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Relative Contributions of Zeaxanthin-Related and Zeaxanthin-Unrelated Types of 'High-Energy-State' Quenching of Chlorophyll Fluorescence in Spinach Leaves Exposed to Various Environmental Conditions[1]

William W. Adams III*[2], Barbara Demmig-Adams[2], and Klaus Winter

Institut für Botanik und Pharmazeutische Biologie, Universität Würzburg, Mittlerer Dallenbergweg 64, 8700 Würzburg, Federal Republic of Germany

ABSTRACT

We have identified two rapidly relaxing components of nonphotochemical fluorescence quenching which suggests that dissipative processes occur in two different sites in the photochemical system of leaves. Under a variety of treatment conditions involving different leaf temperatures, photon flux densities (PFD), exposure times, and in the presence of 5% CO_2 or 2% O_2, no CO_2, the components of nonphotochemical fluorescence quenching were characterized with respect to their sensitivity to dithiothreitol (DTT, which completely inhibits zeaxanthin formation), the effect on instantaneous fluorescence, and the rapidity of relaxation upon darkening. Under most circumstances the DTT-sensitive component (associated with a quenching of instantaneous fluorescence and correlated with zeaxanthin) represented the majority of the rapidly relaxing portion of fluorescence quenching. A DTT-insensitive (zeaxanthin-independent) component, which also relaxed rapidly upon darkening but was not associated with a quenching of instantaneous fluorescence, became proportionally greater in an atmosphere of 2% O_2 and no CO_2, at elevated leaf temperatures, and to some degree during the induction of photosynthesis (1 minute after the onset of illumination). A third component which was also DTT-insensitive and was sustained upon darkening, was largely suppressed in 2% O_2, O% CO_2. We conclude that, under conditions favorable for photosynthesis, energy dissipation occurred mainly in the chlorophyll antennae whereas, under conditions less favorable for photosynthesis, a second dissipation process, probably in or around the reaction center of photosystem II, also developed. Furthermore, evidence is presented that the zeaxanthin-associated dissipation process prevents sustained inactivation of photochemistry by excessive light.

In a previous paper (7), the components of nonphotochemical fluorescence quenching were characterized under conditions that were optimal for photosynthesis, *i.e.* in the steady-state at optimal leaf temperatures in the presence of saturating CO_2. Under such conditions, most of the rapidly relaxing 'high-energy-state' quenching in leaves was inhibited by DTT, whereas in isolated chloroplasts a combination of two types of high-energy-state quenching could be distinguished (DTT-sensitive and DTT-insensitive). Thus, the question remained as to whether the DTT-insensitive type of energy-quenching observed in isolated chloroplasts occurs mainly in the isolated system, or if it can also be found to a substantial extent *in vivo* under certain conditions in intact leaves.

In the present paper, we present evidence that a DTT-insensitive and rapidly relaxing type of nonphotochemical fluorescence quenching does occur in leaves under conditions where linear photosynthetic electron flow is limited, *i.e.* at unfavorable (particularly high) temperatures, during the induction of photosynthesis, and in the absence of CO_2.

MATERIALS AND METHODS

Spinacia oleracea L. (spinach) was grown during the winter of 1989 in a naturally lit greenhouse supplemented with artificial light of 100 to 200 μmol photons m^{-2} s^{-1} under a 12 h photoperiod. Cut leaves were pretreated by placing them in a growth cabinet (25°C) at a PFD of 40 μmol photons m^{-2} s^{-1} with their petioles in distilled water (controls) or in a solution of 3 mM DTT for a minimum of 90 min (1, 7). At the end of such a pretreatment, no zeaxanthin could be detected in either the control or DTT-treated leaves (7). Simultaneous measurements of O_2 evolution and Chl fluorescence, and the calculation of parameters derived from Chl fluorescence, were as described previously (7, 9).

k_D[3] values of 12.5 corresponding to an $F_V/F_M = 0.85$ were subtracted from all calculated k_D values (9). This procedure yielded k_D values in darkness between 1.3 and 3.0 for leaves kept between 5 and 30°C, 3.7 for leaves kept at 35°C, and 7.7 for leaves kept at 40°C. These values are included in the k_D values calculated from the quenching of F_M in the light.

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[2] Present address: Department of Environmental, Population, and Organismic Biology, University of Colorado, Boulder, CO 80309-0334.

[3] Abbreviations: k_D, rate constant for radiationless energy dissipation in the antenna Chl; F_o, yield of instantaneous fluorescence; F_M, maximum yield of fluorescence induced by pulses of saturating light; F_V, variable yield of fluorescence; Q, electron acceptor of PSII; q_P (or 1-q_P), quenching coefficient for photochemical fluorescence quenching.

RESULTS

Temperature Dependence of the DTT-Sensitive and DTT-Insensitive Components of Fluorescence Quenching

Photosynthetic O_2 evolution in these spinach leaves, which had reached a steady-state rate after 10 min of illumination, exhibited a typical response to temperature, with an optimum between 27.5 and 32°C (Fig. 1A). In this set of data, photosynthesis tended to be somewhat lower in those leaves treated with DTT relative to the untreated leaves. The approximate reduction state of Q, $1\text{-}q_P$, was considerably higher in the DTT-treated leaves than in the untreated leaves at temperatures between 18 and 37°C (Fig. 1B), consistent with a lesser degree of radiationless energy dissipation in DTT-treated leaves. At 42°C, PSII was fully reduced in both the control and DTT-treated leaves, and at leaf temperatures of 13.5°C or lower, PSII was fully reduced in the control leaves but not quite fully reduced in the DTT-treated leaves.

