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Photosynthesis in *Chondrus crispus*: The contribution of energy spill-over in the regulation of excitonic flux

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ABSTRACT

Chondrus crispus is a species of red algae that grows on rocks from the middle intertidal into the subtidal zones of the North Atlantic coasts. As such, it has to cope with strongly variable abiotic conditions. Here we studied the response of the photosynthetic apparatus of this red alga to illumination. We found that, as previously described in the case of the unicellular alga *Rhodella violacea* (E. Delphin et al., Plant Physiol. 118 (1998) 103–113), a single multi-turnover saturating pulse of light is sufficient to induce a strong quenching of fluorescence. To elucidate the mechanisms underlying this fluorescence quenching, we combined room temperature and 77 K fluorescence measurements with absorption spectroscopy to monitor the redox state of the different electron carriers in the chain. In addition, we studied the dependence of these various observables upon the excitation wavelength. This led us to identify energy spill-over from Photosystem II to Photosystem I rather than a qE-type non-photochemical quenching as the major source of fluorescence quenching that develops upon a series of 200 ms pulses of saturating light results, in line with the conclusion of Ley and Butler (Biochim. Biophys. Acta 592 (1980) 349–363) from their studies of the unicellular red alga *Porphyridium cruentum*. In addition, we show that the onset of this spill-over is triggered by the reduction of the plastoquinone pool.

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1. Introduction

Chondrus crispus is a red macroalga living in the intertidal zone and widely represented on the rocky shores of the North Atlantic Ocean. Its economical and ecological importance has made it a historical model. It has been harvested for ages for its cell wall polysaccharides, the carrageenans, used in the food industry as thickening and gellifying molecules.

The phylogenic position of *Chondrus* among red algae makes it an interesting organism from the evolutionary point of view. According to the hypothesis of a unique primary endosymbiosis, all Archaeplastida are the result of a single event, where a eukaryotic cell has phagocytized an ancestor of current cyanobacteria, gaining the capacity to perform photosynthesis. Red algae are the sister group of the green lineage, which contains green algae and land-plants. This position gives them noticeable properties, particularly concerning their photosynthetic apparatus. On the contrary to green organisms, red algae have non-appressed thylakoid membranes [1], with evenly distributed photosystems (PS) I and II. Like cyanobacteria, their PSII antenna is an extrinsic protein complex known as the phycobilisome (PBS) [2], composed of several blue and red pigments. A membrane bound Chl containing complex (LHCr for red-LHC), distantly related to green LHCI [3], acts as the light harvesting system of their monomeric PSI.

Photosynthesis allows plants, algae and some bacteria to transform CO_2, into organic matter, $(CH_2O)_n$, using solar energy. Light fluence and quality are not constant during the day and these organisms have developed different strategies to ensure an optimal energy capture. Their optimum photosynthesis is, in actual fact, a trade-off between a large absorption cross section to maximize the excitonic flux and a finely tuned excitation quenching to minimize the harmful effects of excess energy. In order to respond to an excess of incoming photon flux, green algae and plants have evolved different strategies whereby the excitonic flux to photosystems is tuned, and three different types of NPQ (for non-photochemical quenching) are commonly distinguished:

– qE is the dominant form of quenching that is associated with the acidification of the lumen [4]. It requires carotenoid deepooxidation via the Xanthophyll cycle [5,6] and the presence of PsbS [7] (or its homolog in unicellular green algae), a transmembrane protein. qE

Abbreviations: Chl, Chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; FR, far red; HA, hydroxylamine; LHC, light harvesting complex; NPQ, non-photochemical quenching; NSW, natural sea water; OCP, orange carotenoid protein; PBS, phycobilisome; PQ, plastoquinone; PS, photosystem; qE, energy-dependent quenching

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leads to the thermal dissipation of light energy. It is a fast relaxing process, a few minutes in the dark, concomitant with the relaxation of the light-built pH gradient.
- qT is the component related to state transition, a lateral displacement of light harvesting antennae along the membrane that contributes to balancing the excitonic flux between PSII and PSI [8]. It is known to be regulated by the redox state of the plastoquinone (PQ) pool and to involve the phosphorylation of the mobile antennae. The relaxation of qT quenching needs more time than qE, at least 20 min for the entire process.
- qI is the long term relaxing (several hours) component of NPQ [9,10].

The molecular mechanisms underlying NPQ in cyanobacteria and green species differ significantly. Cyanobacteria possess neither the enzymatic machinery required for the (de)phosphorylation identified in plants and green algae [11] nor the one involved in the xanthophyll cycle. Instead, the orange carotenoid protein (OCP) [12], a light-activated protein, bears a carotenoid and acts as an excitation and heat dissipator. In addition, state transitions occur on a much shorter time scale in cyanobacteria [11] although they also would be controlled by the redox state of the PQ pool. Yet instead of mobile membrane-bound LHCII antennae, the process would imply a migration of PBS on the external surface of thylakoids between PSII and PSI [11] and a change in spill-over between the two photosystems [13]. The occurrence of the latter regulation is supported, for instance, by the study of Mc Connell et al. who showed that, upon state transitions, the excitation energy absorbed by chlorophylls on the one hand, and by phycobilin pigments, on the other hand, is redistributed in an independent manner [14].

Some red algae live in the intertidal zone, which is a very dynamic environment. They have to cope with changing fluence and quality of light many times a day, depending on the tidal height, wave action, varying shading from other organisms and the cloud cover. However, the mechanisms related to the regulation of light capture and photoprotection in red algae are not yet fully understood (see [15,16] for reviews), especially the phenomenon known as NPQ, which has been widely described in green organisms [9,10].

According to their genomes [17,18], red algae do not possess any of the proteins characterized to date as being involved in NPQ in other photosynthetic species, like those involved in the xanthophyll cycle, PsbS, or OCP. However, studies have shown that NPQ does occur. In *Rhodella violacea* and *Porphyridium cruentum*, unicellular extremophile red algae, the maximum fluorescence yield undergoes a pronounced decrease upon illumination. In *R. violacea*, this quenching was interpreted as being triggered by the light-induced ΔpH [19,20] and independent of protein phosphorylation [21].

Ley & Butler showed, using absorption and fluorescence spectra of frozen cells, that, in *P. cruentum*, most of the energy absorbed by the PBS is transferred to PSII but that energy transfer occurs from PSII to PSI. They estimated that, in the open state (i.e. when the primary quinone electron acceptor in PSII is oxidized), about half of the energy is transferred to PSI. This fraction increases to near unity when PSII photochemical traps are closed [22]. In addition, they observed that preilluminating the cells with green light resulted in an increased photooxidation rate of P_{700} when compared to that observed in dark-adapted cells and that this specific photochemical enhancement was only observed when exciting PBS [23], suggesting that, although PBS is specifically connected to PSII, energy transfer may occur from PSII to PSI which requires a physical association between PSI and PSII [24]. This physical contact, controlled by a redox parameter would account for the modulation of the PSII fluorescence yield [25]. More recently, Yokono and coworkers, using time resolved fluorescence at −196 °C on *P. cruentum* and macroalgae, also suggested the occurrence of an energy spill-over between PSII and PSI [26], thereby accounting for the excitation quenching observed under more physiological conditions.

