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# pH Dependent chlorophyll fluorescence quenching in spinach thylakoids from light treated or dark adapted leaves

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**Abstract**

The pH dependence of maximum chlorophyll fluorescence yield ($F_m$) was examined in spinach thylakoids in the presence of nigericin to dissipate the transthylakoid pH gradient. 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea (DCMU) was present to eliminate photochemical quenching. Thylakoids were prepared from dark adapted leaves ('dark' thylakoids) or preilluminated leaves ('light' thylakoids). In the latter there had been approximately 50% conversion of the xanthophyll violaxanthin to zeaxanthin, while no conversion had occurred in the former. In the presence of a reductant such as ascorbate, antimycin A sensitive quenching was observed (half maximal quenching at 5 $\mu$M), whose pH dependence differed between the two types of thylakoid. Preillumination of leaves resulted in more quenching at pH values where very little quenching was observed in 'dark' thylakoids (pH 5–7.6). This was similar to activation of high-energy-state quenching (qE) observed previously (Rees D, Young A, Noctor G, Britton G and Horton P (1989) FEBS Lett 256: 85–90). Thylakoids isolated from preilluminated DTT treated leaves, that contained no zeaxanthin, behaved like dark thylakoids. A second form of quenching was observed in the presence of ferricyanide, that could be reversed by the addition of ascorbate. This was not antimycin A sensitive and showed the same pH dependence in both types of thylakoid. The former type of quenching, but not the latter, showed similar low temperature fluorescence emission spectra to qE, and was considered to occur by the same mechanism.

*Abbreviations:* DCMU – 3(3′,4′-dichlorophenyl)-1,1-dimethylurea; DTT – dithiothreitol; EDTA – Ethylenediaminetetra-acetic acid; $F_0$ – dark level fluorescence yield; $F_m$ – maximum fluorescence yield; $F_v/F_m$ – ratio of variable to total fluorescence yield; Hepes – 4-(2-hydroxyethyl)1-piperazineethanesulphonic acid; Mes – 2-(N-morpholino) ethanesulfonate; pH – transthylakoid pH gradient; PS I – Photosystem I; PS II – Photosystem II; $Q_A$ – primary stable electron acceptor of Photosystem II; qE – high-energy-state fluorescence quenching

## Introduction

There are a number of processes which affect the supply of excitation energy to the Photosystem II (PS II) reaction centre. These have been detected and studied mainly by measurement of room temperature chlorophyll fluorescence, which is an indicator of the level of excitation of chlorophylls associated with PS II. The most significant of these processes is excitation dissipa-

tion associated with the formation of the transthylakoid pH gradient (pH) and is termed high-energy-state quenching, qE (Briantais et al. 1979, Krause et al. 1982, Quick and Horton 1984). qE appears to be very important for the control of PS II yield (Horton and Hague 1988, Weis and Berry 1987, Krause and Laasch 1987, Krause et al. 1988, Genty et al. 1989, Horton et al. 1990) and as a protection against the damaging effects of excess light (Krause and Behrend 1986, Oxborough and Horton 1988). There are a number of hypotheses about the mechanism of qE. There is growing evidence that the excitation quenching occurs in the light-harvesting complexes (Genty et al. 1989, Demmig-Adams 1990, Rees et al. 1990, Ruban et al. 1991, Horton et al. 1991). However, there are alternative suggestions that dissipation occurs at the reaction centre (Weis and Berry 1987) and it has been postulated that at least part of the observed quenching may be due to a stimulation of charge recombination (Schreiber and Neubauer 1989).

