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Rapid report

Comparison of state 1–state 2 transitions in the green alga *Chlamydomonas reinhardtii* and in the red alga *Rhodella violacea*: effect of kinase and phosphatase inhibitors

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Abstract

In the green alga *Chlamydomonas reinhardtii*, state transitions occur upon phosphorylation of the light-harvesting complex. The protein kinase inhibitor staurosporine, and the phosphoprotein phosphatase inhibitor NaF, suppress state 2 and state 1 transitions, respectively. By contrast, in the red alga *Rhodella violacea* none of these inhibitors has any effect, suggesting that, in red algae, the mechanisms of redistribution of excitation energy are independent of protein phosphorylation.

Keywords: Red alga; Kinase inhibitor; Phosphatase inhibitor; State transition

Several chloroplast phosphoproteins involved in photosynthesis have been described by Bennett [1]. This author subsequently demonstrated the physiological role of membrane phosphorylations in regulating the distribution of absorbed energy between the two photosystems (PS) in pea chloroplasts [2,3]. This regulation, namely state transitions, was initially studied in the green alga *Chlorella* by Bonaventura and Myers [4]. State transitions occur when incident light drives one photosystem momentarily faster than the other, each having a distinct pigment system with specific absorption characteristics. To reach an optimal photosynthesis rate, higher plants and green algae are able to redistribute absorbed excitation energy and thereby balance Photosystem I and II turnovers: state 1 is observed when light is absorbed predominantly by the PS I antenna and the light absorbed preferentially by PS II antenna leads to state 2 [5]. Bennett et al. [3] have demonstrated that the reversible phosphorylation of LHC II is accompanied by changes in chlorophyll fluorescence yield. The fluorescence emission related to PS II is quenched in state 2 as compared to state 1. During the transition from state 1 to state 2, the phosphorylated part of the LHC II becomes dissociated from PS II, moves away from PS II and supplies excitation energy to PS I [6–9]. The LHC II-kinase is regulated by the redox state of the plastoquinone pool: its reduction leads to kinase activation and its oxidation to kinase inactivation [2,10–12]. The LHCII-phosphatase is continuously active [11].

In red algae and cyanobacteria, fluorescence changes related to state transitions were also observed [13–16]. However, the PS II antenna of these organisms is a large extramembrane-complex of phycobiliproteins (phycobilisome) and therefore, it is very different from the LHC [17]. This suggests that the mechanism of state transitions is likely to be somewhat different between plants and phycobilisome-containing organisms. The possible involvement of a reversible phosphorylation event was initially proposed by Allen et al. [18]. These authors observed the phosphorylation of several polypeptides in vitro under state 2 light conditions in *Synecoccochus* 6301. They proposed a model based on the mechanism of state transitions known for green plants: the phycobilisomes become dissociated from PS II when a polypeptide from the phycobilisome and another from the membrane are phosphorylated. However, subsequent studies [19–21] did not confirm this hypothesis. The results of Biggins et al. [22] were different from those described by Allen and its co-workers: in *Porphyridium cruentum*, a red alga, Biggins et al. [22]

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did not detect any change in protein phosphorylation during the conversion of cells between state 1, state 2 and darkness.

To determine whether a kinase-phosphatase system is involved in state transitions in red algae, we have tested the action of a protein kinase inhibitor, staurosporine, and a protein phosphatase inhibitor, NaF, on state 1–state 2 transition and on state 2–state 1 transition, respectively, in vivo, in an unicellular marine red alga, *R. violacea*. As a control, we have studied the effect of these inhibitors on state transitions in the green alga *C. reinhardtii*, in which the involvement of protein phosphorylations has already been demonstrated [23].

R. violacea and *C. reinhardtii* were grown as described in [23] and in [24] respectively, at 20°C under 40 μE m^{-2} s^{-1} white light, with a 16 h light/8 h dark photoperiod. Exponential phase cells were collected from 3- to 4-day-old cultures of *R. violacea* and from 1- to 2-day-old cultures of *C. reinhardtii*. We characterized the 77 K fluorescence emission spectra of the two types of cell on a Hitachi F-3010 Fluorescence Spectrophotometer. For all the experiments, 4 ml of cell suspension were submitted to the different conditions described below. Then, 150 μl (*R. violacea*) or 200 μl (*C. reinhardtii*) were quickly filtered, the filters were immediately frozen in liquid nitrogen and fluorescence emission spectra were run.

