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Aggregation of Light-Harvesting Complex II leads to formation of efficient excitation energy traps in monomeric and trimeric complexes

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Abstract Non-photochemical quenching (NPQ) protects plants against photodamage by converting excess excitation energy into harmless heat. In vitro aggregation of the major light-harvesting complex (LHCII) induces similar quenching, the molecular mechanism of which is frequently considered to be the same. However, a very basic question regarding the aggregation-induced quenching has not been answered yet. Are excitation traps created upon aggregation, or do existing traps start quenching excitations more efficiently in aggregated LHCII where trimers are energetically coupled? Time-resolved fluorescence experiments presented here demonstrate that aggregation creates traps in a significant number of LHCII trimers, which subsequently also quench excitations in connected LHCIIs.
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Keywords: LHCII; Non-photochemical quenching; Photosystem II; Picosecond fluorescence; Time-correlated single photon counting

1. Introduction

During photosynthesis solar energy is captured by pigments and stored by a series of events that convert the pure energy of light into biochemical energy. Primary reactions of this process take place in photosynthetic systems in the thylakoid membrane. These systems are highly organized: reaction centres (RCs) are surrounded by antennae that transfer absorbed light energy to the RCs. The antenna system consists of various pigment-binding proteins. In higher plants the main antenna complex is light-harvesting complex II (LHCII), binding chlorophyll (Chl) *a*, Chl*b*, and xanthophylls.

Too much light can be damaging, and higher plants respond to conditions where the absorbed light exceeds the photosynthetic capacity via several photoprotective mechanisms. One of the most significant of them is the ΔpH-induced enhancement of non-radiative energy dissipation in the photosystem II antenna, registered as non-photochemical chlorophyll fluorescence quenching (NPQ) [1]. This process causes a decrease of the singlet excited-state lifetime (quenching) of Chl*a* by turning excess excitation energy into heat [2].

NPQ is mainly triggered by a ΔpH across the photosynthetic membrane and the dynamic control is achieved by the regulatory role of the xanthophyll-cycle carotenoids (violaxanthin, antheraxanthin and zeaxanthin) and the PsbS protein [3,4]. Random aggregation of isolated LHCII leads to fluorescence quenching that resembles NPQ [5,6] and therefore aggregated LHCII seems to be a good model system for studying NPQ, although this does not necessarily mean that similar aggregates are also present in thylakoid membranes. A substantial amount of recent work has focused on NPQ-associated events in LHCII [7–12]. However, no consensus has been reached about the physical nature of the energy dissipation. Moreover, it is still unknown whether LHCII aggregation leads to the formation of quenchers, as proposed in for instance [11,13] or that increased connectivity between trimers upon aggregation leads to efficient quenching by a small population of permanently quenched trimers [13,14]. In the latter case excitations would be transferred from unquenched trimers to quenched trimers, leading to accelerated depopulation of the excited state. These two scenarios have been visualized schematically in Fig. 1. To discriminate between them we studied the excited-state lifetimes of LHCII of spinach in different states of aggregation: monomers, trimers and aggregates. We demonstrate that excitation traps are indeed being created upon aggregation of trimeric LHCII. These traps do not only quench excitations in the trimer in which they are located, due to excitation energy transfer between trimers, but these traps also quench excitations originating in complexes that do not contain traps themselves. The fluorescence quenching in monomers was found to be even stronger than that in trimers, suggesting an intramonomeric origin of this process.

2. Materials and methods

Trimeric and monomeric LHCII were prepared from spinach as described before [15]. The proteins were suspended in a 20 mM HEPES buffer (pH 7.6) in 0.03% (0.6 mM) β-dodecylmaltoside (β-DM). Aggregates were obtained by lowering the β-DM concentration to 0.0003% (0.6 μM), i.e. far below the critical micelle concentration (~0.15 mM).

Steady-state absorption spectra were recorded on a Varian Cary 5E spectrophotometer. Steady-state fluorescence emission spectra (430 nm excitation) were recorded on a Spex-Fluorolog 3.2.2 spectrofluorimeter (Jobin-Yvon).