In this paper we reinvestigate the mechanisms of excitation quenching in red algae using the macroalga *C. crispus* as a model. As previously described in the case of the unicellular alga *R. violacea* [19,20] a single multi-turnover saturating pulse of light is sufficient to induce a strong quenching of fluorescence. To elucidate the mechanisms underlying this fluorescence quenching, we combined the room temperature and 77 K fluorescence measurements and absorption spectroscopy to monitor the redox state of the different electron carriers in the chain. We thereby characterized the dependence of this quenching on the illumination regime and analyzed the changes in the PSI and PSII antenna size induced by the quenching.

This leads us to propose that, along the lines of the model that emerged from the studies of *P. cruentum* (see above), on the one hand, the excitonic flux at the level of PSII is regulated by the extent of energy spill-over toward PSI and that, on the other hand, this spill-over is triggered by the reduction of the PQ pool.

2. Materials & methods

2.1. Biological material

Non-fertile gametophytes of *C. crispus* Stackhouse (Gigartinales, Rhodophyta) were collected near the port of Bloscon in Roscoff, Brittany (France) and were kept in a thermostated natural sea water (NSW) bath at 13 °C in 12 h/12 h light/dark regime under a light fluence of 20 μE m^{-2} s^{-1}.

Algae were dark adapted for 1 h before measurements. The time course of fluorescence changes was analyzed with a thallus cut to the size of the sample holder. During the experiments, the samples were kept in a small volume of NSW.

2.2. Functional analysis

Fluorescence and absorption changes were measured using a JTS spectrophotometer (Biologic) in which fluorescence is sampled using short (4 μs duration) flashes with negligible actinic effect. Fluorescence yield F_M is measured at the end of a 200 ms pulse of saturating red light that induced a full reduction of Q_A. If not stated otherwise, actinic green light was provided by a LED peaking at 520 nm (half band ~25 nm) that excites both chlorophyll and PBSs. Experiments were performed using green detecting flashes at 554 nm that excite preferentially the PBSs, except in Fig. 7 in which the kinetics of fluorescence changes were measured using detecting flashes at 554 and 420 nm.

F_0: fluorescence of a dark adapted (at least 1 h) material detected by a weak and short flash of green light. F_S: fluorescence measured right before a saturating pulse. F_M and F'_M: maximum fluorescence, measured immediately after a saturating pulse, respectively on a dark adapted and a preilluminated material.

P_{700} redox state was measured as changes in the absorbance at 705 nm (detecting flash 15 μs duration). P_{700} oxidation was induced by a far red (FR) actinic illumination provided by a LED peaking at 740 nm. The absorption corresponding to the fully oxidized P_{700} was measured by superimposing to the FR light a pulse of saturating red light (20 ms) that induces a full oxidation of P_{700}. The absorption changes associated with the oxidation of c-type cytochrome were measured at 555 nm. As described in more details in the text, the actinic light was provided by green light LED and Wratten filters # 16 and # 44A (see Fig. S1).

Fluorescence emission spectra were measured with a USB2000 Ocean Optics spectrophotometer. The disks of *C. crispus* thalli were in a metallic sample holder that was immerged in liquid nitrogen. The sample holder was connected to a Y-shaped light guide that conducts the excitation light to the sample and collected the emitted fluorescence. The exciting light was provided by a Schott KL 1500 lamp and the wavelengths selected using appropriate filters.

3. Results & discussion

3.1. A fluorescence collapse induced by a train of saturating light pulses

We first assessed the variation of the maximum fluorescence yield under conditions usually promoting photoprotective processes, i.e. high light conditions (k_i PSII ~ 1000 s^{-1}). As shown in Fig. 1, the fluorescence yield $(F'_M - F_0) / F_0$, measured after 200 ms pulses of saturating light, steadily decreased from 2.4 for the dark adapted state to 0.7 after a few minutes of illumination. Thus, as in *R. violacea* [19] fluorescence quenching processes develop in *C. crispus*. Notably, after ~200 s of illumination the difference between F'_M and F_S progressively increased, likely indicating the activation of the CO_2 assimilation cycle as described by [27].

A remarkable observation made by Delphin et al. [20] with *R. violacea* is that a mere train of light pulses, in the absence of any continuous illumination, is sufficient to induce a strong quenching. As shown in Fig. 1, the same applies to *C. crispus*. Actually, we observed that the pulse train induced an even more pronounced decrease of the $(F'_M - F_0) / F_0$ level than the superposition of pulses to a continuous light. Moreover, a single pulse of 200 ms duration (i.e. shorter than the 800 ms pulse used in [20]) was sufficient to promote a large fraction of this quenching as evidenced by the decrease of $(F'_M - F_0) / F_0$ observed upon the second pulse in the series (~1.25 to be compared to 2.4 for the dark adapted state). Finally, the overall time course of NPQ was similar in the presence or absence of the continuous exciting light. To our knowledge this is specific to red algae suggesting that the process governing the onset of NPQ in this part of the photosynthetic realm is unique. As a first step to characterize the origin of the quenching we compared its sensitivity to nigericin, a H^+/K^+ exchanger commonly used to dissipate transmembrane ΔpH. Fig. 2 shows the consequences of the addition of nigericin at two different concentrations, 7 and 30 μM, both being, to our experience, larger than the concentration required to reach saturation of the uncoupling effect in whole leaves from land-plants (2 μM). First we note that the $(F_M - F_0) / F_0$ measured upon the first pulse increased upon the addition of nigericin, showing that i) the dark adapted state is constitutively quenched to a certain extent, and ii) the extent of this quenching decreases upon the addition of nigericin. Since the hydrolysis of ATP by the chloroplast ATP synthase builds up a ΔpH in the dark [28,29], the effect of nigericin addition on the F_M of the dark-adapted state is consistent with the model proposed by Delphin et al. [19] according to which the quenching depends on the lumenal pH. Accordingly, the quenching observed after the first pulse, in the absence of nigericin, would stem from the light-induced increase of the lumenal pH, as proposed by Delphin et al. Yet, several pieces of data are not fully consistent with this framework. First, although the rationale for the fact that uncouplers relief a ΔpH induced quenching is that they promote a faster relaxation of the light-induced ΔpH, the remaining quenching observed after a series of pulses still relaxes slowly even at the highest nigericin concentration used as illustrated in Fig. 2 by the very moderate recovery of $(F'_M - F_0) / F_0$ after 30 s of relaxation in the dark. Second, a single pulse of 200 ms duration is sufficient to induce a pronounced quenching but yet seems unlikely to allow enough photochemical turnovers to promote a marked acidification of the lumen. Third, as shown in Fig. 3, which displays the development and relaxation of the quenching on longer time scales, the quenching had hardly relaxed after several

