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Excitation-energy quenching in aggregates of the LHC II chlorophyll-protein complex: a time-resolved fluorescence study

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Picosecond time-resolved fluorescence emission spectroscopy at 80 K was used to investigate excitation-energy migration in LHC II isolated from spinach chloroplasts. Time-resolved spectra were obtained for solubilised LHC II trimers and for LHC II in a semi-crystalline aggregated state. In solubilised LHC II, the main decay lifetime is about 4.3 ns. Upon aggregation, the main decay lifetime becomes about 110 ps, indicating the presence of a highly-efficient energy-quenching process. Several red-shifted chlorophyll species can be resolved in aggregated LHC II, but none of these appears to be directly involved in energy quenching. The zeaxanthin/violaxanthin ratio has no influence on the time-resolved fluorescence emission spectrum for LHC II in these states. We suggest that a quenching process similar to that observed in aggregates of isolated LHC II is the basis of the ΔpH-dependent quenching (q_E) observed in intact thylakoid membranes.

Introduction

Photosynthesis in vivo is subject to a number of rapid regulation mechanisms which function to maximise the efficiency of photosynthetic electron transport while minimising photodamage due to excess excitation of the light-harvesting complexes and reaction centres. One of these mechanisms, known as ΔpH-dependent or high-energy-state quenching (q_E), decreases the intrinsic efficiency of Photosystem II by increasing the rate of non-radiative dissipation of excitation energy in response to acidification of the thylakoid lumen [1]. The quenching results in a decrease in the PS II fluorescence yield both at room temperature and at liquid nitrogen temperature (77 K). There have been two main proposals concerning the site of action of q_E: (a) Quenching takes place in inactivated PS II reaction centres [2]. (b) Quenching takes place by non-radiative dissipation of excitons in the PS II light-harvesting antenna [3,4]. In case (b), the species responsible for non-radiative de-excitation has been suggested to be either a carotenoid, zeaxanthin [3], or a chlorophyll species formed when LHC II (the major light-harvesting chlorophyll-protein of Photosystem II) is aggregated [4]. The q_E observed as a result of thylakoid lumen acidification has been related to effects observed in vitro upon dialysis of detergent-solubilised LHC II, leading to the formation of an aggregated state [4-6]. It was shown that the aggregation of isolated LHC II decreased the fluorescence yield at 77 K by a factor of 10 or more [4,5]. The 77 K fluorescence emission spectrum was also changed, with an increase in the relative contribution of a band at 700 nm [4,5]. Comparable effects were observed to accompany fluorescence quenching by the q_E mechanism in the intact system [6,7]. Here, we examine the effects of aggregation on the properties of isolated LHC II in more detail using picosecond time-resolved fluorescence emission spectroscopy. The measurements were performed at 80 K in order to improve the spectral resolution of different Chl species. We compare disaggregated and aggregated LHC II and we also compare LHC II preparations containing different ratios of the carotenoids zeaxanthin and violaxanthin. We find that aggregation leads to the formation of a very efficient energy-quenching process. Several Chl

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Abbreviations: Chl, chlorophyll; FWHM, full width at half maximum; LHC II, light-harvesting chlorophyll a/b binding protein of PS II; PS, photosystem; q_E, ΔpH-dependent or high-energy-state quenching of chlorophyll fluorescence; τ, fluorescence lifetime ($1/e$).

species with red-shifted fluorescence maxima can be resolved in the aggregated state. None of these species appear to be directly involved in the quenching process, however. We also find that the zeaxanthin/violaxanthin ratio has no influence on the energy transfer properties of LHC II in these states, suggesting that zeaxanthin is not an acceptor of excitons from Chl and is not directly involved in the energy quenching process.

Materials and Methods

LHC II was prepared from spinach as described in Ref. [8]. LHC II at 0.35 mg Chl/ml was solubilised in 0.5% octylglucoside + 0.5% digitonin to generate disaggregated LHC II trimers [4,5]. The aggregated state was induced by dialysis against 5 mM Tricine (pH 7.8) [4,5]. Prior to the isolation, spinach leaves were dark-adapted or light-treated as in Ref. 9. Samples of LHC II in 5 mM Tricine buffer (pH 7.8) were diluted to 35 μg Chl/ml and rapidly frozen by immersion in liquid nitrogen in 0.1-mm cuvettes. During the measurement, the sample was maintained at 80 K in a gas-cooled cryostat (Leybold VSK 4-300). Excitation light entered at the back face of the cuvette (almost 180° detection geometry).