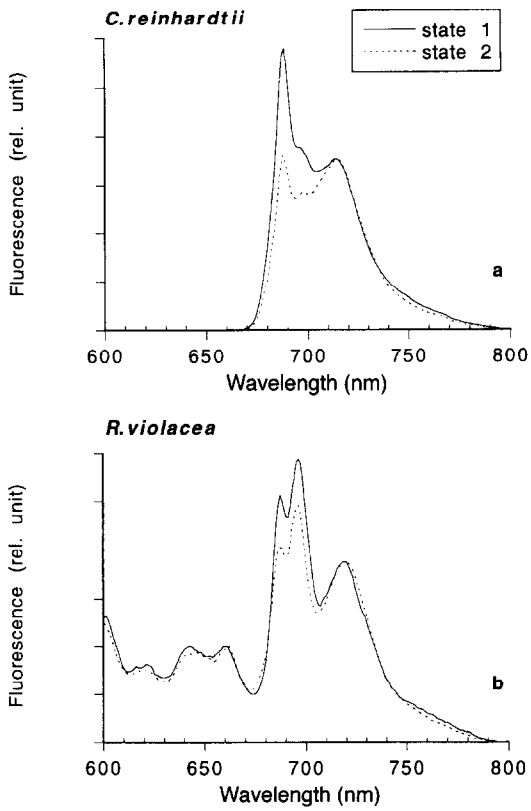

Fig. 1. 77 K emission spectra of *C. reinhardtii* (a) and *R. violacea* (b) in state 1 (solid line) and state 2 (dashed line). The excitation wavelength was 440 nm for (a) and 560 nm for (b), 3 nm slit width. Spectra were normalized to the PS I related peak. For other details, refer to the text.

Table 1
State 1 and State 2 conditions and the resulting fluorescence emission ratio *F*(PS II)/*F*(PS I), in *C. reinhardtii* and *R. violacea*

	Treatment	State	*F*(PS II)/*F*(PS I)
C. reinhardtii	Far-red light, 10 min	State 1	1.63
	Dark/anaerobic conditions, 15 min	State 2	1.01
R. violacea	Far-red light, 5 min	State1	1.60
	Green light, 5 min	State 2	1.30

This ratio is calculated from the height of the peaks from the spectra shown in Fig 1.

Fig. 1 shows the 77 K fluorescence emission spectra of *C. reinhardtii* (a) and *R. violacea* (b) cells in state 1 (solid line) and state 2 (dashed line) conditions. In *C. reinhardtii*, fluorescence emission around 685 nm and at 695 nm are related to the light-harvesting complex (LHC II) and to the core antenna of PS II, respectively [25]. The PS I related peak rises at 715 nm [25]. In *R. violacea*, the peaks at 685 nm and 695 nm are also related to PS II. The first peak derives from the phycobilisome terminal emitter and the second from the Chl *a* core-antenna of PS II [26]. The third main peak, observed at 720 nm, is due to the Chl *a* antenna of PS I [27]. In addition, *R. violacea* shows fluorescence emission from phycobiliproteins: phycocyanin at 643 nm and allophycocyanin at 661 nm [26]. The spectra are arbitrarily normalized to the PS I related peak. State transitions, thus, appear as a relative decrease (state 2) or increase (state 1) of the fluorescence emission related to PS II. The fluorescence emission ratios *F*(PS II)/*F*(PS I) (*F*688/*F*715 for *C. reinhardtii* and *F*695/*F*720 for *R. violacea*), which were calculated from the spectra, as the ratio between the height of the peaks, are presented in Table 1.

Transition to state 2 is obtained in *C. reinhardtii* by reducing the plastoquinone pool [23]. This can be achieved by an anaerobic treatment. We thus adapted cells to darkness under anaerobic conditions for 15 min, as described by Bulté et al. [28]. In these conditions, the peaks related to PS II were at their lowest level (relative to *F*715). On the other hand, 10 min of far-red light (> 720 nm) illumination [23,28] allowed to bring *C. reinhardtii* cells to state 1. In this state, cells were characterized by a high fluorescence emission from PS II Chl *a* (Fig. 1a). The fluorescence emission spectra of *R. violacea* cells are presented in Fig. 1b. State 1 (high PS II fluorescence) was established with 5 min far-red (> 720 nm) illumination, far-red light being preferentially absorbed by Chl *a* PS I [29]. A 5 min incubation in green light (550 nm), predominantly absorbed by phycobilisomes [29], brought *R. violacea* cells to state 2 (low PS II fluorescence).

