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Photoacoustic detection of oxygen evolution and State 1–State 2 transitions in cyanobacteria

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Photoacoustic detection of modulated oxygen evolution was obtained from algae in vivo. In the cyanobacterium *Nostoc muscorum*, during State 1 to State 2 transition, the effective absorption cross-section of PS II measured at 580 and 620 nm, decreased by 10–15% concomitant with an equal increase in the cross-section of Photosystem I. Upon incubation of *Nostoc* cells with a specific inhibitor of phosphatases (NaF) which blocks dephosphorylation of proteins, State 1 was abolished and only State 2 could be observed. These results suggest that in organisms containing phycobiliproteins as efficient antennae and which are missing both light-harvesting chlorophyll *a*/*b* protein complex and grana thylakoids the level of protein phosphorylation controls the distribution of excitation energy between the two photosystems.

Introduction

Photoacoustics has been used in recent years to measure photosynthetic energy storage and oxygen evolution in leaves and lichens [1,2]. The ability to monitor in a whole organism the enhancement of the quantum yield of modulated oxygen evolution upon addition of PS I background light is of interest because it can provide information about the in vivo interactions between the two photosystems and about their molecular organization [3–5]. A striking case is the regulation of electron transport in response to varying light spectral composition at light-limiting conditions (State 1–State 2 transitions). In green plants, control of distribution of absorbed excitation energy is achieved by reversible phosphorylation of LHC polypeptides and their migration and reversible association with either PS II or PS I resulting in changing the absorption cross-section of each photosystem [3–12]. In cyanobacteria and red algae containing phycobilisomes [13] instead of LHC, the mechanism of State–State 2 transitions is still unknown and appears to be controversial. It was previously reported that State 1–State 2 transitions do not involve a change in protein phosphorylation level [14] and result from a change in the extent of energy transfer between PS II and PS I [15,16]. In a contrary report, light-dependent phosphorylation of several polypeptides was demonstrated; however, the transitions were obtained only between light and dark states [17]. Most studies on State 1–State 2 transitions in cyanobacteria and red algae [14–19] employed fluorescence method which can be influenced by unrelated effects (e.g., Kautsky transitions).

Abbreviations: PS I and PS II, Photosystem I and II; LHC, light-harvesting chlorophyll *a*/*b* protein complex; Chl, chlorophyll.

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Up to now, the photoacoustic signal resulting from modulated oxygen evolution in free living algae has eluded detection. In algal suspension, only one photoacoustic component was observed [20] the photothermal signal, generated by conversion of absorbed modulated light into modulated heat. Therefore, I searched for conditions in which modulated oxygen evolution could be monitored in algae, photoacoustically, in order to examine the mechanism of light distribution between the two photosystems in cyanobacteria.

In this work, I report the first observation of a photoacoustic signal generated by modulated oxygen evolution in algae. In order to try and elucidate the mechanism of light state transitions in blue green algae, I analysed the Emerson enhancement of oxygen evolution and determined quantitatively the distribution of excitation energy in each state, showing that during a transition from State 1 to State 2 a decrease of at least 10–15% in the rate of excitation delivery to PS II was matched by a corresponding gain in PS I. I found that in the presence of NaF, a specific inhibitor of phosphatases, an optimal State 2 was achieved while the transition to State 1 was blocked. It is suggested that changes in the distribution of excitation energy during State 1–State 2 transitions in blue green algae (cyanobacteria) containing phycobiliproteins are dependent on the level of protein phosphorylation.

Materials and Methods

Nostoc muscorum no. 7119 was grown in a growth medium [21] at 26 °C at a light intensity of 15 W/m^2. Cells were harvested by centrifugation at 4 °C for 10 mins at $4000 \times g$. The resuspended pellet was passed through a 1 cm diameter (Whatman's no. 1) filter paper. Paper discs covered with 17 mg algal wet weight corresponding to a layer of 200 μm at the concentration of 20 μg Chl/cm^2, were used. In some experiments, only 0.04 mg algal wet weight was layered on a filter paper corresponding to a thickness of 0.5 μm. Algal samples were inserted into the photoacoustic cavity connected to a microphone which has been previously depicted in Ref. 1.

