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Mechanism of ΔpH-dependent dissipation of absorbed excitation energy by photosynthetic membranes. II. The relationship between LHCII aggregation in vitro and qE in isolated thylakoids

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A comparison has been made between the changes in absorption spectra and chlorophyll fluorescence emission occurring upon the induction of non-photochemical dissipation of excitation energy (qE) in isolated spinach thylakoids and those accompanying the aggregation of detergent-solubilised spinach light-harvesting complex (LHCII). In support of a recent hypothesis for the mechanism of qE (Horton et al. (1991) FEBS Lett. 292, 1–4), it was found that absorbance changes at 530 nm were associated with qE and LHCII aggregation. Antimycin A inhibited these changes and prevented LHCII aggregation, as indicated by the electrophoretic mobility of the complex and its low-temperature fluorescence spectrum. An antimycin-insensitive partial aggregation of LHCII was associated with an absorbance change at 505 nm. Low concentration of detergent caused disaggregation of LHCII and the reversal of qE. These data are discussed in terms of the relationship between structural change in LHCII and the mechanism of non-photochemical quenching of chlorophyll fluorescence in thylakoids.

Introduction

Non-photochemical dissipation of absorbed excitation energy in the photosynthetic membranes of higher plants is induced under conditions of excess light; it is usually measured as non-photochemical quenching of chlorophyll fluorescence (for review see Refs. 1, 2 and the preceding paper [27]). The principle mechanism of quenching is dependent upon the acidification of the thylakoid lumen, resulting from light-dependent proton translocation, and is called 'qE' [3]. Whilst the physiological role of qE is understood [4], little is known of its molecular mechanism. It has been hypothesised recently that reversible changes in the structure of LHCII are responsible for qE [5] and that these changes resemble those resulting from LHCII aggregation in vitro. In the preceding paper the spectral changes observed upon LHCII aggregation in vitro were described. It was shown that the decrease in fluorescence yield was associated with the formation of an unusually long wavelength pigment species, emitting at 700 nm (F700), correlating with changes in absorption, with bands at 685 nm, 660 nm and 505–515 nm accompanying F700 formation, and that, in theory, the unique features of the aggregated state of LHCII provide an explanation for the basic mechanism of qE. Previous work has shown that qE is associated with selective quenching of F700 [6]. To test the LHCII model for qE further it was necessary to discover whether the absorption changes accompanying LHCII aggregation also accompany qE. Conversely, it is also necessary to explore whether the characteristic features of qE (induction at low pH [7], inhibition by antimycin A [8] and light-activation [9]) can be attributed to properties of LHCII.

In this paper, a number of comparisons between LHCII aggregation and qE are made. The similarities in each case provide further support for the participation of LHCII in energy dissipation by thylakoid membranes.

Materials and Methods

Thylakoid membranes were prepared from intact spinach chloroplasts, as described previously [10], and illuminated to induce qE as described in Ref. 9. Chlorophyll fluorescence was measured using a Walz fluorimeter, exactly as described by Noctor et al. [9]. LHCII was prepared from spinach, as in the preceding

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paper. Dialysis of LHCII, solubilised by 0.1% of octylglucoside and 0.1% digitonin at 35 μg Chl/ml, was carried out in dialysis tubing (size 2–18/32″, Medicell International) for 16 h at 25°C against 5 mM tricine buffer (pH 7.8), containing 0–10 μM antimycin. Fluorescence and absorption spectra were also determined, as described in the preceding paper. Electrophoresis under non-denaturing conditions followed the methods developed by Thornber [11], except that a 3% acrylamide gel was utilised to allow analysis of larger aggregates. The sample concentration in this case was increased to 0.35 mg Chl/ml and the concentrations of detergents and antimycin were, therefore, correspondingly increased to 0.8% and 50 μM respectively.

