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An easily reversible structural change underlies mechanisms enabling desert crust cyanobacteria to survive desiccation

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ABSTRACT

Biological desert sand crusts are the foundation of desert ecosystems, stabilizing the sands and allowing colonization by higher order organisms. The first colonizers of the desert sands are cyanobacteria. Facing the harsh conditions of the desert, these organisms must withstand frequent desiccation–hydration cycles, combined with high light intensities. Here, we characterize structural and functional modifications to the photosynthetic apparatus that enable a cyanobacterium, *Leptolyngbya* sp., to thrive under these conditions.

Using multiple *in vivo* spectroscopic and imaging techniques, we identified two complementary mechanisms for dissipating absorbed energy in the desiccated state. The first mechanism involves the reorganization of the phycobilisome antenna system, increasing excitonic coupling between antenna components. This provides better energy dissipation in the antenna rather than directed exciton transfer to the reaction center. The second mechanism is driven by constriction of the thylakoid lumen which limits diffusion of plastocyanin to P_{700}. The accumulation of P_{700}^{+} not only prevents light-induced charge separation but also efficiently quenches excitation energy.

These protection mechanisms employ existing components of the photosynthetic apparatus, forming two distinct functional modes. Small changes in the structure of the thylakoid membranes are sufficient for quenching of all absorbed energy in the desiccated state, protecting the photosynthetic apparatus from photoinhibitory damage. These changes can be easily reversed upon rehydration, returning the system to its high photosynthetic quantum efficiency.

1. Introduction

Photosynthetic organisms in strongly fluctuating environments such as arid regions and intertidal zones often face desiccation (*e.g.*, [1–4]). During the process of desiccation, continued photosynthetic activity may lead to the production of reactive oxygen species (ROS) and to subsequent damage to the photosynthetic apparatus [5,6]. The ability to cope with cycles of hydration and desiccation provides photosynthetic organisms in arid regions with a significant advantage in harsh and otherwise uninhabitable niches.

This challenge has been met by various species in the plant kingdom which evolved unique mechanisms for coping with the extreme conditions of the desert. In many desiccation tolerant vascular plants antioxidant metabolism is increased during desiccation [1,2]. In addition, photosynthetic activity is decreased or completely shut down *via* morphological changes in leaves and cell walls or through the accumulation of antioxidants and compatible solutes [1]. In lower order photoautotrophs such as lichens and mosses it has been reported that following desiccation, light energy is dissipated rather than converted into electrochemical energy owing to the accumulation of a quencher. The identity of the quencher has been proposed to be P_{680}^{+} [7,8] or P_{700}^{+} [9] but the evidence remains indirect.

Desiccation tolerance is essential in deserts, which cover about 40% of the earth's surface [10]. The harsh desert environment imposes on its inhabitants minimal amounts of water, extreme temperatures and high irradiance. Sand crust biological communities play an important

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role in stabilizing shifting sands and in preventing desertification, but are extremely sensitive to stresses such as physical disturbance and environmental changes [10–12]. The first colonizers of unstable desert sands are often cyanobacteria. Once there, they contribute to the stabilization of the sand and prevention of erosion. They produce large amounts of extracellular polymeric substances (EPS) which serve as a matrix for sand particles, assist in retention of moisture and provide the soil with organic carbon, nitrogen and other bioavailable compounds [12]. Such stabilization and fertilization of the sand allow succession and finally colonization of arid regions by vascular plants. Desert crust cyanobacteria serve as the foundation of entire ecosystems [10,13] and are thus important agents in the prevention of desertification.

In cyanobacteria excitation energy is captured by phycobilisome (PBS) light harvesting complexes, which transfer energy to chlorophyll *a* containing photosystems I (PSI) and II (PSII). The PBSs are attached to the thylakoid membrane surface and are composed of pigmented phycobiliproteins which harvest light and non-pigmented linker proteins which contribute to the stability and to the structure of the complex. In the cyanobacterial photosynthetic apparatus PBSs extend the spectral range of light absorbed by reaction center (RC) chlorophylls (Supplemental Fig. 2A) [14–16].