The excitation pulse for picosecond time-resolved fluorescence emission spectroscopy was generated by a synchronously-pumped and cavity-dumped dye laser system (Spectra Physics, Mountain View, CA) [10], using sulforhodamine 101 as the laser dye. The dye laser was tuned to 663 nm. The pulse-width as measured by autocorrelation was 13 ps (FWHM). The repetition rate was 4 MHz and the mean power at the sample was normally 0.6 mW. The beam diameter was 1 mm and the pulse energy was about 150 pJ, corresponding to only one incident photon for 7000 Chl molecules per pulse, or about one absorbed photon for every 300 000 Chl per pulse. Exciton annihilation and other multiphoton effects [11] were, therefore, negligible.

Fluorescence was detected by a far-red-sensitive microchannel plate photomultiplier (Hamamatsu R2809U-05) screened by a double monochromator (bandwidth 4 nm). Fluorescence decays were recorded by single-photon timing. The channel resolution of the time-to-amplitude converter was 5.2 ps and decays were recorded in 2048 channels, corresponding to a time-window of about 10 ns. Data were accumulated up to 30 000 counts in the peak channel for aggregated LHC II and up to 20 000 counts for disaggregated LHC II. The system temporal response function was obtained by detecting scattered light from the sample to 40 000 peak channel counts. Fluorescence at the excitation wavelength was negligible in comparison to scattered light. The system temporal response function was about 50 ps wide (FWHM), allowing lifetime components down to about 5 ps to be resolved after deconvolution. Fluorescence decays were recorded at wavelengths from 680 nm to 710 nm. Steady-state fluorescence emission spectra (recorded under the same conditions as used for measuring the kinetics) and replicate fluorescence decays recorded at the beginning and end of the measurement were similar, indicating little or no photodamage to the sample during the course of the measurement. Fluorescence decays were analysed by an iterative deconvolution procedure (global lifetime analysis) in which a set of decays recorded at different emission wavelengths were analysed together in terms of a sum of exponential terms convoluted with the system temporal-response function [10]. The quality of the fits was assessed by global χ^2 values and plots of weighted residuals. The results are presented as decay-associated spectra, in which the pre-exponential term (or fluorescence amplitude) of each kinetic component is plotted as a function of fluorescence emission wavelength [10]. To compensate for fluctuations in the intensity of the laser, the total fluorescence amplitudes at different emission wavelengths were corrected relative to one another by reference to steady-state fluorescence emission spectra recorded on the same equipment.

Results

The properties of the LHC II preparations used in this study are summarised in Table I. As previously reported [4], aggregation of LHC II causes both fluorescence quenching and an increase in the relative contribution of a red-shifted fluorescence peak at 700 nm at 77 K. LHC II from light-treated leaves had a much higher content of zeaxanthin relative to violaxanthin, but did not show any significant differences in the steady-state fluorescence spectra, at least at the extreme states of aggregation and disaggregation used in this study (Table I).

Fig. 1 shows decay-associated spectra for disaggregated LHC II from dark-adapted spinach leaves at 80

TABLE I

Properties of LHC II preparations

F_{680}, Relative fluorescence yield at 680 nm (80 K) with excitation at 663 nm, as obtained from the average fluorescence lifetime at 680 nm; F_{700}/F_{680}, ratio of 700 nm fluorescence to 680 nm fluorescence (80 K) with excitation at 663 nm; Z/V, molar ratio of zeaxanthin to violaxanthin determined by HPLC as in Ref. 9.

LHC II from:	State	F_{680}	F_{700}/F_{680}	Chl a/b	Z/V
Dark-adapted spinach	Disaggregated	1.00	0.20	1.33	0.06
	Aggregated	0.08	1.34		
Light-treated spinach	Disaggregated	1.08	0.17	1.36	3.83
	Aggregated	0.08	1.50		

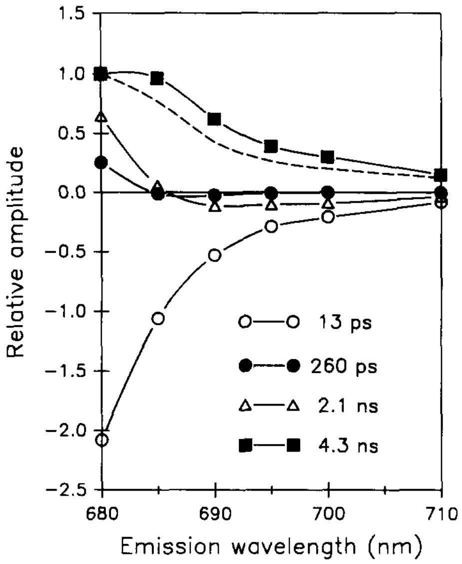

Fig. 1. Decay-associated spectra for disaggregated LHC II from dark-adapted spinach leaves. Fluorescence lifetimes (1/*e*) and amplitudes are accurate to within about ±10%, except for the fastest component, where the error is probably greater. Broken line, steady-state fluorescence emission spectrum recorded under the same conditions as the fluorescence kinetics.