Results

Light-induced absorbance changes in thylakoid membranes in the 500–550 nm region are complex due to the potential influence of a number of processes. However, changes due to cytochromes and C550 are much smaller than those being examined here [12]. The electrochromic bandshift at P518 is also negligible during continuous illumination and is observed only as a transient upon turning on the light [13]. The de-epoxidation of violaxanthin is not observed in our isolated spinach thylakoids in the absence of added ascorbate and, therefore, a potential contribution of ΔA at 505 nm due to zeaxanthin formation can also be ignored [12,14].

Fig. 1 shows the light-minus-dark absorption difference spectrum accompanying reversible light-induced qE. Two absorption bands were observed: one band occurs in the region between 500–560 nm, with a peak at approx. 520 nm (spectrum 2). This band is similar to that previously reported for 'light scattering' associated with qE in thylakoids [15] and leaves [16]. This light scattering is inhibited by antimycin A [5] and is, therefore, associated with the events giving rise to qE, rather than with ΔpH per se. In the previous paper we reported a difference spectrum for aggregation of LHCII showing a peak at 510 nm. An LHCII aggregation spectrum is shown for comparison in Fig. 1 (spectrum 1). Spectral changes in the red band were also seen upon formation of qE, with a positive band at 685 nm (spectrum 3). Again there is some similarity to the LHCII aggregation spectrum.

Direct comparison between the spectra for LHCII and for chloroplast thylakoids is hindered by differences in the overall optical properties of the samples. As discussed in the preceding paper, these differences will cause varying degrees of spectral distortion due to light scattering and sieve effects. In an attempt to correct for the differences between thylakoids and LHCII, the difference spectra in Fig. 1 have been divided by their respective absorption spectra. The

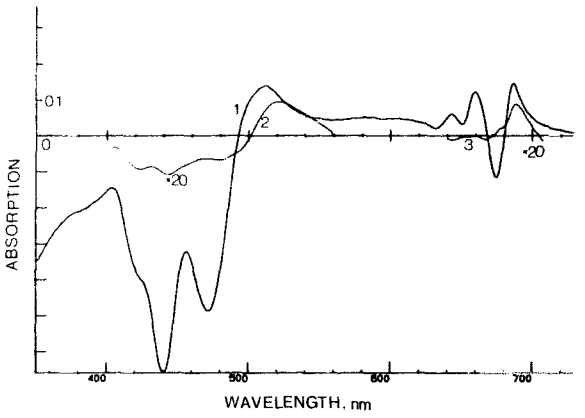

Fig. 1. Absorption difference spectra between 400 and 560 nm (spectrum 2) and between 640 and 705 nm (spectrum 3) upon formation of qE. For spectrum 2, data was recorded during the final 16 s of a 5 min illumination at an intensity of 100 μmol/m^2 per s (l) and following a subsequent 7 min dark period (d); the difference l minus d was calculated. Actinic light was defined by a Corning 2-58 filter and the photomultiplier protected by Corning 4-96 and Cyan T400-570 filters. For spectrum 3, the l spectrum was recorded in darkness within 7 s of the end of the 5 min illumination period. For comparison a difference spectrum for LHCII aggregation is shown; spectra were recorded after solubilisation in 0.1% octyl glucoside and 0.1% digitonin (1), and after dialysis. Spectra of qE are multiplied by 20.

results of this manipulation are shown in Fig. 2. Spectrum A shows the qE difference spectrum divided by a thylakoid absorption spectrum and it displays a sharp band at 530 nm. The corrected difference spectrum for LHCII aggregation also shows a peak at 530 nm. It should be noted that the bandwidth of the LHCII aggregation spectrum is greater than the qE spectrum and there is a distinct shoulder in the former at approx. 500 nm (spectrum B).