Our study focused on the photosynthetic apparatus of *Leptolyngbya* sp. isolated from desert sand crusts in the Nizzana region in Israel. These organisms were shown to be able to decrease photosynthesis to zero when desiccated and regain activity immediately upon rehydration [6,17–19]. In this paper we describe mechanisms that allow such dynamic changes in excitation energy transfer and in photochemical reactions. These mechanisms provide the necessary protection required under natural environmental conditions of desiccation and intense light.

2. Materials and methods

2.1. Preparation of cultures for measurements

Nizzana (N.W. Negev, Israel, 34° 23′ E; 30° 56′ N; elevation 190 m a.s.l.) *Leptolyngbya* sp. cultures were incubated in YBG11 medium in shaking flasks [20], at 30 °C and 60 μmol photons m^{-2} s^{-1} provided by fluorescent lamps. Cells were homogenized and placed on nitrocellulose filters as described in [17]. "Artificial crust" filters were left to air dry at room temperature for at least 2 h prior to spectroscopic measurements. The minimal amount of time required for reaching the stable desiccated state was 2 h. In hydrated samples, measurements were either made prior to desiccation or following rehydration of the filter with distilled water. The strain used here was originally referred to as a *Microcoleus* sp. [18], however, further bioinformatics analyses suggested that it is closer to *Leptolyngbya* sp. [18].

2.2. Spectroscopy

Fluorescence quantum yield (QY) and steady-state absorption were measured using an absolute PL quantum yield spectrometer (Quantaurus-QY — Hamamatsu photonics, Hamamatsu City, Japan). The samples were placed inside the integrating sphere and illuminated at wavelengths between 400–700 nm at 10 nm intervals. Both scattered light and emission were collected through the entire visible spectrum. An empty filter (wet/dry) was measured prior to sample measurement as reference.

Fluorescence decay kinetics were measured using a time-correlated single-photon-counting (TCSPC) approach with an Edinburgh Instrument Lifetime Spectrometer (FLS920) equipped with a Fianium SC400 supercontinuum laser monochromatized at 620 nm. The emission from the sample was collected at a right angle, through a long-pass filter and a monochromator to suppress the scattering from the excitation source, and collected using a Hamamatsu H10720 high speed PMT. Samples were excited at 620 nm, and detection was between 650–710 nm [21,22]. Room temperature steady-state fluorescence was measured using a Fluoromax3 spectrofluorometer (Jobin Yvon). Slow P700 photo-oxidation kinetics were measured *in vivo* using a Joliot-type spectrophotometer (JTS-10, Bio-Logic, Grenoble, France) as previously described [23].

Time-resolved optical transients were monitored with a homebuilt pump-probe spectrometer, previously described in [24]. The actinic flash was provided by a SLOPO (Continuum, SLOPO green) pumped by frequency doubled Nd-YAG Laser (Continuum, Surlite). The excitation wavelength was 700 nm and the bandwidth of the actinic pulse was ~7 ns. The transients were probed between 427–530 nm by the output of an Optical Parametric Oscillator (OPO), pumped by a frequency tripled Nd-YAG laser (Continuum, Surelite). The excitation (pump) flashes were fired with a frequency of 0.2 Hz, which allow the cellular electron transfer chain to return to the dark-adapted condition between flashes. Kinetic traces were acquired by scanning the pump-probe sequence from short to long delays, followed by a reverse (long to short) series and each pump-probe point was sampled twice [24]. Three to four of these sequences were averaged depending on the signal-to-noise at a given wavelength.

