

Rapid quenching of chlorophyll excited states in cyanobacteria, even in the absence of reaction centers

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Introduction

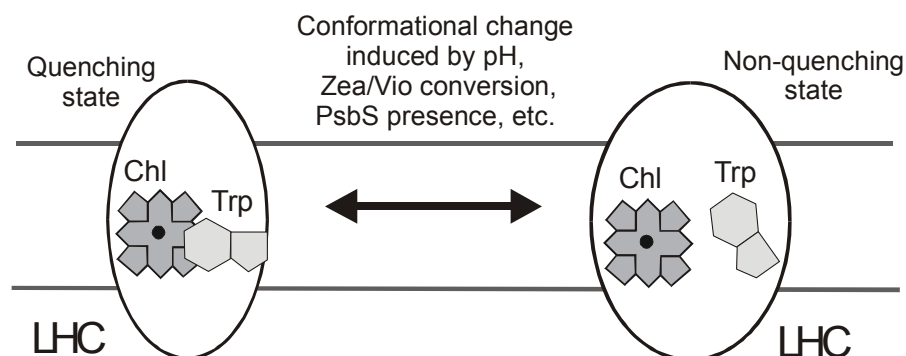
With the development of oxygenic photosynthesis that is powered by chlorophylls in excited state, organisms must have learned to avoid or minimize the formation of highly reactive oxygen species (such as singlet oxygen) that will result from an illuminated mixture of chlorophyll and oxygen. For example, excited chlorophyll will form triplets, which can react with ground-state (triplet) oxygen to form ground-state chlorophyll and the very reactive singlet oxygen that can easily oxidize many biological compounds. A common solution to this problem is to have chlorophylls bound to proteins in close vicinity to carotenoids or other triplet quenchers. Carotenoids (e.g., β -carotene) are efficient quenchers of chlorophyll triplets presumably because the level of their excited triplet state lies below that of chlorophyll (Cogdell and Frank, 1987).

A related issue is how -if energy trapping in open reaction centers is not feasible- excited singlet chlorophyll is converted to the ground state before damage can occur. The quenching mechanism of chlorophyll excited singlet states *in vivo* remains poorly understood, even though such quenching clearly occurs and is referred to as non-photochemical quenching. If carotenoids are directly involved in this quenching, the energy transfer from chlorophyll to carotenoid is likely to involve the exchange (Dexter) mechanism, which requires close proximity of the donor and acceptor molecules (Frank et al., 1994). The reason to invoke carotenoids in non-photochemical quenching primarily was the view that an interconversion between zeaxanthin and violaxanthin (xanthophyll cycle) was an important regulator in determining the degree of non-photochemical quenching (Demmig-Adams, 1990): zeaxanthin would be a much more efficient singlet chlorophyll quencher than violaxanthin. However, the energy values of the excited states of zeaxanthin and violaxanthin relative to that of chlorophyll are still a matter of debate (see Frank, 2001). Alternatively, violaxanthin and zeaxanthin have been proposed to control quenching indirectly by inducing structural changes in antenna proteins that promote quenching (see Wentworth et al., 2000) but the mechanism of this quenching remains unclear.

Studies on plant and algal mutants that are unable to perform the xanthophyll cycle have shown that this cycle is certainly not the sole regulator of non-photochemical quenching. Other mutants that are impaired in other branches of carotenoid synthesis also have shown changes in non-photochemical quenching as well (Niyogi et al., 1997; Pogson et al., 1998). Moreover, mutants lacking specific LHC components have been found to be greatly impaired in non-photochemical quenching: a mutant lacking PsbS, a LHC-like protein, essentially lacked non-photochemical quenching (Li et al., 2000) but was normal in its carotenoid levels (Peterson and Havir, 2000). A working hypothesis developed on the basis of these observations is that PsbS is needed for non-photochemical quenching, possibly sensing the

luminal pH, and that the xanthophyll cycle is a major determinant for the lifetime of the chlorophyll excited state (Li et al., 2000; Müller et al., 2001). However, this hypothesis does not address the molecular mechanism of non-photochemical quenching, and it also is not generally applicable: cyanobacteria have neither PsbS nor a xanthophyll cycle, yet they do show reversible non-photochemical quenching that is independent of state transitions (data not shown).