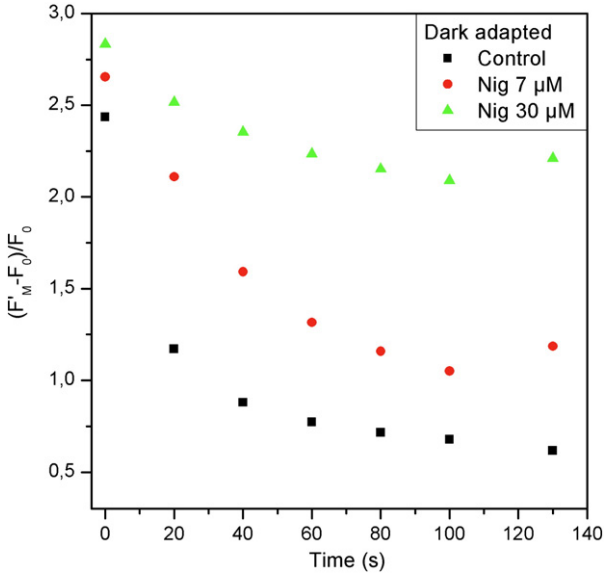

Fig. 2. Fluorescence changes $(F'_M - F_0) / F_0$ induced by a series of pulses in the presence or absence of nigericin (Nig). The concentrations of Nig were 7 μM (circles) and 30 μM (triangles). A thallus, dark adapted for more than 1 h, has been illuminated by a train of 6 pulses 20 s apart. At the end of the series, a last pulse is fired after 30 s of darkness. The 3 experiments have been performed using contiguous fragments of the same thallus.

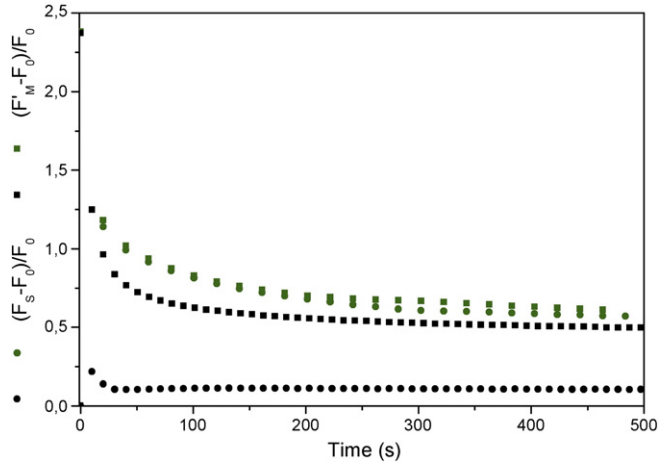

Fig. 1. Fluorescence changes measured on a thallus of *Chondrus crispus*. Green symbols: a thallus, dark adapted for more than 1 h, has been illuminated with green light (k_i PSII ~ 1000 s^{-1}) to which pulses of saturating light (200 ms duration) were superimposed allowing one to probe the F'_M values. Black symbols: the thallus, dark adapted for more than 1 h has been illuminated by a train of saturating pulses (0.1 Hz). The two experiments were performed using contiguous fragments of the same thallus.

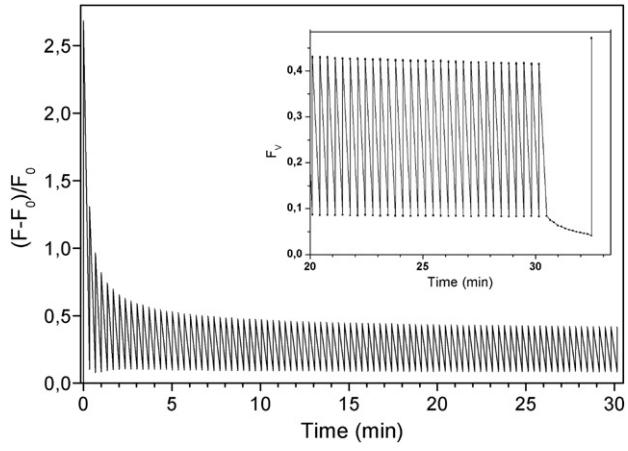

Fig. 3. Fluorescence changes measured on a dark adapted thallus illuminated for 30 min by a train of pulses. Pulse duration: 200 ms. Dark intervals between pulses: 20 s. The curve displays the fluorescence measured immediately before and after the pulse ($(F_S - F_0) / F_0$ and $(F'_M - F_0) / F_0$), respectively. The inset shows the last pulses of the series on an expanded time scale. At the end of the pulse series, the fluorescence decay is measured during the following 2 min of darkness. A last pulse is fired at the end of this period of darkness to probe the recovery of $(F'_M - F_0) / F_0$.

minutes of darkness following a 30 min illumination. We estimated the half-time of the relaxation of the quenching as ~15 min, and this makes it a much slower process than the relaxation of the light-induced ΔpH in land-plants [30]. Even though the life-time of the lumenal acidification is not known in red algae it is unlikely to be as long-lived as the quenching reported here, in as much as the building up of a light-induced proton motive force is expected to fully activate the ATP synthase [31,32] which, in turn, should result in a short-lived proton gradient [33]. We thus conclude that, in addition to the lumenal pH, another parameter likely contributes to determining the occurrence and the extent of the quenching in *C. crispus*.

As mentioned in the introduction, there are three types of hypothesis that one should consider to account for fluorescence quenching [34]: a change in the absorption cross section of PSII resulting from the migration of a mobile antenna from PSII to PSI, the formation of a quencher within the PSII photochemical units, and, finally, an increased connectivity between PSII and PSI photochemical units whereby excitation spills over to the photochemical trap of lowest energy i.e. PSI.