The photoacoustic set-up consisted of a light source (xenon, 450 W, d.c.). Light was focused into a monochromator (Bausch and Lomb, 10 nm bandpass) and modulated with a chopper (Laser precision). Background nonmodulated light was obtained from a d.c. operated projector with either a wide-band (400–680 nm) light of photosynthetically saturating intensity for cyanobacteria (60 W/m^2), or with a far-red (710 nm) interference filter (Ditric optics) which was used as a PS I light for the State 1–State 2 transitions. The modulated and bakcground lights were combined by using a double-branched light guide and focused on the sample in the photoacoustic cell. The photoacoustic signal was analysed by a lock-in amplifier in the two-phase mode, allowing simultaneous recording of the in-phase and quadrature (90° out-of-phase) components of the signal [1].

The photoacoustic signal can be described as a vector quantity. It is the sum of two components arising from modulated heat (photothermal signal) and modulated oxygen evolution [1–5]. The photothermal signal in a photochemically active sample is smaller than the maximum signal because a fraction of the absorbed light energy is stored in photosynthetic products. The maximum photothermal signal is used as a reference signal. The sample is self-referenced by adding a strong nonmodulated background light to the modulated beam. A maximum photothermal signal is obtained in this case because the background light saturates photosynthesis by 'closing' the reaction centers, resulting in an almost complete conversion of absorbed modulated light into heat. Concomitantly, at photosynthetic saturation, the modulated component of oxygen evolution is eliminated.

The photothermal signal and the oxygen signal may have a different phase with respect to the modulated light. In order to separate each component, the following procedure is performed as in Ref. 1. In the presense of photosynthetically saturating background light, only the maximum photothermal signal is present. a rigid axis rotation is performed about the origin by changing the phase on the lock-in amplifier so that the total amplitude of the photothermal signal is projected only along the in-phase mode. Upon switching off the saturating light, the modulated oxygen signal will appear, if present. The oxygen vector can have projections on the in phase mode where it

will now add up to the photothermal signal and on the quadrature mode (90° out of phase) where it will be now the only component. The total amplitude of the oxygen signal is obtained as a vectorial sum of its in-phase and quadrature components, taking into account the measured photochemical energy storage yield which is measured separately at high frequency of modulation [1–5].

The effect of NaF and NaCl on the photoacoustic signal during State 1–State 2 transitions, was monitored in the following way. Algal suspension was adapted to State 1 by illumination with 710 nm light at an intensity of 10 W/m^2 for 5 min. NaF was added to a final concentration of 5 mM and algae were incubated for 20 min with NaF under 710 nm irradiation. Algae were transferred to a filter paper under far-red light and the Emerson enhancement was measured photoacoustically with a low intensity of 7 W/m^2 modulated 620 nm light to which an excess of non-modulated 710 nm light with an intensity of 20 W/m^2 was added. In an alternative experiment, *Nostoc* cells were adapted to State 2 by illumination with 620 nm light (interference filter - Ditric optics), at an intensity of 3.2 W/m^2 for 5 min. They were then treated with 5 mM NaF under the 620 nm irradiation, and Emerson enhancement was measured photoacoustically as above. Control experiments with 5 mM NaCl or in algae resuspended only in growth medium were carried out in the same way. All these experiments were also repeated with modulated 580 nm light at the intensity of 7.5 W/m^2. Phycobiliproteins were determined in crude extracts as in Ref. 22 and chlorophyll as in Ref. 23.