Total nonphotochemical fluorescence quenching, or the calculated rate constant k_D, exhibited an antiparallel pattern to that of the rate of photosynthesis between 13.5 and 42°C, *i.e.* k_D was lowest in the leaves with the highest rates of photosynthesis (at 27.5°C) and increased at lower and higher temperatures (Fig. 2A). At the intermediate temperatures, the quenching of fluorescence in control leaves was, to a large extent, reversible within 5 min of darkening (Fig. 2A). However, at leaf temperatures below 23°C and above 32°C, a greater level of fluorescence quenching was still present 5 min after darkening of the leaf. Upon lowering of the temperature from 27.5 to 13.5°C the level of fluorescence quenching, reflecting alternative pathways of dissipation, increased with decreasing rates of photosynthesis. In contrast, both photosynthesis and the development of nonphotochemical fluorescence quenching were strongly inhibited at temperatures below 13.5°C (8.8°C).

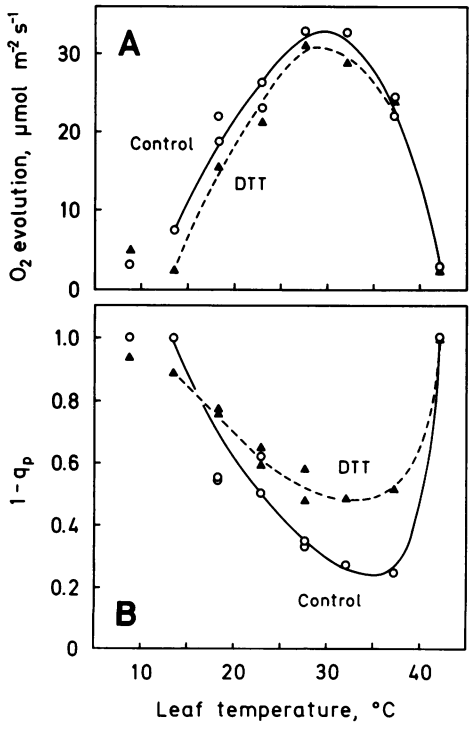

Figure 1. Response of photosynthetic O_2 evolution (A) and the approximate reduction state of Q, $1\text{-}q_P$ (B), in untreated (control) and DTT-treated spinach leaves following illumination with 880 μmol photons m^{-2} s^{-1} for 10 min at different leaf temperatures.

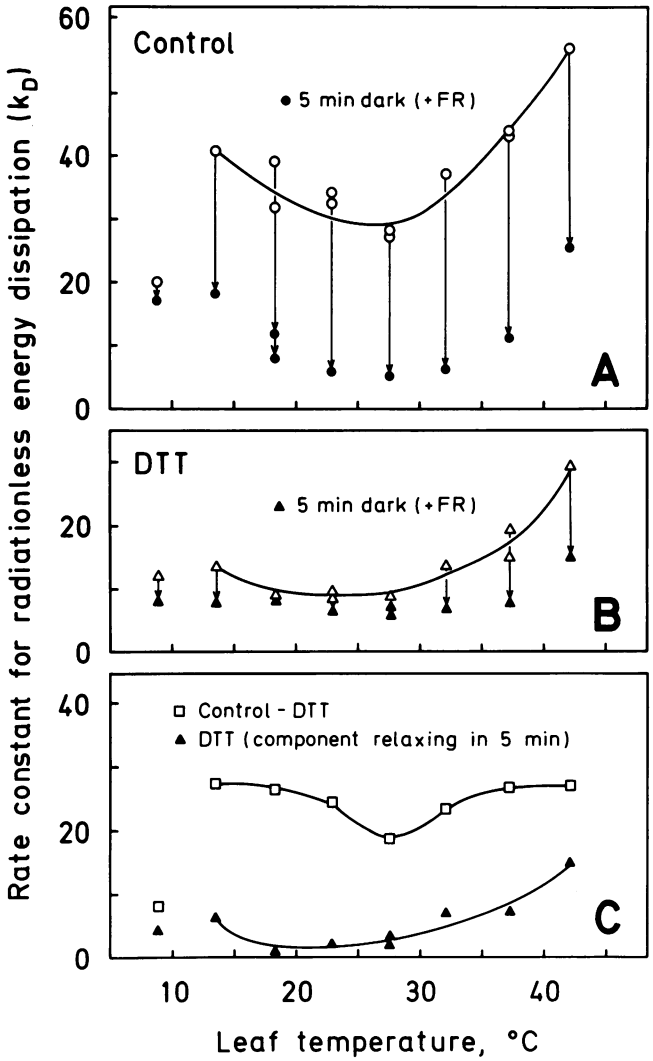

Figure 2. Response of the rate constant for radiationless energy dissipation, k_D, in untreated (control, A) and DTT-treated (B) spinach leaves following illumination with 880 μmol photons m^{-2} s^{-1} for 10 min at different leaf temperatures. The relaxation of fluorescence quenching 5 min after return to darkness (with low intensity, far red background light) is indicated by the arrows and solid symbols in A and B. k_D (control) − k_D (DTT-treated leaves) in C were calculated using mean values (where available) from A and B. The rapidly relaxing component of the DTT-insensitive quenching (C) was obtained by subtracting k_D calculated from F_M determined after 5 min in darkness from k_D determined in the light, all in DTT-treated leaves (B).