EPR spectra were recorded at 15 K in whole cells using an ESR300D X-band spectrometer (Bruker), using a TE_{102} resonator equipped with a front grid for sample illumination within the cavity. When illuminated, a tungsten–halogen lamp (800 W) was used to illuminate the sample within the cavity. White light was filtered. The temperature was controlled with a helium cryostat (Oxford Instruments, UK) [25]. Cells were recovered after thawing the EPR tubes and the chlorophyll contents of the samples were measured and found to be unchanged (2.1 ± 0.15 μg chl). For the chlorophyll measurement, we had to sonicate the cells after suspending them in 1.5 ml of growth medium.

2.3. Thin-section transmission electron microscopy

Exponentially grown *Leptolyngbya* sp. cells were cryo-immobilized in an HPM 010 high-pressure freezer (BAL-TEC AG, Lichtenstein), freeze-substituted (Leica EM AFS, Vienna, Austria) in dry acetone containing 2% glutaraldehyde and 0.1% tannic acid for 60 h at −90 °C, and then slowly warmed up to 0 °C. Following acetone rinses, the samples were incubated in 0.1% uranyl acetate and 1% OsO_4 for 1 h at room temperature. Samples were then washed with dry acetone, infiltrated with increasing concentrations of epon over 6 days and polymerized at 60 °C (the procedure is detailed in [26,27]). Sections were cut using an Ultracut UCT microtome (Leica, Vienna, Austria) and post-stained with 5% uranyl acetate in isobutanol-saturated double-distilled water (DDW) and Reynold's lead citrate in isobutanol-saturated DDW [26,27].

3. Results

Leptolyngbya sp. isolated from biological sand crusts near Nizzana (NW Negev, Israel) is capable of transitioning quickly between desiccated and hydrated states. Measurements of P_{700} photo-oxidation indicate that the photosynthetic apparatus shuts down completely in the desiccated state and is able to regain full functionality within minutes following re-hydration of the sample (Supplemental Fig. 1). In order to protect the photosynthetic apparatus in the desiccated state certain desiccation tolerant photosynthetic organisms reduce their effective absorption cross-section, either by reorganizing pigments or by accumulating photoprotective pigments (*e.g.*, [19,28–30]). This is not the case for Nizzana *Leptolyngbya*. Supplemental Fig. 2A shows measurements of the absorption cross-section of desiccated and hydrated *Leptolyngbya*. The total absorption of both states is similar. In the hydrated state, absorbed energy is distributed between photochemistry, heat and fluorescence [22]. In the desiccated state photochemistry does not occur (Supplemental Fig. 1) [6,18,19,31] and the energy is

distributed between heat and fluorescence. The quantum yield (QY) of fluorescence was measured parallel to absorption (Supplemental Fig. 2B). The integrated fluorescence QY in the desiccated state was ~⅓ of fluorescence QY in the hydrated state. Although the fluorescence of the desiccated state was substantial, heat must account for the bulk of energy dissipation in the absence of photochemistry. In our search for protective mechanisms by which Negev desert *Leptolyngbya* dissipate light energy and avoid photodamage in the desiccated state, we studied changes occurring in both PBS antennae and RCs.

Characterization of fluorescence kinetics of desiccated *Leptolyngbya* was performed using time-correlated single-photon counting (Fig. 1A). Time-resolved fluorescence of the PBS antennae was measured following excitation at 620 nm (phycocyanin absorption peak). The desiccated sample exhibited a significant decrease in fluorescence life-times throughout the entire measured range. In addition, it appears that fluorescence in desiccated *Leptolyngbya* has shifted to longer wavelengths, around 700 nm (Fig. 1A & B). Fluorescence emission at 700 nm has not been associated with PBS or PS fluorescence [32]. In studies exposing cyanobacteria to high light, shortening of PBS fluorescence life-time was recorded, but a fluorescence emission band at 700 nm was not reported (for a recent review see [33]). In steady state measurements, the excitation spectrum of the 700 nm band indicates that PBSs are the main absorbing pigments responsible for fluorescence at this wavelength (Fig. 1B).