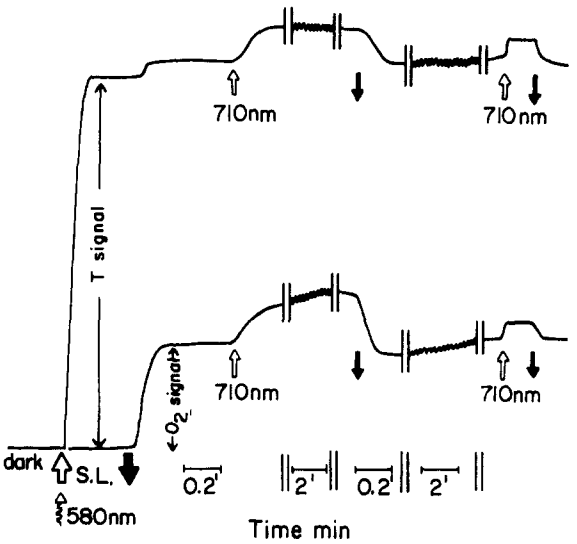

Fig. 1. State 1–State 2 transitions in *Nostoc muscorum*, monitored by changes in the far-red induced Emerson enhancement. Modulated light, 580 nm, 7.5 W/m^2. Far-red light, 710 nm, 20 W/m^2. Modulation frequency, 12 Hz. The photoacoustic signal in the presence of saturating background light (S.L.) of 400–680 nm, 60 W/m^2, was adjusted to zero on the quadrature mode (bottom, Fig. 1), so that the photothermal signal T was present only in the in-phase mode (top, Fig. 1).

Results and Discussion

Characterization of the oxygen signal

Self-referencing of the sample was obtained by alternating photoacoustic measurements in the presence and absence of a photosynthetically saturating, continuous background beam. Fig. 1 (top) depicts the in-phase photoacoustic signal upon simultaneous illumination with 580 nm light modulated at 12 Hz and non-modulated broad band (400–680 nm) saturating light. In this experiment, the phase angle with respect to an instrumental reference was adjusted to obtain a zero-quadrature signal (Fig. 1, bottom). Since in the presence of saturating light, photosynthesis is saturated, only the photothermal (T) contribution to the photoacoustic signal appears as an in-phase component [1]. A new signal was exhibited upon switching off the saturating light, consisting of a large quadrature component and a small in-phase component. The new signal disappeared upon incubation of the algae with DCMU (not shown). At a higher frequency of modulation (100 Hz) the new signal vanished and only changes in the photothermal signal were detected. The new photoacoustic signal observed here in cyanobacteria is similar in all criteria tested to modulated oxygen evolution signal previously observed in intact leaves and lichens [1,2]. Modulated oxygen evolution was also similarly detected photoacoustically in other algal species (*Dunaliella salina, Chlamydomonas reinhardtii*, unpublished results) and in isolated chloroplasts from Tobacco.

The ratio of the oxygen signal to the photothermal signal was found to be in the range of 0.1–0.3

(arbitrary units) compared to that in intact leaves of 1.5–3.0 (arbitrary units). The oxygen signal in algae is at least 10-times weaker than in leaves probably due to the difference in diffusion path of the oxygen and thermal signals in the two biological systems. The ratio of the oxygen signal to photothermal signal in the range of 10–200 μm did not show a strong dependence on the algal sample thickness. It appears that in algae, the oxygen signal is generated from the top part of the sample up to a depth of 1–10 μm and is then strongly damped, while most of the thermal signal travels all the way through the algal layer with a much smaller damping. The stronger attenuation of the oxygen signal is due to the fact that the diffusion coefficient of oxygen in water is about 2 orders of magnitude smaller than the corresponding thermal diffusivity of heat conduction. In an intact leaf a different situation prevails due to the presence of air spaces, and a 1 μm diffusion distance from the chloroplast to cell boundary [1].