Time-correlated single photon counting was performed with a home-built setup, as described elsewhere [16]. In brief, samples were excited with vertically polarized 430 nm pulses of 0.2 ps duration at a repeti-

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Abbreviations: β-DM, *n*-dodecyl-β,D-maltoside; Chl, chlorophyll; CI, confidence interval; LHCII, light-harvesting complex II; NPQ, non-photochemical quenching; PSII, photosystem II; RC, reaction centre

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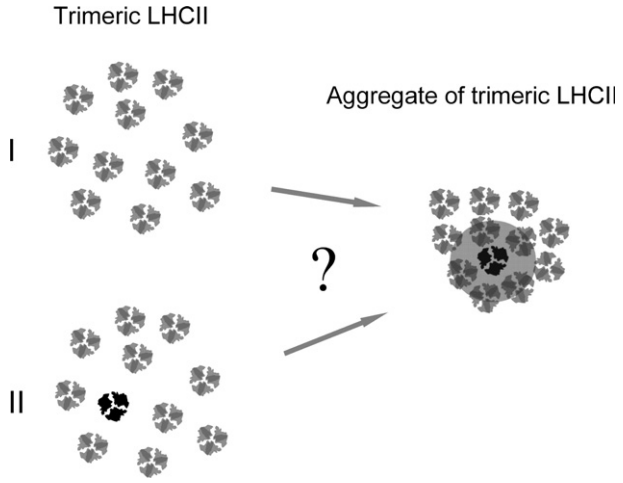

Fig. 1. Two scenarios of aggregation-induced fluorescence quenching of LHCII: (I) quencher formation upon aggregation, and (II) existing quenchers start quenching excitations originating in LHCII without quenchers, due to efficient transfer between trimers in aggregates. LHCII trimers with and without quenchers (black and light grey images, respectively) and LHCII trimers that are affected by the quencher (grey circular semitransparent area) are shown.

tion rate of 3.8 MHz. The excitation density was reduced to obtain a count-rate below 30 000 per second (sub-pJ pulse energy) and care was taken to minimize data distortion [17]. The instrument response function (~30 ps FWHM) was obtained with pinacyanol iodide in methanol, with 10 ps fluorescence lifetime. Fluorescence was detected at right angle with respect to the excitation beam in 10 measuring sequences of two times 10 s through a vertical or horizontal polarizer and through a 665 nm long-pass filter (Schott). Detection through band-pass filters at 635 nm, 701 nm and 721 nm gave identical results. Individual photons were detected by a microchannel plate photomultiplier, and arrival times were stored in 4096 channels of a multichannel analyzer. The channel spacing was 5 ps or 1.25 ps (0.6 mM β-DM) and 3.0 ps (6 μM β-DM).

Fluorescence decay curves (parallel + 2 × perpendicular) were fitted to a sum of exponentials, convoluted with the instrument response function [18]. The quality of a fit was judged from the χ^2-value and by visual inspection of the residuals and the autocorrelation thereof. The number of exponentials was considered sufficient if the addition of one extra decay component did not significantly improve the fit. Confidence intervals were calculated by exhaustive search.

3. Results and discussion

Absorption spectra of trimeric and monomeric LHCII are shown in Fig. 2. These spectra resemble previous results (e.g. [19–21]). The broader absorption bands for monomeric LCHII indicate a more disordered/less rigid (and therefore less unique) environment of the pigments [22]. Upon aggregation the light scattering increases which causes an apparent increase of the absorption. This becomes more pronounced upon going to the blue. The absorption decreases around 490 nm (absorption of lutein/neoxanthin [23]). The changes in absorption of LHCII upon aggregation have been discussed extensively by Naqvi et al. [21].

Trimeric and monomeric LHCII both show multi-exponential fluorescence decay curves (Fig. 3). The fitting results are given in Table 1. For trimeric LHCII the decay is nearly mono-exponential (3.81 ns, 86%). A small fraction of the trimers shows a shorter decay time (1.96 ns, 11%), and only a very small fraction decays much faster, i.e. is heavily quenched

