Plant Physiol. (1994) 106: 1647–1658

# Intrathylakoid pH in Isolated Pea Chloroplasts as Probed by Violaxanthin Deepoxidation[1]

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**Light-driven violaxanthin deepoxidation was measured in isolated pea (*Pisum sativum*) chloroplasts without ATP synthesis (basal conditions) and with ATP synthesis (coupled conditions). Thylakoids stored in high salt (HS) or low salt (LS) storage medium were tested. In previous experiments, HS thylakoids and LS thylakoids were related to delocalized and localized proton coupling, respectively. Light-driven deepoxidase activity was compared to the pH dependence of deepoxidase activity established in dark reactions. At an external pH of 8, light-driven deepoxidation indicated effective pH values close to pH 6 for all reaction conditions. Parallel to deepoxidation, the thylakoid lumen pH was estimated by the fluorescent dye pyranine. In LS thylakoids under coupled conditions the lumen pH did not drop below pH 6.7. At pH 6.7, no deepoxidase activity is expected based on the pH dependence of enzyme activity. The results suggest that deepoxidation activity is controlled by the pH in sequestered membrane domains, which, under localized proton coupling, can be maintained at pH 6.0 when the lumen pH is far above pH 6.0. The extent of violaxanthin conversion (availability), however, appeared to be regulated by lumenal pH. Dithiothreitol-sensitive nonphotochemical quenching of chlorophyll fluorescence was dependent on zeaxanthin and not related to lumenal pH. Thus, zeaxanthin-dependent quenching—known to be pH dependent—appeared to be triggered by the pH of localized membrane domains.**

This work brings together two threads of recent developments in chloroplast physiology: (a) the pH-regulated enzymatic formation of the presumably photoprotective xanthophylls antheraxanthin and zeaxanthin and (b) the occurrence of two acidic pools, membrane localized and lumenal, that can form under energized conditions. The question of concern herein is whether there is any specificity for one or the other acidic pool for activating the xanthophyll conversions. The interest in the deepoxidation of violaxanthin to zeaxanthin stems from the probable involvement of zeaxanthin in a mechanism that facilitates safe dissipation of excess excitation energy under light-stress conditions (for reviews, see Demmig-Adams, 1990; Demmig-Adams and Adams, 1992, 1994; Pfündel and Bilger, 1994).

Activation of deepoxidation by low pH values was first shown by Hager (1966). Hager (1969) found the optimum for deepoxidation at pH 4.8 in isolated chloroplasts and at pH 5.2 for the isolated enzyme. It is well known that deepoxidation in vivo is triggered by light and it has been assumed that the lumen space, known to become acidic under illumination, is the sole acidic determinant of violaxanthin deepoxidase activity (Hager, 1975). However, recent evidence indicates that there are two experimentally separable acidic loci in thylakoids, a membrane-localized domain and the lumen space (cf. Dilley et al., 1987; Rottenberg, 1990; Dilley, 1991). Protons of the localized domains can be sequestered from the lumen by a poorly understood barrier to proton diffusion (cf. also Theg and Homann, 1982; Pfister and Homann, 1986). Moreover, the sequestered protons can be delivered from the localized domains to the $CF_0$-$CF_1$ energy-coupling complex without equilibrating with the lumen (Theg et al., 1988; cf. the reviews by Dilley et al., 1987; Dilley, 1991).

It is of particular interest for matters of regulation of chloroplast function that the evidence suggests a facile shifting between localized and delocalized proton gradient coupling keyed to the level of membrane energization and dependent on a $Ca^{2+}$ interaction with the ATP-synthase subunit III protein, which provides a type of gating mechanism (Chiang and Dilley, 1987, 1989; Chiang et al., 1992; Wooten and Dilley, 1993; Zakharov et al., 1993). It was suggested that localized proton coupling is the normal condition (Dilley, 1991). Over-excitation or inadequate turnover of the ATP-synthase, as in limitation of ADP supply, results in spillover of localized protons into the lumen and its subsequent acidification (Horner and Moudrianakis, 1986; Dilley et al., 1987; Dilley, 1991). It is precisely such over-energization conditions that induce the photoprotective response that was related to

[1] This work was supported in part by a Feodor-Lynen-Fellowship of the Alexander von Humboldt-Foundation granted to E.E.P. and in part by a grant from the U.S. Department of Energy and the U.S. Department of Agriculture to R.A.D.

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Abbreviations: $\Delta A_{505}$, change in absorbance at 505 nm; $\Delta A_{505}(t)$, absorbance change at 505 nm as a function of time; $\Delta A_{505}\infty$, asymptote value of $\Delta A_{505}(t)$; $\Delta F450$ and $\Delta F405$, light minus dark differences of fluorescence excited at 450 and 405 nm, respectively; $^1k$, first-order rate constant of $\Delta A_{505}(t)$; HS, high salt; LHC II, light-harvesting complex II; LS, low salt; MV, methyl viologen; $\Delta pH$, proton gradient across thylakoid membrane; $\Delta\psi$, electrical potential difference across the thylakoid membrane; qE, energy-dependent fluorescence quenching; $SV_N$, Stern-Volmer quenching.

zeaxanthin formation (reviewed by Demmig-Adams, 1990; Demmig-Adams and Adams, 1992, 1994; Pfündel and Bilger, 1994).

An obvious question concerns the role of sequestered domain versus lumen acidification in the induction of zeaxanthin formation. Recently, the pH dependence of violaxanthin deepoxidation in isolated thylakoids was evaluated in more detail with the essential finding being that above pH 6.3 there is virtually no enzyme activity and that below pH 6.3 enzyme activity rises sharply to the maximum at pH 5.8 (Pfündel and Dilley, 1993). If violaxanthin deepoxidation can be light activated under localized coupling conditions with a lumenal pH higher than 6.3, then the deepoxidase activity probably reflects the acidity of membrane-sequestered domains and provides a way to estimate the pH of these domains.

Here we report measurements of light-induced violaxanthin deepoxidation in thylakoids known to be functioning in either the localized or in the delocalized energy-coupling mode. In these thylakoid preparations, violaxanthin deepoxidation was investigated under basal or coupled conditions that attain quite different lumen pH values. The results indicate two levels of control, with the deepoxidase activity being related to the pH of the sequestered domains and the availability of the enzyme substrate violaxanthin being associated with the acidity of the lumen.

## MATERIALS AND METHODS

### Plant Material and Chloroplast Isolation

Pea (*Pisum sativum* cv Little Marvel) plants were grown in moist vermiculite in a growth chamber for 16 to 20 d as described by Pfündel and Dilley (1993). Plants were illuminated for a 12-h photoperiod with a photon flux density of 450 $\mu$E $m^{-2}$ $s^{-1}$. The temperature was maintained at 15 and 20°C for dark and light conditions, respectively.

Prior to chloroplast isolation, plants were dark adapted for 8 to 12 h so that all zeaxanthin was converted to violaxanthin. Chloroplasts were isolated as described elsewhere (Ort and Izawa, 1973) under reduced light. The thylakoids were resuspended in "low-salt" medium containing 200 mM sorbitol, 5 mM Hepes-KOH (pH 7.5), 3 mM $MgCl_2$, and 0.5 g $L^{-1}$ BSA or in "high-salt" medium in which sorbitol was replaced by 100 mM KCl. In the present paper, thylakoids stored in high-salt and low-salt medium are referred to as HS thylakoids and LS thylakoids, respectively.

Four parts of HS or LS thylakoid suspension at 3 to 4 mg Chl $mL^{-1}$ were mixed with one part of pure ethylene glycol as a cryoprotectant and stored in liquid nitrogen until used. Prior to the experiments, thylakoids were quickly thawed, stored on ice, and used within 3 h. The average activity loss after a freeze-thaw cycle was 15% as measured by the ATP yield per single-turnover flash.