We first tested the hypothesis of a lateral migration of the PBSs which could, as proposed in cyanobacteria [35], be preferentially associated with PSII or PSI, depending on the redox state of the plastoquinone. One way to assess the occurrence of putative changes in the relative excitonic connectivity between PBSs and PSII or PSI is to specifically excite PBSs and to probe the relative extent of PSII and PSI fluorescences. As shown in the inset of Fig. 4, the low temperature fluorescence emission spectrum displays no contribution of PBS when excited in blue, as expected since PBS hardly absorb at this wavelength. Notably, the contribution of fluorescence emitted by PSII photochemical units when excited at 420 nm drastically decreased thus showing that this wavelength preferentially excites PSI, most presumably via LHCr, a membrane bound Chl containing complex that acts as the light harvesting system of monomeric PSI [24]. Conversely, when exciting at 554 nm, the low temperature emission spectrum clearly displayed the emission from PBSs, in the 650 nm region [24], and those ascribed to PSII at 685 and 695 nm [24,25]. We repeated the experiments shown in Fig. 1 where the quenching was induced by a series of saturating pulses (0.1 Hz)

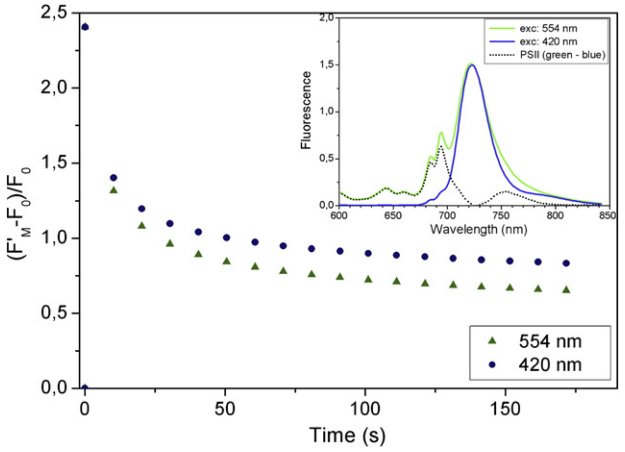

Fig. 4. Fluorescence changes induced by a series of pulses separated by 10 s of dark, measured using blue or green detecting flashes. Green symbols: the fluorescence yield was measured using detecting flash at 554 nm. Blue symbols: the fluorescence yield was measured using detecting flash at 420 nm. $(F'_M - F_0) / F_0$ ratio is lower under blue detecting flashes than under green detecting flashes excitation (1.8 and 2.49 respectively) owing to the larger contribution of PSI fluorescence in conditions where only Chl's are excited. Variable fluorescence $(F_M - F_0) / F_0$ measured using blue detecting flashes has been normalized to that obtained with green detecting flashes. Inset: fluorescence emission spectra measured at 77 K: a dark adapted thallus has been frozen in liquid nitrogen and the fluorescence emission spectra measured using green (green line) or blue (blue line) excitation. Fluorescence emission at 725 nm has been normalized for the two spectra. Dotted line: difference between the spectra obtained using green and blue excitation. This spectrum reflects the PBS + PSII emission spectra.

and the fluorescence was probed by weak flashes exciting preferentially either PBSs (554 nm) or LHCr (420 nm). As shown in Fig. 4, both antenna systems underwent a quenching of similar extent (see below for a discussion of the slightly more pronounced quenching when using green detecting light), thus showing that it does not specifically affect either one. This argues against a dramatic change in the connectivity between PBSs and PSII since, in this case, the quenching would be specifically observed when exciting in their absorption band, i.e. at 554 nm. We are thus, at this stage, left with two remaining types of hypothesis: the occurrence of spill-over or the light-induced formation of a quencher.

For energy spill-over to occur from PSII to PSI, the two photochemical units must be excitonically coupled. In this case, an exciton within the PSII antenna has a given probability to be transferred to – and trapped by – the PSI photochemical unit. This probability is expected to increase when PSII centers are unable to trap the incoming excitation flux owing to their reduced Q_A, so that an increase in the PSI antenna size occurring upon the reduction of Q_A is commonly considered to be the signature of energy spill-over. We thus measured the dependence of the PSI turnover rate on the redox state of Q_A, following the steps of Ley and Butler in their study of the excitonic connectivity between PSII and PSI photochemical units in the red alga *P. cruentum* [23]. To assess this rate, we measured, at room temperature, the extent of the oxidation of the electron donors to PSI induced by short and weak light pulses. We submitted the sample to a 200 to 400 μs weak pulse of actinic light to induce a limited number of PSI turnover. As a probe of this turnover number, we used the relative extent of cytochrome *f* and/or cytochrome c_6 oxidation 300 μs afterwards i.e. after completion of the oxidation phase of cyt *c* and *f*. As shown in the Supplementary data (Fig. S2) no significant re-reduction of cytochrome *f* occurred at this delay time. After normalization to the full oxidation extent, measured after a saturating actinic pulse, the fraction of cytochrome oxidized by the short and weak pulse did not exceed 10%. We checked that it was linearly related to the duration of the pulse, or in other terms a reliable measure of the number of PSI photochemical turnovers. These conditions being satisfied, the extent of cytochrome oxidation provides a measure that is proportional to the number of photons absorbed by the PSI photochemical units and thus, the light intensity being kept constant, to its absorption cross section. In order to assess whether PBS can transfer excitonic energy to PSI we used two different types of exciting light, hereafter denoted as A and B, as described in the Material & methods section. A-type pulses (in the 530–560 nm region, see the spectra in Fig. S1) preferentially excited PBSs whereas B-type pulses (in the 510–540 nm region) excites Chl, carotenoids and PBSs. We then compared the extent of cytochrome oxidation in the absence and presence of DCMU (30 μM) and hydroxylamine (3 mM) to induce the stable reduction of Q_A^- (see Table 1A). In both cases, nigericin was present in order to relax the quenching as much as possible (see above). With both the A and B type pulses, the fraction of oxidized cytochrome increased in the presence of Q_A^-, as expected in the spill-over hypothesis. Moreover, this increase was more pronounced when exciting more specifically PBSs (two fold for the A type

Table 1
Fraction of oxidized cyt c_{556} and cyt *f* induced by a short and weak pulse. A-type pulses (in the 530–560 nm region, see the spectra in Fig. S1) preferentially excited PBSs whereas B-type pulses (in the 510–540 nm) region excites Chl, carotenoids and PBSs.