There is now considerable evidence that xanthophylls, particularly constituents of the xanthophyll cycle, are involved in qE quenching, either directly, or as part of the control mechanism. (Demmig et al. 1987, 1988, Demmig-Adams et al. 1989, Demmig-Adams and Adams 1990, Demmig-Adams 1991). The xanthophylls, zeaxanthin and violaxanthin are interconverted via the intermediate antheraxanthin in reactions catalysed by enzymes of the 'xanthophyll cycle' located at the thylakoid membrane. Stress conditions and the formation of the transthylakoid pH gradient tend to favour the de-epoxidation of violaxanthin to zeaxanthin. In vivo experiments on leaves showing a direct correlation between excitation dissipation and levels of zeaxanthin led to the hypothesis that zeaxanthin was necessary for qE formation, and could act as a direct quencher of chlorophyll excitation (Demmig et al. 1987, 1988, Demmig-Adams et al. 1989, Demmig-Adams and Adams 1990, Demmig-Adams 1990). Contrary to this hypothesis, during experiments on thylakoids isolated from spinach leaves treated to manipulate the zeaxanthin levels, we have demonstrated qE formation in the absence of zeaxanthin. However, although maximum qE formation was similar, we found that thylakoids containing zeaxanthin were capable of qE formation at lower pH values than thylakoids in which zeaxanthin was absent (Rees et al. 1989, Noctor et al. 1991). We therefore suggested that zeaxanthin functions physiologically as a 'quenching amplifier'. Following our observations it was hypothesised that there are two mechanisms of qE, one of which functions in the presence, and the other in the absence of zeaxanthin (Adams et al. 1990, Demmig-Adams and Adams 1990). It was claimed that these two mechanisms could be distinguished by the fact that the latter does not result in any quenching of dark level fluorescence, $F_0$. Contrary to this hypothesis we have shown that in thylakoids the relationship between quenching of maximum fluorescence, $F_m$ and of $F_0$ is unchanged whether zeaxanthin is present or absent (Noctor et al. 1991).

In earlier studies on qE it was demonstrated that fluorescence quenching could be induced by exposing uncoupled thylakoids to low pH (Wraight et al. 1972, Mills and Barber 1975, Briantais et al. 1979). It was assumed that the quenching was by the same mechanisms as qE. From these studies it was concluded that qE is controlled by the pH of the lumen and not the transthylakoid pH gradient per se.

If quenching induced by pH changes in uncoupled thylakoids occurs by the same mechanism as qE this would provide a system that could be more easily manipulated than a coupled system and would therefore be a powerful tool for obtaining information about the molecular mechanism of qE. In this paper we therefore consider pH-dependent quenching in uncoupled thylakoids and consider the important question as to how this quenching may relate to qE quenching in vivo. As we found that the characteristics of quenching depended on the redox conditions used, we consider the effect of low pH in the presence of either ascorbate or ferricyanide. We compare thylakoids isolated from leaves that have been pretreated to manipulate the levels of zeaxanthin as in our previous studies (Rees et al. 1989, Noctor et al. 1991), and demonstrate that there is a clear difference in the behaviour of thylakoids that do or do not contain zeaxanthin.

## Materials and methods

Spinach plants were grown for 4–6 weeks in a greenhouse under supplemented light with a 12 h photoperiod. After 24 h dark adaptation, leaves were floated on water at 25 °C with their cut petioles under water and either light treated with 200 $\mu$E $m^{-2}$ $s^{-1}$ for 50 min under an atmosphere of 98% $N_2$, 2% $O_2$ or dark adapted under the same conditions. A 10 min equilibration period was allowed before treatment.

For dithiothereitol (DTT) treatment leaves were placed overnight upright in a beaker containing 1 mM DTT solution. Air was fanned across the leaf surface in order to increase the transpiration rate and aid uptake of DTT. During subsequent preillumination, leaves were floated on a 1 mM DTT solution. Controls were treated identically but in the absence of DTT.

Chloroplasts were isolated as described in Noctor et al. 1991. Measurements were carried out at 20 °C using thylakoids at 35 $\mu$g chlorophyll $ml^{-1}$ in a volume of 1.2 ml. Thylakoids were prepared immediately before each measurement by breaking chloroplasts osmotically by incubation for 30 s in 30 mM $MgCl_2$, 0.5 mM EDTA, 10 mM Hepes (pH 7.6). An equal volume of medium containing 660 mM sorbitol buffered at the appropriate pH was then added. The buffers used were either 20 mM of each of Hepes, Mes and citrate (Figs. 3, 4, 6), or 50 mM of each of Hepes and Mes (Figs. 1, 2, 5). 1 $\mu$M nigericin was present for all measurements. $F_m$ fluorescence levels were measured after the addition of 50 $\mu$M DCMU in the presence of 300 $\mu$E $m^{-2}$ $s^{-1}$ light. When indicated 5 mM ascorbate or 3 mM $K_3Fe(CN)_6$ were included.