### Flash-Driven ATP Formation

Photophosphorylation at 18°C was driven by single-turnover flashes (approximately 10 $\mu$s duration at half intensity) delivered at a frequency of 5 Hz (Beard and Dilley, 1986, 1988). Flashes delivered by a xenon lamp (FZ 200, EG&G Electronics, Salem, MA) were filtered by a Schott RG 630 (Schott, Mainz, Germany) filter and guided by fiber optics to a water-jacketed cuvette containing 800 $\mu$L of assay buffer mixture. ATP formation was monitored by luciferin-luciferase chemiluminescence as described in the above references. The photomultiplier tube (EMI 9558Q, Emitronics, Inc., Plain View, NY) was protected from the red actinic light by a Corning 4–96 blue glass filter (Corning, NY).

The reaction medium contained 10 mM sorbitol, 50 mM Tricine-KOH (pH 8.0), 3 mM $MgCl_2$, 2 mM $KH_2PO_4$, 0.1 mM ADP, 5 mM DTT, 5 $\mu$M diadenosine pentaphosphate (to inhibit adenylate kinase), 0.1 mM MV, 100 nM nonactin (to dissipate transthylakoid electrical potential), thylakoids equivalent to 15 $\mu$g Chl $mL^{-1}$, and 10 $\mu$L of the LKB luciferin-luciferase kit prepared as specified by Beard and Dilley (1986). ADP was purified on a Dowex AG-1 column to remove contaminating ATP. The concentration of pyridine, if present, was 5 mM. In some experiments the osmotic strength of the reaction medium was increased by replacing 10 mM sorbitol with 100 mM sorbitol and 35 mM ascorbate. Prior to some experiments under high-osmotic conditions, thylakoids were osmotically shocked in 1 mL of cold, distilled water for 15 s followed by addition of 2 mL of 1.5-fold-concentrated reaction medium.

### Violaxanthin Deepoxidation

Violaxanthin deepoxidation was measured at 18°C in an Aminco DW-2 spectrometer (SLM Instruments, Inc., Urbana, IL) in dual-wavelength mode as the change in $A_{505}$ with 540 nm as reference wavelength (Siefermann and Yamamoto, 1974). For light-driven deepoxidation, actinic light from a 150-W projection lamp was passed through 2.5 cm of 3% $CuSO_4$ and a Schott RG 695 red glass filter and was delivered at a right angle to the measuring beam. The quantum flux density at the surface of the cuvette was 60 $\mu$E $m^{-2}$ $s^{-1}$. The photomultiplier tube was shielded against actinic light by a Corning CS 4–96 blue glass filter.

Prior to the experiments, the thylakoids were osmotically shocked as described above. The final Chl concentration was 20 and 25 $\mu$g $mL^{-1}$ for light-driven and dark reactions, respectively. Light-driven deepoxidation was carried out under coupled conditions permitting photophosphorylation (+0.2 mM ADP with a hexokinase/Glc trap to recycle the ATP to ADP) and under basal conditions without ADP. In both cases, the assay medium contained 100 mM sorbitol, 50 mM Tricine-KOH (pH 8.0), 35 mM ascorbate, 5 mM Glc, 3 mM $MgCl_2$, 2 mM $KH_2PO_4$, 0.1 mM MV, 100 nM nonactin, 50 units catalase/$\mu$g Chl, 15 units superoxide dismutase/$\mu$g Chl, and 5 units hexokinase/$\mu$g Chl. In experiments at pH 7, Tricine was replaced by 50 mM Hepes-KOH. Controls in which deepoxidation was inhibited by 2 mM DTT were run to obtain the contribution of light scattering to the $\Delta A_{505}$ signal.

Violaxanthin deepoxidation in the dark was done as described by Pfündel and Dilley (1993). The reaction mixture contained 100 mM sorbitol, 3 mM $KH_2PO_4$, 3 mM $MgCl_2$, 500 nM nigericin, 500 nM nonactin, and 50 mM citrate(KOH) or 50 mM Mes(KOH) for pH values <5.8 or >5.8, respectively. Dark deepoxidation reactions were initiated by the addition of ascorbate to a final concentration of 50 mM. Ascorbate

stock solutions were prepared from the free acid and titrated to pH 7 with NaOH.

Dark and light-driven reactions followed first-order kinetics during the initial 10 min after the reaction start. Rate constants and the asymptote values of the kinetics were estimated by nonlinear regression using the Marquardt-Levenberg algorithm.

### Chl Determination

Total Chl concentrations were determined in 80% acetone according to Arnon (1949) and corrected as described by Porra et al. (1989).

### Pigment Chromatography

Pigment extraction with 100% acetone and reversed-phase HPLC of the pigments was done according to Gilmore and Yamamoto (1991a) with slight modifications as described by Pfündel and Dilley (1993). Xanthophylls and Chls were separated isocratically on an Spherisorb ODS-1 column with a solvent mixture acetonitrile:methanol:0.1 M Tris-HCl buffer, pH 8.0 (72:8:3, v/v) and quantified using the conversion factors as determined for this solvent mixture (Gilmore and Yamamoto, 1991a). The flow rate was 2 mL $min^{-1}$ and the detection wavelength was 440 nm.

### Chl Fluorescence

Chl fluorescence was measured at 18°C with a PAM Chl fluorimeter (Walz, Effeltrich, Germany) according to Schreiber et al. (1986). Actinic light, sample treatment, Chl concentration, and the pH 8 reaction medium were as described for light-driven violaxanthin deepoxidation. Saturating white flashes of 500 ms from a KL-1500 saturation pulse lamp (Walz) operated at maximum intensity were delivered at a frequency of 0.017 Hz through the PAM fiber optics to a water-jacketed, custom-made glass cuvette.

Nonphotochemical quenching of maximum fluorescence ($SV_N$) was treated in analogy to Stern-Volmer quenching, where $SV_N$ is given by $(F_m/F_m') - 1$ (Gilmore and Yamamoto, 1991b). Here, $F_m$ is the maximum fluorescence intensity when all PSII reaction centers are closed and all nonphotochemical quenching processes are at a minimum (dark state), and $F_m'$ is the fluorescence intensity when all PSII centers are closed in any light-adapted state (see Kooten and Snel, 1990).

### pH Determination by Pyranine Fluorescence

The fluorescent dye pyranine (8-hydroxy-1,3,6-pyrenetrisulfonic acid) was loaded and measured as described in detail by Renganathan et al. (1993). Dye loading into either LS or HS thylakoids was by incubation of thylakoids equivalent to 1 mg Chl $mL^{-1}$ at 15°C in a pH 6 buffer containing 1 mM pyranine, 200 mM sorbitol, 20 mM Mes(KOH), 3 mM $MgCl_2$, and 20 mM KCl. After two washing steps to remove external dye, an osmotic shock was given (see "Flash-Driven ATP Formation"), and the fluorescence of pyranine-loaded thylakoids was recorded at pH 8 with a dual-wavelength SLM 8000 spectrofluorimeter. The Chl concentration was 10 $\mu g$ $mL^{-1}$. Sample treatment, actinic light intensity, and reaction medium were as described for light-driven violaxanthin deepoxidation except that actinic light was passed through a Corning CS 2–64 red glass filter.

