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Mechanism of ΔpH-dependent dissipation of absorbed excitation energy by photosynthetic membranes. I. Spectroscopic analysis of isolated light-harvesting complexes

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It has been proposed that the increase in thermal dissipation of excitation energy by thylakoid membranes at high light intensity is dependent upon the formation of an aggregated state of the light-harvesting complexes of Photosystem II (LHCII) (Horton et al. (1991) FEBS Lett. 292, 1–4). Therefore, a study of the spectroscopic changes occurring upon LHCII aggregation in vitro has been undertaken. Aggregation of LHCII, brought about by the removal of detergent, is associated with quenching of chlorophyll fluorescence and the appearance of the major emission band at 700 nm at 77 K (F700). Aggregation is associated with absorption bands with maxima at approx. 510 nm, 660 nm and 690 nm; each of these bands preferentially sensitises F700. The temperature-dependence of the emissions at 680 nm and 700 nm is very different; whereas the yield of F680 saturated at 240 K, that of F700 was not saturated at 77 K. The results are discussed in terms of the unusual and unique properties of aggregated LHCII, providing the basis for energy dissipation in vivo.

Introduction

Photosynthesis is dependent upon the presence of light-harvesting pigments, which transfer absorbed excitation energy to the photosynthetic reaction centres. In higher plants there are typically 2–300 chlorophylls serving each centre. These light-harvesting chlorophylls are organised into discrete pigment-protein complexes. In the case of Photosystem II, these include the proximal antenna proteins CP47 and CP43 and the distal antenna LHC's – LHCIIa, b and c [1,2]. The fact that the quantum yield of photosynthetic O_2 evolution in vivo approaches the theoretical maximum value results, in part, from the efficiency of this light-harvesting system. The physiological role of this, in allowing plant growth in low light, is exemplified by the frequently observed increase in the ratio of light-harvesting:reaction centre pigments when photosynthetic organisms grow in such conditions [3].

However, for varying periods of time, plants are exposed to light intensities far greater than can be used with maximum quantum efficiency. When the capacity for energy utilisation is progressively saturated the quantum efficiency declines. In full sunlight, the quantum yield of O_2 evolution is typically only 20% of that under strictly light-limiting conditions [4].

It is now well-established that when the rate of photon absorption approaches the maximum rate of photosynthesis, regulatory feedback mechanisms are induced. These mechanisms bring about an increase in the rate of dissipation of absorbed excitation energy as heat. An active decrease in quantum efficiency of photosynthesis results. Experimentally this was first identified as the observation that PS II reaction centres do not become closed to the extent expected from the decrease in quantum efficiency of O_2 evolution or CO_2 fixation [5]. There have been several suggestions as to how centres could be kept open as the light intensity increases. In most conditions, it would appear that the major mechanism involves non-photochemical, thermal dissipation of excited chlorophyll molecules rather than the occurrence of futile photochemical reactions [6]. Thus, the quantum efficiency of photosynthesis can be quantitatively accounted for by the increase in thermal dissipation, measured as the non-photochemical quenching of chlorophyll fluorescence, qN. This indicates that, at high light intensities, there is a fundamental change in the functioning of the photosynthetic membrane – absorbed energy is now being dissipated

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with high efficiency rather than being made available for trapping by the reaction centre [7]. The effect of this regulatory mechanism is to bring about dissipation of excess light, in effect 'feeding back' to offset the deleterious results of high light. The mechanism is, therefore, photo-protective, designed to protect against damage to the photosynthetic membrane that would occur at high excitation energy levels. This damage may be the general form of photo-oxidation or, more specifically, damage to the PS II reaction centre, resulting from excessive rates of excitation.

There have been many investigations of qN, in chloroplasts, protoplasts, algal cells and leaves. The major component of qN in high light is induced in response to the formation of the ΔpH [8]. This process has been called high-energy-state quenching or qE and has been shown to result from acidification of the thylakoid lumen [9,10]. However, neither the mechanism nor the location of the quenching is known. It has been proposed that quenching results from the conversion of the reaction centre into an inactive quenched state [5]. This state may result from ΔpH-dependent inhibition of electron donation to $P680^+$. We have observed such quenching upon acidification of PS II particles and thylakoids but have shown this to be different from qE [10,11]. Alternatively, it has been suggested that qE results from a dissipative process in the PS II antenna; this idea is supported by the effect of qE on the F_O level of fluorescence [12,13]. Indirect evidence is provided by the strong correlation that exists between qE and the conversion of the carotenoid violaxanthin to zeaxanthin [14]; these pigments are known to be associated with the LHC's rather than reaction centres [15,16]. Whilst it has been suggested that zeaxanthin has an obligatory role in qE formation [17], we have correlated the content of this carotenoid to light-activation of qE; i.e., the increase in sensitivity of qE to change in ΔpH that occurs following pre-illumination to induce zeaxanthin synthesis [18,19].