In isolated thylakoids, antimycin A prevents qE formation in a process that is independent of its well-known inhibition of PS I cycle electron transport [8]. The concentration for half-maximal inhibition is somewhat variable and depends on the source of antimycin and the chlorophyll concentration. Fig. 3 shows a titration of qE by antimycin A in spinach thylakoids; the I_{50} is approx. 100 nM. Antimycin A has been shown to inhibit LHCII aggregation in vitro [5]. Fig. 3 shows the titration by antimycin A of the $F680/F700$ ratio, which decreases upon aggregation. An I_{50} for antimycin A of approx. 200 nM is seen, remarkably similar to that for inhibition of qE.

The effect of antimycin A on LHCII aggregation was probed by examining the retardation of LHCII migration upon polyacrylamide gel electrophoresis. Electrophoresis in a mild detergent mixture allows unaggregated LHCII to be separated as two bands (Fig. 4), which are thought to represent the trimeric

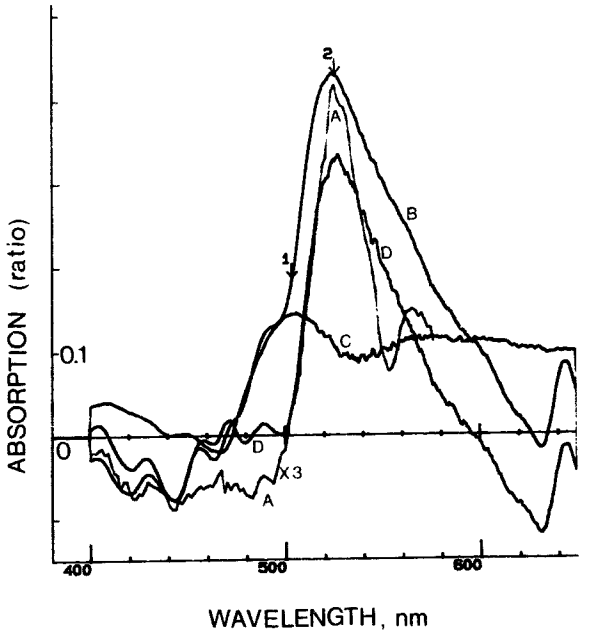

Fig. 2. Corrected difference spectra for qE and LHCII aggregation. For qE (spectrum A), the data in Fig. 1 were manipulated to give (I − d)/I. For LHCII aggregation (spectra B and C), the general form was aggregated minus unaggregated divided by unaggregated. Spectrum B was obtained from LHCII aggregated by 16 h dialysis against 5 mM tricine (pH 7.8) and spectrum C following partial aggregation by dialysis against 10 μM antimycin. Spectrum D is the difference of B minus C.

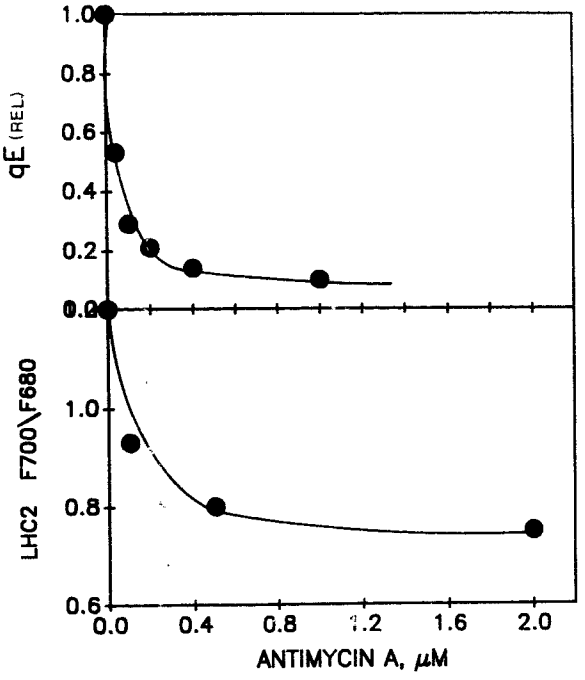

Fig. 3. Effect of antimycin A concentration on qE and LHCII aggregation. qE was measured as described in Ref. 9 with antimycin A added before illumination to the concentration shown. LHCII aggregation was induced by dialysis, as described in the text, with the varying antimycin A concentrations added to the dialysis mixture. Chlorophyll fluorescence emission spectra were recorded, as described in the text, and the ratio of emission intensities at 700 nm and 680 nm used as an index of aggregation. For unaggregated LHCII this ratio is 0.39.