The conditions used for the treatments with inhibitors of kinases and phosphatases were first determined in *C. reinhardtii*. It had already been demonstrated that the LHC II complex is phosphorylated in a Thr residue during the

Table 2
Effect of the protein phosphatase inhibitor, NaF, on the state 2-state 1 transition: comparison of the F(PS II)/F(PS I) ratio in presence and absence of NaF, in *C. reinhardtii* and *R. violacea*

	Treatment	State	F(PS II)/ F(PS I)
C. reinhardtii	Dark/anaerobic conditions, 15 min + NaF, 60 min	State 2	1.01
	Dark/anaerobic conditions, 15 min + NaF, 60 min → far-red light, 10 min	State 2	1.06
	Dark/anaerobic conditions, 15 min-NaF → far-red light, 10 min	State 1	1.61
R. violacea	Green light, 5 min + NaF, 60 min	State 2	1.27
	Green light, 5 min + NaF, 60 min → far-red light, 5 min	State 1	1.60
	Green light, 5 min-Naf → far-red light, 5 min	State 1	1.65

Ratio calculated as in Table 1, from spectra not shown.

transition to state 2 [23]. Up to now, the LHC II kinase was indirectly inhibited by oxidation of the plastoquinone pool in the presence of DCMU [30,31]. We decided to directly prevent protein kinase activity by treating cells with staurosporine, a non-specific inhibitor of Ser/Thr and Tyr protein kinases. Staurosporine is an indole carbazole, derived from certain fungi, which interacts at the ATP-binding site of protein kinases [32]. We determined that the time of incubation required to suppress state 2 transition in *C. reinhardtii* was 90 min with 100 nM of staurosporine. Conversely, the transition to state 1 is induced by the dephosphorylation of LHC II [23]. The action of the phosphoprotein phosphatase inhibitor, NaF, has already been characterized. NaF was shown to block green algae, plant cells and thylakoids in state 2 [2,31,33–35]. Indeed, we observed that the state 2 to state 1 transition is prevented in *C. reinhardtii* after at least 60 min incubation with 100 mM NaF (data not shown).

C. reinhardtii cells were adapted to state 2 (low $F688/F715$ ratio) by a 15 min incubation in darkness under anaerobic conditions. After transfer to far-red light for 10 min, *C. reinhardtii* cells were brought to state 1. However, when cells were kept 60 min in presence of NaF under state 2 conditions and then transferred to conditions required for obtaining a state 1 transition, they remained in state 2 (Table 2). Shorter incubations in the presence of NaF led to partial inhibition of the state transition. Alternatively, cells adapted to state 1 and then incubated in darkness under anaerobic conditions showed a transition to state 2. In contrast, when cells were adapted to state 1 in presence of staurosporine as described above, they remained in this state after subsequent transfer to state 2 conditions (Fig. 2a and Table 3).

The results in Tables 2 and 3 show that state transitions were inhibited neither by NaF nor by staurosporine in *R. violacea*. Cells were in state 2 when submitted to green light. After 60 min in the presence of NaF they were transferred to far-red light for 5 min leading to state 1, similarly to cells illuminated directly with far-red light with no treatment, thus indicating that NaF had no effect on state 1 transition (Table 2). Higher concentration of the inhibitor (200 mM) or a longer incubation time (2 h) could not prevent the conversion of cells to state 1 (data not shown). Alternatively, when state 1 transition was established by a 5 min exposure to far-red light, ulterior transition towards state 2 occurred under green light in the absence or presence of staurosporine (Fig. 2b and Table 3). The inhibitor concentration was increased 10-times with no additional effect on the state 2 transition (data not shown).

The present study was carried out to determine whether a phosphorylation event is involved in state transitions in phycobilisome-containing organisms, since this possibility remains controversial (for review, see [36]). To our knowledge, only one report has been published about a possible involvement of a kinase-phosphatase system in red algae.

Table 3
Effect of the protein kinase inhibitor, staurosporine, on the state 1–state 2 transition: comparison of the F(PS II)/F(PS I) ratio in presence and absence of staurosporine, in *C. reinhardtii* and *R. violacea*

	Treatment	State	F(PS II)/ F(PS I)
C. reinhardtii	Far-red light, 10 min + staurosporine	State 1	1.60
	Far-red light, 10 min + staurosporine → dark/anaerobic conditions, 15 min	State 1	1.55
	Far-red light, 10 min − staurosporine → dark/anaerobic conditions, 15 min	State 2	1.06
R. violacea	Far-red light, 5 min + staurosporine	State 1	1.64
	Far-red light, 5 min + staurosporine → green light, 5 min	State 2	1.28
	Far-red light, 5 min − staurosporine → green light, 5 min	State 2	1.27

Ratio calculated as in Table 1, from spectra shown in Fig 2.

