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

Adam M. Gilmore and Harry Y. Yamamoto*

University of Hawaii, Department of Plant Molecular Physiology, 3190 Maile Way, St. John 503, Honolulu, Hawaii 96822

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 (SVN) calculated as \( \frac{F_0}{F} - 1 \) where \( F \) is the fluorescence intensity with all PSII reaction centers closed in a nonenergized, dark-adapted state and \( F_0 \) 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 SVN. Under light-induced saturating \( \Delta \)PH, constitutive-SVN reached steady-state in about 1 minute whereas zeaxanthin-SVN continued to develop for several minutes in parallel with the slow kinetics of violaxanthin deepoxidation. SVN above the constitutive level and relative zeaxanthin concentration showed high linear correlations at steady-state and during induction. Furthermore, \( F_0 \) quenching, also treated as Stern-Volmer quenching (SVN) and calculated as \( \frac{F_0}{F} - 1 \), showed high correlation with zeaxanthin and consequently with SVN, \( F_0 \) and \( F_0 \) are fluorescence intensities with all PSII reaction centers in nonenergized and energized states, respectively. These results support the view that zeaxanthin increases SVN above the constitutive level in a concentration-dependent manner and that zeaxanthin-dependent SVN occurs in the pigment bed. Preforming zeaxanthin increased the rate and extent of SVN, indicating that slow events other than the amount of zeaxanthin also affect final zeaxanthin-SVN expression. The redox state of the primary electron acceptor of photosystem II did not appear to determine SVN. Antimycin, when added while chloroplasts were in a dark-adapted or nonenergized state, inhibited both zeaxanthin-SVN and constitutive-SVN induced by linear and cyclic electron transport. These similarities, including possible constitutive \( F_0 \) quenching, suggest that zeaxanthin-dependent and constitutive SVN 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 \( q_N \) (23). The light-doubling technique (6) and advances in instrumentation (23) have enabled resolution of various \( q \) components (see ref. 13 for a review). The major component is \( \Delta \)PH-dependent quenching, \( q_N \) (15), which also appears to depend on the redox state of a membrane component (20). Recently, \( q_N \) has been related to “down regulation” of photochemistry at PSII (32).

The mechanism for \( q_N \) 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 \( q_F \) (9, 10). Zeaxanthin-dependent \( q_N \) appears to have a photoprotective function (3).

Whether \( q_N \) comprises more than one component is controversial. The results of several laboratories support the view that zeaxanthin-dependent \( q_N \) adds to an underlying zeaxanthin-independent \( q_N \) (3, 9, 10). Other studies conclude instead that zeaxanthin sensitizes \( q_N \) to \( \Delta \)PH and that, at saturating \( \Delta \)PH, zeaxanthin does not increase total \( q_N \) (18). Both \( q_N \) (5) and zeaxanthin-dependent \( q_N \) (3, 9) have been correlated with \( F_0 \) quenching (\( q_0 \)) which, according to the Butler-Kitajima model (7), suggests quenching occurs in the pigment bed.

---

*Abbreviations: \( q_N \): coefficient for nonphotochemical quenching; \( q_F \): coefficient for energy-dependent nonphotochemical quenching; \( Q_N \): 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, plastocyanine; \( q_N \): coefficient for photochemical quenching; FQR, ferredoxin-quinone reductase; VAZ, the sum of the xanthophyll cycle pigments violaxanthin, antheraxanthin, and zeaxanthin; ASC, ascorbate; \( F_{F0} \), fluorescence intensity with all PSII reaction centers closed in nonenergized; dark adapted state; \( F_{F0} \), fluorescence intensity with all PSII reaction centers closed in an energized state; \( F_{F0} \), fluorescence intensity with all PSII reaction centers open in nonenergized state; \( F_{F0} \), fluorescence intensity with all PSII reaction centers open in energized state.
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 Bjorkman (3), because it allowed quantitation of concentration-dependent quenching. The results suggest that at saturating $\Delta pH$, 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 $\mu$mol 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 mM with reaction medium. Chloroplast concentration was equivalent to 30 $\mu$g 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 spectrophotometer 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 $\mu$mol m$^{-2}$ s$^{-1}$ and 2960 $\mu$mol m$^{-2}$ s$^{-1}$, respectively, unless otherwise stated. This actinic light intensity was saturating for $\Delta pH$ 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 $SV_o$. 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 $\Delta SV_N$, calculated as $SV_N$ with zeaxanthin formed minus $SV_N$ with zeaxanthin formation completely inhibited with $\geq$1 mM DTT (33). $\Delta SV_o$ was resolved in a similar way as $\Delta SV_N$. $q_P$ (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 $\mu$L containing about 90 $\mu$mol 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 $\mu$mol 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 DBMB restored the original $F_m$ (Fig. 1A and B). DBMB 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
was not inhibited with DBMIB or when linear flow was uninterrupted by DCMU, the latter Fₘ was used; when DBMIB or DCMU was present, the former Fₘ was used for calculations. Since DCMU and reduced DBMIB abolish ΔpH, these inhibitors also enabled resolution of SVE from ΔpH-independent SVN. However, only data employing DBMIB are strictly quantitative because, as previously mentioned, DCMU does not fully restore Fₘ.