and monomeric states [11,17,18]. If the detergent is dialysed from the sample prior to electrophoresis, the aggregated LHCII does not enter the gel, as expected from the presence of the large supramolecular aggregates shown in the preceding paper. As seen before [5] and as indicated in Fig. 3 antimycin A does not inhibit LHCII aggregation completely. Electrophoresis of LHCII dialysed in the presence of antimycin A gives an unexpected result (Fig. 4). Nearly all the LHCII enters the gel but runs as a broad band band of mobility less than that of unaggregated LHCII. This suggests that LHCII can exist in a partially aggregated state, formed from a relatively small number of LHCII trimers.

Absorption spectra are consistent with this observation that LHCII, aggregated in the presence of antimycin A, is not simply a mixture of fully aggregated and unaggregated complexes (Fig. 2). A corrected difference spectrum between the dialysed (+antimycin A) and undialysed samples has a strong maximum at approx. 505 nm (spectrum C), whereas the spectrum of dialysed (−antimycin A) minus dialysed (+antimycin A) has a peak near 530 nm (spectrum D). This is consistent with the above assertion that the aggregation of LHCII consists of two processes: one giving rise to absorption at 505 nm and a second with a maximum

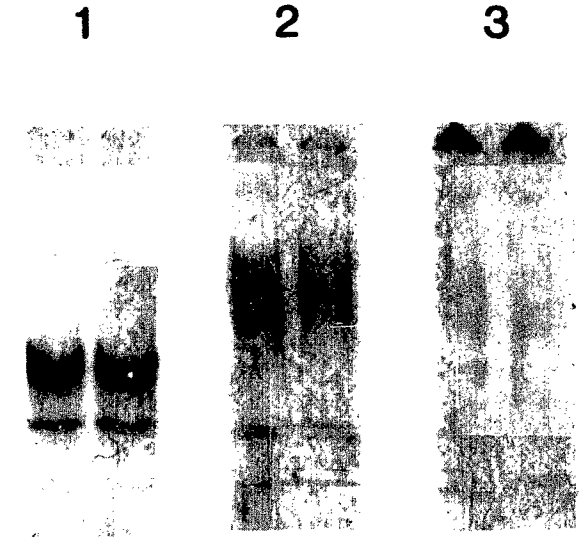

Fig. 4. Electrophoretic mobility of LHCII solubilised, as described in Materials and Methods at 0.35 mg Chl/ml (1) and after subsequent dialysis against 5 mM tricine (pH 7.8) in the presence (2) and absence (3) of 50 μM antimycin A. Electrophoresis was carried out under non-denaturing conditions, as described in the text.