In leaves treated with DTT, only a relatively small degree of nonphotochemical fluorescence quenching developed between 8.8 and 27.5°C (Fig. 2B). However, between 27.5 and 42°C, this DTT-insensitive component exhibited a several-fold increase. Subtraction of the DTT-insensitive component

of k_D from that of the total k_D obtained in the control leaves yielded a rather temperature-insensitive curve with a depression between 18 and 37°C (Fig. 2C). The rapidly relaxing portion of fluorescence quenching in the DTT-treated leaves, obtained by subtracting k_D after 5 min dark from k_D in the light, increased continuously between 18 and 42°C (Fig. 2C).

DTT-Sensitive and DTT-Insensitive Components of Nonphotochemical Fluorescence Quenching during the Induction of Photosynthesis at Different Temperatures and PFDs

The induction kinetics of the DTT-sensitive and DTT-insensitive components of nonphotochemical fluorescence quenching, expressed as k_D, upon exposure of spinach leaf discs to 880 μmol photons m^{-2} s^{-1} at different temperatures are depicted in Figure 3. The induction of O_2 evolution was very similar in untreated and DTT-treated leaves (not shown). Both DTT-sensitive and DTT-insensitive components of fluorescence quenching developed more rapidly with an increase in temperature. The DTT-sensitive component exhibited a particularly pronounced increase between 15 and 35°C, with respect to that portion which developed within, for example, 1 min (Fig. 3, insets). After 10 min the magnitude of the DTT-sensitive component was in the same range at temperatures between 15 and 35°C but was very low at 5°C. The DTT-insensitive component had a similar magnitude after 10 min at all temperatures between 5 and 25°C. At 35°C, however, its magnitude was almost twice as great as that observed at the lower temperatures. This additional portion of the DTT-insensitive quenching present at 35°C was rapidly reversible upon darkening, whereas the portion present at all other temperatures was rather sustained. It should be pointed out that leaf temperatures rose somewhat during the first 2 min of illumination (e.g. from 35 to 37°C), equilibrating between 3.8°C (preillumination temperature = 5°C) and 2°C (preillumination temperature = 35°C) above the initial leaf temperature within 5 to 8 min.

The nonphotochemical quenching of fluorescence which had developed after only 1 min of illumination at 25°C in control leaves did not show the pronounced response to PFD (Fig. 4) which was observed during steady-state photosynthesis (after 10 min illumination; see 7). After 1 min of illumination a considerably greater (and rapidly reversible) amount of nonphotochemical fluorescence quenching had developed at low PFDs (Fig. 4A) than was still present under steady-state conditions (7), whereas less had developed at higher PFDs than was present under steady-state conditions. A large portion of this fluorescence quenching in untreated leaves relaxed within 2 to 5 min.

In leaves treated with DTT, the quenching of fluorescence exhibited almost no response to PFD (Fig. 4B), and the magnitude of this quenching at 1 min was similar to that observed after 10 min illumination (7). However, in contrast to the DTT-insensitive fluorescence quenching observed at 10 min, up to 50% of this component of quenching relaxed within 5 min upon return to darkness (Fig. 4B). Consequently, the calculated difference in k_D between untreated and DTT-treated leaves following 1 min illumination also exhibited less pronounced increases with increasing PFD than after 10 min (Fig. 4C; *cf.* with ref. 7).

Furthermore, the changes in F_o following 1 min illumination corresponded to the changes in k_D (Fig. 5). There was a difference in F_o between untreated and DTT-treated leaves at low PFD, in contrast to the situation existing after 10 min illumination (7). Furthermore, the difference in F_o between the two groups of leaves after 1 min at high PFD was smaller than after 10 min (*cf.* ref. 7 and Fig. 5). A net increase in F_o was observed in all of the leaves treated with DTT, and F_o in the untreated leaves rose to the level of that present in the DTT-treated leaves within 5 min upon return to darkness (Fig. 5).