	Pulse A	Pulse B	F_i/F_0	F_m/F_0
A				
Control + nigericin (30 μM)	4.91	5.45	1	3.81
DCMU + HA + nigericin (30 μM)	9.88	8.81	3.75	3.82
Ratio	2.01	1.61		
B				
Control dark-adapted	3.6	5.87	1	3.48
Control pre-illuminated	10.0	10.8	1.2	1.53
Ratio	2.78	1.83		

pulse with respect to 1.61 fold for the B type ones), as expected if the additional excitonic input observed when Q_A is reduced, stems from the excitonic coupling between PBSs and PSI. Altogether these data support the occurrence of a noticeable spill-over from PSII photochemical units to PSI ones, in good agreement with the conclusions reached by Ley and Butler with the unicellular red alga *P. cruentum* [22,23]. As put forward by these authors such an energy transfer may account for the rather unusually low F_M/F_0 ratio, 3.8, observed despite the addition of nigericin which, as shown above, relieves part of the quenching (even though the F_M/F_0 ratio observed in *P. cruentum* by Ley and Butler [22] was, 1. 85, i.e. significantly lower than reported here for *C. crispus*). The present conclusion that in dark-adapted *C. crispus*, a significant energy spill-over may occur from PSII to PSI and that it likely accounts for the fluorescence quenching is also consistent with the conclusion drawn by Yokono et al. [26] who studied the fluorescence emission spectra and fluorescence lifetimes in several macroscopic red algae. They also suggested that the extent of this energy spill-over would be linearly correlated to the relative absorbance of PBS with respect to that of Chl binding protein and this may account for the interspecies variability of the F_M/F_0 ratio mentioned above.

Although it makes this hypothesis more plausible, the occurrence of spill-over in the dark-adapted state does not necessarily imply that the light-induced quenching stems from an increased spill-over. To address this issue we compared the PSI absorption cross section in conditions of potentially minimal spill-over, i.e. oxidized Q_A and dark-adapted thalli and of potentially maximal spill-over, i.e. after induction of a strong quenching (F_M/F_0) by a train of 40 saturating pulses (Table 1B). In the latter conditions, the extent of cytochrome f oxidation was assessed 500 ms after the last saturating pulse in the series in order to allow for the complete relaxation of the primary and secondary PSI electron donors and acceptors. Importantly, 500 ms was not enough to allow for the complete reoxidation of Q_A (we estimate from the fluorescence yield changes that about 40% of Q_A^- was still reduced at this time so that 60% of PSII units were not promoting spill-over). The preillumination induced an increase of the PSI antenna size 2.78 and 1.76 folds with A and B type pulses, respectively. Thus, as described above, the increase in the PSI absorption cross section is more pronounced when exciting more specifically PBSs. In addition and even more importantly, the increase in the PSI absorption cross section is larger than in dark-adapted conditions (2.78 versus 2, and this even though Q_A is not fully reduced in the preilluminated case), as expected if indeed fluorescence quenching stems from spill-over. Thus, a significant fraction of the excitonic energy absorbed by PBSs is efficiently transferred to PSI and this efficiency increases when i) Q_A is reduced and ii) when fluorescence is quenched. These various characteristics are those expected in the spill-over model and, taking into account the fact that Q_A was reduced in only ~40% of PSII, one would expect that, when quenching is maximum and Q_A is fully reduced, the relative contribution of spill-over in the excitonic flux to PSI is $1.78/0.4 = 4.45$ fold larger than that of the PSI antenna (we note that, using the same relative units, when quenching is minimal and Q_A is fully reduced, the relative contribution of energy transfer from PSII to PSI would be equal to one). Our conclusions regarding *C. crispus* are thus consistent with those reached earlier by Ley and Butler with *P. cruentum*. Indeed, not only did they evidence the occurrence of a significant energy transfer from PSII to PSI photochemical units in dark adapted samples [22,23] but they showed that the extent of this energy spill-over can be modulated by exciting preferentially PSII (state II inducing light) or PSI (state I inducing light) [25]. The parameters that trigger these changes yet remain to be identified.

3.2. A fluorescence quenching controlled by the redox state of the plastoquinone pool

We have observed that, in *C. crispus*, a single saturating pulse is sufficient to promote a significant quenching as previously observed by Delphin et al. in *R. violacea* [20]. To gain further insights into the mechanism behind this process, we focused on the characterization of the quenching induced by a single saturating pulse. In the experiments described below (Fig. 5A and B) we applied a 200 ms light-pulse and probed its effect with several observables described below. One methodological difficulty in such an experiment is that the complete relaxation of the quenching induced by a pulse has to be reached before applying another pulse. Ley and Butler [25] reported that, in *P. cruentum*, energy spill-over, which, according to the results presented above, is responsible for most of the fluorescence quenching, decreases upon illumination with light exciting specifically PSI. We checked that this applies to *C. crispus* as well and that full relaxation of the quenching could be achieved by applying a weak continuous illumination of FR light ($\lambda \geq 720$ nm, 2 min), as illustrated for example in Fig. 5B. As pointed out in [25], this mere finding hints at the redox state of the electron carrier upstream of PSI, i.e., under all likelihood, the plastoquinones, including Q_A, being responsible for the modulation of the fluorescence yield.

We first analyzed the time course of the fluorescence quenching after the pulse. Fig. 5A shows the relative fluorescence yield measured just before $((F_S - F_0) / F_0$, blue symbols) and after $((F'_M - F_0)/F_0$, black symbols) a second saturating pulse applied at various delay time following the first one. As can be seen, these two parameters underwent a marked decrease in the first tens of seconds following the triggering pulse. The maximum of the quenching was observed at ~60 s and decreased as the time in the dark was further increased. As regard to $(F_S - F_0) / F_0$, it continuously decreased with increasing delays between pulses. $(F'_M - F_S)$ increased similarly indicating the progressive, but sluggish, reoxidation of Q_A. Yet, 4 min was not sufficient for F_S to reach the F_0 level, suggesting that the reoxidation of the plastoquinone pool in the dark is a very slow process.

As noted above, the finding that FR illumination promotes the reversion of the quenching, suggests that the extent of the latter is related to the redox state of the electron transfer chain upstream of PSI. To further characterize the kinetics of this FR induced recovery, we first submitted the sample to one saturating pulse. It was then kept in the dark for 60 s to allow the quenching to develop completely and then submitted to FR illumination of various durations. The variation of $(F'_M - F_0) / F_0$ as a function of this duration is shown in Fig. 5B (red symbols) and is compared to the evolution of the quenching induced by a single pulse (black symbols). The increase of $(F'_M - F_0) / F_0$ upon FR illumination first displayed a lag-phase, ~1 s, and then rose to reach the $(F_M - F_0) / F_0$ value with a half-time of ~5 s. A 5 fold increase in the FR intensity expectedly shortened the lag phase but did not significantly accelerate the relaxation of the quenching thus showing that it is kinetically limited by a non-photochemical process (not shown). As regard to the parameter(s) that trigger(s) the quenching, the observation that FR illumination enhances the relaxation of the quenching does not argue for a contribution of the lumenal pH. Indeed, the FR-induced turnovers of PSI likely contribute to building up – rather than dissipating – a transmembrane proton motive force. On the contrary it supports the hypothesis that the quenching is controlled by the redox state of plastoquinol or Q_A.