Fluorescence excitation was switched at a frequency of 250 Hz between 450 and 405 nm. Changes in pyranine fluorescence intensity during a 300-s illumination period were recorded at 511 nm. The fluorescence signal in dye-loaded samples was corrected for background fluorescence of nonloaded controls. From the corrected data, the $\Delta F450$ was calculated. Similarly, the $\Delta F405$ was obtained. Calculated ratios of $\Delta F450$:$\Delta F405$ were transformed into pH changes using a calibration curve obtained with uncoupled, dye-loaded thylakoids (Renganathan et al., 1993).

## RESULTS

### Flash-Driven ATP-Synthesis

The effect of pyridine on the number of flashes required to start ATP formation has been used by Beard and Dilley (1986, 1988) to show delocalized and localized modes of proton coupling. After storage in liquid nitrogen and under the osmotic conditions of Beard and Dilley, the average pyridine-dependent extension of the onset lag of ATP formation was 10.6 and 1.8 flashes for HS- and LS-stored thylakoids, respectively. (Table I, experiment A). Thylakoids were also tested under experimental conditions similar to those established by Siefermann and Yamamoto (1974) to record violaxanthin deepoxidation by the $\Delta A_{505}$ (Table I, experiment C). At variance with Beard and Dilley, the $\Delta A_{505}$ assay medium had higher osmotic strength and contained ascorbate, and osmotically shocked thylakoids were used.

Higher osmotic strength reduced the pyridine-dependent flash extensions in HS and LS thylakoids by about 50% (Table I, experiment B). The difference between HS and LS pretreatment, however, remained statistically significant. This is also valid for osmotically shocked samples (Table I, experiment C). No consistent differences in the ATP flash yield were observed between the various experimental conditions of Table I.

### pH Determination by Pyranine Fluorescence

Under conditions of continuous illumination, pyranine fluorescence-detected pH changes were introduced as a criterion for localized and delocalized coupling by Renganathan et al. (1993). Table II shows the results of pyranine lumen pH determinations that were carried out in parallel with light-driven deepoxidation at pH 8. At an external pH of 8.0, the lumen pH in the LS coupled case was pH 6.7. For HS basal, LS basal, and HS coupled reaction conditions, the fluorescence ratio changes corresponded to fully protonated pyranine (cf. Renganathan et al., 1993). Since the –OH group of pyranine is expected to be mostly protonated below pH 6.0 ($pK_a = 7.3$), the pH in the latter three cases must be $\leq 6.0$.

### Light-Induced $\Delta A_{505}(t)$ at pH 8

Under the four reaction conditions used for pyranine pH determination (Table II), violaxanthin deepoxidation was measured by the light-induced $\Delta A_{505}$. $\Delta A_{505}$ changes were

**Table I.** *Onset lags and flash yield of ATP formation in HS and LS thylakoids after storage in liquid nitrogen*

Data of HS and LS thylakoids that were stored in liquid nitrogen prior to experiments in the presence of 20% (v/v) ethylene glycol are shown. ATP-formation was driven by single turnover flashes and measured by the luciferin/luciferase assay as described in "Materials and Methods." Onset lags of ATP formation in the absence and presence of 5 mM pyridine, the calculated pyridine-dependent onset lag extensions, and ATP flash yields are listed (All values are ± SD). The significance of the pyridine effect was confirmed by the *t* test (P values are shown). Onset lags were determined by extrapolating the steady rise in the luminescence signal back to the baseline (cf. fig 1D of Beard and Dilley, 1986). The assay medium for experiments B and C contained 100 mM sorbitol, 50 mM Tricine-KOH (pH 8.0), 35 mM ascorbate, 3 mM $MgCl_2$, 2 mM $KH_2PO_4$, 0.1 mM ADP, 5 mM DTT, 5 μM diadenosine pentaphosphate, 0.1 mM MV, and 100 mM nonactin. Ten millimolar sorbitol and no ascorbate was used in experiment A. The data of experiment C were taken after the thylakoids were osmotically shocked and brought back to the osmotic strength used in experiment B.

| Pretreatment | Reaction Medium | | Onset Lag of ATP Formation (flashes) | Onset Lag Extension by Pyridine (flashes) | *t* Test on Pyridine Effect | ATP Yield per Flash (nmol/mg Chl) |
|---|---|---|---|---|---|---|
| | Osmotic strength | Additions | | | | |
| | | | Experiment A | | | |
| HS | Low | −Pyridine | 62.4 ± 3.4 ($n$ = 10) | | | 0.42 ± 0.07 |
| | | +Pyridine | 73.0 ± 2.1 ($n$ = 10) | 10.6 ± 3.8 | | 0.39 ± 0.09 |
| LS | | −Pyridine | 50.3 ± 5.4 ($n$ = 11) | | | 0.54 ± 0.18 |
| | | +Pyridine | 51.9 ± 4.8 ($n$ = 11) | 1.8 ± 1.5 | P< 0.001 | 0.51 ± 0.18 |
| | | | Experiment B | | | |
| HS | High | −Pyridine | 58.4 ± 8.6 ($n$ = 7) | | | 0.32 ± 0.08 |
| | | +Pyridine | 63.6 ± 9.5 ($n$ = 7) | 5.1 ± 2.1 | | 0.28 ± 0.08 |
| LS | | −Pyridine | 51.8 ± 7.6 ($n$ = 7) | | | 0.38 ± 0.07 |
| | | +Pyridine | 52.4 ± 9.1 ($n$ = 7) | 0.7 ± 2.2 | P = 0.002 | 0.35 ± 0.03 |
| | | | Experiment C | | | |
| HS, osmotically shocked | High | −Pyridine | 50.9 ± 5.8 ($n$ = 8) | | | 0.40 ± 0.12 |
| | | +Pyridine | 55.8 ± 5.4 ($n$ = 8) | 4.9 ± 2.0 | | 0.31 ± 0.14 |
| LS, osmotically shocked | | −Pyridine | 47.0 ± 8.4 ($n$ = 8) | | | 0.50 ± 0.25 |
| | | +Pyridine | 47.9 ± 8.5 ($n$ = 8) | 0.9 ± 1.1 | P < 0.001 | 0.45 ± 0.24 |

**Table II.** *Determination of ΔpH by the rate constant of $\Delta A_{505}(t)$ and ΔpH values obtained with the pyranine technique*

ΔpH values obtained for HS and LS thylakoids assayed under basal and coupled conditions are given. pH determination by the $\Delta A_{505}(t)$ rate constant (external pH= 8), pH values were derived from the rate constants of $\Delta A_{505}(t)$ at pH 8 (Fig. 2) using the $^1k$ calibration curve in Figure 5. Values in parentheses correspond to SD values. Since the calibration curve is nonlinear, the estimated positive deviation may differ from the negative one. pH determination by pyranine fluorescence (external pH = 8), ratio changes (±SD) of pyranine fluorescence were measured under similar conditions as violaxanthin deepoxidation at pH 8, and were converted into pH values as described in Renganathan et al. (1993). Except for LS-coupled reaction conditions, the ratio changes correspond to fully protonated pyranine and exact pH determination was not feasible (ΔpH ≥ 2). For a comparison, ΔpH values of earlier work (Renganathan et al., 1993) determined at an external pH of 8 and of 8.9 are shown.

| Parameter | Thylakoids and Reaction Conditions | | | |
|---|---|---|---|---|
| | HS basal | LS basal | HS coupled | LS coupled |
| | pH determination by the $\Delta A_{505}(t)$ rate constant (external pH = 8) | | | |
| ΔpH | 2.1 (−0.03 /+ 0.03) | 2.1 (−0.09 /+ 0.06) | 2.0 (−0.02 /+ 0.03) | 2.0 (−0.02 /+ 0.04) |
| | pH determination by pyranine fluorescence (external pH = 8) | | | |
| $\Delta F450/\Delta F405$ | 2.9 ± 0.4 (n = 6) | 3.4 ± 1.0 (n = 9) | 3.5 ± 0.6 (n = 6) | 2.0 ± 0.1 (n = 9) |
| ΔpH | ≥2.0 | ≥2.0 | ≥2.0 | 1.3 ± 0.1 |
| | pH determination by pyranine fluorescence (data from Renganathan et al., 1993) | | | |
| ΔpH (external pH = 8.0) | >2.0 | >2.0 | >2.0 | 1.0 |
| ΔpH (external pH = 8.9) | 2.9 | 2.5 | 2.3 | 1.8 |

recorded in the absence and presence of 2 mM DTT, a deepoxidase inhibitor (Fig. 1). A concentration of 2 mM DTT prevented zeaxanthin formation completely, as established by HPLC (data not shown), permitting determination of $\Delta A_{505}$ changes not related to deepoxidation reactions.