Excitation energy distribution between PS II and PS I in Nostoc

The Emerson enhancement was used to characterize the imbalance of photon delivery to the two photosystems during various light adaptations. The enhancement of modulated oxygen evolution yield by the addition of continuous far-red background light to the exciting short-wavelength modulated light is known as Emerson enhancement [24]. In cyanobacteria short-wavelength modulated light (580 nm) is absorbed to a greater extent by PS II than by PS I because the phycobiliproteins harvesting this light transfer their energy primarily to PS II reaction centers [13]. Therefore, at limiting light intensity of 580 nm, the reaction centers of PS II remain partially closed and a smaller oxygen evolution signal is observed initially as seen on the left side of Fig. 1. Addition of excess non-modulated far-red light which is primarily absorbed by PS I removes this limitation. The effect of PS I light is to draw electrons from PS II resulting in an increase of the modulated oxyen evolution signal (Fig. 1). Further irradiation with excess background, 710 nm light in addition to the low intensity 580 nm modulated light for 2 min resulted in adaptation to State 1. A large decline of modulated oxygen evolution was observed upon turning off the 710 nm light, both in the quadrature and in-phase modes (Fig. 1). State 1 results from irradiation that over-excites PS I. In this state, short-wavelength light is initially distributed so that PS II receives excessive excitation relative to PS I. Therefore, the large imbalance of energy distribution between the two photosystems results in a large decline in the oxygen signal immediately upon removal of the 710 nm light. Adaptation to State 2 was followed by 2 min illumination with modulated 580 nm light alone which is primarily absorbed by phycocyanin (since phycoerythrin was absent in this species). State 2 results from adaptation of an initial over-excitation of PS II in which excess energy is redistributed to PS I until a balance distribution is approached. Therefore, after adaptation to State 2, addition of far-red light caused only a small increase in the oxygen signal (Fig. 1, right). This is a demonstration of State 1–State 2 transitions in *Nostoc,* by the photoacoustic method, which had been previously shown in an intact leaf [3–5].

The distribution of excitation energy between the two photosystems was determined by calculating the enhancement in each adaptation state. The extent of Emerson enhancement, E, is equal to the ratio of oxygen signals in the presence and absence of 710 nm light. Examining several *Nostoc* species, I found E to be in the range of 1.6–2.0 and 1.1–1.2 in State 1 and State 2, respectively. The fractions of short-wavelength light distributed to PS II and PS I are denoted by β and α, respectively. In the absence of far-red light, modulated oxygen is limited by the rate of light absorption by PS I and therefore is proportional to α. In the presence of excess far-red light, PS I is not limiting and in this case, modulated oxygen is proportional to β. If we assume that in State 1, there is no appreciable direct energy transfer from PS II to PS I, then, the enhancement ratio is equal to β/α as analysed in Ref. 3. For the example shown in Fig. 1, from the enhancement ratio E_1 of 1.6 in State 1, β_1 was found to be 0.62 and α_1 was found to be 0.38, assuming that $\alpha_1 + \beta_1 = 1.0$. The ratio of the oxygen signals in the presence of far-red light in State 1 and State 2, respectively, is proportional to the ratio of β_1 (State 1) to β_2 (State 2). From this ratio, β_2 in State 2 was

calculated to be 0.52. From the enhancement ratio of 1.1 in State 2 and assuming $E_2 = \beta_2/\alpha_2$, in State 2, it was calculated that α_2 in State 2 is 0.47. These results suggest that in a typical transition from State 1 to State 2 in the cyanobacterium, the fraction of light absorbed by PS II decreased from 0.62 to 0.52 and that absorbed by PS I increased from 0.38 to 0.47. Almost no loss in excitation occurs in State 2 as judged by the sum $\alpha_2 + \beta_2 = 0.99$. If, in contrary, it is assumed that there is direct energy transfer ('spill-over') from PS II to PS I in State 1, then $E = 2\beta/(\alpha + \beta)$ as shown in Ref. 3. Assuming $\alpha_1 + \beta_1 = 1.0$ in State 1, and from E_1 of 1.6 in State 1, β_1 will be 0.8 and α_1 will be 0.2, while β_2 and α_2 will be 0.55 and 0.45, respectively. However, preliminary analysis of enhancement saturation curve in State 1 (not shown) suggest that there is no appreciable direct energy transfer in State 1, and therefore the assumption of $E = \beta/\alpha$ is taken here. Similar results were obtained also in the lichen *Peltigera membranacea* containing a *Nostoc*. Distributions of excitation energy of 620 nm light between PS I and PS II in State 1 as well as in State 2 were similar to those obtained for 580 nm light, respectively, since 580 and 620 nm are primarily absorbed by phycocyanin.