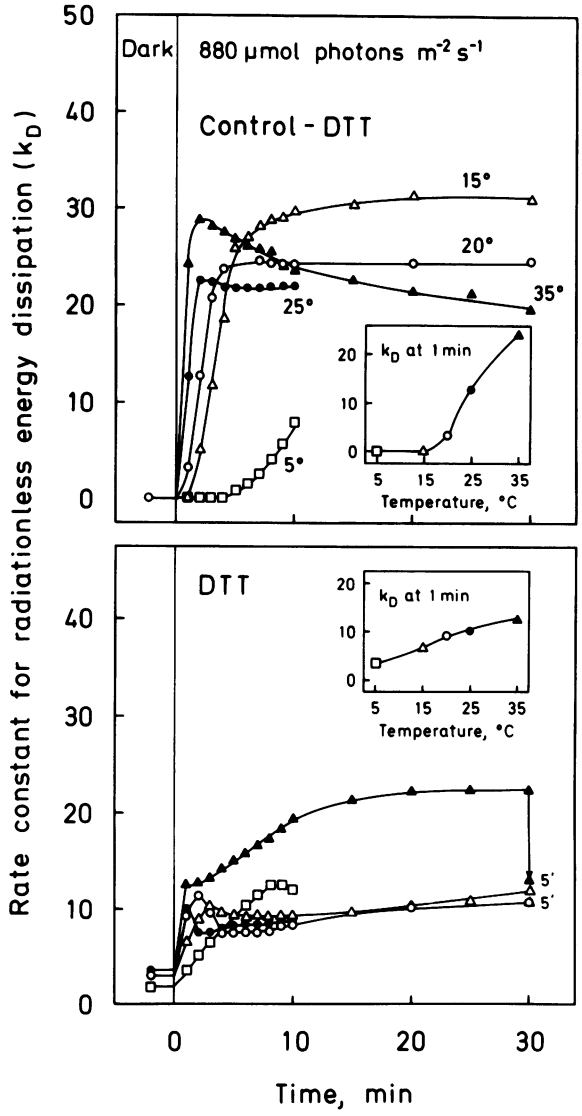

Figure 3. Time course of the induction of the DTT-sensitive and the DTT-insensitive components of nonphotochemical fluorescence quenching (expressed as k_D) upon exposure of spinach leaves to 880 μmol photons m^{-2} s^{-1} at different temperatures. The numbers indicate the temperature prior to illumination. k_D values determined after 5 min of darkness subsequent to 30 min of illumination are also shown for the leaves treated with DTT at 15 and 35°C.

Effect of DTT on Nonphotochemical Fluorescence Quenching in 2% O_2, 0% CO_2 at Various PFDs

For comparative purposes, the induction and relaxation kinetics of nonphotochemical fluorescence quenching (k_D) for

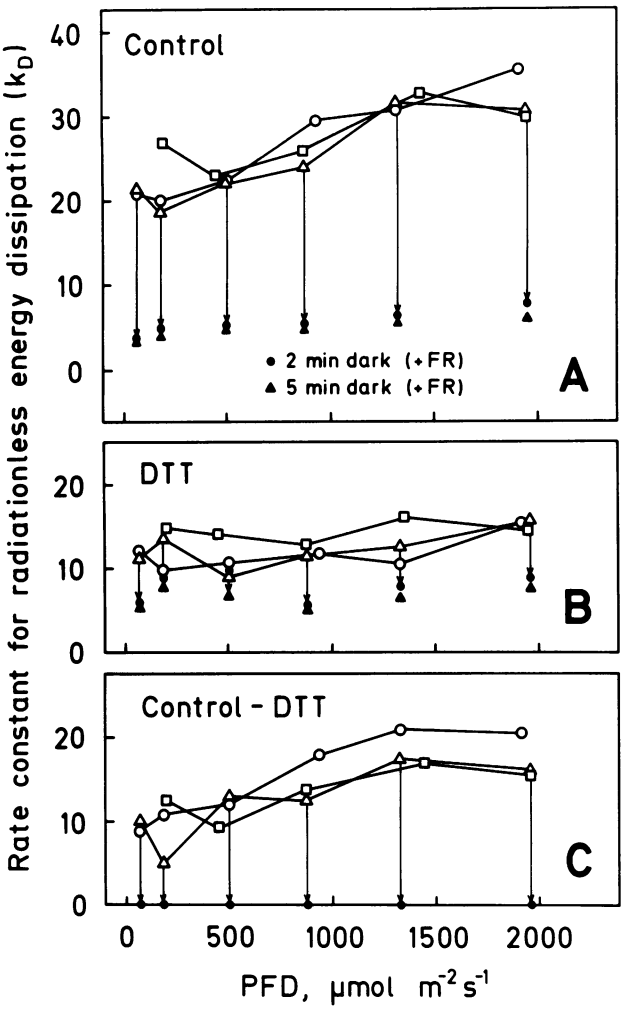

Figure 4. Effect of DTT on the rate constant for radiationless energy dissipation k_D in spinach leaves exposed to various PFDs in 5% CO_2 at 25°C for 1 min. Depicted are (A) k_D in control leaves, (B) k_D in DTT-treated leaves, and (C) the calculated difference of k_D (control leaves) − k_D (DTT-treated leaves). The k_D values were calculated from F_M obtained in a pulse of saturating light given after 1 min at each PFD (open symbols, each of which represents a separate experiment), and 2 and 5 min after switching to low intensity far red light (closed symbols).