At an external pH of 8 and in the absence of DTT, the $\Delta A_{505}(t)$ after 900 s of illumination was 1.13 (mg Chl mL)$^{-1}$ for HS-stored thylakoids under basal reaction conditions (Fig. 1A). This value was decreased by 13, 34, and 47% for LS thylakoids under basal conditions, HS thylakoids under coupled conditions, and LS thylakoids under coupled conditions, respectively (Fig. 1, B–D).

In the presence of DTT, the $\Delta A_{505}$ of HS thylakoids was only 0.32 (mg Chl mL)$^{-1}$ after 900 s of light (Fig. 1A). A similar change was found for HS thylakoids under coupled conditions (Fig. 1C). Compared to the HS values, the corresponding values in LS-stored thylakoids were slightly decreased (Fig. 1, B and D).

The DTT-sensitive component of $\Delta A_{505}(t)$ as calculated from the data of Figure 1 followed first-order kinetics during the first 10 min (Fig. 2). The $^1k$, corresponding to the deepoxidase activity (Siefermann and Yamamoto, 1974), and $\Delta A_{505}\infty$, corresponding to the violaxanthin availability, were

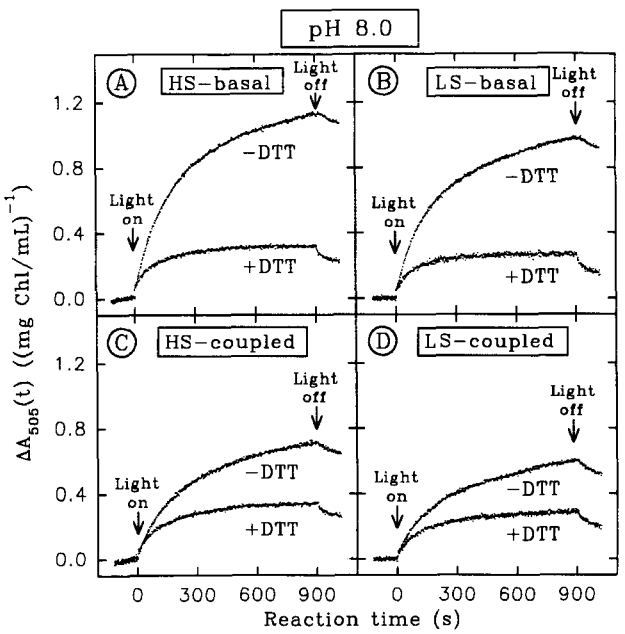


**Figure 1.** Light-induced $\Delta A_{505}(t)$ at pH 8. The $\Delta A_{505}$ in the presence (+DTT) and absence (−DTT) of 2 mM DTT is shown. HS- (A and C) and LS-stored thylakoid (B and D) were tested under basal (A and B) and coupled (C and D) reaction conditions (Chl = 20 $\mu$g mL$^{-1}$). All $\Delta A_{505}$ values in this figure and also in Figures 2 to 5 are normalized to 1 (mg Chl mL)$^{-1}$. Red light of 60 $\mu$E m$^{-2}$ s$^{-1}$ was turned on and off as indicated. Each trace is the mean of at least three experiments. The reactions were carried out at pH 8 in a buffer solution containing 100 mM sorbitol, 50 mM Tricine-KOH, 35 mM ascorbate, 5 mM Glc, 3 mM $MgCl_2$, 2 mM $KH_2PO_4$, 0.1 mM MV, 100 nM nonactin, 50 units catalase/mg Chl, 15 units superoxide dismutase/mg Chl, 5 units hexokinase/mg Chl. In coupled conditions, 0.2 mM ADP was present.

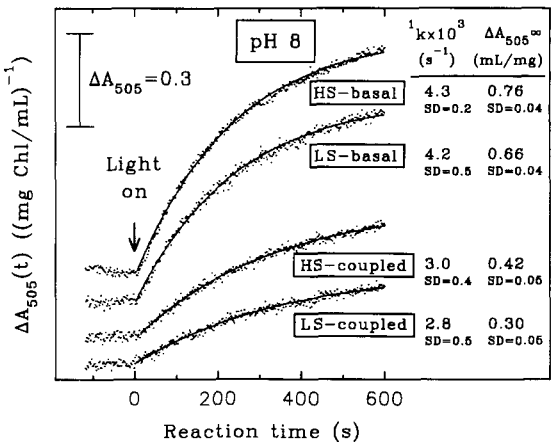


**Figure 2.** DTT-sensitive light-induced $\Delta A_{505}(t)$ at pH 8. $\Delta A_{505}(t)$ were derived from the data of Figure 1 by calculating the difference of $\Delta A_{505}(t)$ in the absence of DTT minus the corresponding $\Delta A_{505}(t)$ in the presence of DTT. Solid lines represent single exponential curves fitted to the $\Delta A_{505}(t)$ kinetics. The parameters $^1k$ and $\Delta A_{505}\infty$ of the fitted functions are displayed.

derived from single exponential functions fitted to the experimental data (Fig. 2).

Under basal reaction conditions, the rate constants of HS and LS thylakoids were similar. This is also valid for HS and LS thylakoids tested under coupled conditions. Coupled reaction conditions yielded $^1k$ that were about 30% lower than the $^1k$ of basal conditions. The $\Delta A_{505}\infty$ of 0.76 (mg Chl mL)$^{-1}$ obtained for HS thylakoids under basal conditions dropped in the sequence LS basal, HS coupled, and LS coupled by 13, 45, and 61%, respectively (Fig. 2).

### Light-Induced $\Delta A_{505}(t)$ at pH 7

At an external pH of 7, the DTT-sensitive $\Delta A_{505}(t)$ was recorded under otherwise identical conditions as used for the pH-8 experiments. In the pH-7 experiments, we chose that pair of experimental conditions that yielded the highest difference in the 505-nm signal at pH 8, namely HS basal and LS coupled (Fig. 3). As observed for light-driven deepoxidation at pH 8.0, $\Delta A_{505}(t)$ at pH 7 followed first-order kinetics during the first 10 min. At variance with the differences between HS basal and LS coupled at pH 8, the $^1k$ and $\Delta A_{505}\infty$ values at pH 7 were similar. $\Delta A_{505}\infty$ values at pH 7 were considerably higher compared to the highest value measured at pH 8 (HS basal).