The critical role of the redox state of the plastoquinone pool is further illustrated in Fig. 6 that shows the kinetics of absorption changes associated with the redox changes of the chlorophyll dimer P_{700}, borne by PSI. These absorption changes were induced by FR illumination and the maximum signal was assessed by using a pulse of saturating light (see [36] and Materials & methods). Expectedly, we observed a bleaching, indicating the oxidation of P_{700}, which reached a steady state level of ~90% showing that the PSII to PSI excitation ratio is smaller than ~1/10 under these illumination conditions. We compared the kinetics of oxidation of P_{700} in the dark-adapted and quenched states. As above, the latter were induced by a single pulse and the oxidation kinetics of P_{700} were measured 1 min, a time where the quenching is

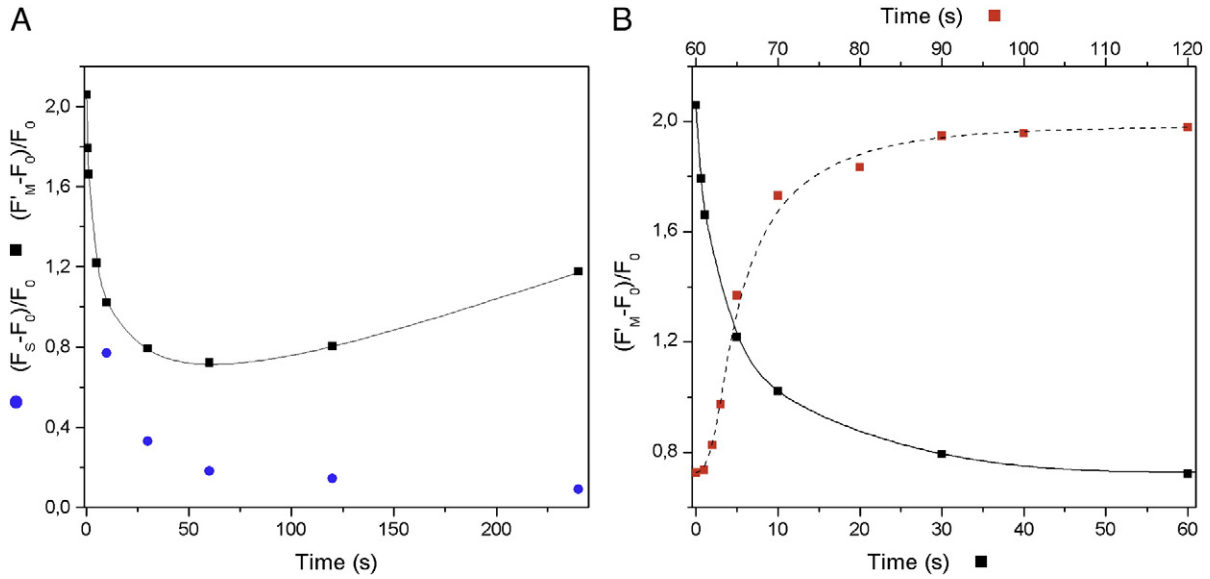

Fig. 5. Fluorescence yield measured as the function of the time of darkness following one saturating pulse. The thallus has been illuminated by a first pulse (200 ms duration) followed by a second pulse given at various time. A: $(F_S - F_0) / F_0$ (blue symbols) and $(F'_M - F_0) / F_0$ (black symbols) are plotted as a function of the dark time between the two pulses. At the end of each experiment, the thallus was illuminated by Far Red (100 μE m^2 s^{-1} for 1 min) in order to restore the F_M level (see text). B: black symbols $(F'_M - F_0) / F_0$: same as in Fig. 5A on an expanded time scale. Red symbols: the thallus has been first illuminated by one pulse. After 1 min of darkness the thallus was submitted to a FR illumination (100 μE m^2 s^{-1}) followed by a second pulse. $(F'_M - F_0) / F_0$ measured after the second pulse is plotted as a function of the duration of the FR illumination.

maximal, and 3 min after the pulse, to probe the redox state of the photosynthetic chain when the relaxation has started to develop.

The most insightful feature in this comparison is the lag-phase that precedes the oxidation of P_{700} 1 min after the pulse. As such, this lag shows that more PSI turnovers are required than in the dark-adapted case to reach a given oxidation state. This may either stem from the electron carrier upstream of P_{700} being more reduced or from a more efficient cyclic electron flow. We favor the former hypothesis for the two following main reasons: i) we have shown above that the fluorescence yield F_S measured 1 min after the pulse is larger than F_0 suggesting that a fraction of Q_A, and hence of the plastoquinone pool, is still reduced, ii) we observed here that, after the lag-phase, the oxidation time-course of P_{700} at 1 min had a similar slope than in the dark-adapted case. Contrary to this, a more efficient cyclic electron flow would, everything being the same, result in more efficient reduction competing with the oxidation and this would be manifested by a gentler slope in the oxidation phase (see [4,37] for a discussion). If the various absolute rates involved remain constant, the time required to reach the half-oxidation of P_{700} is proportional to the number of turnovers undergone by one PSI during this time laps. Roughly, it is two-fold larger 1 min after the pulse than in the dark adapted state. This shows that the overall pool of electron donor to PSI, including plastoquinone, is larger in the former case than in the latter. In addition, the fact that this results in the appearance of a lag-phase shows that the additional electron carriers have a more negative redox potential than those available in the dark-adapted state making plastoquinol good candidates. Assuming 6 plastoquinols, or 12 electron equivalents, in the pool, the 200 ms duration of the lag-phase translates into a maximal PSI turnover-rate of ~60 s^{-1}. The observation that the oxidation kinetics of P_{700} was similar 1 min and 3 min after the pulse shows that the reoxidation of the plastoquinone pool in the dark is very slow as already discussed above on the basis of the data shown in Figs. 1 and 2. This, again, contrasts with plants [38] or unicellular algae [39] where the Plastid Terminal Oxidase PTOX drives the reoxidation of the plastoquinones much more quickly.

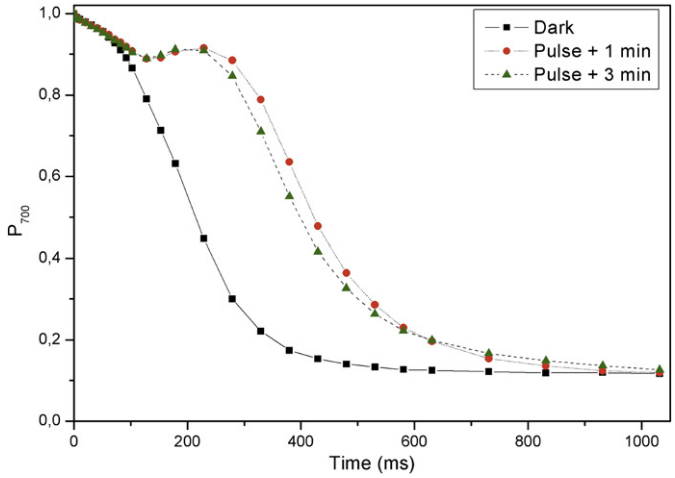

Fig. 6. The P_{700} redox changes upon a FR illumination. Intensity of far red illumination: 500 μE m^{-2} s^{-1}. Black symbols: dark adapted thallus. Red symbols: the thallus has been submitted to a 200 ms saturating pulse and, after 1 min of dark, submitted to FR illumination. Green symbols: the thallus has been submitted to a 200 ms saturating pulse and, after 3 min of darkness, submitted to FR illumination.