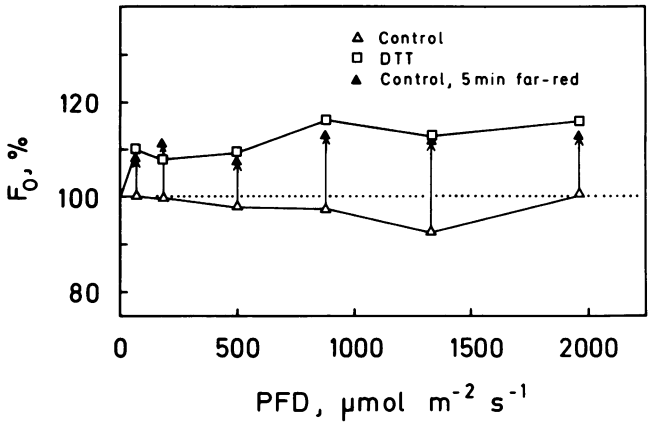

Figure 5. Changes in F_o in control and DTT-treated spinach leaves following 1 min illumination at various PFDs at 25°C. The F_o levels in the control leaves 5 min after switching to low intensity far red light are represented by the tip of the arrows.

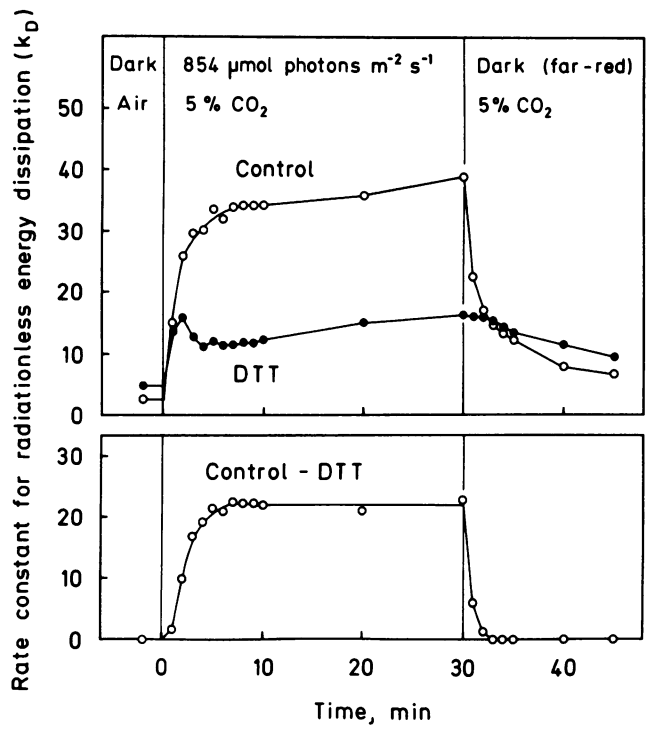

Figure 6. Changes in the rate constant for radiationless energy dissipation k_D in control and DTT-treated spinach leaves upon exposure to 854 μmol photons m^{-2} s^{-1} in 5% CO_2 at 25°C for 30 min, and during a subsequent period of recovery in 5% CO_2 under low intensity far red light at 25°C.

an untreated and a DTT-treated spinach leaf exposed to similar degrees of excessive light, either at high PFD at 5% CO_2 or at a lower PFD in 2% O_2, 0% CO_2, were characterized (Figs. 6 and 7). As was shown in a previous paper (7), the larger portion of k_D at 854 μmol photons m^{-2} s^{-1} in 5% CO_2 was DTT-sensitive and relaxed relatively rapidly upon darkening, whereas the quenching which developed in the leaf treated with DTT relaxed rather slowly upon darkening. The calculated difference in k_D between the control and the DTT-treated leaves decreased to zero within 3 min of darkening. This subtraction, as with all of those shown previously, is made under the assumption that the quenching which occurred in the leaf treated with DTT also occurred in the untreated leaf. Since a somewhat greater degree of quenching remained in the DTT-treated leaf relative to the control leaf 3 to 15 min after darkening, this assumption may not always be correct, particularly after long exposure times.

The relative proportions of the DTT-sensitive and the DTT-insensitive components of fluorescence quenching which developed during illumination between 5 and 30 min in 2% O_2, 0% CO_2 at a low PFD were similar to those which developed in 5% CO_2 at the higher PFD (*cf.* Figs. 6 and 7). The relaxation kinetics upon darkening, however, were different. Whereas the fluorescence quenching which developed in 5% CO_2 in the DTT-treated leaf relaxed very slowly upon darkening (Fig.