### Relation between the DTT-Sensitive $\Delta A_{505}(t)$ and Violaxanthin Deepoxidation

To confirm the validity of the $A_{505}$ increase as a quantitative measure for violaxanthin deepoxidation, DTT-sensitive $\Delta A_{505}$ values were plotted against corresponding increases of antheraxanthin and zeaxanthin as quantified by HPLC (Fig. 4). Figure 4 includes samples representing all four pH-8 reaction conditions of Figure 2. A linear relationship between $\Delta A_{505}$ and formation of antheraxanthin + zeaxanthin was observed

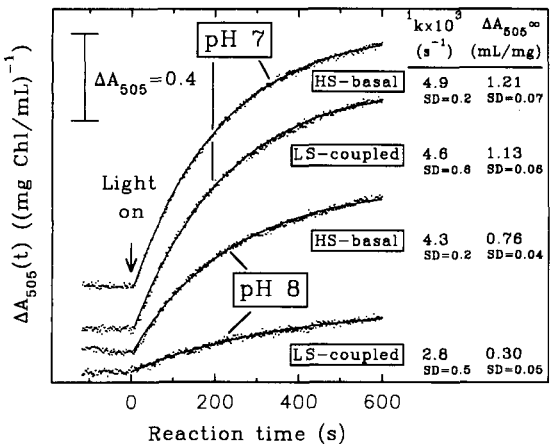


**Figure 3.** Comparison of the DTT-sensitive $\Delta A_{505}(t)$ at pH 7 and pH 8. The $\Delta A_{505}(t)$ at pH 7 were calculated from $\Delta A_{505}(t)$ without DTT minus $\Delta A_{505}(t)$ in the presence of DTT. The reaction conditions were similar to those described for Figure 1, except that 50 mM Hepes was used as the buffer substance. The pH-8 traces were taken from Figure 2. Solid lines represent single exponentials fitted to the $\Delta A_{505}(t)$ kinetics. The parameters $^1k$ and $\Delta A_{505}\infty$ of the fitted functions are shown.

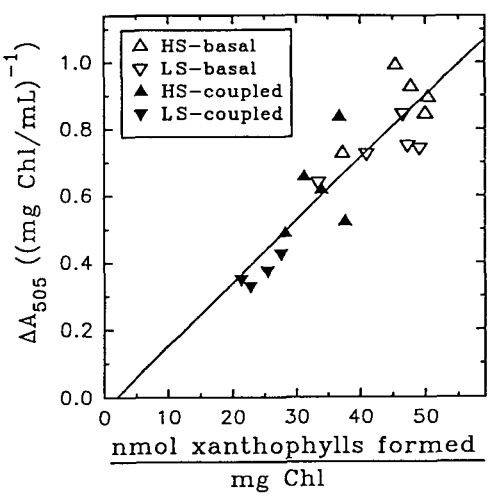


**Figure 4.** Comparison of values of the DTT-sensitive $\Delta A_{505}$ with the formation of antheraxanthin and zeaxanthin. Deepoxidation reactions in HS-stored (△, ▲) and LS-stored (▽, ▼) chloroplasts were carried out under basal (△, ▽) and coupled (▲, ▼) reaction conditions as described in Figure 1. Reactions were terminated by 5 mM DTT after 10 to 30 min of illumination. Subsequently, the pigments were extracted and analyzed by HPLC. The straight line corresponds to the first-order regression to the experimental data ($r = 0.891$). The in vivo difference extinction coefficients as obtained by curve fitting were 13.2 and 18.7 $mM^{-1}$ $cm^{-1}$ for the conversion of violaxanthin to antheraxanthin and of violaxanthin to zeaxanthin, respectively.

(regression coefficient $r = 0.89$). In vivo difference extinction coefficients were 13.2 and 18.7 $mM^{-1}$ $cm^{-1}$ for the conversion of violaxanthin to antheraxanthin and of violaxanthin to zeaxanthin, respectively.

### pH Dependence of $\Delta A_{505}(t)$

The pH dependence of violaxanthin deepoxidation was measured in HS- and LS-stored thylakoids (Fig. 5). Assays were carried out in the dark with uncoupler present (nigericin and nonactin) to dissipate $\Delta$pH and $\Delta\psi$. The 505-nm kinetics followed first-order kinetics during the first 10 min after the start of the reaction (not shown). No significant differences were observed between HS-stored and LS-stored thylakoids for $^1k$ and $\Delta A_{505}\infty$ values of $\Delta A_{505}(t)$. As has already been shown (Pfündel and Dilley, 1993), $^1k$ was zero for pH values higher than 6.3 and showed a plateau value of $5 \times 10^{-3}$ $s^{-1}$ between pH 5.1 and 5.6. $\Delta A_{505}\infty$ values ranged between 0.9 and 1.25 (mg Chl $mL)^{-1}$ for pH values lower than 6.1 and did not show a significant pH-dependent trend. Some lower values were observed around pH 6.2.

### Light-Induced $\Delta A_{505}(t)$ in Relation to the pH Dependence of the Dark $\Delta A_{505}(t)$

$^1k$ of DTT-sensitive $\Delta A_{505}(t)$ were transformed into pH values using the pH dependence established in Figure 5. At an external pH of 8, this transformation yielded pH values of 5.9 for HS basal and LS basal and 6.0 for HS coupled and LS coupled thylakoids. Those values correspond to $\Delta$pH predictions of 2.1 for the former two and 2.0 for the latter two cases (Table II). The rate constants at an external pH of 7 (Fig. 3) coincided with the maximum values of the dark

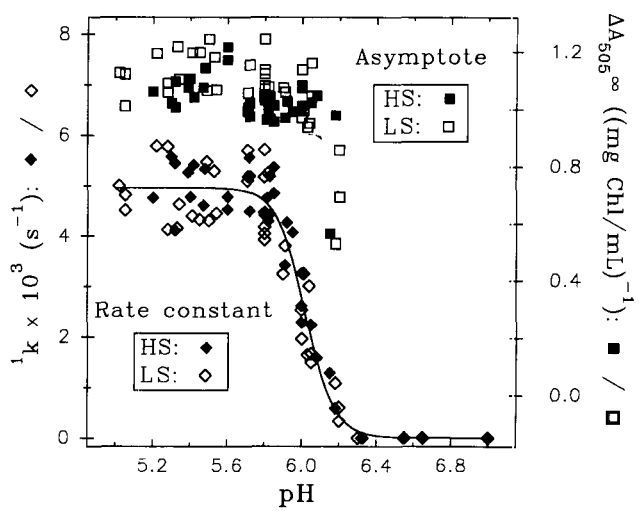


**Figure 5.** pH dependence in HS and LS thylakoids of the parameters $\Delta A_{505}\infty$ and $^1k$. The initial 10 min of the dark $\Delta A_{505}(t)$ kinetics of HS stored (filled symbols) and LS stored (open symbols) were analyzed as described for the light-driven reaction (Figs. 2 and 3). In all reactions, 500 nM nigericin and 500 nM nonactin were present. Deepoxidation was started by the addition of ascorbate to a final concentration of 50 mM (see "Materials and Methods"). ◇, ◆, $^1k$. □, ■, $\Delta A_{505}\infty$.

reactions (Fig. 5). Thus, accurate pH values cannot be derived from the pH-7 experiments. The $\Delta A_{505}\infty$ values from Figure 2 for the pH-8 light-driven reaction were considerably below the maximum extent observed in dark experiments (Fig. 5), whereas light-driven $\Delta A_{505}(t)$ at pH 7 (Fig. 3) yielded asymptotes that agreed with the dark maxima.

### $SV_N$ at pH 8

Under the conditions of light-driven deepoxidation at pH 8 (Fig. 1), the time course of $SV_N$ during 360 s of illumination was determined (Fig. 6). For all four reaction conditions, fluorescence quenching in the presence of DTT was significantly lower compared to quenching in experiments without the deepoxidase inhibitor. Fluorescence quenching sensitive to DTT was calculated in analogy to the calculation of DTT-sensitive $\Delta A_{505}$. Except for the HS basal case (Fig. 7A), DTT-sensitive $SV_N$ was linearly related to DTT-sensitive $\Delta A_{505}$ for all reaction conditions (Fig. 7, B–D). The slopes of the regression lines increased in the order LS basal, HS coupled, and LS coupled. However, a statistically significant difference existed only between LS basal and LS coupled reactions (Fig. 7D, inset).