Increases in pH were brought about by the additions of aliquots of 2 M KOH. During this procedure the pH was monitored by a Jenway semi-micro glass combination electrode inserted into the reaction cuvette.

Room temperature chlorophyll fluorescence was measured by a Walz fluorimeter. Fluorescence quenching coefficients were calculated as follows: Ferricyanide dependent quenching = ($F_m$ in the presence of ascorbate and ferricyanide – $F_m$ in the presence of ferricyanide only)/($F_m$ in the presence of ascorbate and ferricyanide). Quenching coefficients in the presence of ascorbate:

$q_{rev}$ = ($F_m$ after pH has been returned to pH 7.6 – $F_m$ at low pH)/($F_m$ after pH has been returned to pH 7.6).

$q_{ant}$ = ($F_m$ at low pH in the presence of antimycin A – $F_m$ at low pH in the absence of antimycin A)/($F_m$ at low pH in the presence of antimycin A)

$q_{tot}$ = ($F_m$ at pH 7.6 – $F_m$ at low pH)/($F_m$ at pH 7.6).

(For $q_{ant}$ and $q_{tot}$, separate samples were used for the two $F_m$ measurements.)

For measurement of low temperature fluorescence spectra, 200 $\mu$l samples were removed, injected into a sample holder consisting of two flat round pieces of glass (diameter 14 mm), sealed by a metal ring, and rapidly frozen in liquid nitrogen. The whole procedure of taking and freezing samples took less than 15 s. During measurements the sample holder was immersed in liquid nitrogen in a purpose built cryostat. Excitation was provided by a Wotan tungsten halogen 150 W lamp defined by 4-46 and a 5-57 Corning filters and a heat filter to give broad band excitation of 50 $\mu$E $m^{-2}$ $s^{-1}$ in the Soret region of chlorophyll absorption centered near 435 nm. Fluorescence was detected by a 1024-channel silicon photodiode detector (Model 1455) via a Jarrell-Ash Monospec 27 monochromator and processed by an EG&G PARC optical multichannel analyser, Model 1461 with EG&G OMA-Vision-PDA data acquisition/analysis software. Spectra were normalised to the signal from fluorescein at 614.81 nm. Due to lack of sensitivity below 590 nm with the experimental design used, this was the wavelength of the maximum fluorescence level from fluorescein.

Pigment analysis by HPLC was carried out as described by Barry et al. (1990). Carotenoids were quantified using a diode-array detector: absorbances at peak wavelengths were integrated and concentrations calculated using a common extinction coefficient of 2500 $mol^{-1}$ $cm^{-1}$. Fractional content of pigments were calculated from data based on a mol:mol basis.

## Results

Figure 1 characterises the effect on room temperature chlorophyll fluorescence yield of exposing spinach thylakoids in the presence of nigericin to low pH. In each fluorescence trace $F_m$ was measured in the presence of DCMU and moderate light. (We did not consider measurement of $F_0$ to be reliable as the intensity of the excitation beam would not necessarily be low enough to ensure that all PS II centres were open in the case of inhibition of $Q_A$ reoxidation at low pH.). Traces a), b) and c) were obtained from thylakoids at pH 7.6, 6.2 and 4.6, respectively, in the presence of 5 mM ascorbate. Under these conditions the $F_m$ level is immediately quenched at low pH. The quenching is almost completely reversible when the pH is returned to 7.6 by the addition of KOH.