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 SVN. We therefore characterized these effects. Figure 2 shows the effects of ascorbate and MV on 1-qₑ (panel A), SVN (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-qₑ was high, SVN was low, little if any SVₐ was observed, and no detectable zeaxanthin was formed (confirmed by HPLC analysis). The addition of MV reduced 1-qₑ markedly and stimulated a low level of constitutive SVₑ, but again no detectable zeaxanthin was formed. Ascorbate and ascorbate + MV stimulated SVₑ was not inhibited with DBMIB or when linear flow was uninterrupted by DCMU, the latter Fₘ was used; when DBMIB or DCMU was present, the former Fₘ was used for calculations. Since DCMU and reduced DBMIB abolish ΔpH, these inhibitors also enabled resolution of SVE from ΔpH-independent SVN. However, only data employing DBMIB are strictly quantitative because, as previously mentioned, DCMU does not fully restore Fₘ.

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 SVN. We therefore characterized these effects. Figure 2 shows the effects of ascorbate and MV on 1-qₑ (panel A), SVN (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-qₑ was high, SVN was low, little if any SVₐ was observed, and no detectable zeaxanthin was formed (confirmed by HPLC analysis). The addition of MV reduced 1-qₑ markedly and stimulated a low level of constitutive SVₑ, but again no detectable zeaxanthin was formed. Ascorbate and ascorbate + MV stimulated SVₑ was not inhibited with DBMIB or when linear flow was uninterrupted by DCMU, the latter Fₘ was used; when DBMIB or DCMU was present, the former Fₘ was used for calculations. Since DCMU and reduced DBMIB abolish ΔpH, these inhibitors also enabled resolution of SVE from ΔpH-independent SVN. However, only data employing DBMIB are strictly quantitative because, as previously mentioned, DCMU does not fully restore Fₘ.

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 SVN. We therefore characterized these effects. Figure 2 shows the effects of ascorbate and MV on 1-qₑ (panel A), SVN (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-qₑ was high, SVN was low, little if any SVₐ was observed, and no detectable zeaxanthin was formed (confirmed by HPLC analysis). The addition of MV reduced 1-qₑ markedly and stimulated a low level of constitutive SVₑ, but again no detectable zeaxanthin was formed. Ascorbate and ascorbate + MV stimulated SVₑ...
Correlation of Zeaxanthin and SVₜ at Steady-State and during Induction

Earlier, we (10) reported that the kinetics and extent of zeaxanthin formation qualitatively correlated with qₑ under both linear and PSI-cyclic electron transport. Here we quantified the steady-state and kinetic relationships between zeaxanthin formation and SVₜ under artificially mediated electron transport. In Figure 3, steady-state SVₜ and zeaxanthin formation were varied with increasing DTT concentrations. DTT inhibited depeoxidase activity (33) but had no effect on the quantum yield of photosynthesis, electron transport rates (4), or ApH (28). Figure 3A shows SVₜ 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 ΔSVₜ. According to classic SV quenching, the relationship between fluorescence-quencher concentration and quenching is directly proportional to the expression (Fₘ/Fₘ') - 1. The SV-type quenching for the two types of electron transport closely fit the same line (r² = 0.992), indicating that induction of zeaxanthin-SVₜ 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 SVₜ units/mmol zeaxanthin mol⁻¹ Chl a.

The quantitative relationship of SVₜ and zeaxanthin during induction was examined using 505 nm absorbance changes to follow zeaxanthin formation continuously. Figure 4 shows SVₜ 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 SVₜ 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 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. DBMIR-reversible SVₜ that developed in the presence of DTT is constitutive SVₜ (Fig. 4A). Likewise, the DBMIR-reversible SVₜ that was DTT sensitive (DTT absent - present) is zeaxanthin SVₜ. Constitutive SVₜ reached steady-state in about 1 min, whereas
zeaxanthin SVₜ developed more slowly and appeared to correlate with zeaxanthin.

Figure 5 shows an SV-type plot of the zeaxanthin-dependent part of nonphotochemical quenching (ΔSVₕ) 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 (r² = 0.997) between relative zeaxanthin concentration and ΔSVₕ during the course of zeaxanthin formation. The SV-type quenching constant was 0.026 ΔSVₕ units/mmol zeaxanthin mol⁻¹ Chl a.