## DISCUSSION

### Flash-Driven ATP Formation and Lumen pH Determination

We investigated the kinetics of light-driven violaxanthin deepoxidation in two different types of thylakoid preparations that are thought to represent delocalized and localized proton gradient energy coupling, respectively. In previous work, the presence of different coupling types was derived from the effects of the membrane-permeable amine buffer pyridine on the number of flashes required to initiate ATP synthesis (Beard and Dilley, 1986) and from measurements of the lumenal pH with the fluorescent dye pyranine (Ren-

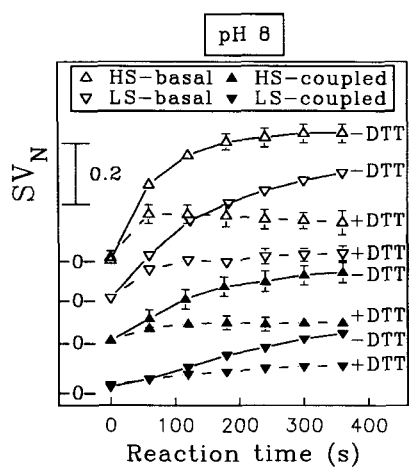


**Figure 6.** $SV_N$ of maximum fluorescence at pH 8. Time courses of $SV_N$ (±SD) in the presence (+DTT) and absence (−DTT) of 2 mM DTT are shown. HS-stored (△, ▲) and LS-stored (▽, ▼) thylakoids were tested under basal (△, ▽) and coupled (▲, ▼) reaction conditions. Experimental conditions were as described in Figure 1.

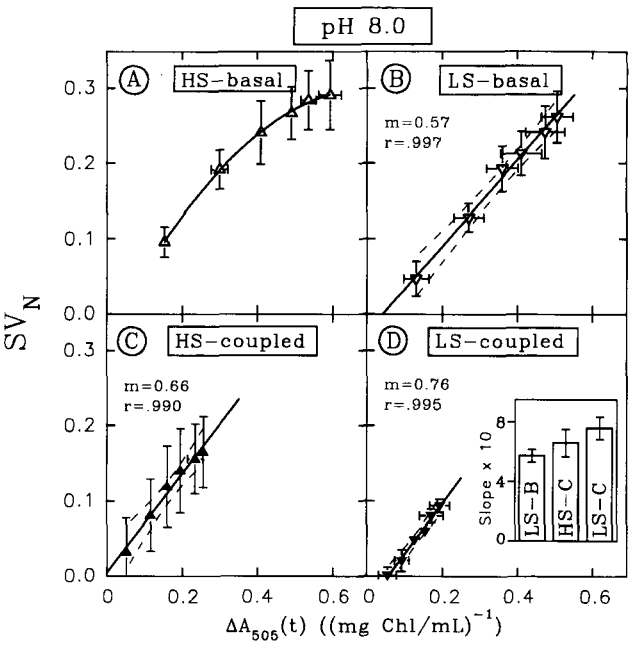


**Figure 7.** Relation between the DTT-sensitive $SV_N$ and the DTT-sensitive $\Delta A_{505}$ at pH 8. Values of $SV_N$ (±SD) as derived from Figure 6 are plotted versus the respective $\Delta A_{505}$ taken from Figure 2. DTT-sensitive fluorescence quenching was calculated as the difference between $SV_N$ in the absence of DTT minus $SV_N$ in the presence of DTT (Fig. 6). Except for the HS-basal reactions, first-order regressions to the data points are shown (solid lines: m, correlation coefficient; r, slope). The values of m ± 2 SE are graphically shown in the inset to D, and 99% confidence intervals of the regressions are indicated as dashed lines.

ganathan et al., 1993). Like these authors, we generated delocalized coupling behavior by storing the thylakoids in a medium containing 100 mM KCl as the osmoticum (HS) and localized coupling by thylakoid storage in a medium in which KCl was replaced by 200 mM sorbitol (LS). In both cases, the reaction media were identical.

A concern raised by a reviewer of this paper is that the two storage conditions used could have different effects on the electric field contribution to the proton motive force or on pyridine entry into the thylakoid lumen, and that such effects could cause the typical differences between HS and LS thylakoids. As reviewed by Dilley (1991), the delocalizing effect of $Ca^{2+}$ chelators in LS thylakoids, measurements of $\Delta\psi$ in HS and LS thylakoids by the electrochromic shift, and quantification of pyridine uptake into HS and LS thylakoids clearly indicate that electric field effects and pyridine uptake are no different for HS and LS thylakoids. Moreover, evidence was provided indicating that the protons sequestered in the localized domains are actually driving ATP synthesis by the chloroplast ATP-synthase (Dilley and Schreiber, 1984; Theg et al., 1988).

Using the pyridine-dependent effects on the number of flashes needed to reach the energetic threshold of ATP formation, we tested for the two different coupling modes in HS and LS thylakoids under conditions used for violaxanthin deepoxidation experiments (Table I). In accordance with

Beard and Dilley (1986), pyridine increased significantly the number of flashes required to initiate ATP synthesis in HS compared to LS thylakoids. Thus, the typical pattern indicating delocalized and localized coupling was present under our reaction conditions.

In HS-stored thylakoids, the pyridine-dependent flash lag extensions in deepoxidation buffer with its higher osmotic strength were reduced by 50% compared to the lower osmotic conditions (Table I, experiment B). Such a reduction is expected owing to a reduced thylakoid lumen volume, which under the higher osmotic conditions allows less pyridine inside the thylakoid. We have observed this effect in previous studies (data not shown).

Violaxanthin deepoxidation was carried out under continuous illumination. Therefore, it was important to test for the presence of localized or delocalized energy coupling status under steady-state conditions. This can be achieved by lumen pH determinations with the pyranine method (Renganathan et al., 1993). For an external pH of 8.9, these authors reported the values of the lumen pH to drop in the series LS coupled, HS coupled, LS basal, and HS basal (data of Renganathan et al. [1993] are shown in Table II). At an external pH of 8, no accurate pH determination for the latter three cases is feasible, since the lumen pH falls below the value of 6. At values < pH 6, the pyranine fluorescence ratio parameter does not change much with pH.

Nonetheless, under the experimental conditions used for violaxanthin deepoxidation at pH 8, the pyranine method unequivocally demonstrated that the lumen pH is much more acidic in the HS-coupled (pH ≤ 6.0) compared to the LS-coupled case (pH = 6.7) (Table II). Consequently, HS and LS thylakoids used for pH-8 deepoxidation experiments differed as would be expected for delocalized and localized proton coupling.

The calculated ΔpH value in LS-coupled thylakoids was 1.3 (Table II). This value is in good agreement with earlier data on LS thylakoids obtained under steady-state illumination. The ΔpH was 1.0 when measured by pyranine fluorescence (Renganathan et al., 1993) and 1.5 when measured with the [$^{14}$C]methylamine distribution method (Renganathan et al., 1991). In the latter case, high rates of ATP formation were reported in LS thylakoids (100 μmol ATP $mg^{-1}$ Chl $h^{-1}$). Those rates were measured in the absence of a hexokinase/Glc trap for ATP. Assuming a stoichiometry of 3 $H^+$/ATP, the transthylakoid ΔpH must be in the range of 2.3 to account for the thermodynamic energy requirements of ATP formation without an ATP trap (see Renganathan et al., 1993, for the relevant calculations and a discussion of this point). Since pH determination with [$^{14}$C]methylamine mainly reflects bulk-to-bulk phase ΔpH, the thermodynamically required ΔpH was suggested to be membrane localized.