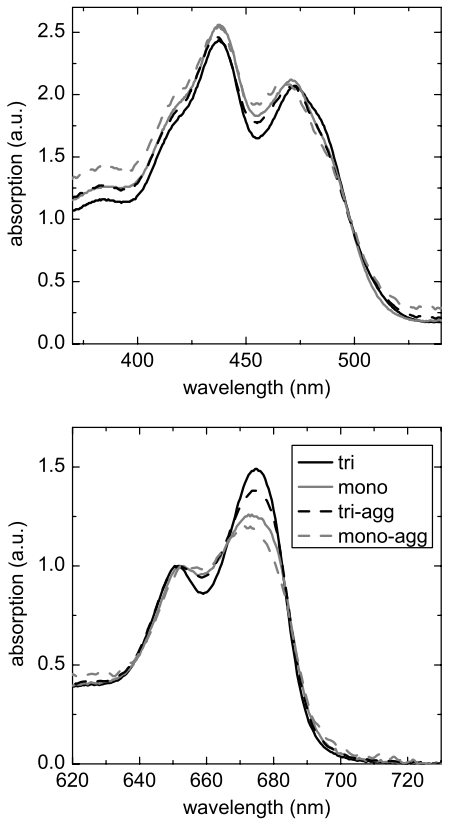

Fig. 2. Absorption spectra of trimeric (black) and monomeric (grey) LHCII, and aggregates (dashed) thereof, in the Soret (upper plot) and Q-band region. Spectra are normalized to the peak around 650 nm.

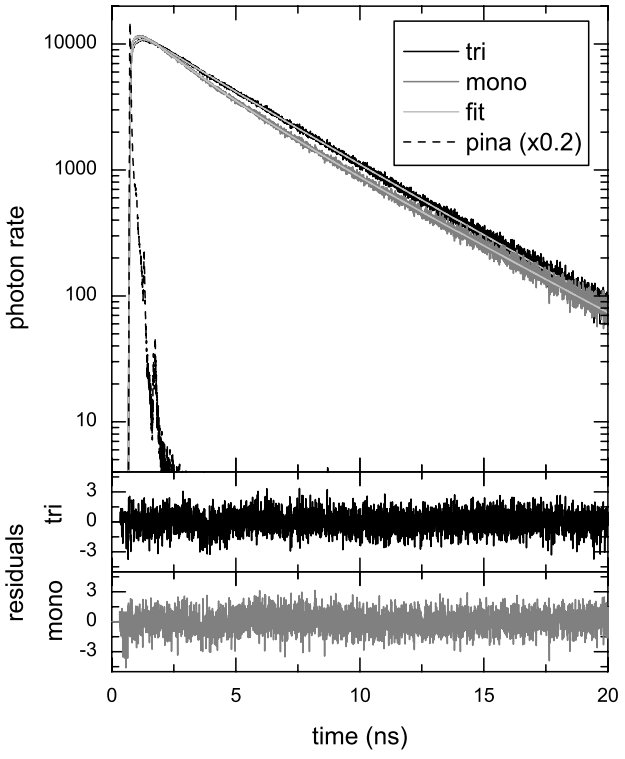

Fig. 3. Fluorescence decay curves of trimeric (black) and monomeric (grey) LHCII and of pinacyanol iodide in methanol (dashed). Excitation was at 430 nm, detection at >665 nm, 5.0 ps/time-channel.

Table 1
Fitted decay times (τ) and relative amplitudes (p) of trimeric and monomeric LHCII and aggregates thereof, with 95% confidence intervals (CI)

	Unaggregated				Aggregated			
	τ (ns)	CI	p	CI	τ (ns)	CI	p	CI
Trimer	0.21	[0.088–0.406]	0.02	[0.016–0.036]	0.033	[0.025–0.039]	0.34	[0.301–0.361]
	1.96	[1.856–2.074]	0.11	[0.108–0.120]	0.18	[0.156–0.195]	0.43	[0.402–0.453]
	3.81	[3.805–3.820]	0.86	[0.858–0.863]	0.51	[0.459–0.558]	0.20	[0.178–0.241]
					1.62	[1.409–1.803]	0.028	[0.024–0.035]
					3.96	[3.413–4.895]	0.002	[0.001–0.004]
Monomer	0.19	[0.139–0.238]	0.14	[0.123–0.167]	0.039	[0.036–0.042]	0.62	[0.588–0.645]
	1.89	[1.775–1.970]	0.38	[0.365–0.403]	0.15	[0.135–0.161]	0.31	[0.288–0.345]
	4.02	[3.962–4.068]	0.47	[0.452–0.499]	0.50	[0.451–0.533]	0.06	[0.052–0.072]
					1.62	[1.409–1.803]	0.007	[0.006–0.008]
					3.96	[3.413–4.895]	0.001	[0.001–0.002]