State 1–State 2 transitions in *Nostoc* in contrast to higher plants could be obtained in less than 30 s (Fig. 2a). A transition from State 1 to State 2 induced by 620 nm modulated light is demonstrated, followed by the time-course of adaptation back to State 1 upon further exposure to 720 nm light, occurring in about 24 s almost to completion (Fig. 2a, right). However, the transition to State 1 may even be faster and not accurately resolved by our apparatus. This is in agreement with other investigations on state transitions in phycobiliprotein-containing organisms [14–16, 18,19]. It was of interest to find out if these fast transitions would be affected by conditions of impaired phosphatase activity in which the level of protein phosphorylation would be expected to be maximal. Biggins et al. [14] have shown the occurrence of twelve phosphorylated polypeptides in the red alga *Porphyridium cruentum* and had similar data in the cyanobacterium *Anacystis nidulans*. However, no changes in phosphorylation level were detected when samples of cells in State 1 and State 2 were compared. *Nostoc* cells were adapted to State 1 and then the cells were incubated with 5 mM NaF, a specific inhibitor of phosphatases [25], under far-red light. The cells were further illuminated in the photoacoustic cell for 1 min with excess non-modulated far-red light in addition to modulated 620 nm light (Fig. 2b). Initially, the state was close to State 1, as suggested by an increase in the oxygen signal observed in the presence of far-red light. Upon switching off the far-red light, the oxygen evolution signal decreased. Further adaptation to State 2 with 620 nm light alone (2 min) resulted in an

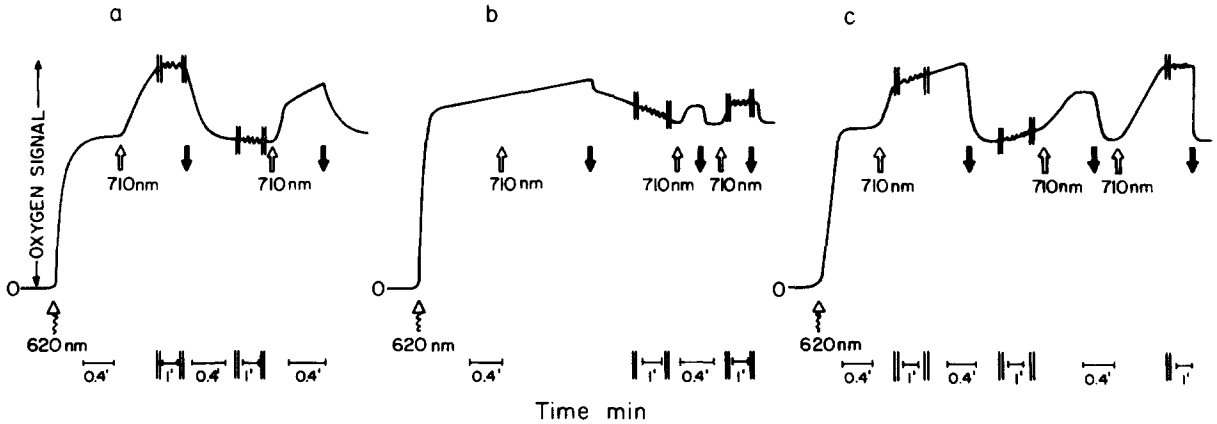

Fig. 2. The effect of NaF on State 1–State 2 transitions. Modulated light, 620 nm, 7 W/m^2. Only the quadrature mode is shown. Other conditions as in Fig. 1. (a) Control, *Nostoc* cells; (b) *Nostoc* cells in the presence of NaF (5 mM); (c) *Nostoc* cells in the presence of NaCl (5 mM).