## The $\Delta A_{505}$ and Violaxanthin Deepoxidation

The light-induced $\Delta A_{505}$ was shown to be quantitatively related to the amount of deepoxidized violaxanthin by Siefermann and Yamamoto (1974, 1975a). In our experiments, part of $\Delta A_{505}$ was not inhibited by 2 mM DTT (Fig. 1). By HPLC assay, no formation of antheraxanthin and zeaxanthin was detected when 2 mM DTT was present (data not shown). Similar observations have been made by Gilmore and Yamamoto (1991b). Thus, $\Delta A_{505}$ contains a component that is independent of deepoxidation.

The DTT-insensitive $\Delta A_{505}(t)$ are possibly related to membrane energization by $H^+$ uptake, which results in conformational changes and altered scattering properties (Dilley and Vernon, 1965). Krause (1974) demonstrated that such scattering changes in broken thylakoids are significant in the presence of $MgCl_2$ but are small without $MgCl_2$ in the assay medium. $Mg^{2+}$ ions were present in our reaction media, but not in those of Siefermann and Yamamoto (1974, 1975a). This would explain why Siefermann and Yamamoto found that the light-induced $\Delta A_{505}$ was caused exclusively by violaxanthin deepoxidation.

To correct for $\Delta A_{505}$ independent of deepoxidation reactions, controls with 2 mM DTT present were subtracted from light-driven $\Delta A_{505}(t)$ kinetics (Figs. 1 and 2). The question arises whether DTT may influence transthylakoid pH by interacting with thylakoid membrane components other than violaxanthin deepoxidase. Since scattering changes were related to the magnitude of the ΔpH (see above), our correction may not be strictly valid in this case. However, DTT applied to isolated thylakoids had no effect on the ΔpH as determined by 9-aminoacridine fluorescence quenching (Richter et al., 1994; Thiele and Krause, 1994). Moreover, the pH-dependent fluorescence quenching component, qE, in isolated chloroplasts was not affected by DTT (Gilmore and Yamamoto, 1991b). Thus, we conclude that DTT does not interfere with ΔpH.

That DTT-corrected $\Delta A_{505}$ changes can be used as a measure for violaxanthin deepoxidation was also supported by comparison of $\Delta A_{505}(t)$ with chromatographically measured pigment changes. $\Delta A_{505}(t)$ after DTT correction were linearly related to the corresponding increases of antheraxanthin and zeaxanthin (Fig. 4). The in vivo difference extinction coefficients derived from Figure 4 were similar to those obtained previously in the absence of scattering changes from dark deepoxidation experiments (Pfündel and Dilley, 1993). In the present work, the coefficients were 13.2 and 18.7 $mM^{-1}$ $cm^{-1}$ for the conversion of violaxanthin to antheraxanthin and of violaxanthin to zeaxanthin, respectively. The corresponding coefficients determined in the previous study were 12.6 and 21.7 $mM^{-1}$ $cm^{-1}$, respectively.

## $^1k$ of the $\Delta A_{505}$

Siefermann and Yamamoto (1974, 1975b) showed that $\Delta A_{505}(t)$ follows first-order kinetics. They introduced $^1k$ as a measure for deepoxidase activity and $\Delta A_{505}\infty$ as a measure for the availability of the enzyme substrate violaxanthin. We found that DTT-corrected $\Delta A_{505}(t)$ also followed first-order kinetics during the first 10 min of illumination (Figs. 2 and 3). To relate the rate constants of light-driven kinetics to pH values effective for enzyme activity, the pH dependence of $^1k$ in dark reactions was characterized in the presence of uncouplers to dissipate ΔpH and $\Delta\psi$ (Fig. 5). In the dark, no significant differences between HS and LS thylakoids were observed. Thus, direct influences of HS and LS storage on enzyme activity and violaxanthin availability can be excluded.

The pH dependence of $^1k$ showed a strong positive cooperativity (Fig. 5), indicative of a possible involvement of thylakoid polymeric complexes in deepoxidase activity regulation. Possible candidates are the LHC II or the ATP-synthase. qE is thought to occur in LHC II and has been shown to be pH dependent (Horton et al., 1991; Noctor et al., 1993). The pH curves of qE, however, did not exhibit the high cooperativity observed for deepoxidase activity. For the ATP synthase, highly cooperative trapping of protons has been reported (Griwatz and Junge, 1992). However, the pK of the ATP proton binding sites was 7.3 and disagrees with the inflexion point at pH 6.0 as observed for the deepoxidation rate constant. We conclude that thylakoid polymeric complexes are likely not involved in deepoxidase activity regulation.

The transformation of $^1k$ of light-driven $\Delta A_{505}(t)$ into pH values with the established pH dependence of $^1k$ of dark reactions requires that deepoxidase activity is regulated in the same way under light and dark conditions. A major difference between light and dark conditions is the presence of a $\Delta$pH in the former but not in the latter case. Since the isolated violaxanthin deepoxidase is highly active at low pH (Hager, 1969; Yamamoto and Higashi, 1978), it is obvious that enzyme activity does not require $\Delta$pH. This is not evidence that $\Delta$pH had an additional stimulating effect on deepoxidase activity under light conditions. Maximum values of light-driven deepoxidase activity, however, did not exceed maximum activities obtained in the dark in the presence of uncouplers (compare Fig. 3 and Fig. 5). Hence, it appears that absolute pH rather than $\Delta$pH is important for deepoxidase activity in light-driven reactions. In addition, no interference of $\Delta\psi$ is expected, since nonactin to dissipate $\Delta\psi$ was present in light and in dark reactions. We conclude that the calibration curve established under dark conditions is suitable to derive pH values from the $^1k$ of light-driven reactions.

With the external pH in the range of 7.2 to 8.0, deepoxidation can be activated by light-driven proton pumping (Hager, 1975; Siefermann-Harms, 1977) or, in the dark, by ATP hydrolysis-dependent proton flow (Gilmore and Yamamoto, 1992). Since deepoxidation requires acidic conditions, it is the pH somewhere behind the thylakoid permeability barrier that is sensed by the deepoxidase. The question is whether it is the pH of the lumen or of membrane-confined domains that regulates activity. This problem can be addressed by comparing the lumen pH determined by the pyranine fluorescence technique with the pH derived from deepoxidation $^1k$. In LS-coupled thylakoids at an external pH of 8, the measured lumen pH was 6.7 (Table II), a level at which no enzyme activity is observed in the pH calibration curve (Fig. 5). Thus, in the LS-coupled case (Fig. 2), lumen acidification does not explain the observed $^1k$ of $2.8 \times 10^{-3}$ $s^{-1}$. From this we conclude that deepoxidase activity responds to concentrations of membrane-sequestered protons.

At an external pH of 8, the $^1k$ values measured under basal conditions were higher compared to those observed under coupled conditions and no differences between HS and LS thylakoids were observed (Fig. 2). The pH values derived from $^1k$ of $\Delta A_{505}$ kinetics were near pH 5.9 for HS-basal and LS-basal conditions and near pH 6.0 for HS-coupled and LS-coupled conditions (Table II). At pH 8, the pyranine method gave light-induced lumenal pH values $\leq$6.0 for HS-basal, HS-coupled, and LS-basal thylakoids and pH 6.7 for LS-coupled thylakoids (Table II). In the LS-stored thylakoids under coupled conditions, the difference between lumen pH value and the pH derived from deepoxidation indicated that the domains in which the proton-binding site that controls deepoxidase activity is located are not in equilibrium with lumen protons. In the three other cases we studied, the lumen appeared to be acidic enough to account for the observed $^1k$ values. Thus, deepoxidase activity control by lumen protons, which are in equilibration with domain protons under those conditions, cannot be ruled out.