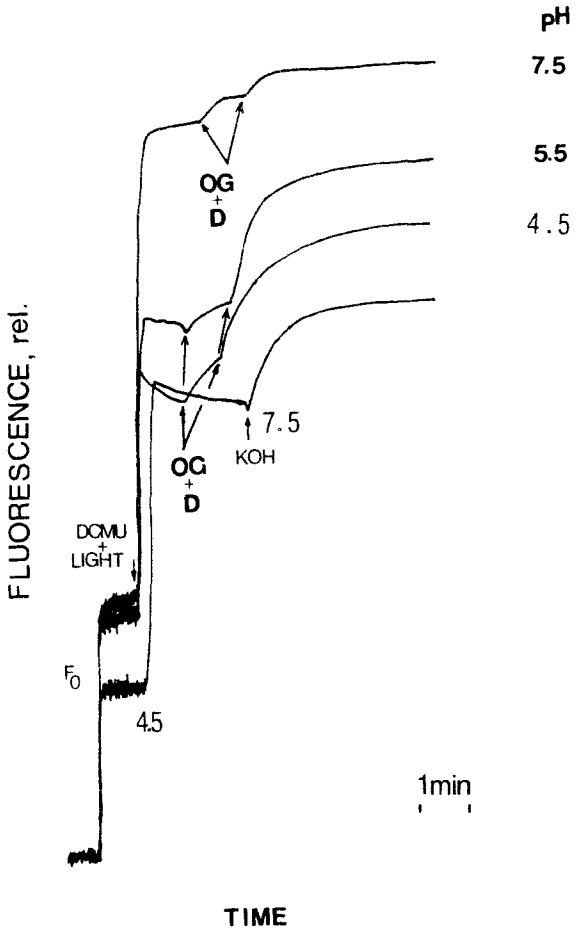

Fig. 5. Reversal of pH-dependent quenching of chlorophyll fluorescence by detergent. Thylakoids were incubated for 2 min at pH 4.5, 5.5 and 7.5 in the presence of ascorbate to induce qE-type quenching, as described in Ref. 7. Illumination was given at an intensity necessary to reach F_m. After 1–2 min either NaOH was added to bring the pH to 7.5 (lower trace), or two additions were made of 0.02% octyl glucoside (OG)+0.02% digitonin (D) (upper traces). Note that the lower trace is displaced downwards for clarity; the initial F_O level was independent of pH.

at 530 nm. Only the formation of the latter is inhibited by antimycin and only the latter is associated with qE.

In some respects the effect of antimycin is similar to that of a rather specific detergent. Fig. 5 shows the results of an experiment that provides support for this idea. It is known that acidification of thylakoids to pH 4.5 causes quenching of chlorophyll fluorescence that resembles qE [3,7,19]. This quenching can be partially reversed by subsequent alkalinisation (lower trace). Addition of a low concentration of octylglucoside at pH 4.5 causes a reversal of the pH-induced quenching (middle trace). This is not a non-specific effect on the pigment bed, since octyl glycoside addition at pH 7.5 does not cause a fluorescence increase (upper trace). Rather, the results in Fig. 5 indicate that detergent can specifically reverse qE.

Discussion

All of the data described in the present paper are consistent with the hypothesis that LHCII is the major site for ΔpH-dependent dissipation of excitation energy in thylakoid membranes and that the mechanism of dissipation is dependent upon the formation of a new chlorophyll/carotenoid species upon aggregation of LHCII. In previous work we have shown that the characteristics of qE in vitro and in vivo are more consistent with energy dissipation in the PS II antenna than with charge recombination in the reaction centres [20]. The suggested role of the pigment zeaxanthin in qE formation [21] provided indirect support for this since xanthophylls are associated with the light-harvesting complexes rather than reaction centres [11]. Recently, selective quenching of fluorescence emission from LHCII was observed during the induction of qE [6]. Furthermore, changes in light scattering were found to correlate with qE rather than being directly due to ΔpH formation [5]. Finally, antimycin A, an inhibitor of qE, was found to be effective in preventing the aggregation of LHCII in vitro [5].