Recently, we have obtained direct evidence for the location of quenching in the LHCII complexes [20]. Fluorescence emission spectra for qE showed a maximum at 680 nm, the emission maximum of isolated LHCII. Furthermore, after light activation, the spectrum had a peak at 700 nm previously associated with aggregation of LHCII in vitro [21]. Light activation was also associated with the appearance of a red-shifted chlorophyll band, again a characteristic of LHCII aggregation [20]. It is well-known that LHCII can spontaneously and reversibly form ordered aggregates in vitro and that this aggregation of LHCII is associated with a large decrease in fluorescence yield [21]. An experimental link between qE and LHCII aggregation was established by the inhibitory effect of antimycin A on both processes [22]. On the basis of this work it has been hypothesized that qE results from energy dissipation in an aggregated form of LHCII [22]. To explore this hypothesis further it is necessary to carry out a more detailed study of the aggregated state of LHCII. Previous work has considered aggregation only in terms of its involvement in grana formation and there has been no analysis of the associated changes in fluorescence emission.

Materials and Methods

LHCII was prepared following solubilisation of spinach thylakoids with Triton X-100 (Sigma), using the procedure of Burke et al. [23]. This procedure yields a Mg^{2+}-induced LHCII aggregate and before use the preparation was dialysed against 5 mm Tricine (pH 7.8) for 16 h at 20°C. Aggregated LHCII prepared in this way contains three polypeptides with apparent molecular masses 24–28 kDa (Fig. 8), has a chlorophyll a/b ratio of 1.2 and has the appearance of ordered two-dimensional arrays (Fig. 2); it is mostly LHCIIb, according to the nomenclature of Peter and Thornber [16]. In experiments involving trypsin treatment, the aggregated LHCII sample was diluted to a final concentration of 100 μg Chl/ml in 5 mM Tricine buffer (pH 7.8) at room temperature. Trypsin (from bovine pancreas, type III, 10000–13000 BAEE units/mg protein, Sigma) was added to a final concentration of 2 μg/ml. Digestion was stopped after 15 min by addition of a trypsin inhibitor (soybean, type I-S, Sigma) to a final concentration of 20 μg/ml. The digested LHCII was centrifuged at $4000 \times g$ for 10 min and the pellet used for spectroscopical measurements after the appropriate dilution. Polypeptide analyses of samples were carried out by SDS-PAGE according to Laemmli [24].

Low-temperature fluorescence was measured using a liquid nitrogen cryostat. The sample concentration was 10 μg chlorophyll/ml and the sample cell thickness was 1 mm. For some experiments the sample temperature was changed by slow recooling (1 K/15 s), the temperature being measured by a Comark electronics thermocouple inserted into the sample through a hole in the cell. Excitation was provided by a Wotan tungsten halogen lamp and defined by 4-96 and 5-57 Corning filters to give broad band excitation in the Soret region. Fluorescence spectra were detected by a 1024-channel silicon photodiode detector (Model 1455) via a Jarrell-Ash Monospec 27 Monochromator and analysed by an EG&G PARC optical multichannel analyser (Model 1461) using EG&G OMA-Vision-PDA data acquisition/analysis software. The mean accumulation time for a spectrum was 14 s. The spectral resolution was 0.3 nm and signal/noise ratio was less than 0.5%. For excitation fluorescence measurements an Applied Photophysics f/3.4 monochromator with a 1200 lines/mm grating was used. The slit width

was 1 mm. A programme was designed in OMA – Vision software for the data acquisition and control of the monochromator drive unit (the step-length was 1 nm). This system allowed the recording and manipulation of several excitation spectra simultaneously for different fluorescence regions for each sample. For 'red' region excitation spectra a Schott filter RG715 was used to cut scattered light from the excitation light beam. No correction was made for light intensity spectral distribution of the excitation light.

Absorption measurements were carried out at room temperature using an Aminco DW2000 dual wavelength spectrophotometer operating in split-beam mode. The sample concentration was 5 μg Chl/ml. The spectral slit width was 1 nm.

Samples for electron microscopy were frozen in liquid nitrogen slush and freeze-fractured at 163 K using a Balzers BAF 400D freeze etching unit. A unidirectional Pt/C replica was laid down under an elevation angle of 45° using electron beam evaporation [25]. Replicas were cleaned overnight on sodium hypochlorite (40% v/v) and washed on double-distilled water prior to mounting on 200 mesh Pioloforcoated copper grids. Grids were examined in a Philips CM 10 transmission electron microscope operated at an accelerating voltage of 100 kV. Electron micrographs were recorded at calibrated magnifications on Agfa Scientia 23 D 56 electron image sheet film.