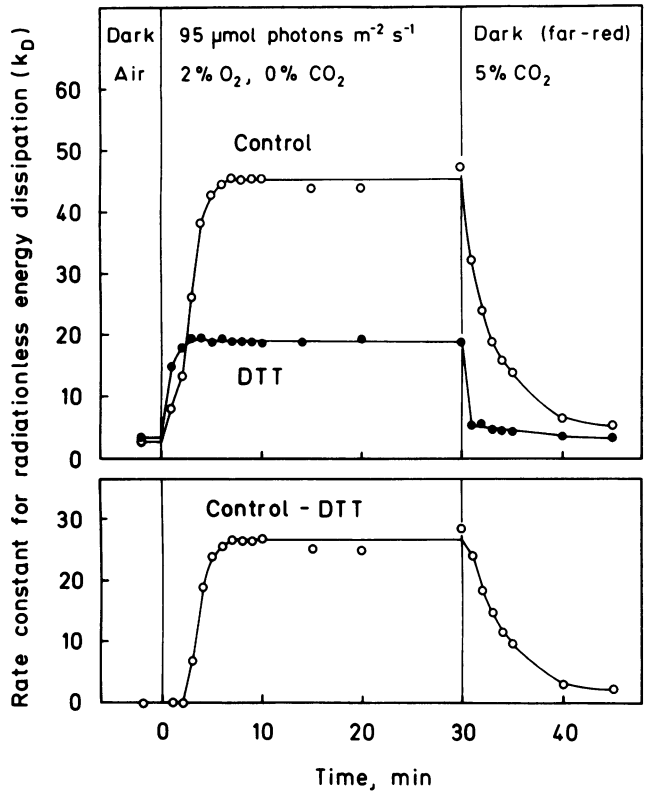

Figure 7. Changes in the rate constant for radiationless energy dissipation k_D in control and DTT-treated spinach leaves upon exposure to 95 μmol photons m^{-2} s^{-1} in 2% O_2, 0% CO_2 at 25°C for 30 min, and during a subsequent period of recovery in 5% CO_2 under low intensity far red light at 25°C.

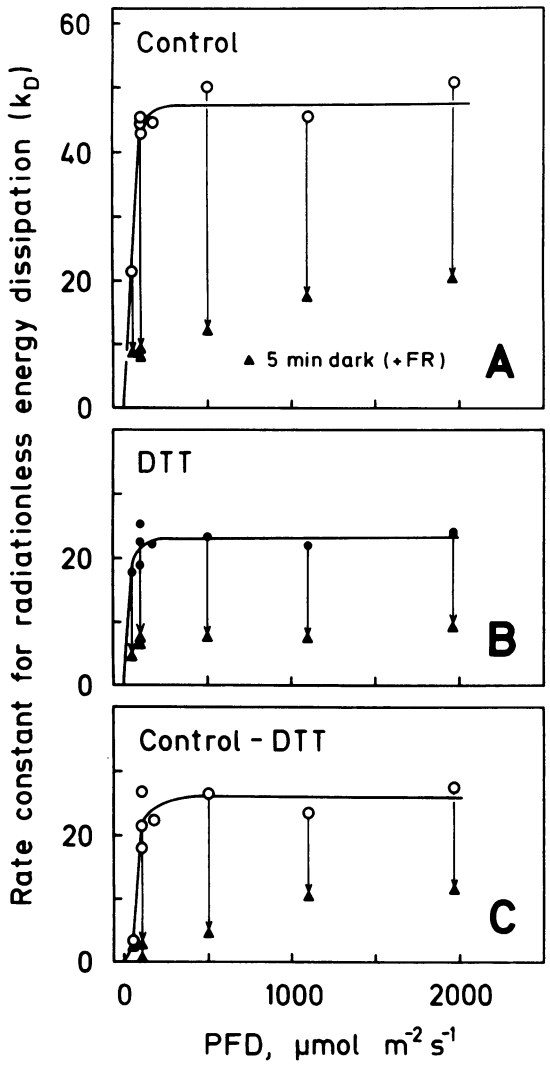

Figure 8. Effect of DTT on the rate constant for radiationless energy dissipation k_D in spinach leaves exposed to various PFDs in 2% O_2, 0% CO_2 at 25°C for 10 min. Depicted are (A) k_D in control leaves, (B) k_D in DTT-treated leaves, and (C) the calculated difference of k_D (control leaves) − k_D (DTT-treated leaves). The k_D values were calculated from F_M obtained in a pulse of saturating light given after 10 min at each PFD, and 5 min after switching to low intensity far red light (indicated by arrows).

6), almost all of the quenching in a similar leaf exposed to a low PFD in 2% O_2, 0% CO_2 relaxed within 1 min upon darkening (Fig. 7). Fluorescence quenching in 2% O_2, 0% CO_2 was induced more rapidly in the DTT-treated leaf than in the control leaf (Fig. 7). Consequently, the calculated difference in k_D between the control and DTT-treated leaves was zero (or negative) during the first 2 min, which underestimated the initial increase in the DTT-sensitive component of nonphotochemical fluorescence quenching.

The response of fluorescence quenching induced in an atmosphere of 2% O_2, 0% CO_2 to PFD is shown in Figure 8. Fluorescence quenching which developed in 2% O_2, 0% CO_2, in both control and DTT-treated leaves, was almost already light saturated at the PFD depicted in Figure 7. Upon return to darkness and 5% CO_2, the quenching of fluorescence relaxed increasingly less rapidly with increasing PFD in the untreated control leaves (Fig. 8A), whereas the relaxation kinetics of fluorescence quenching in leaves treated with DTT exhibited little response to the previous PFD. The DTT-sensitive component of fluorescence quenching was also associated with a quenching of F_o (Table I). However, this quenching component relaxed more slowly following exposure to light in 2% O_2, 0% CO_2 than after exposure to light n air (*cf.* with 7). The calculated difference in F_o relaxed concomitantly with the calculated difference in k_D (Table I). There was no increase in F_o in the DTT-treated leaves between 0 and 5 min, during which time the majority of the quenching present in these leaves during actinic illumination relaxed. There was, however, an increase in F_o which developed initially in the DTT-treated leaves and which relaxed extremely slowly. Upon transfer to low intensity far red light in 5% CO_2, a similar increase in F_o in the untreated leaves also became apparent.