In the preceding paper we showed that aggregation of LHCII was associated with the appearance of absorption bands at 500–540 nm and 685 nm. In the present paper, a similar 685 nm band was found to be associated with qE. The properties of the 500–540 nm band appear to be more complex; partial aggregation in the presence of antimycin A give rise to the formation of a 505 nm band but the more extensive aggregation that occurs in the absence of antimycin is accompanied by formation of a band at 530 nm. In the preceding paper [27] it was shown from fluorescence excitation spectra, which are free from spectral distortion, that this band was narrow and showed a maximum at 505–507 nm; this suggests that only the 505 nm band is an electronic transition and that the longer wavelength band is 'light scattering'. In fact, this 530 nm band resembles the 530 nm change that we have shown to accompany qE, and which has been previously reported upon energisation of isolated chloroplasts [15] and leaves [16]. These data suggest that the 505 nm change represents a form of 'pre-aggregation' of LHCII, whereas qE is associated with more extensive aggregation and an absorbance change at 530 nm. The inhibitory effect of antimycin A on this aggregation, on 530 nm formation in LHCII, on 530 nm changes in thylakoids and on qE itself provides strong support for this interpretation. It should be pointed out that the sizes of the absorption changes that occur upon LHCII aggregation are about 20-times that recorded for qE (see scales on Fig. 1). The extent of

fluorescence quenching (expressed as $\Delta F/F_{quenched}$) is 19 for LHCII aggregation and 0.9 for qE. The ratio of quenching to ΔA is very similar, consistent with the linear relationship that we have observed between $\Delta F/F_{quenched}$ and ΔA_{530} for isolated chloroplasts and leaves obtained from a variety of species (Ruban and Horton, unpublished data).

It is appropriate, perhaps, to further speculate on the 505 nm band formed upon LHCII aggregation. In the previous paper its electronic nature was shown by its preferential sensitization of F700 at 77 K, the species whose presence correlates with energy dissipation. The 505 nm band probably arises from LHCII-associated xanthophyll and it is possible that a pre-requisite for qE is the formation of a xanthophyll/chlorophyll interaction that is enabled by a partial or limited degree of LHCII aggregation. It is interesting that enzymatic de-epoxidation of violaxanthin to zeaxanthin also results in the formation of a band at 505 nm [22] and evidence exists that zeaxanthin is required for qE [21]. Our own data indicate that zeaxanthin is not required for qE but potentiates or activates its formation [9,14]. Perhaps LHCII aggregation perturbs the environment around violaxanthin enough for it to adopt a configuration similar to zeaxanthin (i.e., giving an absorbance change at 505 nm); de-epoxidation would achieve the same result and could be envisaged to favour the aggregation of LHCII, hence explaining the activating role for zeaxanthin. The involvement of a unique xanthophyll/chlorophyll interaction within an aggregated LHCII to form qE, therefore, explains both light activation and the impressive list of observations that correlate zeaxanthin level with energy dissipation [21], and yet is also consistent with the observation that qE can be formed in the absence of zeaxanthin [9,14].

The data described in this paper establish in other ways the principle that qE is associated with macroscopic changes within the thylakoid membrane. Previous work has suggested that LHCII exists as macrodomains in vivo [23], with supporting evidence recently coming from biochemical analysis of thylakoid membranes [24]. It has been known for many years that light scattering changes accompany ΔpH formation [15], although it is only recently that these have been specifically associated with qE [5]. Theoretical analysis suggests that scattering at 530 nm results from macroscopic changes in the thylakoid membrane [25]. In this paper we have established that similar light scattering can be induced upon aggregation of LHCII into large two-dimensional arrays. Furthermore, it has been found that antimycin A inhibits this aggregation, explaining its inhibitory effect on qE. Low concentrations of detergent were also found to reverse pH-dependent quenching of fluorescence in thylakoid membranes. It appears, therefore, that the fundamental functional change in the thylakoid membranes that reduces photosynthetic efficiency by creation of new thermal dissipative pathway in conditions of excess light results from a 'state' change in the LHCII complexes; domains of semi-crystalline LHCII form sinks for excitation energy. Although we propose that quenching is caused by an alteration in pigment properties, this alteration results from pH-dependent changes in the protein conformation. The adoption of this kind of dissipative state will, of course, be especially dependent upon many factors, such a lumen pH, xanthophyll composition, density of complexes, lipid composition and ionic environment, readily explaining the significant differences in the properties of qE that have been observed in different plant species [26].

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