There are other explanations that can provide a molecularly more satisfactory mechanism for non-photochemical quenching. Quenching can result from either chlorophyll aggregation (difficult to envision *in vivo*) or from a “futile cycle” of electron transfer consisting of charge separation and charge recombination. An interesting possibility for such a cycle involves electron transfer between chlorophyll and a nearby tryptophan residue. As indicated in Vavilin et al. (1999), a set of photosystem II (PS II) mutants has been generated in *Synechocystis* sp. PCC 6803 that have essentially constitutive and complete non-photochemical quenching while PS II activity remains quite normal. This set of mutants carry a Trp (rather than a Phe residue present in wild type) at position 191 of the D2 protein, which presumably is very close to one of the chlorophylls in the reaction center complex. Introduction of a Trp residue at position 191 causes a highly efficient quenching of excitations, and this has been interpreted by Trp oxidation by excited chlorophyll followed by a charge recombination to form chlorophyll in the ground state. The efficiency of this quenching process is expected to be very much dependent on the precise distance between Trp and chlorophyll. Extrapolating this phenomenon to the antenna complex, it is feasible that upon acidification of the lumen (and neutralization of acidic amino acid residues in LHC polypeptides) the conformation of LHC polypeptides changes (either directly or because of a carotenoid interconversion) causing a Trp residue to move in closer proximity to a chlorophyll, and that upon excitation of this chlorophyll rapid energy dissipation will occur by charge separation and recombination between chlorophyll and Trp. This interpretation provides a mechanistic explanation of the xanthophyll cycle and why certain LHCs are needed for non-photochemical quenching. If zeaxanthin is present in a particular LHC then a Trp in one of these LHC proteins is expected to be closer to a chlorophyll and therefore excitation is quenched, whereas with violaxanthin present this quenching does not occur due to a different relative positioning of Trp and the chlorophyll. If a protein such as PsbS is present in the LHC complex, the conformation of the remaining LHCs is such that non-photochemical quenching can occur when the luminal pH drops (due to a repositioning of a Trp vs. a chlorophyll in one of the remaining LHCs). In the absence of PsbS, the orientation of such a Trp residue vs. chlorophylls may be different and non-photochemical quenching would not occur. A schematic representation of this phenomenon is shown in Fig. 1 below.



In *Synechocystis* sp. PCC 6803, β -carotene is the primary carotenoid associated with reaction center complexes, and the peripheral antenna system is the phycobilisome, which does not have carotenoids associated with it. Plant-type LHC complexes are absent in this

organism. We have monitored energy transfer and excitation lifetimes in *Synechocystis* mutants that lacked either PS I or both PS I and PS II; the latter mutant also lacked ApcE, which is the protein linking phycobilisomes to the thylakoid membrane. The majority of the excitations is quenched very efficiently in these mutants, showing that even in a system without reaction centers, LHC, or PsbS excitation quenching is an efficient process. This is in line with the hypothesis that chlorophyll excitation quenching is mediated through interaction of chlorophyll with common components in proteins (rather than with specific carotenoids), and certainly lends further credence to our working hypothesis that small movements in proteins modulating the distance and relative orientation between Trp residues and chlorophylls may be major determinants in chlorophyll excitation quenching.

Materials and methods

The *Synechocystis* sp. PCC 6803 mutants used in this study were grown at 30°C and at a light intensity of 4 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ in liquid BG-11 medium that was supplemented with 10 mM glucose and 5 mM TES-NaOH buffer (pH 8.2). Air was bubbled through the culture.

Time-resolved fluorescence of intact cells was measured at room temperature in a single-photon-timing spectrometer as described by Bittersmann and Vermaas (1991). Excitation at 432 nm was provided by a Tsunami mode-locked Ti:sapphire laser equipped with a model 3980 frequency doubler/pulse selection unit and continuously pumped by a Millennia diode-Nd:YVO₄ cw laser (Spectra Physics). The ~2 ps excitation pulses were delivered at a frequency of 4.2 MHz. The slitwidth of the fluorescence detection system was set at 8 nm. The full width at half maximum of the instrument response function at this configuration was about 60 ps.

Steady-state fluorescence emission and excitation spectra were recorded using a SPEX Fluorolog spectrofluorometer. To measure the steady-state fluorescence emission in the PS I-less strain in F₀ state, cells were continuously pumped through a flow cuvette. Steady-state fluorescence in F_m state was recorded in the presence of DCMU without pumping.