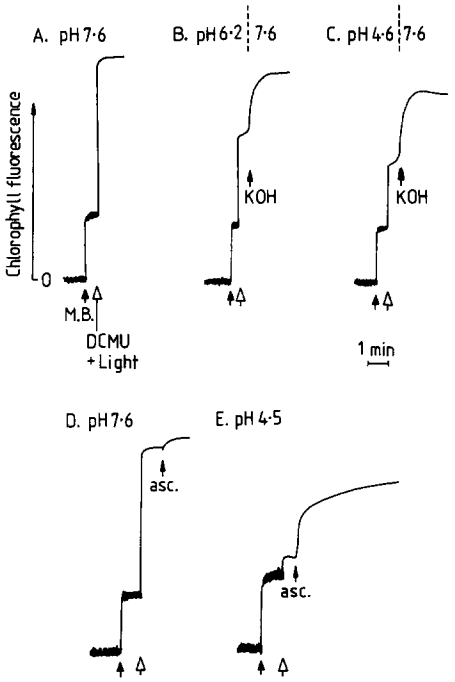


*Fig. 1.* Room temperature chlorophyll fluorescence traces obtained from spinach thylakoids (35 $\mu$g chl ml$^{-1}$). Chloroplasts were isolated from light treated leaves and broken in the chamber as described in *Materials and methods*. 1 $\mu$M nigericin was present throughout. The measuring beam (M.B.) of the fluorimeter was turned on where indicated by the closed arrow. 50 $\mu$M DCMU and moderate light were applied where indicated by the open arrow. (a) In the presence of 5 mM ascorbate at pH 7.6. (b) As (a), but with an initial pH of 6.2. The pH was increased to 7.6 by the addition of KOH at the point indicated. (c) As (b), but with an initial pH of 4.6. (d) In the presence of 3 mM $K_3Fe(CN)_6$ at pH 7.6. 5 mM ascorbate was added where indicated. (e) As (d) at pH 4.5.

In the presence of an oxidant such as ferricyanide additional pH dependent quenching is observed, which can be reversed by the addition of a reductant such as ascorbate (Fig. 1d) or dithionite (not shown). Some increase in fluorescence is observed on the addition of ascorbate even at pH 7.6 (Fig. 1c). This is probably due to the reduction of plastoquinone which acts as a quencher in its oxidised form (Vernotte et al. 1979). We would expect this to contribute to quenching at all pH values observed.

It is noticeable that both forms of quenching reverse quite slowly, with half times ranging from seconds to minutes.

The characteristics of both forms of quenching were investigated further to determine how they may relate to in vivo qE.

Although the mechanism is unknown, it has been shown that antimycin A inhibits qE formation in thylakoids without affecting the magnitude of the transthylakoid pH gradient (Oxborough and Horton 1988). We found that the addition of antimycin A also inhibits the pH dependent quenching observed in the presence of ascorbate. Antimycin A inhibition is seen whether reversible quenching, or the initial quenching with respect to the control at pH 7.6 is considered. Figure 2 shows the concentration

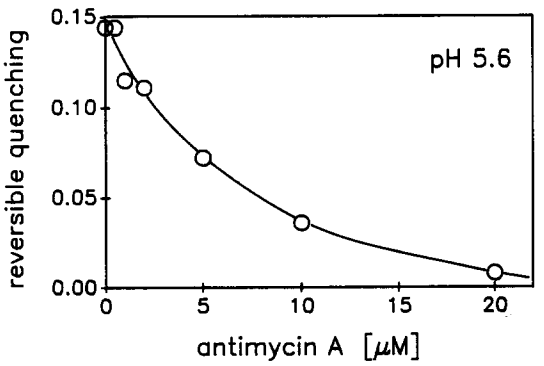


*Fig. 2.* The inhibition by antimycin A of pH quenching in the presence of 5 mM ascorbate. The thylakoids were initially exposed to pH 5.6. Quenching of $F_m$ is expressed as that reversible when the pH is raised to 7.6 by the addition of KOH. In all cases this resulted in an $F_m$ level very close to the control $F_m$ level obtained in thylakoids initially at pH 7.6. Antimycin A was added to the thylakoids in the breaking medium at pH 7.5, before the pH was decreased to 5.6.

dependence of the inhibition measured at pH 5.6. In this case half-maximal inhibition of quenching occurred at 5 $\mu$M antimycin A and almost complete inhibition of quenching was observed at 20 $\mu$M antimycin A. In some cases when lower pH values were used, slightly higher concentrations were necessary for maximal inhibition. These are higher concentrations than that needed to inhibit qE in chloroplasts (1 $\mu$M) (Oxborough and Horton 1987). However, in both this cases, and for light dependent qE the inhibition only occurs if antimycin A is present before quenching is initiated, i.e., before the thylakoids are exposed at low pH in this case. At antimycin A concentrations greater than 20 $\mu$M a decrease in fluorescence was observed when the pH was returned to 7.6, followed by slow quenching. It is not clear whether this effect occurs only at high antimycin A concentrations, or whether the effect is masked by an increase in fluorescence at the lower antimycin concentrations. The phenomenon was not seen if antimycin A was added to thylakoids initially at pH 7.6. Experiments described below were carried out using antimycin A at a concentration of 20 $\mu$M or greater, and therefore may underestimate reversible quenching. We found no effect of antimycin A on ferricyanide dependent quenching (results not shown).