Results

Sucrose density-gradient centrifugation of Triton-solubilised thylakoids yields an LHCII band which aggregates upon addition of Mg^{2+}, so that it can be readily sedimented. This isolated LHCII has a low fluorescent yield with an emission maximum at 700 nm (Fig. 1A, spectrum 2). After incubation with detergent (0.2% octyl glucoside + 0.2% digitonin) for 2 min a large increase in yield occurs and the spectrum now has a maximum at 680 nm (spectrum 1). This transformation is readily reversible; dialysis for 16 h to remove the detergent results in a quenching of fluorescence, the dialysed form emitting at 700 nm (spectrum 3) and being transformed back to the high fluorescent state by further addition of detergents. By applying different detergent concentrations it is possible to get a continuous set of spectra corresponding to different aggregation states. For example, spectrum 4 in Fig. 1A is clearly an intermediate state with a reduced yield and a strong shoulder at 700 nm. The inset in Fig. 1A shows that for different aggregation states there is a linear relationship between the quenching of fluorescence at 680 nm and the relative contribution of the 700 nm component.

In Fig. 1B, spectra 1 (unaggregated) and 2 (aggregated) have been normalised to show the distinct difference in band-shape. Subtraction of spectrum 1 from

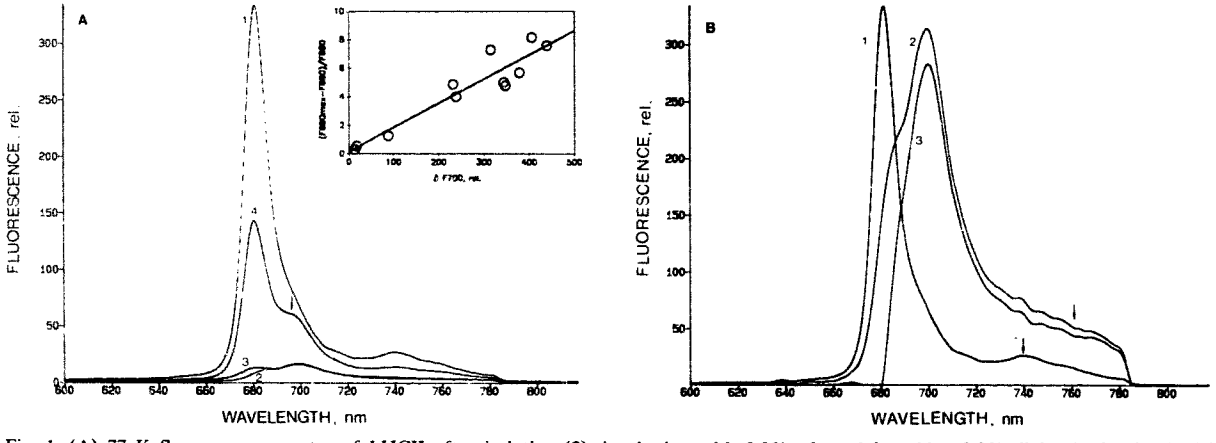

Fig. 1. (A) 77 K fluorescence spectra of LHCII after isolation (2), incubation with 0.2% of octylglucoside + 0.2% digitonin for 2 min (1), subsequent dialysis for 16 h at 25°C against 5 mM tricine buffer (pH 7.8) (3), and resolubilisation with 0.1% detergents (4). Arrow shows the shoulder at 700 nm. (B) Spectra of solubilised (1) and dialysed (2) samples normalised to the same amplitude to show the difference in the shape and the percentage of satellite fluorescence at 740 nm and 760 nm, respectively (see arrows). (3). Calculated spectrum of F700 achieved by subtraction of spectrum 1 from spectrum 2 (spectra were normalised to 680 nm). Inset: Plot of quenching of LHCII fluorescence at 77 K expressed as $(F680_{max} - F680)/F680$ against relative increase in amplitude of F700, expressed as $(F700 \cdot F680_{max}/F680) - F700_{max} \cdot F680_{max}$ and $F700_{max}$ refer to fluorescence amplitudes at 680 nm and 700 nm after disaggregation of dialysed LHCII with a saturating level of detergent. $F680$ and $F700$ values were obtained by addition of varying levels of detergent.