At this stage, we can tentatively propose that the quenching is directly controlled by the redox state of the plastoquinone pool and/or Q_A. To discriminate between these two candidates, we studied the extent of quenching as a function of the duration of the saturating pulse. Fig. 7 shows the variation of the fluorescence yield measured after one pulse of varying duration (black symbols). This corresponds to the so-called OJIP curve [40] and displays its characteristic feature: i) a fast initial rise developing in the 1–2 ms time range, which is assigned to the reduction of Q_A, ii) following this fast component, a further progressive fluorescence increase developing in the tens of ms time range and assigned to the reduction of the plastoquinone pool, and, lastly, iii) a slower component, in the 40–100 ms time range associated with the reduction of the PSI electron acceptors [41,42]. In Fig. 7, in green symbols, is also shown the maximum fluorescence yield measured 1 min after the first pulse, again as a function of the duration of this pulse. Although 2 ms of saturating light

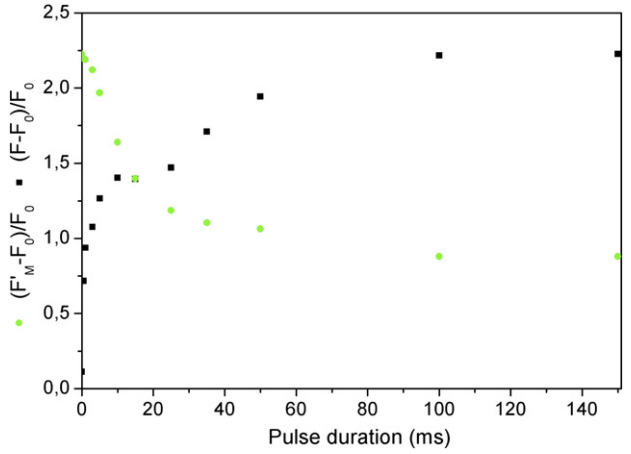

Fig. 7. Fluorescence changes as a function of the duration of the pulse of light. The sample was submitted to a pulse of varying duration. The fluorescence yield changes resulting from this illumination were probed immediately after this pulse and are depicted by the black symbols, $(F - F_0) / F_0$, as a function of the duration of the pulse. A second pulse of 250 ms duration of saturating light was applied 60 s afterwards, in order to probe the changes in F'_M, as a function of the duration of the first pulse (Green symbols: $(F'_M - F_0) / F_0$).

intensity is sufficiently long to reduce Q_A in all the PSII reaction centers (see [43] for example), we observed hardly any quenching after a 1 or 2 ms long pulse, showing that the reduction of Q_A alone is not sufficient to promote the quenching. Notably, the quenching had almost fully developed (~85%) after a 30 ms pulse and its increase paralleled in time the reduction of the plastoquinone pool. This suggests that the parameter triggering the quenching is the redox state of the plastoquinone pool rather than that of Q_A.

To further assess this conclusion, we attempted to tune the redox state of the plastoquinone pool in the dark. To this end we relied on the chlororespiratory chain [44–46] which involves a NADPH:plastoquinone oxidoreductase (NDH) on the one hand and a plastoquinol oxidase (PTOX) on the other hand. Under anoxic conditions, one expects the latter to be inhibited and thus the plastoquinone pool to be reduced in the dark owing to the activity of the former. We noted above that the long-lasting high fluorescence level, F_S, observed after a pulse was indicative of a sluggish PTOX activity. Despite this sluggishness the plastoquinone pool is mostly oxidized in dark-adapted thalli from *C. crispus* suggesting that the NDH activity is even lower than its PTOX chlororespiratory accomplice. Consistent with this, anoxic conditions did not induce any change in F_0 nor in F_M that would reflect the reduction of Q_A or of the plastoquinone pool in the dark. We thus resorted to a different strategy to achieve the reduction of the plastoquinone pool without soliciting PSII. It has been reported that, in land-plants, illumination enhances the chlororespiratory influx of electrons into the plastoquinone pool [47,48]. Even though the molecular rationale for this observation is presently unknown, we reasoned that the same may apply to *C. crispus*. We thus first imposed anoxic conditions by flushing argon and then illuminated the sample by a weak FR light. Expectedly, this protocol maintained the plastoquinone pool in its oxidized state in the light as checked by the time course of P_{700} redox changes. Accordingly, when probed 1 s after the end of the FR preillumination, F'_M was equal to F_M in aerobic conditions and only slightly lower in anoxic ones (see Table 2). Consistent with the empirical assumption that the chlororespiratory influx is enhanced by the FR illumination, the F'_M value was lower when probed 60 s after the preillumination and this applied both in aerobic and anoxic conditions, although to a smaller extent in the former case than in the latter. In aerobic conditions this decrease in the F'_M value reversed 240 s after the end of the preillumination, likely owing to the PTOX activity. Contrary to this, the quenching continued to develop in anoxic conditions. In addition, 240 s after the end of the preillumination, the oxidation

Table 2
$(F'_M - F_0) / F_0$ measured in aerobiosis and anaerobiosis.
For anoxic condition. the thallus has been incubated for more than 1 h in anaerobiosis.

$F'_M - F_0 / F_0$ in *Chondrus crispus* in aerobiosis and anaerobiosis.		
Time after 10 min of Far Red illumination	Aerobiosis	Anoxic
1 s	2.18	1.92
60 s	1.88	0.99
240 s	2.15	0.77

time course of P_{700} showed, in anoxic conditions, the signature described above of a fully reduced plastoquinone pool. Hence the quenching develops upon the reduction of the plastoquinone pool even when neither PSII nor PSI turns over. Moreover the fact that, in anoxic conditions, it develops in the dark (or at least in the dark period following the FR illumination) argues against any triggering event that would relax rather than to develop in the dark, such as a transmembrane ΔpH.