(0.21 ns, 2%). Similar results were obtained before (e.g. [24,25]) although the percentages differ somewhat. For monomeric LHCII similar decay times are observed but the fraction of fast components is higher (38% 1.89 ns and 14% 0.19 ns). Moreover, the fitting results depend slightly on the time interval used for fitting, indicating a broader distribution of decay times than for trimeric LHCII. This is probably related to the increased disorder/reduced rigidity of the monomeric unit, as also reflected by broadening of the absorption spectrum (Fig. 2).

Upon aggregation the fluorescence decay becomes considerably faster as can be seen in Fig. 4. The results of the fitting of these decay curves depend on the fitted time-range, starting values of the fitted parameters, and number of decay times. In all cases, at least five decay times are needed, as observed before [26]. Two of these are above 1 ns and the corresponding amplitudes are very small. They are possibly due to non-aggregated monomers or trimers. For the discussion below these components are not relevant and for the fitting of the decay curves we assumed these lifetimes to be equal for aggregated monomers and trimers. The fitting results are presented in Table 1. The large heterogeneity of lifetimes is probably due to the fact that the aggregates are random/disordered. More ordered aggregates of plant light-harvesting complexes show less decay components [27]. This demonstrates clearly that the organization of the aggregates can modulate the lifetime.

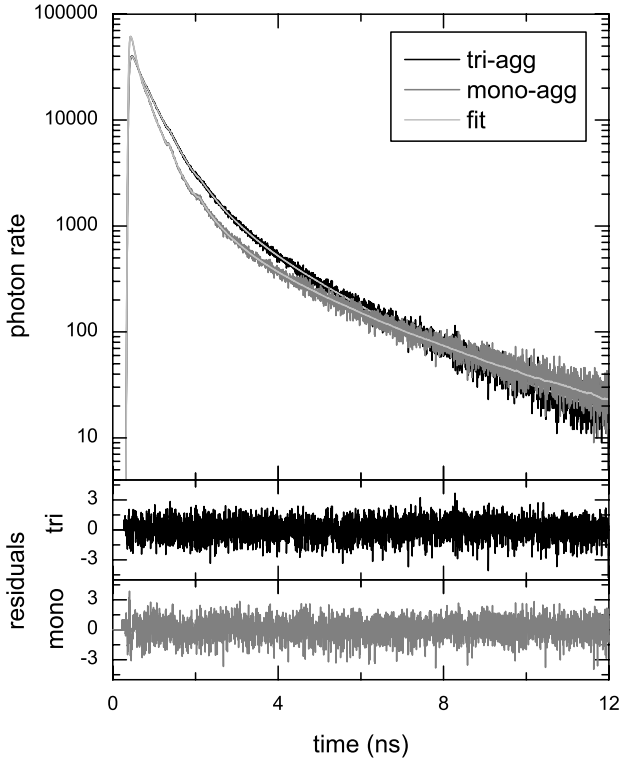

Fig. 4. Fluorescence decay curves of aggregates of trimeric (black) and monomeric (grey) LHCII. Excitation was at 430 nm, detection at >665 nm, 3.0 ps/time-channel.

The fitted values for the amplitudes and lifetimes can be used to calculate the relative fluorescence quantum yield of monomers, trimers and aggregates (Table 2). These yields are rather precise, do not depend significantly on the variation in the fitting values, and are very close to the relative quantum yields obtained from the steady-state measurements. The latter observation means that there is no indication for the presence of components that are even faster than the shortest fitted decay components.

The two >1 ns-lifetimes (1.6 ns and 4.0 ns) are very similar to those before aggregation, and they have very small amplitudes (<3%). The remaining lifetimes are much faster, reflecting severe quenching. The dominating lifetimes are of the order of 100–200 ps and several tens of ps. The 100–200 ps is similar to that of the small fraction observed for trimers and monomers in ''unaggregated'' LHCII, suggesting that in those preparations a small fraction of aggregates or quenched monomers/trimers is present.