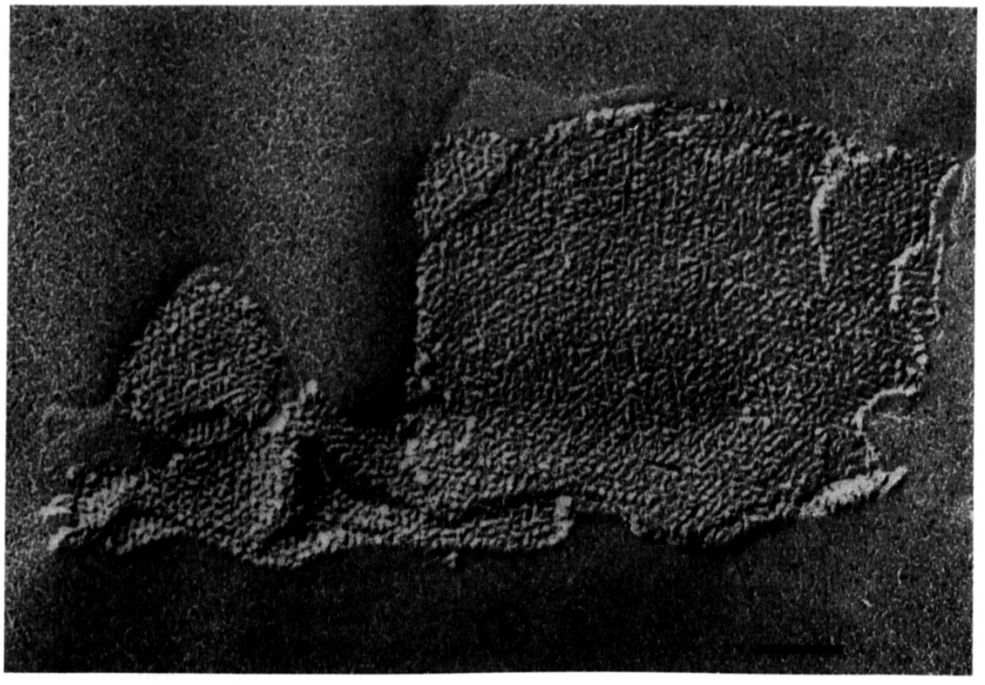

Fig. 2. Electron micrograph of dialysed LHCII obtained as described in Materials and Methods. Ordered arrays of particles show a periodicity of around 12 nm. Arrows indicate that the different rows, comprising an array, are arranged at an angle of 60° or 120° relative to each other. Arrowhead shows the direction of shadowing and bar represents 100 nm.

2 shows the band-shape of F700; the band-width at half-maximum is 50–60% larger for F700 than F680. It should also be noted that the satellite emission band of F680 is at 740 nm, whereas, that for F700 is less distinct but around 760 nm.

Previous work has shown that dialysis induces LHCII aggregation [21,26]. This was confirmed in the present experiment by the observation that the dialysed LHCII could be quickly sedimented by centrifugation at 1000–4000 × g and showed zero mobility on a non-denaturing polyacrylamide gel (not shown). Electron microscopical examination of dialysed LHCII showed it to be in the form of ordered two-dimensional arrays of particles with a periodicity of about 12 nm (Fig. 2). This organisation of LHCII upon dialysis has been reported previously and results from association of LHCII trimers into supramolecular aggregates [27,28].

Aggregation of LHCII also results in changes in the absorption spectrum. A difference spectrum aggregated-minus-unaggregated yields three positive bands at 515 nm (a), 660 nm (b) and 685 nm (c) (Fig. 3). Aggregation results in a significant increase in light scattering and the introduction of 'sieve effects'; hence, it can be seen that aggregation leads to a net loss of absorption. The spectral distortion resulting from these effects cannot be easily corrected for. Therefore, fluorescence excitation spectra were used to confirm the existence of these absorption bands and to show the extent of distortion in the absorption spectra.

Soret band spectra for the excitation of fluorescence emitted between 670 nm and 720 nm strongly resembled the absorption spectra of Fig. 3 (Fig. 4). A difference spectrum between aggregated and unaggregated LHCII again revealed the positive band at 507 nm, although with a narrower band width than the 515 nm band in Fig. 3. In addition to confirming the electronic

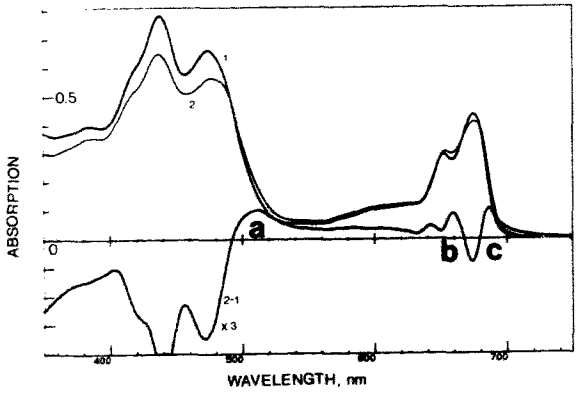

Fig. 3. Room temperature absorption spectra of solubilised (1) and dialysed (2) LHCII. Three positive bands in the difference spectrum are shown. 505–540 nm (a), 640–660 nm (b) and 685–690 nm (c).