To investigate further the relationship between the two types of pH dependent quenching and light dependent qE, we compared their fluorescence characteristics at 77 K. Figure 3 compares the spectra of quenching for both 'light' (A) and 'dark' (B) thylakoids. Fluorescence emission between 680 and 695 nm arises from PS II, while the longer wavelength emission centered at 735 nm arises primarily from PS I (Rijgersberg et al. 1979). For both 'light' and 'dark' thylakoids ferricyanide dependent quenching resulted in more quenching of PS I than PS II fluorescence, whereas the characteristics of pH quenching in the presence of ascorbate were very similar to those of light dependent qE quenching.

We compared the characteristics of both forms of quenching in thylakoids isolated from leaves with or without prior illumination at 200 $\mu$E $m^{-2}$ $s^{-1}$ for 50 min under 98% $N_2$, 2% $O_2$. This treatment results in conversion of more than 50% of the violaxanthin to zeaxanthin.

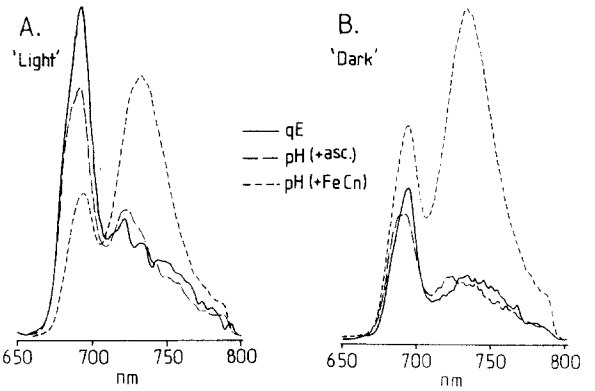


*Fig. 3.* 77 K fluorescence spectra of qE (solid lines), pH dependent quenching in the presence of ascorbate (long dashes), Ferricyanide dependent quenching (short dashes), in (A) 'light' thylakoids, (B) 'dark' thylakoids. qE quenching was induced by exposure of 50 $\mu$E $m^{-2}$ $s^{-1}$ with 100 $\mu$M methylviologen as electron acceptor. The spectra shown are the difference between the quenched state after 5 min light in the absence and presence of 1 $\mu$M antimycin A to inhibit qE formation. The spectrum for pH dependent quenching in the presence of ascorbate was obtained as the difference between spectra obtained at pH 4.4 in the absence and presence of 50 $\mu$M antimycin A which inhibits the quenching. The spectrum for ferricyanide dependent quenching was obtained as the difference between spectra obtained in the presence of 3 mM ferricyanide at pH 4.4 before and after the addition of 5 mM ascorbate. Other conditions are given in *Materials and methods*.

Figure 4 shows the pH dependence of ferricyanide dependent quenching (i.e., that reversed by the addition of ascorbate as in Fig. 1e). The pH characteristics of the quenching in thylakoids prepared from dark adapted leaves ('dark' thylakoids) and from preilluminated leaves ('light' thylakoids is very similar and is only significant below pH 5.

The pH dependence of quenching in the presence of ascorbate is quite different. In Fig. 5, pH dependent quenching in the presence of ascorbate has been measured in two ways; as the quenching reversed on the addition of KOH to bring the pH back to neutral (Fig. 5a) or as the quenching with respect to the fluorescence level at that pH in the presence of 20 $\mu$M antimycin A (Fig. 5b). In both cases the quenching is expressed with respect to $F_m$, as the $F_0$ measurement is considered to be unreliable. In addition, the total quenching of $F_m$ with respect to that at neutral pH has been calculated (Fig. 5c). As for