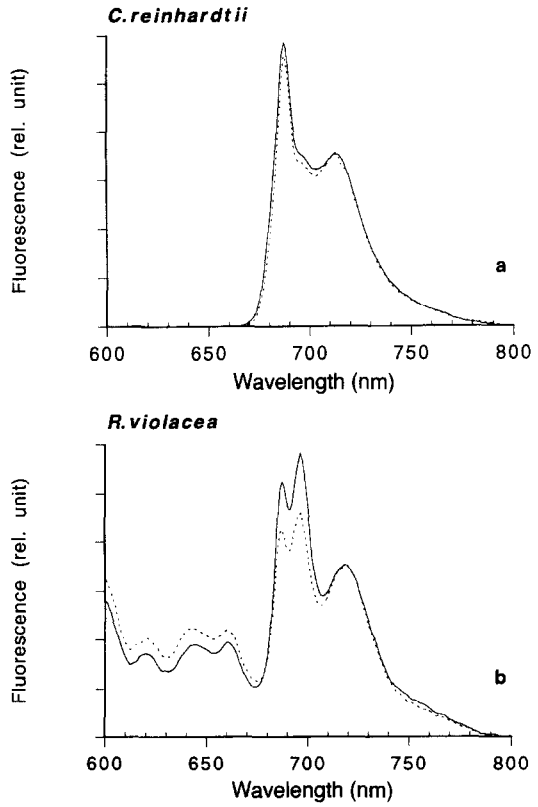

Fig. 2. Effect of the inhibitor of protein kinase staurosporine, on the state 1–state 2 transition in *C. reinhardtii* (a) and *R. violacea* (b). Solid line: cells adapted to the requested state 1 conditions and incubated for 90 min with staurosporine. Dashed line: the above cells transferred to state 2, still in presence of staurosporine. The excitation wavelength was 440 nm for (a) and 560 nm for (b), 3 nm slit width. For other details, refer to the text.

Biggins et al. [22] analyzed the phosphorylation of polypeptides in *Porphyridium*, in cells incubated for several hours in a medium containing $[^{32}P]P_i$, and converted to state 1 or state 2. Comparison of the pattern of labelled proteins isolated from state 1 and state 2 samples did not reveal any difference in phosphorylation. Similar results were obtained with the cyanobacteria *Synechococcus* 6301 [22] and *Calothrix* 7601 [37] and with *Prochloron* [37]. In these organisms, labelling of proteins with $[^{32}P]P_i$, in vivo and in vitro, suggested that protein kinases were present in thylakoids. However, the labelling was light-independent. By contrast, Allen et al. [18] observed, in vitro in *Synecococchus* 6301, a light-dependent phosphorylation of several polypeptides, with most obvious labelling of a 18.5 kDa and a 15 kDa protein. The 18.5 kDa protein mainly present in the soluble fraction was identified as a phycobilisome polypeptide, whereas the 15 kDa protein was found exclusively in the thylakoid fraction. However, in subsequent work, Sanders and Allen [19] could not observe the dephosphorylation of the 15 kDa protein in the dark. In addition, Harrison et al. [20] showed that phosphorylation and dephosphorylation of the 18.5 kDa protein did not follow the changes in fluorescence related to state transitions.

In this work, we have studied the effect of protein kinase and phosphatase inhibitors on low temperature fluorescence spectra to answer the open question about phosphorylation involvement in state transitions, in red algae. We have demonstrated that, in the presence of NaF or staurosporine, state transitions took place in *R. violacea*, as indicated by variations in the emission ratio F(PS II)/F(PS I), while in *C. reinhardtii* cells submitted to similar treatments, state transitions, that are known to depend on protein phosphorylations, were abolished. In addition, McCormac et al. [31] have recently shown that, in vivo, in whole leaf, NaF suppresses dephosphorylation and fluorescence changes related to state 1 transition, while the presence of DCMU inhibits the LHC II-kinase activity and triggered the transition to state 1. We have then concluded that the mechanisms leading to state transitions in red algae are different from those governing state transitions in green plants and algae, and that protein phosphorylations are not involved in the former organism.

In this study, the protein kinase activity was directly inhibited by the inhibitor of protein kinases, staurosporine. In previous publications, the kinase activity was abolished by oxidizing the plastoquinone pool since the LHC II kinase is regulated by the redox state of the plastoquinone pool. We showed that staurosporine can be used to directly inhibit the LHC II kinase. This compound, originally used in animal cells, can also prove to be useful to reveal other protein phosphorylations in plant cells.

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