Photochemical responses were measured by pump-probe spectroscopy (Fig. 2). To minimize the contribution of PBSs to the spectra, the excitation wavelength was set to 700 nm allowing direct excitation of Photosystem I [34,35]. In the hydrated state several transient features developed following pump excitation. Bleaching at 433 nm and induced absorption at 455 nm, 10 ns after excitation, are characteristics of $P700^+$ (arrows in Fig. 2A) [34,36]. Absorption changes in the 450–530 nm range result from the electrochromic shift associated with carotenoids. In the sub-μs time range, kinetic changes of the absorption profile in this wavelength range are attributed to electron transfer from phylloquinones to FeS clusters [37]. The lifetime of the bleaching at 433 nm, in the hundreds of μs time range (Fig. 2A and C), suggests slow electron donation to P_{700}^+, as commonly observed in cyanobacteria [34,38–40]. Importantly, about a third of the initial absorption changes remained 10 ms after the actinic flash demonstrating the initial light induced radical pair has been stabilized by successive electron transfer reactions. Consistent with this, a closer examination of the transient absorption changes at various wavelengths (*e.g.*, 433 nm and 485 nm, Fig. 2C) shows that these kinetics vary with wavelength. This implies that different processes, with different spectroscopic characteristics and time constants, take place in the hydrated sample.

In the desiccated state, spectral features were observed at 433 nm, 485 nm and 520 nm (Fig. 2B). However, they differed from the light induced absorption changes observed in the hydrated sample in at least two respects. Firstly, the peak at 450 nm, characteristic of $P700^+$, was not observed. Secondly, these absorption changes decayed with similar kinetics throughout the entire spectrum (Fig. 2B and D), suggesting that in this case a single process takes place. Based on the absence of the spectroscopic signature of $P700^+$ this process is unlikely to be the evolution, as a function of time, of a radical pair state. The most straightforward hypothesis is that the light-induced absorption changes reflect the decay of an excited state [41,42].

If no radical pair is formed in the desiccated sample, one expects the dependence of the light-induced absorption changes upon excitation energy to be significantly different. Whereas the formation of a radical pair will be determined by the absorption cross section of the light-harvesting pigments that transfer their excitation to the photochemical trap, the formation of the excited state will depend on the absorption cross section of the isolated excited pigments (in this case chlorophyll since the excitation wavelength was 700 nm). We thus analyzed the dependence of bleaching at 432 nm on excitation energies (Fig. 2E). In the hydrated sample, the absorption changes detected at 10 ns displayed two components: 1) A first component characterized by a steep dependence upon energy (0–0.3 mJ range). 2) A second component (0.3–7 mJ range), the amplitude of which increased with energy but more sluggishly and did not reach saturation. The behavior of this second component is typical of excited states which may form on any pigment in the thylakoid membrane. In the desiccated sample, absorption changes detected at 10 ns display a non-saturable component. In the hydrated sample, a light-induced absorption change persisted 100 μs after the flash and saturated in amplitude at energy levels lower than 1 mJ. This component, which corresponds to the first component observed steeply increasing when detecting at 10 ns, is attributed to charge separation in the RC and is expected to saturate once all RCs are excited. This component is completely absent from the desiccated sample. In the hydrated sample, the occurrence of saturable and non-saturable components indicates that two processes are at work: charge separation that saturates at low energy and the non-saturable formation of an excited state decay associated with energy dissipation. The desiccated sample completely lacks the saturable component displaying only the non-saturable one, showing that all of the excitation energy is dissipated and that photochemistry does not take place.