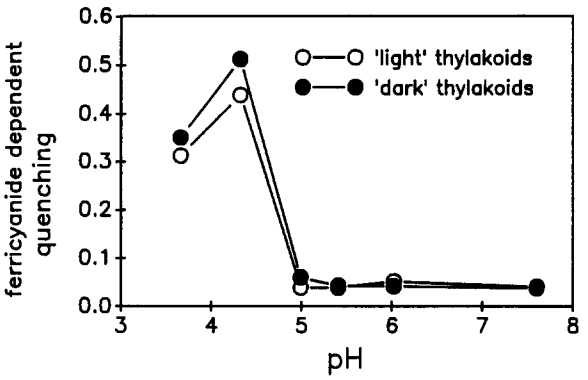


*Fig. 4.* A comparison of pH dependent quenching in 'light' and 'dark' thylakoids in the presence of 3 mM $K_3Fe(CN)_6$. The treatment of spinach leaves prior to chloroplast isolation is described in *Materials and methods*. The quenching, expressed with respect to $F_m$, was calculated as that reversed on the addition of 5 mM ascorbate. The following parameters were measured from 'dark' and 'light' thylakoids, respectively: $F_v/F_m$; 0.79, 0.75; zeaxanthin/(zeaxanthin + antheraxanthin + violaxanthin); 0, 0.523. The $F_m$ in 'light' thylakoids was 83% of that in 'dark' thylakoids.

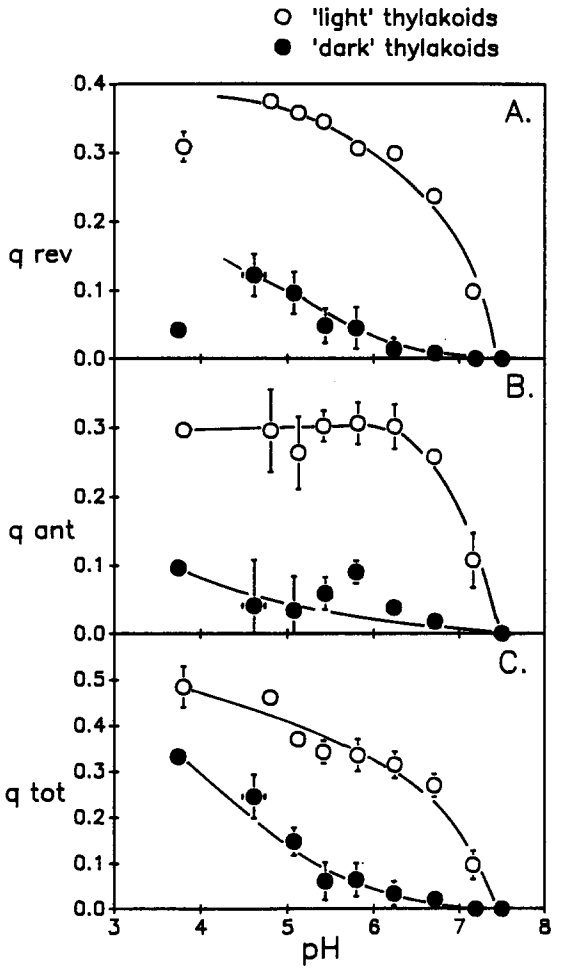


*Fig. 5.* A comparison of pH dependent quenching in 'light' (open symbols) and 'dark' thylakoids (closed symbols) in the presence of 5 mM ascorbate. Quenching is expressed with respect to $F_m$, and was calculated as follows: (a) $q_{rev}$ by reversal when the pH was increased to 7.6 by KOH addition. (b) $q_{ant}$ by comparison with samples in which quenching had been inhibited by the addition of 20 $\mu$M antimycin A. (c) $q_{tot}$ as total quenching compared with controls at pH 7.6 Each data point is the mean ±s.e. of measurements on 3 different thylakoid preparations. The following parameters were measured from the thylakoids: 'Dark' thylakoids: $F_v/F_m$; 0.77 ± 0.01 (mean ±s.e.). zeaxanthin/(zeaxanthin + antheraxanthin + violaxanthin); 0. 'Light' thylakoids: $F_v/F_m$; 0.72 ± 0.01. $F_m$ was 84% ± 1% of that in 'dark' thylakoids. zeaxanthin/(zeaxanthin + antheraxanthin + violaxanthin); 0.460 ± 0.025.

pH dependent qE, there is a clear difference in the pH dependence of quenching in 'light' thylakoids and 'dark' thylakoids, however this quenching is measured. The characteristics are very similar to those for qE. 'Dark' thylakoids show very little quenching above pH 5, whereas 'light' thylakoids show quenching at all pH values observed below 7.6, and have attained almost maximal quenching by pH 5.