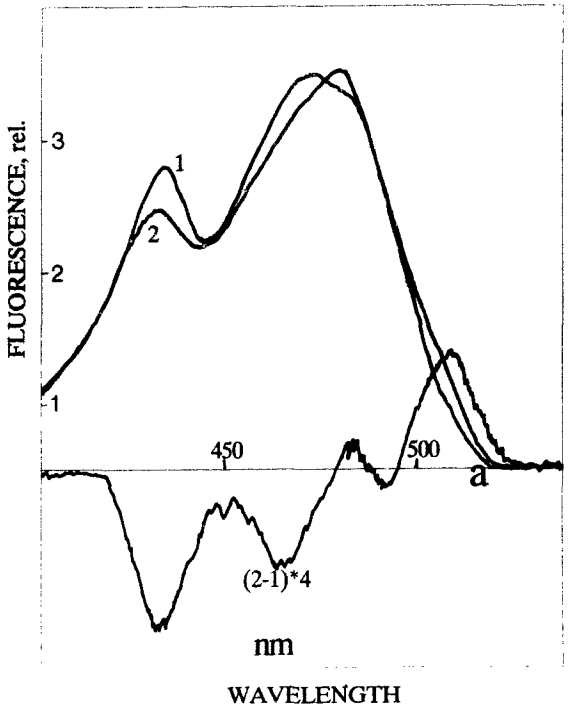

Fig. 4. 77 K excitation fluorescence spectra in the Soret band region for solubilised (1) and dialysed (2) LHCII. Whole fluorescence spectra were measured in this experiment, then the area between 670 nm and 720 nm was calculated and taken as the fluorescence parameter for the Y-axis. Compare band (a) with a similar band (a) in Fig. 3.

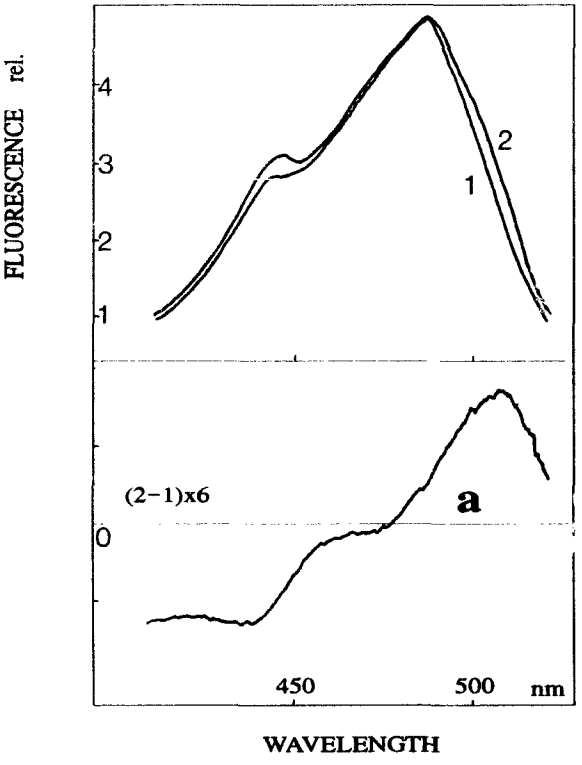

Fig. 5. 77 K excitation fluorescence spectra of dialysed LHCII in the Soret region for F680 (1) and F700 (2). For F680 and F700, the excitation spectra of the areas 670–685 nm and 695–705 nm, respectively, were taken respectively from the same set of fluorescence spectra. Note band (a) as seen in Fig. 3.

nature of this band, these data suggest that F700 is preferentially excited by absorption in this region. This suggestion was confirmed by recording excitation spectra for F680 and F700 on the same LHCII sample (Fig. 5). The spectra for excitation of F680 and F700 were similar, but a clear difference was apparent at wavelengths greater than 500 nm. A difference spectrum for excitation of F700 minus that for F680 shows a band with a maximum at about 505 nm. A further demonstration of preferential transfer from the 510 nm band to F700 is shown in Fig. 6. Excitation at 510 nm gives rise to a higher F700/F680 ratio than excitation at 435 nm.

A similar approach was taken to explore bands (b) and (c) of Fig. 3. Since these occur in the 650–700 nm region, excitation of the long-wavelength satellite bands at 740 nm and 760 nm were used, corresponding to F680 and F700, respectively (see Fig. 1). Fig. 7 shows that both the 655 nm and 685 nm bands preferentially excite the F760 satellite of F700.