Further information on the function of PSI was obtained from Electron Paramagnetic Resonance (EPR) spectroscopy (Fig. 2F). As expected, in the hydrated state, illumination at 15 K induced the photo-accumulation of P_{700}^+. Saliently, in the desiccated sample, the P_{700}^+ signal was observed before illumination. Subsequent illumination showed that the P_{700}^+ signal intensity in the dark reached values near its maximal extent. These results indicate that in a large portion of PSIs, P_{700} remained stably oxidized in the desiccated state. It is important to note that the absorption of P_{700}^+ is red shifted (820 nm, [43])

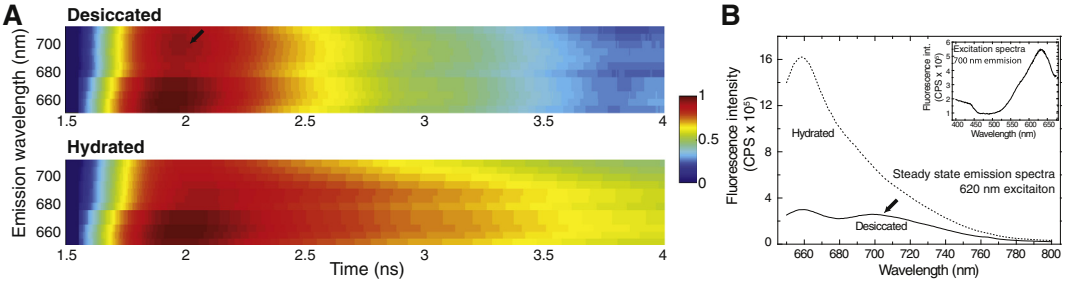

Fig. 1. Fluorescence kinetics. (A) Time correlated single photon counting of *Leptolyngbya* samples before (desiccated) and after (hydrated) rehydration at room temperature. Excitation was provided by a 620 nm laser, targeting the PBS absorption band. The ns resolution of these measurements detects the decay phase of the fluorescence. Data at 670 nm is missing due to a strong instrumental optical interference biasing the detection. The results are presented on a false color scale. Similar results were obtained in two independent measurements. (B) Steady state room temperature fluorescence of a sample before and after rehydration. Both time-resolved (A) and steady state (B) fluorescence measurements exhibit a fluorescence band at 700 nm in the desiccated state (indicated by arrow). The excitation spectrum of the 700 nm emission feature is presented in the insert in panel B.