Comparison of Fig. 5c with Figs. 5a and b suggests that below pH 5 additional irreversible quenching occurs in both types of thylakoids. This may well be a consequence of exposing the stromal side as well as the lumenal side of the thylakoid membrane to low pH. It is also possible that, as irreversible pH effects become more pronounced with decreasing pH, they may obscure the characteristics of the reversible effect, resulting in the decrease in reversible quenching at the lowest pH values (Fig. 5a). We would predict that, if this effect could be eliminated, reversible pH dependent quenching in 'dark' thylakoids would attain the same levels as in 'light' thylakoids at low pH, analogous to the characteristics of qE.

It is known that thylakoids tend to aggregate below pH 4.8. This may affect our measurements, so that the data obtained at the lowest pH values should be interpreted with caution. This data was included to ensure that we covered the complete range of pH values to which the lumenal side of the thylakoid membrane could be exposed in vivo. There is some dispute about the maximum possible values of the trans-

thylakoid pH gradient. Using quenching of 9-aminoacridine fluorescence, Briantais et al. (1979) calculated a lumenal pH as low as 4 in chloroplasts, but they suggested that there may be an artefact leading to an overestimation of the pH gradient in that case.

As observed previously, light treatment also resulted in the conversion of the xanthophyll violaxanthin to zeaxanthin, such that zeaxanthin increased from 0% to 43–52% of total xanthophyll cycle components. (The levels of xanthophylls for the individual experiments are given in the figure legends.) In addition, the light treatment led to a decrease in the dark adapted ratio of variable to total fluorescence, $F_v/F_m$ ($F_v/F_m$ values are given in figure legends).

The importance of zeaxanthin in the activation of qE has been previously supported by the effect of dithiothreitol (DTT) which inhibits zeaxanthin formation (Demmig-Adams et al. 1990, Bilger et al. 1989, Adams et al. 1990, Noctor et al. 1991). We have shown that DTT feeding of leaves prior to illumination inhibits both zeaxanthin formation and qE activation (Noctor et al. 1991). Figure 6 compares the pH dependence of quenching in the presence of ascorbate in thylakoids isolated from leaves that were light treated with and without prior DTT feeding. As

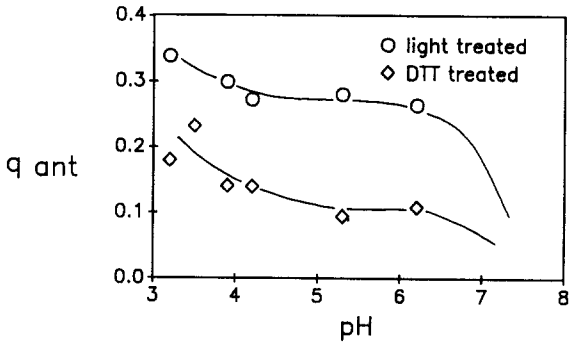


*Fig. 6.* A comparison of pH dependent quenching in the presence of 5 mM ascorbate in thylakoids isolated from light treated leaves (circles) or from leaves which were light treated after feeding with DTT (diamonds). Further details of the treatments are given in *Materials and methods*. Quenching is expressed with respect to $F_m$, and was calculated using 50 $\mu$M antimycin A (as for Fig. 4b). The following parameters were measured from control and 'DTT' thylakoids, respectively: $F_v/F_m$; 0.75, 0.73. $F_m$ of control thylakoids was 98% of that of 'DTT' thylakoids. Zeaxanthin/(zeaxanthin + antheraxanthin + violaxanthin); 0.463, 0.

observed previously, 'DTT' thylakoids contain only trace amounts of zeaxanthin, and show quenching characteristics similar to 'dark' thylakoids. Thus, the quenching was much lower than in 'light' thylakoids at the higher pH values, but rose to similar values at the lower pH values. However, unlike the results shown in Fig. 5, a small, but significant amount of quenching was observed in both sets of thylakoids even above pH 6.