Further data correlating these three absorption bands with F700 emission arose following trypsin treatment of LHCII. It has been reported that mild trypsin treatment of aggregated LHCII cleaves a N-terminal 2 kDa peptide from the surface that is stromally exposed in situ [21]. Fig. 8 shows the expected increase in electrophoretic mobility of the LHCII polypeptides that results from this cleavage by trypsin. Trypsin treat-

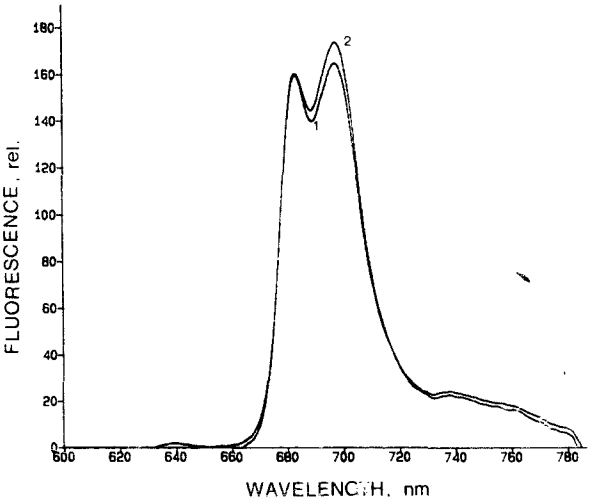

Fig. 6. 77 K fluorescence spectra of dialysed LHCII for excitation in 435 nm (1) and 510 nm (2) regions.

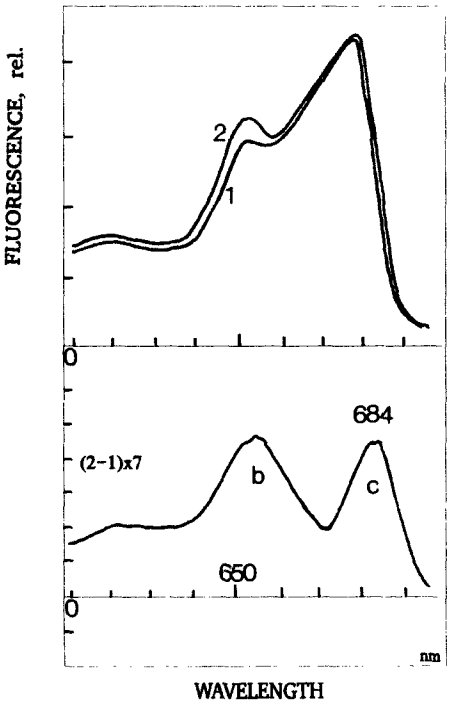

Fig. 7. 77 K excitation spectra of satellite fluorescence for dialysed LHCII. 1, satellite of F680 from 730 to 745 nm; 2, satellite of F700 from 755 to 770 nm (see Fig. 1B). Compare bands (b) and (c) with those in Fig. 3.

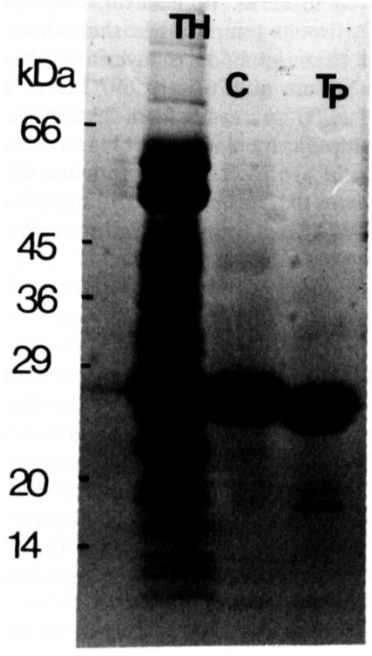

Fig. 8. Polypeptide gel of thylakoids (TH), dialysed LHCII (C) and pellet (Tp) after trypsin treatment and centrifugation (see Materials and Methods). The low molecular weight band, at near 20 kDa, is the trypsin inhibitor.

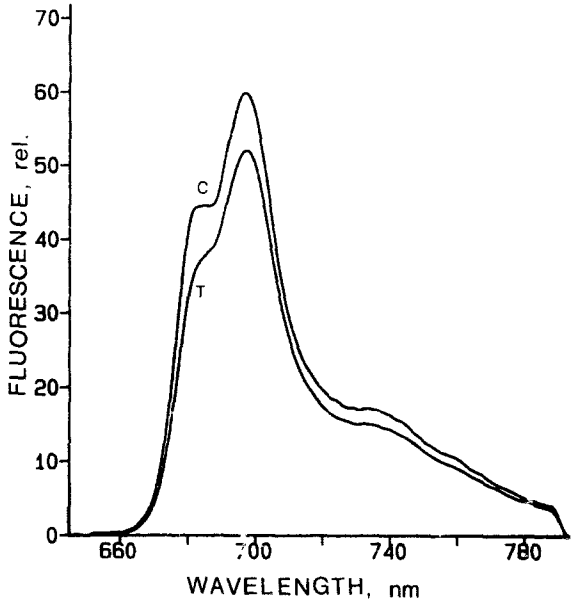

Fig. 9. 77 K fluorescence spectra of LHCII before (C) and after (T) trypsin treatment.

ment resulted in a further increase of the F700 species relative to F680 (Fig. 9). Trypsin treatment also causes changes in the absorption spectrum (Fig. 10). The difference spectrum between trypsin-treated and control strongly resembles that shown for LHCII aggregation in Fig. 3, with the three bands at 510 nm, 660 nm and 690 nm. The fact that trypsin treatment does not result in decreased aggregation also shows that the N-terminus is not involved; in fact, after trypsination, LHCII seems to be more strongly aggregated, as judged from the fluorescence ratio.