K. The steady-state fluorescence emission spectrum recorded on the same equipment is shown in addition to the time-resolved components. We found it necessary to use four exponential components to obtain satisfactory fits to this data. The principal decay component of Chl *a* has a lifetime (1/*e*) of 4.3 ns and has an emission maximum between 680 nm and 685 nm. The second main component has negative amplitude and, therefore, represents a fluorescence rise rather than a fluorescence decay. This component has a lifetime of 13 ps. This term probably reflects mainly energy transfer from Chl *b* to Chl *a*. At our excitation wavelength of 663 nm, there will be substantial excitation of Chl *b*. Two further components with lifetimes of 260 ps and 2.1 ns were resolved (Fig. 1). Both these components have a positive amplitude at 680 nm, but a negative amplitude at longer emission wavelengths, suggesting that they represent equilibration of excitons between the bulk Chl *a* pool (emitting maximally at 680 nm) and species emitting at longer wavelengths.

The effect of aggregation on the decay-associated spectra for LHC II from dark-adapted spinach leaves is shown in Fig. 2. We found that five exponential components were necessary to obtain satisfactory fits to these data. The fastest kinetic component resolved has a lifetime of 22 ps and is a fluorescence rise in the emission range from 680 nm to 710 nm (Fig. 2). Like the 13 ps component seen in disaggregated LHC II (Fig. 1), this most probably represents mainly Chl *b* to Chl *a* energy transfer. The decay component of largest amplitude has a lifetime of 110 ps and an emission maximum at 680 nm. This component has negative amplitude at emission wavelengths above 690 nm, suggesting that the chlorophylls from which this kinetic component originates can transfer energy to species emitting at longer wavelengths. Note, however, that the

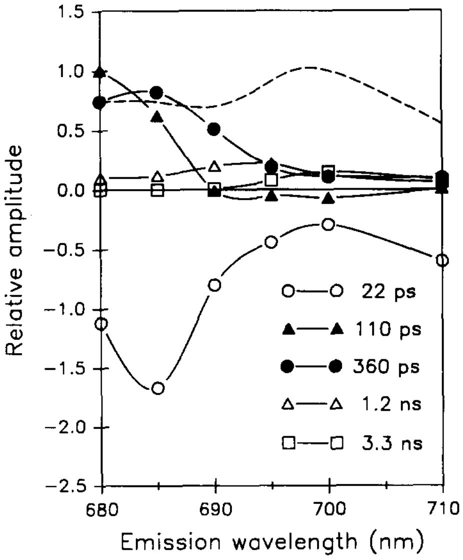

Fig. 2. Decay-associated spectra for aggregated LHC II from dark-adapted spinach leaves. Fluorescence lifetimes (1/*e*) and amplitudes are accurate to within about ±10%, except for the fastest component, where the error is probably greater. Note that the amplitude of the fastest component at 710 nm is clearly erroneous: the total fluorescence amplitude cannot be negative. Broken line, steady-state fluorescence emission spectrum recorded under the same conditions as the fluorescence kinetics.

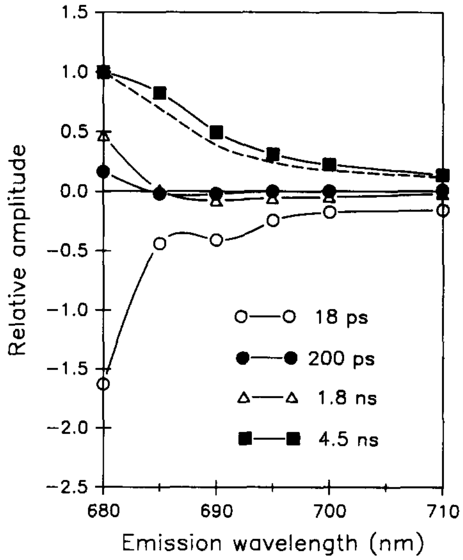

Fig. 3. Decay-associated spectra for disaggregated LHC II from light-treated spinach leaves. Fluorescence lifetimes (1/*e*) and amplitudes are accurate to within about ±10%, except for the fastest component, where the error is probably greater. Broken line, steady-state fluorescence emission spectrum recorded under the same conditions as the fluorescence kinetics.