Emerson enhancement ratio of 1.12. Distribution of excitation energy was calculated from the enhancement ratio and found to be equal to 0.52 for β and equal to 0.47 for α. Subsequently, addition of excess 710 nm light in the presence of 620 nm modulated light for 2 min did not induce a large enhancement ratio characteristic of State 1, but resulted in an enhancement ratio of 1.12 with energy distribution identical to State 2. These results suggest that an irreversible transition to State 2 was induced by NaF. The same experiment was carried out with cells adapted to State 2 and then treated with NaF under short wavelength illumination, showing similar resutls (not shown). It did not matter if the NaF-treated cells were adapted to excess PS I illumination or to PS II illumination. In both cases only one state could be achieved, with a minimal extent of enhancement characteristic of an optimal State 2. Emerson enhancement is an indicator of the degree of over-excitation in PS II relative to PS I. Therefore, the presence of NaF induced an almost perfect balance of excitation between the two photosystems. In order to check that the naF effect was not merely a salt effect, I examined the distribution of excitation energy between PS II and PS I in the presence of 5 mM NaCl. *Nostoc* cells treated with NaCl were initially adapted to State 1 followed by adaptation to State 2 and then further adaptation to State 1 (Fig. 2c). All the states displayed Emerson enhancement ratios similar to ratios of control, untreated *Nostoc* cells. Thus, it appears that the NaF effect was specific and not just a salt effect. In addition, the oxygen evolution signals in control and in either NaF- or NaCl-treated cells in the absence of far-red light were almost identical, suggesting that NaF did not cause any inhibition of oxygen evolution. The possibility that NaF (5 mM) had any effect on the carbon cycle appears unlikely, since incubation of *Nostoc* cells with azide (0.5 mM), a known inhibitor of the Calvin cycle in whole cells, caused a decline of 40% in the oxygen evolution signal with no apparent change in the State 1–State 2 transitions (not shown), which is in contrast to the NaF effect. Therefore, it is suggested that the inhibition of phosphatase activity blocked the transition to State 1 in *Nostoc,* and therefore resulted in a much better balance of excitation distribution between PS II and PS I, which was probably due to the attainment of maximum phosphorylation level. This conclusion suggests that in cyanobacteria which contain phycobiliproteins, State 1–State 2 transitions are controlled by the level of protein phosphorylation. These results are also in agreement with the observations of Allen et al. [17] who detected light-dependent phosphorylation of polypeptides of 18.5 and 15 kDa in the cyanobacterium *Synechoccocus* 6301. However, in the latter case, the changes in distribution of excitation energy were obtained by transitions between light and dark states which were characterized as State 2 and State 1, respectively, by fluorescence criteria. Using dark and light states is in contradiction with the data of Fork and Satoh [15] who claimed that the dark state in *Synechoccosus lividus* is State 2. In this work, the light state transitions are better defined by strictly illuminating with excess PS I light (710 nm) or PS II light (580 and 620 nm) for attaining State 1 and State 2, respectively, and characterizing them by Emerson enhancement ratios of oxygen evolution.

Cyanobacteria do not have either LHC or grana thylakoids. In higher plants, the slow transition times (7 min) may be necessary for the diffusion of LHC from granal to stromal regions and vice versa. In cyanobacteria, since lateral heterogeneity does not exist, the transitions are fast and involve protein phosphorylation, which probably occurs in seconds. It is possible that phosphorylation of certain polypeptides cause their dissociation from PS II and reversible attachment to PS I, so that increased excitation energy transfer to PS I occurs as previously suggested [17] which is consistent with this work, showing that PS I absorption cross-section increases and that of PS II decreases (as reflected in changes of β and α). In a previous investigation [26] it was found that the effective absorption cross-section per reaction center of Photosystem II for O_2 production by *P. cruentum* in State 1 was 50% larger than in State 2. However, it was not possible to distinguish between a change in the degree of energy transfer from PS II to PS I or actual redistribution of antenna components between PS I and PS II [26]. This is still an open question and will be dealt with in more depth in future work.

Pigment analysis of this *Nostoc* species showed

that phycoerythrin was absent and the ratio of phycocyanin to allophycocyanin was 1.5 : 1. In algae, it was shown that the photothermal signal of a thin sample (0.5 μm) is proportional to the absorbed photon energy [27]. The photoacoustic oxygen signal divided by the thermal signal and by wavelength of a thin sample is then proportional to the quantum yield of oxygen evolution [1–3]. The relative quantum yield of oxyge evolution was calculated for a thin sample (0.5 μm) of *Nostoc* cells. It was found that relative O_2 quantum yields were in the range of $0.12 \cdot 10^{-3}$ at 580 and 620 nm corresponding to absorption of phycocyanin, and about the same at 650 nm corresponding to the absorption of allophycocyanin, but only about 0.05 at 680 nm corresponding to the absorption of chlorophyll *a*. These results suggest that phycocyanin and allophycocyanin are the major light harvesting pigments in cyanobacteria, distributing their energy equally between PS II and PS I in State 2, while chlorophyll *a* transfers energy mostly to PS I, in agreement with previous observations [28].

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