In our analysis we initially focus on the results for aggregated trimers. The two main decay components are 25–40 ps (30–36%) and 150–200 ps (40–45%). How can these decay rates be related to quenching processes in the aggregate? Singlet

Table 2
Fluorescence quantum yields (ϕ_f^{rel}) and average lifetime ($\langle\tau\rangle^*$) of trimeric and monomeric LHCII and aggregates thereof

	Unaggregated		Aggregated	
	Trimer	Monomer	Trimer	Monomer
ϕ_f^{rel} (st.st.)[a,b]	1	0.68	0.05	0.02
ϕ_f^{rel} (time-res.)[b,c]	1	0.75	0.07	0.03
$\langle\tau\rangle^*$ (ns) [d]	–	–	0.191	0.101

[a]From steady-state data.
[b]Relative to unaggregated trimeric LHCII.
[c]From time-resolved fluorescence.
[d]Calculated without >1 ns lifetimes.

excitations can move from one Chl to another. This can be described as a diffusion process that leads to excitation equilibration [28]. Singlet–singlet annihilation studies showed that the excitation equilibration time in trimers is 30–50 ps and that for an aggregate of N trimers it is roughly equal to N^* 30 ps [28]. From these numbers it can be predicted what the effect of quenchers will be on the fluorescence decay times.

For the sake of argument it is first assumed that the quenchers are extremely efficient (quenching rate >1 ps^{-1}). In this case the fluorescence lifetime equals the time it takes for an excitation to reach the quencher, after which it is immediately quenched. Thus one would expect a lifetime of 30–50 ps (the excitation equilibration time) for quenched trimeric LHCII. A similar lifetime (25 ps) is for instance observed for a large fraction of LHCII trimers under high hydrostatic pressure [29]). In aggregates, the presence of one efficient quencher per 4 trimers would thus lead to a diffusion-limited lifetime of 120–200 ps. The largest part of the fluorescence decay for aggregates (of trimers) is described by lifetimes of 33 ps (34%) and 150 ps (43%). This demonstrates the presence of a high concentration of quenchers: 33 ps corresponds to 1 quencher per trimer, 150 ps corresponds to 1 quencher per ~4 trimers These results are in sharp contrast to the case of isolated trimers where only 2% of the complexes show a 210 ps decay component. These results unequivocally demonstrate the fact that a large amount of quenchers is created upon aggregation. In the case of less efficient quenchers the number of quenchers needs to be even higher to explain the short lifetimes.

The three <1 ns decay components probably reflect a broad distribution of lifetimes originating from heterogeneity of the quencher concentration in the aggregates. In such case it is better to consider the amplitude-weighted average fluorecence lifetime ($\langle\tau\rangle$). The >1 ns components probably originate from non-aggregated LHCII, so the relevant parameter is in fact not $\langle\tau\rangle$, but $\langle\tau\rangle^*$: the average lifetime calculated from only the <1 ns components. For aggregates of trimeric LHCII $\langle\tau\rangle^* = 191$ ps (Table 2), reflecting the presence of at least one quencher per 5 trimers.

Also upon aggregation of monomers substantial quenching is observed and the amplitude of the fastest components is even higher than for trimers. This is in agreement with other experiments that showed that monomeric LHCII is more easily quenched than trimeric LHCII (e.g. [30,31]). This can be relevant for NPQ, because excess light can lead to monomerization of LHCII in thylakoid membranes [32]. The fluorescence lifetimes of aggregated monomers strongly resemble those of aggregated trimers. This points at domains with the same concentration of quenchers in both types of aggregates, however these domains are present in different amounts (Table 1). Again it should be concluded that quenchers are created upon (random) aggregation.

In conclusion, we find that only a very small fraction of trimeric LHCII is in a quenched state. This fraction increases substantially upon aggregation. These quenchers can then also trap excitations that arise in trimers that do not contain quenchers themselves. Aggregates of monomeric LHCII contain even a larger fraction of quenchers in comparison to the trimer. This fraction further increases upon aggregation, even more than for trimeric LHCII.

From the present data we cannot conclude what the nature is of the created quencher. It was argued before that structural changes of LHCII upon aggregation lead to quenching similar to NPQ in vivo [5]. It was for instance shown that aggregation leads to a change in interaction between Lutein 1 and Chl*a* [33] and it was speculated that this might lead to a change of the excited-state lifetime [34]. However, it remains uncertain whether such a mechanism is also present here.

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