Results and discussion

Fluorescence lifetime spectroscopy can monitor the fate of excited pigments in an *in vivo* system. Fig. 2 presents fluorescence lifetimes in a strain lacking PS I (*psaAB*⁻), the genes for the PS II chlorophyll-binding proteins CP47 and CP43 (*psbB*/*psbC*⁻), and the gene for ApcE (*apcE*⁻) linking phycobilisomes to the thylakoid. This strain lacks significant accumulation of the PS II reaction center proteins D1 and D2, and still carries about 3% of the amount of chlorophyll present in the wild type (Shen and Vermaas, 1994). This chlorophyll may be associated with pigment carrier proteins (He and Vermaas, 1999), with small Cab-like proteins (SCPs) (Funk and Vermaas, 1999), or with other reservoir/regulator proteins in the cell. In this strain, upon 432 nm excitation the predominant chlorophyll fluorescence lifetimes are 100-200 and 600-700 ps, which are much shorter than the 3-4 ns lifetimes typically associated with free chlorophyll or with isolated antenna complexes (e.g. Crimi et al., 2001). A 1.4 ns lifetime component was observed as well, but this appeared to originate mainly from phycobilins according to the spectrum shown in Fig. 2 (maximum amplitude at 660-670 nm). Indeed, upon 590 nm excitation (exciting phycobilins) this 1.4 ns lifetime becomes the major component in the deconvoluted spectrum (data not shown). Therefore, the majority of

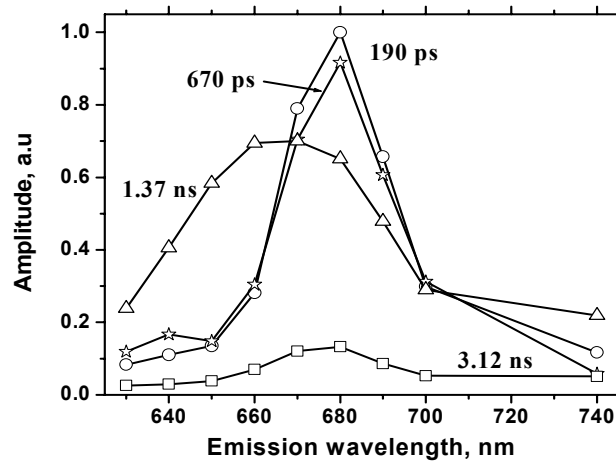


Fig. 2. Decay-associated spectra measured in intact cells of the PS I-less/CP43-less/CP47-less/ApcE-less strain of *Synechocystis* sp. PCC 6803 at room temperature. The global χ^2 value of the fitted data was 1.062, indicating that the calculated lifetimes provided a good fit to the experimental data.

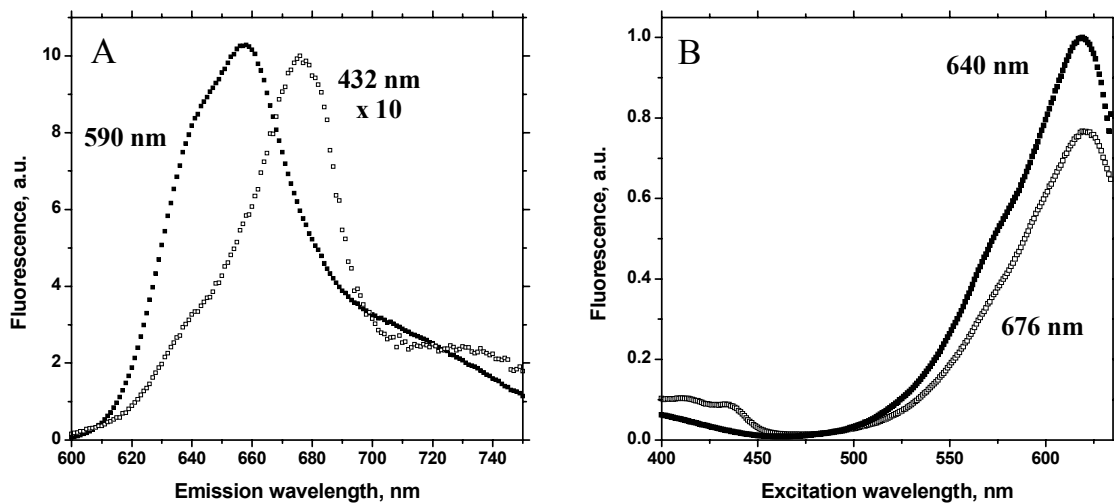


Fig. 3. Room-temperature fluorescence emission and excitation spectra of intact cells of the PS I-less/CP43-less/CP47-less/ApcE-less strain of *Synechocystis* sp. PCC 6803. (A) Emission spectra recorded upon excitation at 432 (open symbols) or 590 (closed symbols) nm. The emission spectrum at 432 nm excitation has been expanded 10-fold in the y-direction to correct for the high fluorescence yield upon 590 nm excitation that is associated with free phycobilisome components in this strain. (B) Excitation spectrum of fluorescence emission at 640 nm (closed symbols) or 676 nm (open symbols).

excitations absorbed by chlorophyll in thylakoids is quenched within 700 ps, even in the absence of reaction centers.

