatively increases nonphotochemical quenching. This contrasts with Noctor *et al.* (18), who reported that zeaxanthin increased the q_E to ΔpH ratio at subsaturating ΔpH and that at saturating ΔpH, q_E was equal with or without zeaxanthin. Since we also used saturating ΔpH conditions, the discrepancy cannot be attributed to a difference in this aspect of the experimental protocol. While other possible reasons for the difference can only be speculated, contrasting data treatment, quenching coefficients *versus* Stern-Volmer, may partly explain the difference. As Demmig-Adams *et al.* (9) discussed previously, whereas q_E is constrained ($0 < q_E < 1$), SV parameters or the equivalent are not similarly constrained. Thus, at high levels of quenching, differences are less apparent as quenching coefficients than as SV quenching.

Demmig-Adams *et al.* (9) reported evidence supporting the view that zeaxanthin quenches fluorescence in the pigment bed and that quenching unrelated to zeaxanthin (presumably constitutive quenching) occurs in the reaction center. Our Stern-Volmer F_o and F_m quenching results support zeaxanthin quenching in the pigment bed but suggest in contrast that constitutive quenching also possibly occurs in the pigment bed. The latter is consistent with constitutive and zeaxanthin-dependent quenching having similar properties as to ΔpH dependency, support by artificial electron transport mediators and antimycin inhibition.

Bilger and Schreiber (5) did not observe q_O quenching at low q_N in *Arbutus unedo,* whereas Rees *et al.* (21), treating nonphotochemical quenching as a single component, postulated that all q_E occurs in the pigment bed. Since neither Bilger and Schreiber (5) nor Rees *et al.* (21) reported zeaxanthin changes, their results cannot be compared directly with the present results. However, zeaxanthin formation in these two studies cannot be excluded. In intact leaf systems, high light intensity alone is sufficient for zeaxanthin formation (34). In isolated intact (type A) chloroplasts, Sokolove and Marsho (28) showed that zeaxanthin formation can occur without addition of exogenous ascorbate.

Antimycin Inhibition of Zeaxanthin SV_E and Constitutive SV_E

The mechanism of antimycin action is unclear. Antimycin inhibits both SV_E types under linear and cyclic electron transport, indicating that the target site is common to both electron transport systems. In mitochondria, the site appears to be in the Cyt b-c_1 region of the electron transport chain (27). For isolated chloroplasts, Moss and Bendall (17) speculated that antimycin inhibits an FQR based on partial inhibition of Fd-mediated but not of chemically mediated cyclic phosphorylation. Oxborough and Horton (20) observed that increasing MV concentrations increased q_E and suggested the effect was mediated by oxidized FQR.

There is as yet no direct evidence that the antimycin target is FQR. Antimycin completely inhibits nonphotochemical quenching only when added to dark-adapted or nonenergized chloroplasts (10, 19). Antimycin does not inhibit electron transport (19) or zeaxanthin formation (10) but does decrease the q_E to ΔpH ratio (18). Available evidence thus appears to suggest three possible alternative mechanisms for conditional inhibition, namely, sensitivity to antimycin of a critical component depending on its redox state, protection against antimycin inhibition by the energized state (ΔpH), or both.

Xanthophyll Cycle and Photoprotection

If energy-dependent nonphotochemical quenching and zeaxanthin have a protective function (3, 14) and the two types of quenching are additive as reported here and in ref. 1, the xanthophyll cycle together with constitutive quenching components appear to form a system that has the potential of responding over a wide time scale and to variable extent. Constitutive quenching can respond rapidly but only to limited extent (Fig. 4A and ref. 1). Zeaxanthin-dependent quenching represents a latent potential. It requires conversion of violaxanthin to zeaxanthin (34), an activity which is also ΔpH dependent and relatively slow (11). Furthermore, the amount of zeaxanthin that can be formed depends on so-called violaxanthin availability, the fraction of the total violaxanthin pool that can form zeaxanthin. Violaxanthin availability is itself affected by the redox state of an intersystem electron transport component, probably PQ (25). On a longer time scale, growth conditions influence the xanthophyll pool size as well as violaxanthin availability (29). Thus, energy-dependent nonphotochemical quenching has the capacity for rapid (constitutive), slow (zeaxanthin formation and availability), and adaptive (pool size) expression. Slowly reversible or irreversible ΔpH-independent zeaxanthin-related nonphotochemical fluorescence quenching has been reported in

leaves given severe treatments (8). Little is known about the mechanism of this form of zeaxanthin quenching, but it may represent a further extension of the overall protective function of zeaxanthin.

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