Although the correlation in Figure 5 is high, close examination of the data shows that zeaxanthin SVₜ 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 SVₜ development (Fig. 5A), and light-scattering changes obscure the results during this period (Fig. 5B). After 1 min, zeaxanthin SVₜ development clearly followed zeaxanthin formation. Importantly, this direct relationship between zeaxanthin and SVₜ 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 SVₜ 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 to determine the contribution of constitutive SVₜ. Figure 6A shows that during the first min of the initial light treatment, the kinetics of SVₜ with DTT present and absent were superimposed. In the second light treatment (Fig. 6B), SVₜ during the first min developed faster with zeaxanthin already formed than with zeaxanthin formation inhibited. As before, subtracting SVₜ with ≥1 mM DTT present from total SVₜ gave ΔSVₕ or zeaxanthin SVₜ (Fig. 6B). The curves clearly show zeaxanthin SVₜ developed faster and to a higher extent when zeaxanthin was preformed (Fig. 6B). The higher SVₜ 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-SVₜ expression. As noted earlier, DTT inhibits zeaxanthin formation without inhibiting ΔpH (28). Because DTT did not inhibit zeaxanthin SVₜ after zeaxanthin was formed, DTT apparently had no observable effect on zeaxanthin SVₜ other than inhibiting violaxanthin deepoxidase activity.

Figure 4. Kinetics of (A) SVₜ 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.

Figure 5. Relationship ΔSVₕ and zeaxanthin concentration during induction. ΔSVₕ was calculated from the data in Figure 4 as the difference between SVₜ in the MV + ASC reaction minus SVₜ in the DTT reaction at corresponding times. Zeaxanthin concentrations were determined from the 505 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.
Zeaxanthin F₀ Quenching

According to Butler and Kitajima (7), nonradiative dissipation of energy in the pigment bed of PSII quenches both Fₙ and F₀ proportionally, whereas energy dissipation in the reaction center quenches only Fₙ. Figure 7A shows the zeaxanthin-concentration dependency of SVₙ and SV₀, and Fig. 7B shows the same data replotted, SVₙ versus SV₀ 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. SVₙ was determined as in the previous experiments. SV₀ 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'ₙ. F'₀ held momentarily then relaxed within 90 s to a steady-state F₀ level.

Both SVₙ and SV₀ increased linearly with zeaxanthin concentration (Fig 7A) and, consequently, proportionally with each other (Fig. 7B). The SVₙ and SV₀ at zero zeaxanthin represents constitutive Fₙ and F₀ quenching, respectively, at the detectable limit of zeaxanthin. The SV-type constants for SVₙ and SV₀ were 0.025 and 0.012 units/mmol zeaxanthin mol⁻¹ Chl α, respectively. The results are consistent with zeaxanthin quenching occurring in the pigment bed. F₀ 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 α down to zero zeaxanthin. Nevertheless, the evidence for constitutive F₀ quenching is less conclusive than for zeaxanthin F₀ quenching, the former being limited to a single point and the latter comprising several measurements. Obtaining further quantitative data for constitutive F₀ quenching, however, may prove difficult given that F₀ quenching is small and constitutive quenching, by its nature, cannot be easily varied.

The Stern-Volmer type constants for SV₀ ranged from...
0.025 to 0.031 SVN 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 F₅ and F₆₅₀ 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 SVₑ and Constitutive SVₑ

We reported previously (10) that antimycin inhibited qₑ 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 SVₑ. Under cyclic electron transport, SVₑ 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 SVₑ 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 SVₑ 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 SVₑ. The rate and extent of this SVₑ were nearly identical for reactions with and without zeaxanthin formation. The slow induction kinetics of this ΔpH-independent SVₑ 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 SVₑ is an artifact, reflecting an inherent sensitivity of isolated chloroplasts to damage, is not excluded.

Antimycin added prior to illumination completely inhibits SVₑ, but its addition during illumination either has no effect or only partly reversing the previously formed SVₑ (10, 19). Accordingly, Figure 9 shows that antimycin added prior to illumination completely inhibited SVₑ; antimycin added during illumination only slightly reversed the partly formed SVₑ. However, after an intervening dark period, SVₑ 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 SVₑ and constitutive SVₑ by a common mechanism.

DISCUSSION

Zeaxanthin Formation and SVₑ 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-
tation of nonphotochemical quenching, $S_V$. 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, $S_V$, and that both could be supported by artificial linear and cyclic electron transport. A few precautions were necessary for accurate quantitation of $S_V$. 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 $S_V$ mechanism under these conditions other than inhibition of the deoxidase 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 $S_V$ 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 $S_V$ 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 $\Delta pH$ ratio at subsaturating $\Delta pH$ and that at saturating $\Delta pH$, $q_e$ was equal with or without zeaxanthin. Since we also used saturating $\Delta pH$ 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 $q_S$ is constrained ($0 < q_S < 1$), $S_V$ parameters or the equivalent are not similarly constrained. Thus, at high levels of quenching, differences are less apparent as quenching coefficients than as $S_V$ 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 $F_0$ 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 $\Delta pH$ dependency, support by artificial electron transport mediators and antimycin inhibition.

Bilger and Schreiber (5) did not observe $q_0$ quenching at low $q_e$ 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 $S_V$ and Constitutive $S_V$.**

The mechanism of antimycin action is unclear. Antimycin inhibits both $S_V$ 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 $\Delta pH$ 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 ($\Delta pH$), 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 $\Delta pH$ 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 inter-system 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 $\Delta pH$-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.

LITERATURE CITED