Recovery of PSII Photochemical Efficiency in Untreated and DTT-Treated Leaves Subsequent to an Exposure to High Light

From Figure 6 it was apparent that there was somewhat more sustained quenching of fluorescence (upon return to darkness) in the leaf treated with DTT than in the control

Table I. *Time Course of Changes in F_O and k_D in Untreated and DTT-Treated Spinach Leaves Subsequent to an Exposure to 98 μmol Photons m^{-2} s^{-1} in 0% CO_2, 2% O_2 for 10 min*

Leaves were returned to low intensity far-red light in 5% CO_2, 21% O_2 subsequent to the exposure to white light.

	Time after Return to Far-Red Light, min		
	0	5	20
F_O, percent of control			
Untreated	88	102	109
DTT treated	116	114	112
Untreated-DTT treated	28	12	3
k_D			
Untreated	47	16	6
DTT treated	19	5	4
Untreated-DTT treated	28	11	2

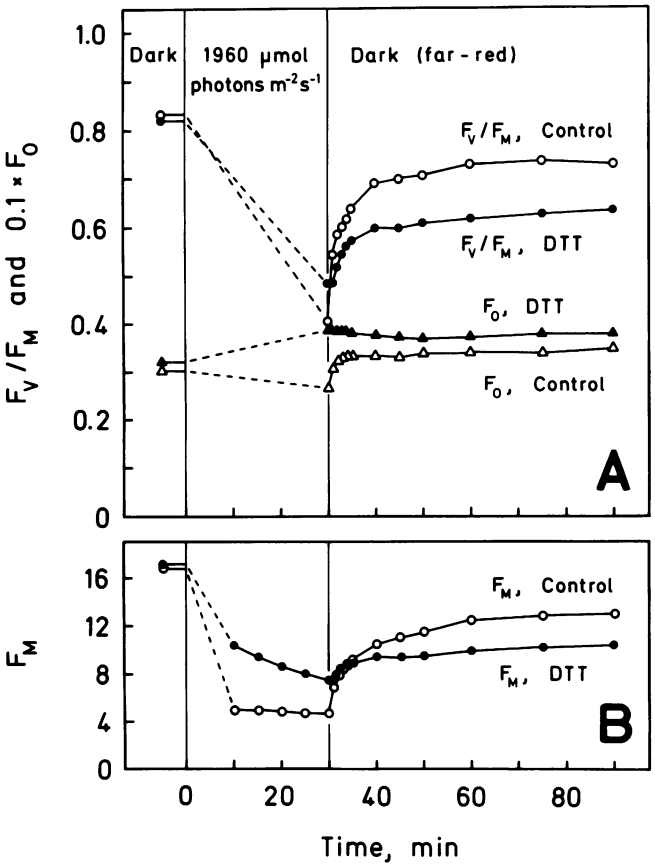

Figure 9. Changes in PSII photochemical efficiency, F_V/F_M, and F_o (A), and in F_M (B) in untreated (control) and DTT-treated spinach leaves following exposure to 1960 μmol photons m^{-2} s^{-1} in 5% CO_2 at 26°C for 30 min, and during a subsequent 60 min period of recovery under low intensity, far red light in 5% CO_2 at 20°C.

leaf following a 30 min exposure to 854 μmol photons m^{-2} s^{-1} in 5% CO_2. Subsequent to a 30 min treatment at 1960 μmol photons m^{-2} s^{-1}, F_M and PSII photochemical efficiency, F_V/F_M (2, 14), initially were lower in the control leaf relative to one treated with DTT (Fig. 9). Within 5 min after darkening, however, both F_M and F_V/F_M had increased considerably more in the control leaf, and after 60 min in darkness F_V/F_M was only 12% below that of the preexposure value in the control leaf, whereas the remaining depression in the DTT-treated leaf was twice as great at 23% below that of the preexposure value. In the control leaf, F_o was decreased relative to the preexposure value immediately following illumination with 1960 μmol photons m^{-2} s^{-1}, but rose rapidly above the preexposure value upon darkening. In the leaf treated with DTT, F_o was strongly increased following the high light exposure, followed by a very small decrease during recovery in darkness such that a greater increase in F_o in the DTT-treated *versus* the control leaf was maintained throughout the recovery period considered here. Numerous experiments using different PFDs, temperatures, and exposure times yielded very similar results.

DISCUSSION

In a previous paper (7), it was reported that the DTT-sensitive (zeaxanthin-associated) quenching of Chl fluorescence accounted for almost all of the 'high-energy-state' quenching in spinach leaves under favorable conditions. Here we have shown that, under conditions which were less favorable for photosynthesis (in 2% O_2, 0% CO_2, at elevated temperatures, and during the first minute of photosynthetic induction), this DTT-sensitive quenching of fluorescence was still a large component of the total quenching. However, there was also a considerable amount of a second rapidly reversible component of fluorescence quenching which was insensitive to DTT (Figs. 2, 4, and 8) and was not associated with a quenching of F_o (Table I). Thus, whereas the DTT-insensitive portion of fluorescence quenching under favorable conditions was largely slowly reversible (Fig. 6; ref. 7), under the less favorable conditions presented in this study this portion was composed of both a slowly relaxing component and a rapidly relaxing component (Figs. 2–4, 7, and 8). Thus, the quenching of fluorescence in spinach leaves under these less favorable conditions was similar to that observed in isolated chloroplasts (*cf.* with 7).