The above data show that fluorescence quenching upon LHCII aggregation is correlated with the formation of pigment species emitting at 700 nm. This species

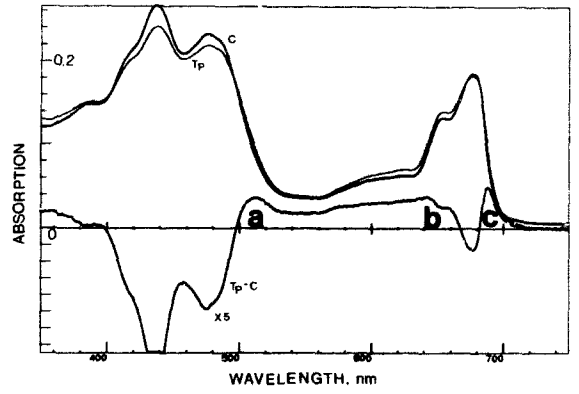

Fig. 10. Room-temperature absorption spectra for LHCII before (C) and after trypsin treatment and centrifugation (Tp, pellet).

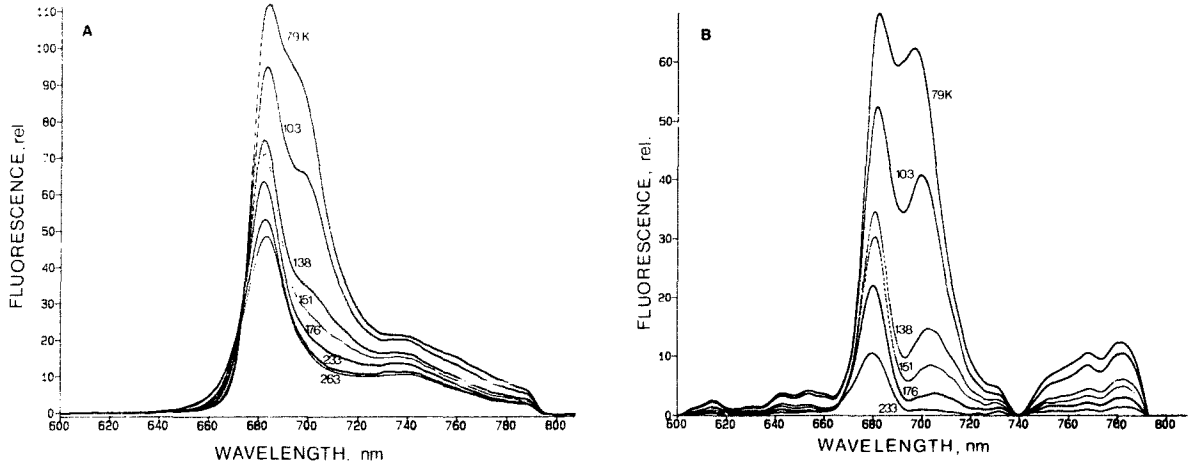

Fig. 11. (A) Temperature-dependence of the fluorescence spectrum of aggregated LHCII. (B) Temperature-dependence of difference between the spectrum at a given temperature and the spectrum at 273 K, after normalisation at 740 nm.

has been observed upon induction of the dissipation of excitation measured as qE [20]. Its occurrence in LHCII provides an opportunity to investigate the possible reason for its long-wavelength emission.

Fig. 11 shows the fluorescence emission spectrum of a partially aggregated form of LHCII, recorded at temperatures from 79 K to 263 K. As mentioned above, it is possible to manipulate the extent of aggregation using different detergent concentrations. For this experiment it was necessary to use a sample with approximately similar fluorescence intensities for F700 and F680 (see, for example, spectrum 4 in Fig. 1A). At temperatures above 150 K, F700 is not clearly detectable. It was found that only at 100 K or below, does a defined F700 shoulder emerge. Difference spectra, after normalisation at 740 nm (the satellite peak of F680) allowed the temperature dependency of the yield, peak position, and bandwidth of F700 to be calculated and compared to F680. In general, F680 parameters are less sensitive to temperature than those of F700. The peak position of F680 is invariant whereas F700 shifts from 705 nm at 176 K to 697 nm at 79 K. The halfwidth of F700 decreases from 25.5 nm to 20.7 nm over this temperature range. In Fig. 12 the yield of F700 and F680 are plotted as a function of temperature. It is clear that, whilst F680 is saturated even at 230 K, F700 is far from saturation even at liquid nitrogen temperature.