Even though the present results point to the redox state as being prominent among the parameters that control the fluorescence quenching, one cannot ignore the simple fact that the addition of nigericin decreases the extent of the quenching. This delicate duet played by the redox state of the intersystem electron carriers and the lumenal pH is further illustrated in Fig. S3 that displays again the experiment presented in Fig. 2 now followed by an FR illumination. Dark-adapted thalli were first exposed to a series of 6 pulses in the presence of nigericin (7 μM). As described above, this induced a quenching of smaller extent than in the absence of uncoupler. As discussed above, Far Red (180 s) efficiently enhanced the relaxation of the quenching but an additional series of 6 pulses given to this far red preilluminated thallus induced a quenching of larger extent than in the dark-adapted case. Thus far red seems to have opposite effects on the quenching since it accelerates not only its relaxation but also its onset when pulses are superimposed. These two opposite effects can be reconciled assuming that Far red illumination induces on the one hand the reoxidation of the plastoquinone pool and this is sufficient to relieve the quenching, but in addition it acidifies the lumen by promoting PSI turnovers and hence facilitates the onset of the quenching when the plastoquinone pool is reduced by the first pulse in the second series. In summary, spill-over would require both a reduced plastoquinone pool and a low lumenal pH but the former would prevail over the latter.

4. Conclusion

At present, there is no consensus for the mechanism of state transitions in phycobilisome-containing cyanobacteria (for review, [49,50]) but the physical displacement of PBS through lateral diffusion is considered as the main mechanism accounting for the light-induced decrease in the PSII fluorescence yield [51]. In contrast, several studies conducted with red algae rather support energy spill-over from PSII to PSI, as a major source of fluorescence quenching [19,23] This is in agreement with Fluorescence Recovery After Photobleaching studies conducted with *P. cruentum* that did not support the occurrence of a large scale diffusion of PBSs in red algae [52]. As will be discussed below, in an effort to build a unified mechanistic view from several experimental observations that arose from our study of the light-induced quenching in dark-adapted thalli of *C. crispus*, we acknowledge that the extent of quenching shows a dependency on the lumenal pH but is caused by an increased sensitization of PSI by PBS –mostly through changes in spill-over – under the redox control of the PQ pool.

We argued that the two processes for regulating the extent of PSI sensitization by a PSII peripheral antenna – that is spill-over and direct change in PSI sensitization by PBS in phycobilisome-containing organisms – are commonly discriminated by comparing the extent

of the excitation quenching when probed with wavelengths preferentially absorbed by PBS or the chlorophyll antennae. This rationale has been followed as well in [14], in their study of the regulation of energy transfer in cyanobacteria. Importantly, we observed, as they did, that the quenching induced by a series of pulses is systematically slightly larger when probed in the PBS absorption wavelength range than in the Chl one (see Fig. 4). As proposed by McConnell et al. this finding supports the view that, under conditions promoting spill-over, a small fraction of the energy absorbed by PBS would by-pass PSII and be directly transferred to PSI. We thus confirmed that this direct change in PBS/PSI connection contributes for a minor part to the light-induced quenching in *C. crispus*. However based on the dependence of the PSI turnover rate on the redox state of Q_A we have argued that, in line with the conclusions drawn by Ley and Butler from their studies of *P. cruentum*, and more recently from studies with multicellular red algae [26], there is already an excitonic coupling between PBS and PSI due to significant energy spill-over in dark-adapted thalli. We further demonstrated that this spill-over increases with the light-induced quenching in *C. crispus*. The increase of this excitonic connectivity allowing direct and indirect energy transfer between PBS and PSI also has been proposed to account for light-induced changes in fluorescence yield in unicellular red algae [25,53] and in cyanobacteria such as *Synechococcus sp.* PCC 7002 and *Synechocystis sp.* PCC 6803 [14].

We have described above that the quenching, which we interpret as resulting from an energy transfer from the closed PSII traps to PSI, displays highly multiphasic kinetics. It involves first a quickly developing component that accounts for half of the total quenching and is followed by slower components that are not fully completed after 30 min of illumination. At least two conditions must be fulfilled for spill-over to occur efficiently: firstly, the PSII photochemical traps must be closed and, secondly, a physical contact between the PSII and PSI photochemical units must exist to allow the excitonic coupling of neighboring pigments. According to Förster theory [54,55], one of the parameters that determine the efficiency of the energy transfer is their distance. Thus, if one assumes that the distance separating a given PSII unit from a given PSI unit is the main parameter determining the time required for them to be in physical contact, then the multiple kinetic components would merely reflect the large distribution of distances between PSII and PSI photochemical units. Alternatively, one may assume that the distance between PSII and PSI units is homogenously distributed, in which case the multiple kinetic components would reflect the heterogeneity of the rate of spill-over promotion. Imaging techniques suggests that PBSs are aligned along the membrane [56] thereby providing support to the spatially homogenous but kinetically heterogeneous hypothesis.

Our data support the idea that the light-induced quenching is mainly controlled by the redox state of plastoquinone, as proposed previously by Ley and Butler in *P. cruentum* [25]. It remains however that nigericin and other proton carriers relieve part of this quenching, an observation that is consistent with those made by Delphin et al. that led them to interpret this quenching as being controlled by the lumenal pH [20]. These apparently conflicting conclusions can be reconciled assuming that the physical contact that, as just discussed, is required for spill-over can be tuned by electrostatics effects involving protonable residues borne by the PSII and PSI protein complexes: at high pH, the unprotonated state(s) would loosen the interactions between PSI and PSII units whereas at lower pH, these would be promoted. This would account for the observation that the extent of quenching induced by a series of saturating pulses is decreased upon the addition of nigericin (Fig. 2). It would also account for the fact that addition of nigericin results in an increase of $(F_M - F_0) / F_0$ ratio. Indeed in the dark, the hydrolysis of mitochondrial ATP generates a ΔpH [29]. In *Chlamydomonas*, the lumenal pH thereby sustained in the dark has been estimated to be ~5.6 [28] and it is, expectedly, larger in the presence of nigericin (~7.2). In the present model the triggering parameter would thus be the reduction of the PQ pool but the extent of the quenching would depend on the lumenal pH as this would contribute to loosening or tightening the connectivity between PSII and PSI units. Finally we emphasize that, according to the present model, the event that triggers the onset of spill-over is the reduction of the PQ pool indeed. This is reminiscent of the lateral redistribution of the LHCII antenna during the state 1–state 2 transition seen in green algae [57–59]. Yet, state transitions imply a cascade of biochemical reactions, such as antenna phosphorylation, which requires several minutes to reach completion and kinetically control the quenching [58]. On the contrary, upon oxidation of the PQ pool in red algae induced by a strong FR illumination the spill-over efficiency decreases much more quickly which argues for the spill-over efficiency being controlled by a totally different mechanism that yet remains to be elucidated.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbabio.2013.04.004.

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