### $\Delta A_{505}\infty$ Values

The $\Delta A_{505}\infty$ estimates of DTT-sensitive $\Delta A_{505}(t)$ kinetics were used as a measure for the availability of the substrate violaxanthin to the enzyme. In light-driven reactions at pH 8, the $\Delta A_{505}\infty$ values decreased markedly in the series HS basal, LS basal, HS coupled, and LS coupled (Fig. 2). Lumen pH as determined by pyranine fluorescence at pH 8.9 was reported to drop also from HS basal to LS coupled (Renganathan et al. [1993]; data of this work are shown here in Table II). The similarity between $\Delta A_{505}\infty$ and lumen pH might indicate that lumen pH controls violaxanthin availability.

However, in the dark, no pH-dependent trend was observed for the $\Delta A_{505}\infty$ values (Fig. 5). Some decreased $\Delta A_{505}\infty$ values at pH > 6.1 were probably caused by inaccurate estimation of $\Delta A_{505}\infty$ in the case of the barely curved $\Delta A_{505}$ kinetics in this pH range (see Pfündel and Dilley, 1993). Thus, a direct effect of lumen protons on availability seems unlikely.

Siefermann and Yamamoto (1974, 1975b) studied the mechanisms regulating deepoxidation and proposed that the size of the violaxanthin fraction that is available for deepoxidation is related to the redox state of "some electron carrier between plastoquinone and the primary acceptor of PSII or plastoquinone itself." They related a more reduced state of the proposed carrier to greater violaxanthin availability. Nishio and Whitmarsh (1993) demonstrated that decreasing the pH below 6.5 leads to inhibition of Cyt *f* reduction. When electrons are donated to the plastoquinone pool by PSII light-driven reactions, a decreased electron flow through the Cyt *b/f* complex will lead to a more reduced state of the plastoquinole pool. Consequently, we suggest that lumenal acidification can increase violaxanthin availability by decreasing the rate of plastoquinol oxidation at the Cyt *b/f* complex.

In contrast to results at pH 8, at pH 7 the $\Delta A_{505}\infty$ values of the HS-basal and LS-coupled cases were similar (Fig. 3). Possibly, lowering the external pH resulted in lowered lumen pH values in the light and slowed down plastoquinol oxidation to a degree that brought about maximum violaxanthin availability even under LS-coupled conditions.

In the dark, i.e. in the absence of rapid PSI-dependent plastoquinol oxidation, the plastoquinone pool can be reduced by 50 mM ascorbate within 15 min (Aristarkhov et al., 1987). In our dark experiments, 50 mM ascorbate was present and could have led to plastoquinone reduction. In osmotically shocked thylakoids, we have observed that 10 min of dark preincubation in the presence of 50 mM ascorbate reduced by

about 50% the complementary area of Chl fluorescence induction curves (data not shown), a measure for the PSII acceptor pool size (Murata et al., 1966). Thus, ascorbate-dependent plastoquinone reduction could explain the high and pH-independent values for violaxanthin availability observed under dark conditions (Fig. 5).

### $\Delta A_{505}$ Changes and Nonphotochemical Quenching

In parallel experiments to the light-driven deepoxidation at pH 8, we have investigated the time course of nonphotochemical quenching (Fig. 6). It is known that nonphotochemical quenching of Chl fluorescence is significantly reduced in the presence of the deepoxidase inhibitor DTT. This has been taken as evidence for the role of zeaxanthin in fluorescence quenching (see Pfündel and Bilger, 1994, for a recent review).

The DTT-sensitive fluorescence quenching was calculated from the time course in the absence of DTT minus the corresponding kinetics in the presence of DTT and was plotted against the DTT-sensitive $\Delta A_{505}$ (Fig. 7). Except for the HS-basal case, linear relationships between the two parameters were obtained for all reaction conditions, indicating an association between the amount of deepoxidized violaxanthin and fluorescence quenching, as already suggested from the work of Demmig et al. (1987) and Gilmore and Yamamoto (1991b). Comparable slopes of first-order regressions to the data points indicate similar quantitative relations for LS-basal, HS-coupled, and LS-coupled thylakoids. Interestingly, under HS-basal conditions a curved relation was obtained. Although the scattering of the data points is too great to allow a firm conclusion, it appears that under certain conditions factors are brought into play that mediate zeaxanthin-related fluorescence quenching. Linear and curved relationships between zeaxanthin content and qE have also been reported in the literature (e.g. Gilmore and Yamamoto, 1991b; Horton et al., 1991).

Fluorescence quenching under the reaction conditions applied is predominantly of the qE type (see Gilmore and Yamamoto, 1991b). This type of quenching increases with decreasing intrathylakoid pH (Horton et al., 1991; Rees et al., 1992; Gilmore and Yamamoto, 1993). The lumen pH under coupled conditions was lower in HS thylakoids than in LS thylakoids (Table II). If the lumen pH is important for qE, one would expect higher quenching at the same zeaxanthin content in the former compared to the latter case or, equivalently, anticipate a larger slope of the regression line in the plots of $SV_N$ versus $\Delta A_{505}(t)$ for HS-coupled compared to LS-coupled thylakoids. However, the slopes of these two cases were similar (Fig. 7). These observations make a direct influence of the lumen acidification state on qE unlikely.

In view of the relatively constant domain pH as reported by deepoxidation $^1k$ values, we suggest that the acidification of the localized proton domains is critical for the development of qE. In experiments with the uncoupling action of the tertiary amine dibucaine, Laasch and Weis (1989), Noctor et al. (1993), and Mohanty and Yamamoto (1994) arrived at similar conclusions.

## CONCLUDING REMARKS

Considering the proposed role of zeaxanthin in energy dissipation, effective control of the deepoxidation reactions is required to restrict zeaxanthin formation to conditions where light absorption exceeds photosynthetic capacity. We infer from our experiments that violaxanthin deepoxidation is controlled on the level of enzyme activity and on the level of violaxanthin availability. The two parameters can be regulated independently, as has also been shown by Siefermann and Yamamoto (1974, 1975b). Deepoxidase activity probably depends on the pH of localized proton domains and violaxanthin availability is controlled by the lumen pH, perhaps via the pH-dependent reoxidation of plastoquinol by the Cyt *b/f* complex.

Localized proton coupling has been proposed to occur under conditions where absorbed light energy does not exceed photosynthetic capacity (Dilley, 1991). Under localized coupling conditions, the relatively high lumen pH (Renganathan et al., 1991, 1993) would keep the availability of violaxanthin at a low level. It has been suggested that high irradiance could trigger the switching from localized to delocalized proton coupling and, consequently, increase thylakoid lumen acidification (Dilley, 1991). The resulting high violaxanthin availability favors zeaxanthin formation and, thus, energy dissipation.

Availability control by shifting between localized and delocalized coupling, regulation of violaxanthin activity by membrane-sequestered protons that are likely driving ATP synthesis under localized conditions (Dilley, 1991), together with the steep pH dependence of deepoxidase activity could allow the rapid onset of deepoxidation at exactly the pH value that exceeds the requirements for ATP synthesis. Such a tight pH control would comply with an irradiance-dependent switch that could increase energy dissipation with little change in $\Delta$pH as suggested by Foyer et al. (1990).

### ACKNOWLEDGMENT

The authors thank Janet Hollister for her always excellent help in the preparation of the manuscript.

Received May 23, 1994; accepted September 19, 1994.


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