## Discussion

In this study we have identified two distinct mechanisms of reversible quenching that occur when thylakoids are exposed to low pH values in the presence of an uncoupler. It is important to consider whether either or both of these mechanisms may relate to qE quenching observed in vivo.

Ferricyanide dependent pH quenching has been observed previously in PS II BBY particles (Crofts and Horton 1991). The characteristics of the quenching in PS II particles is very similar to that observed here in thylakoids, e.g., it is only significant at pH values below 5.5, and it is insensitive to antimycin A. Crofts and Horton concluded that the quenching is an artefact unrelated to in vivo qE, from the observations that it did not affect $F_0$, it was light dependent and tightly controlled by redox state. In this study we did not accurately assess the effect on $F_0$, but the conclusions of Crofts and Horton are supported by the fact that unlike for qE, we saw no activation of this quenching in the presence of zeaxanthin, and the low temperature characteristics were distinctly different from those of qE. We cannot discount the possibility, however, that this mechanism contributes to qE in vivo at high values of the transthylakoid pH gradient. In support of this there is evidence of some redox control of qE in pea thylakoids (Oxborough and Horton 1987). Possible mechanisms of this quenching are discussed by Crofts and Horton.

The quenching observed in the presence of ascorbate (i.e., without the need for an oxidant) is probably the same as that observed previously under conditions when no electron acceptors were present (Wraight et al. 1972, Mills and

Barber 1975, Briantais et al. 1979). The characteristics of the quenching with respect to pH in these early studies were similar to those which we observed in 'dark' thylakoids, which is consistent with the fact that no prior light treatment of the plant material was indicated. It has been suggested that at pH values below about pH 5, the quenching occurs by a different mechanism (Mills and Barber 1975, Briantais et al. 1979), thus the quenching was reported to be magnesium dependent above pH 5, but magnesium independent below pH 5. We could find little magnesium dependency of reversible quenching at any pH value, and therefore have no evidence that we are looking at more than one mechanism, although we accept that additional irreversible quenching may occur at the lower pH values.

The quenching in the presence of ascorbate had a number of characteristics similar to qE in vivo. Firstly, although the concentrations needed were considerably higher, the quenching could be inhibited by the addition of antimycin A. Secondly, as for qE, antimycin A was only effective at preventing the formation of the quenching, and had no effect if added after quenching had occurred. Thirdly, conditions leading to the conversion of violaxanthin to zeaxanthin resulted in an activation of quenching with very similar characteristics to those previously observed for qE. Fourthly, low temperature fluorescence spectra were very similar (Fig. 3).

We suggest that the reversible quenching observed in the presence of ascorbate at low pH occurs by the same mechanism for qE, and could therefore be used as an easily manipulated system for probing the qE mechanism. The advantage of this system is that quenching can be studied after treatments which lead to uncoupling of the thylakoid membrane. For example, selective removal of pigments by solvents or detergent.

One conclusion that can already be drawn from this study relates to our previous data showing activation of qE with respect to the magnitude of the transthylakoid pH gradient as measured by quenching of 9-aminoacridine fluorescence (Rees et al. 1989, Noctor et al. 1991). One possible explanation of that data is that 9-aminoacridine does not accurately measure the pH gradient that qE reflects, i.e., qE is controlled by localised proton domains, and accessibility of these domains is affected by leaf treatment. Exposing thylakoids to low pH in the presence of nigericin should eliminate localised domains. Therefore the fact that we still see an activation effect argues strongly for the hypothesis that there is a real change in the sensitivity of qE to pH.

It has been suggested that at least some of the observed qE quenching is a photochemical phenomenon that arises due to a stimulation of charge recombination such that it is impossible to fully reduce $Q_A$ even with a very bright pulse of light (Schreiber and Neubauer 1989). Even if such a mechanism does contribute to qE in vivo, it seems unlikely that it is responsible for the quenching we observe here. When considering the fluorescence induction kinetics of chloroplasts in saturating light, Neubauer and Schreiber (1987) identified a slow component of fluorescence rise ($I_1$–$I_2$) that they suggested may indicate the inhibition of a pathway of rapid $Q_A$ oxidation. Their results, however, indicated that such a pathway would be inhibited by DCMU. We would therefore not expect it to contribute to the quenching observed in these experiments.

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