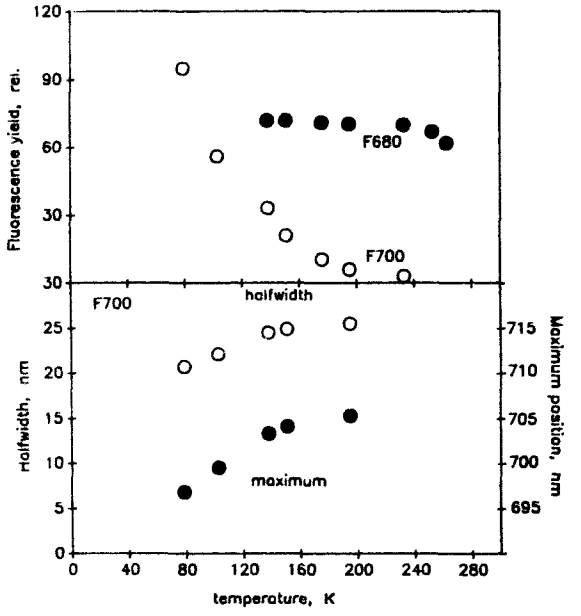

Fig. 12. Temperature-dependence of spectral parameters of F700 and F680 in the spectrum of aggregated LHCII. The upper graph shows the fluorescence yields for F680 (●) and F700 (○). The lower graph shows the bandwidth at half-maximum (○) and wavelength maximum positions (●) for F700.

Discussion

In recent work we have presented evidence that LHCII is the site of a major part of the dissipation of energy that is causing qE [20]. Moreover, a link has been made between LHCII aggregation and qE [22]. In this paper the remarkable flexibility of the properties of LHCII have been examined. We suggest that these properties provide an explanation for the fine control of the balance between excitation trapping and dissipation in vivo.

LHCII in vitro displays a change in fluorescence yield of greater than 20-fold upon reversible aggregation, a change which is from the native trimeric LHCII to supramolecular 2-dimensional arrays. By recording absorption difference spectra and fluorescence excita-

tion spectra we have shown the formation of three absorption bands, at 505–515 nm, 660 nm and 685–690 nm upon aggregation. These absorption spectral changes are consistent with a pigment aggregation involving chlorophyll *a*, chlorophyll *b* and carotenoid. This pigment aggregate appears to preferentially sensitize a long wavelength F700 emission, also diagnostic of LHCII aggregation. In its aggregated state, LHCII is profoundly altered – the yield of fluorescence decreases, heat dissipation increases, new absorption bands appear at 505–515 nm, 660 nm and 685 nm, and fluorescence is emitted at 700 nm at 77 K. As shown previously [20,22], and in the following accompanying paper, all of these features are associated with non-photochemical quenching in thylakoid membranes.

The yield of F700 emission was found not to be saturated at 77 K, an unusual property that can be explained by the effects of LHCII aggregation. The increase in fluorescence at low temperature is caused by the suppression of molecular vibrations. The vibronic energy, or mean phonon energy can be calculated from the temperature dependency by the formula $E = 2kT_m$ [29], where k is the Boltzman constant and T_m is the temperature which corresponds to a maximum slope of the temperature-dependence curve in Fig. 12. Using this formula, it is found that the mean phonon energy is in the range of 50–100 cm^{-1}. This value indicates the involvement of low frequency vibrations in establishing this unusual temperature dependency; low frequency vibrations would result from vibrations within the large LHCII aggregate.

It is possible that the mechanism of fluorescence quenching could be based upon strong interactions between electronic excitation and these vibrational movements. The evaluated electron-phonon coupling strength (S) can be calculated from the formula $S = E_m/kT_m$ [30], $2E_m$ corresponding to the F700 Stokes shift, the difference in energy of C685 and F700, about 300 cm^{-1} [29]. The data for F700 yield a value of 3, showing that there would be a high probability of dissipation of excitation energy through low frequency vibration. However, further work is required to elucidate the pathway of energy dissipation in aggregated LHCII.

In conclusion, the changes in fluorescence yield of LHCII upon aggregation provide an explanation for the induction of energy dissipation in vivo. The principle that the physical properties of bound pigments are modulated by the apo-protein in pigment-protein complexes is well accepted. We present here an extension to this principle; that physiological regulation of the properties of the pigments results from structural modification of the apo-protein (in the case of qE by protonation-dependent conformational change). There is, in fact, evidence that LHCII in vivo can exist as supramolecular assemblies [31,32], but it remains to be established whether light activation or qE itself are associated with any changes in the extent of aggregation/disaggregation.

Acknowledgements

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