The fact that the DTT-sensitive and one DTT-insensitive component of nonphotochemical fluorescence quenching relaxed rapidly suggests that there may be two different regulatory processes which contribute to high-energy-state quenching (Fig. 8). Since the DTT-sensitive, zeaxanthin-correlated component was associated with a quenching of F_o, we have previously suggested that this reflects radiationless energy dissipation in the antenna Chl (3, 4, 6, 14). We now suggest that the second rapidly relaxing component of nonphotochemical fluorescence quenching (*i.e.* DTT-insensitive) observed in this study (which is not associated with F_o quenching) may be related to a dissipative process in or around the reaction center such as a PSII cycle, possibly accompanied by a decrease in the rate of electron donation from the water splitting complex (8, 13, 15). This component represents a large portion of the high-energy-state quenching in isolated chloroplasts (7) and also represents an appreciable portion of the fluorescence quenching observed in leaves in 2% O_2, 0% CO_2, at higher temperatures, and during the induction of photosynthesis. We therefore suggest that the ΔpH or high-energy state of the thylakoid membrane induces both kinds

of high-energy state quenching, one representing dissipation processes in the antenna Chl and the other associated with PSII centers. Thus, the zeaxanthin-associated process would be subject to two levels of control: first, through the known control of zeaxanthin formation via the xanthophyll cycle (12), and second through a special state of the antenna complexes induced, *e.g.* by the ΔpH which would render zeaxanthin effective as a quencher of fluorescence. Furthermore, the identification of an additional dissipation process with different properties (DTT sensitivity, F_o effect) supports the conclusion that zeaxanthin mediates the remaining component of high-energy-state quenching and negates the concern that DTT might independently inhibit quenching associated with the reaction center.

The difference in the magnitude of the DTT-sensitive quenching between 1 and 10 min upon exposure to light is consistent with the known characteristics of zeaxanthin formation through violaxanthin de-epoxidation. During the induction of photosynthesis, there is a transient accumulation of zeaxanthin (16) which probably leads to the greater magnitude of the DTT-sensitive quenching at low PFD after 1 min than after 10 min. The lesser magnitude of this component at high PFD after 1 min than after 10 min is also consistent with the kinetics of zeaxanthin formation (10, 12, 16).

The rapidity of the development of the DTT-sensitive fluorescence quenching was strongly temperature dependent (Fig. 3), which probably resulted from the temperature dependence of the enzymatic conversion of violaxanthin to zeaxanthin. The final, steady-state levels of DTT-sensitive quenching exhibited less of a dependence on temperature, with a minimum at the temperature (27.5°C) at which the largest fraction of energy was dissipated via photosynthesis (Figs. 1 and 2C). Since very little quenching of fluorescence (*i.e.* radiationless energy dissipation) developed at 8.8°C (Fig. 2C), one would expect damage to occur under such conditions where the absorbed energy can be dissipated neither via photosynthesis nor deexcitation in the antenna Chl (11). Illumination at elevated temperatures (37°C, and particularly 42°C, when photosynthesis approached 0) resulted in a rather pronounced increase in the magnitude of the second rapidly relaxing and DTT-insensitive quenching process. This is consistent with the previous suggestion that there is a stimulation of a PSII cycle or of charge-recombination, associated with a decreased rate of electron donation from the water splitting complex, at elevated temperatures (15).

This component of fluorescence quenching was very large in DTT-treated leaves illuminated in 2% O_2 and no CO_2 (Figs. 7 and 8). At the same time, there was very little slowly reversible quenching present under these conditions. We do not know the nature of this slowly reversible, DTT-insensitive component of fluorescence quenching. Since this type of quenching develops at low PFD (7), it is unlikely that this component is indicative of photoinhibitory damage. It is interesting to note that phosphorylation of the light-harvesting Chl-protein complex of PSII has been observed in low light in air but not in 2% O_2, 0% CO_2 (5). It is possible that this slowly reversible, DTT-insensitive component of fluorescence quenching is associated with the phosphorylation of the light-harvesting complex, as there is little of this component in either 2% O_2, 0% CO_2 or after 1 min of illumination (Figs. 4 and 8).

In spite of the fact that DTT inhibited one component of fluorescence quenching, a greater degree of quenching was sometimes observed subsequent to an exposure to an excess of light in leaves treated with DTT relative to untreated leaves (*e.g.* Fig. 6). A greater degree of sustained 'photoinhibitory inactivation' of photochemistry could actually be expected to occur in leaves which experience overexcitation of PSII, as indicated by the higher reduction state of Q in DTT-treated leaves (Fig. 9 and ref. 7). Exposure to even greater levels of excessive light lead to some degree of sustained depression of PSII photochemical efficiency in DTT-treated zeaxanthin-free leaves but not in untreated zeaxanthin-forming leaves (Fig. 9). This supports a photoprotective role of the zeaxanthin-associated dissipation process in the antenna Chl.

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