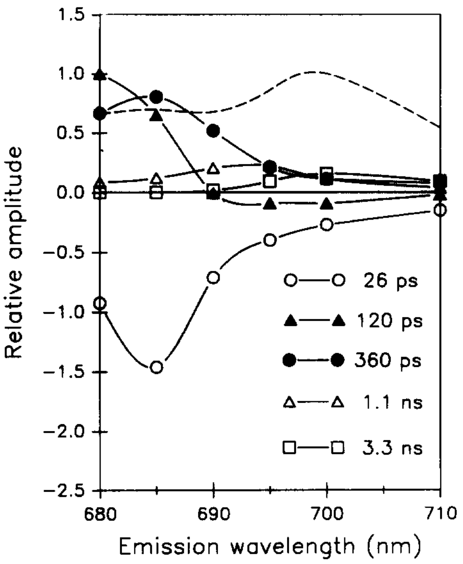

Fig. 4. Decay-associated spectra for aggregated LHC II from light-treated spinach leaves. Fluorescence lifetimes (1/*e*) and amplitudes are accurate to within about ±10%, except for the fastest component, where the error is probably greater. Broken line, steady-state fluorescence emission spectrum recorded under the same conditions as the fluorescence kinetics.

amplitude of the rise at long wavelengths is very much less than the amplitude of the decay at 680 nm (Fig. 2). This indicates that energy transfer to red-shifted Chl species is not the major sink for energy from this species. Three further decay components can be resolved: these all appear to originate from red-shifted Chl species (Fig. 2). Species with emission maxima at about 685, 695 and 700 nm have decay lifetimes of 360 ps, 1.2 ns and 3.3 ns, respectively (Fig. 2).

We also obtained decay-associated spectra for LHC II from light-treated spinach leaves, which has a higher content of the carotenoid zeaxanthin (Table I). In the disaggregated state, the decay-associated spectra do not differ greatly from those for LHC II from dark-adapted leaves (Figs. 3 and 1). In the aggregated state, the two sets of spectra are almost identical (Figs. 4 and 2).

Discussion

The data presented here show that aggregation of LHC II in vitro leads to the formation of an efficient energy-quenching process which greatly decreases the fluorescence lifetime of Chl *a* at 80 K. This is in accord with previous reports that the Chl *a* fluorescence lifetime at room temperature is decreased when LHC II is aggregated [11,12]. Under our conditions, however, the main Chl *a* fluorescence-decay lifetime in aggregated LHC II was about 110 ps (Fig. 2), which is considerably faster than previously reported [11,12]. Our fluorescence decays were obtained at very low pulse-energies, so it is clear that annihilation of excitons migrating over large domains in the aggregate [11] plays no part in the energy-dissipating process which we observe.

Our decay-associated spectra give some further information on the energy transfer processes in aggregated LHC II. On the basis of their fluorescence decay lifetimes, three chlorophyll species, red-shifted relative to the bulk Chl *a* pool, could be resolved (Fig. 2). One of these species, emitting maximally at about 700 nm and with a decay lifetime of 3.3 ns (Fig. 2), is the origin of most of the 700 nm peak seen in steady-state fluorescence emission spectra at low temperature [4,5]. This species was postulated to be involved in the energy-quenching mechanism [4]. This postulate makes two clear predictions: (a) The 700 nm species should be the major acceptor of energy from the bulk Chl. (b) The 700 nm species should have a decay lifetime much faster than the main decay lifetime in disaggregated LHC II, which is about 4.3 ns (Fig. 1).

However, our kinetic results show that neither of these predictions is fulfilled. There is a 110-ps rise term at 700 nm, indicating energy transfer from the bulk Chl *a* pool to the 700 nm emitting species (Fig. 2). However, this rise term has very low amplitude relative to the 110-ps decay at 680 nm (Fig. 2), showing that the 700 nm species receives only a small proportion of the energy transferred away from the 680 nm emitting Chl *a*. Furthermore, the decay lifetime of the 700 nm species is 3.3 ns (Fig. 2), which is only slightly faster than in disaggregated LHC II (Fig. 1).