The fluorescence emission and excitation spectra of the PS I-less/CP43-less/CP47-less/ApcE-less strain of *Synechocystis* sp. PCC 6803 are presented in Fig. 3. As expected, excitation in the blue region primarily excites chlorophyll (emitting around 680 nm), and the fluorescence yield is low, in accordance with a short excitation lifetime. Phycobilin excitation is not transferred to chlorophylls due to the absence of the ApcE linker and the yield of fluorescence emission around 660 nm is high, in accordance with the long lifetime of phycobilin emission in this strain. A significant amount of phycobilin emission still occurs at 675-680 nm, thus accounting for the substantial phycobilin contribution (around 620 nm) to 676 nm fluorescence emission. Light absorbed by carotenoids does not clearly contribute to the chlorophyll emission around 676 nm (Fig. 3B). This is in line with the realization that the contribution of carotenoid absorption to light harvesting in cyanobacterial thylakoids is negligible, suggesting that energy transfer between chlorophyll and carotenoid is small in this system.

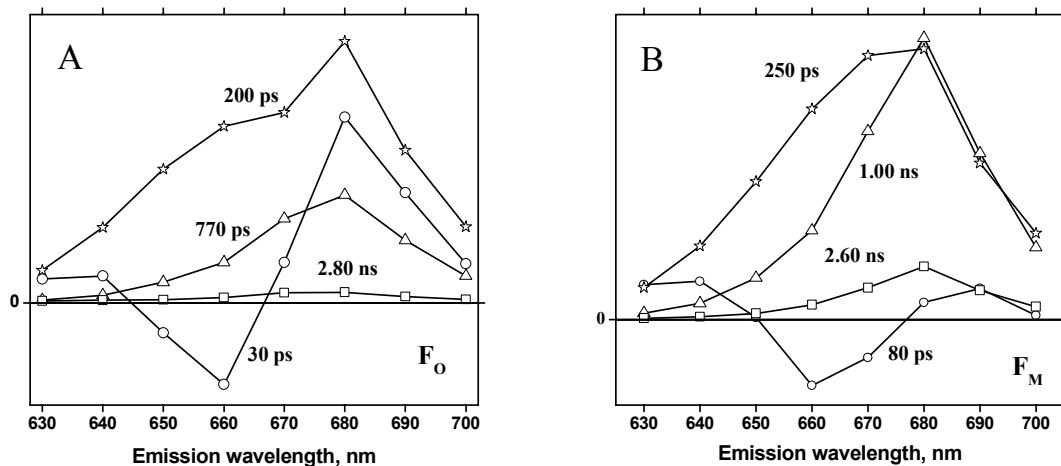


Fig. 4. Decay-associated spectra of fluorescence emission measured in intact PS I-less cells of *Synechocystis* sp. PCC 6803 at room temperature. (A) Open PS II centers (cells kept in darkness); (B) Closed PS II centers. The negative amplitude of a fast component in both decay-associated spectra signifies energy transfer from phycobilisomes to chlorophyll. Excitation was at 432 nm.

The results obtained with the PS I-less/CP43-less/CP47-less/ApcE-less strain of *Synechocystis* sp. PCC 6803 were compared to what was seen in a strain that is PS I-less, but that retains PS II and has functional interconnection between phycobilisomes and the thylakoid membrane. In the PS I-less strain, room-temperature fluorescence emission peaks around 680 nm and is increased by about 3-fold if PS II reaction centers are closed (not shown). Fluorescence lifetimes of the PS I-less strain are presented in Fig. 4. Comparing decay-associated spectra of PS I-less strains under conditions corresponding to open and closed PS II reaction centers, the increase in fluorescence yield upon closure of PS II centers is due primarily to a major increase in a 0.8-1 ns fluorescence lifetime component. Moreover, a small component with a 2.6 ns lifetime appears upon closure of PS II reaction centers.

These results imply that also in cyanobacteria that retain a normal PS II complex the excitation lifetime is short (≤ 1 ns) even if PS II centers are closed. Therefore, excitations are quenched efficiently. The nature of the quenching is as yet unknown, but based on the fact

that fluorescence yields were not affected much in systems in which the *crtR* gene for β -carotene hydroxylase (converting β -carotene to zeaxanthin) had been inactivated (data not shown), it is unlikely that zeaxanthin or other carotenoids are involved in this quenching. A possible quenching of excited chlorophyll by electron transfer between this pigment and amino acid residues such as Trp is very attractive, but remains to be proven.

Acknowledgements

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