A second red-shifted Chl species has an emission maximum at 695 nm. This species has a faster decay lifetime of 1.2 ns (Fig. 2). However, the low amplitude of the 110 ps rise term at 695 nm again suggests that this species is only a minor acceptor of energy from the bulk Chl *a* pool. The third red-shifted Chl species has an emission maximum at 685 nm and a decay lifetime of 360 ps. Energy transfer from the 680-nm emitting Chl to this species would probably not be resolvable because of the small spectral difference, which would not allow the fluorescence rise at 685 nm to be distinguished from the fluorescence decay at 680 nm. However, the 685-nm emitting species cannot be the main acceptor of energy from the 680 nm species. If this were the case, the 110 ps energy-transfer step would change the fluorescence yield and emission maximum very little and, thus, no separate 110 ps component would be resolved. It, thus, appears that none of the red-shifted species which we could resolve is involved in the energy-quenching mechanism. The quenching species appears to be the major acceptor of excitons from the 680 nm emitting Chl *a* pool, so that energy transfer to the quencher is responsible for most of the 9 ns^{-1} rate constant for de-excitation of these chlorophylls. However, we cannot resolve any fluorescence from the quenching species or from any intermediate energy acceptor.

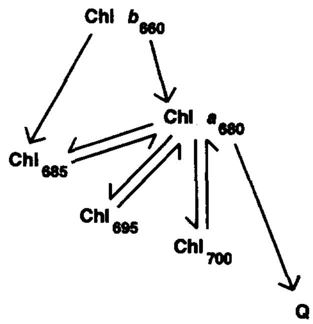

Fig. 5. Scheme for energy transfer pathways in aggregated LHC II. Q: energy-quenching species. Chl b_{660}, bulk Chl *b* pool with fluorescence maximum at 660 nm; Chl a_{680}, bulk Chl *a* pool with fluorescence maximum at 680 nm. Chl_{685}, Chl_{695}, Chl_{700}: red-shifted Chl species.

The red-shifted Chl species all show decay lifetimes somewhat faster than the decay lifetime in disaggregated LHC II, although in the case of the 700 nm-emitting species the difference is relatively small (Figs. 1 and 2). A simple explanation would be that these species can decay faster because of the possibility of back-transfer to the 680 nm emitting Chl *a*, which can then transfer the excitons to the quenching species. This scheme is illustrated in Fig. 5. The relative rate constants for forward and reverse energy transfer will depend on the ambient temperature and the concentrations of the red-shifted species. The relatively low yields for forward energy transfer to the 695-nm and 700-nm emitting species suggest that these species are not very efficiently coupled in terms of energy transfer to the bulk of the short-wavelength chlorophylls. Furthermore, the absorption spectra suggest that these species are present only in low concentrations [5]. It seems very unlikely that either species will be an effective trap for excitons at room temperature; from the Boltzmann equation, the probability of an exciton being located on the 700 nm species at equilibrium will be about 380-times less at 293 K than at 80 K. This would explain why the 700 nm fluorescence peak from aggregated LHC II is seen only at low temperatures [4,5].

The LHC II from light-treated spinach had a much higher content of the carotenoid zeaxanthin (Table I) as a result of the conversion of violaxanthin to zeaxanthin via the xanthophyll cycle [3,9]. However, our decay-associated spectra for LHC II from light-treated spinach (Figs. 3 and 4) are very similar to those from dark-adapted spinach (Figs. 1 and 2). This indicates that zeaxanthin is not an efficient acceptor of excitons from chlorophyll, nor is it involved in the energy quenching process which we observe in aggregated LHC II. It has been proposed that zeaxanthin could be directly involved in the non-radiative dissipation of excitons in the PS II antenna [3].

Because we cannot resolve any fluorescence from the quenching species, our results give no direct information on the mechanism of the non-radiative de-excitation process involved. However, it is likely that the quenching species is a non-fluorescent pigment aggregate formed when LHC II becomes aggregated [4,12]. The red-shifted Chl species which we observe may be formed in a similar way. It appears, however, that some red-shifted species are present even in the disaggregated state, because equilibration of excitons towards longer wavelengths can be observed (Fig. 1). No separate decay components can be resolved from the red-shifted species in the disaggregated state, presumably because the exciton lifetime is about 4.3 ns throughout the chromophore matrix (Fig. 1).

It has been proposed that the aggregation state of LHC II in the thylakoid membrane may be controlled by factors including the transmembrane pH gradient and the content of zeaxanthin, and that a quenching process similar to that observed in aggregated LHC II in vitro may be the basis of high-energy state quenching in vivo [4,6]. Our present results show that the quenching process in aggregated LHC II would be rapid enough to compete with trapping at PS II in the intact system. A recent study suggests that the effective rate constant for exciton trapping at PS IIα (the species of PS II with which most LHC II is associated) is about 3 ns^{-1} with open reaction centres [13]. We observe de-excitation of the bulk Chl *a* pool with a rate constant of about 9 ns^{-1} in aggregated LHC II (Fig. 2). It is clear that this could provide an effective mechanism for down-regulation of PS II in vivo.

Acknowledgements

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