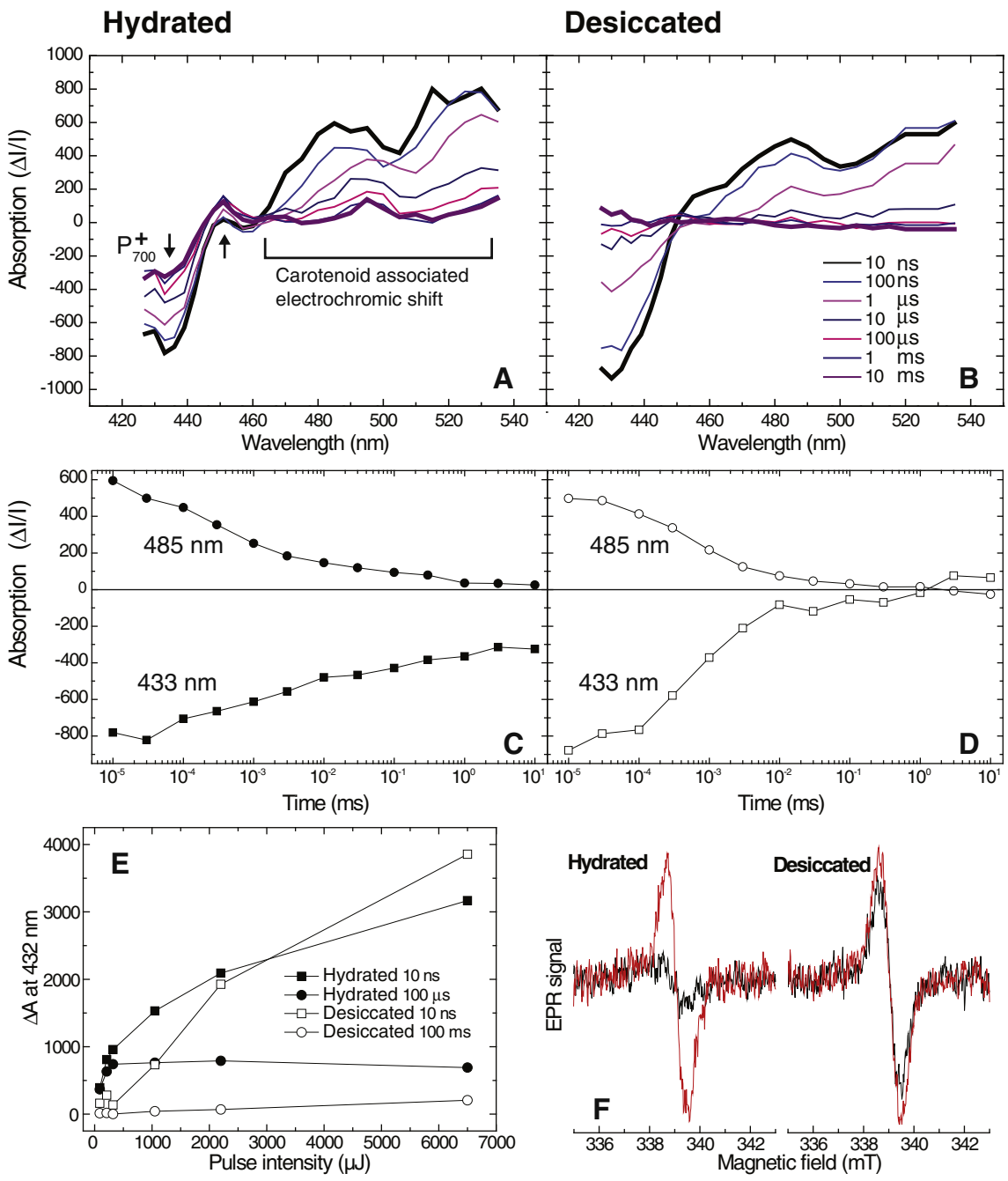

Fig. 2. Transient absorption changes in *Leptolyngbya* samples. (A) & (B) spectral evolution following pump-excitation at 700 nm. The P^{+}_{700} feature and the carotenoid associated bands are marked. Thicker lines indicate first and last time points following pump excitation. Panels (C) and (D) present the kinetics of the decay at 433 nm (P_{700} region) and 485 nm (carotenoid region). The complete decay of the signal in the dry sample by 10 µs was apparent in three independent experiments. (E) Saturation curve of the absorption changes measured at 432 nm. (F) *In vivo* P^{+}_{700} EPR spectra. EPR spectra of hydrated and desiccated samples measured at 15 K (microwave frequency, 9.503 GHz; modulation, 0.2 mT; averages of 2 scans). The black spectrum was recorded in darkness and the red spectrum was recorded after 3 min. of continuous illumination at 15 K. The relative intensity of the dark signal was <10% in the hydrated sample and 77% in the desiccated sample, as compared to the light signal.

making it a very efficient quencher, dissipating absorbed light energy in the desiccated state, whereas in the hydrated state P^{+}_{700} is quickly re-reduced, to allow photochemical conversion of light energy.

TEM images point to structural differences between hydrated and desiccated *Leptolyngbya* samples (Fig. 3). Hydrated samples appear to be better organized and structured than desiccated samples. While average distances between adjacent lumenal compartments (repeat distance) did not change drastically, the distribution of these distances in the desiccated samples was significantly wider. In many hydrated samples we observed an organized rod antenna structure that was missing from all of the desiccated samples (Fig. 3A & C). An additional structural change observed in the desiccated samples is a substantial (~50%) decrease in the lumenal width.

4. Discussion

In this paper we characterized protection mechanisms utilized by a desert crust cyanobacterium. The *Leptolyngbya* sp. used in this study is capable of surviving harsh desert conditions using existing components of the photosynthetic apparatus. Altering the structure and function

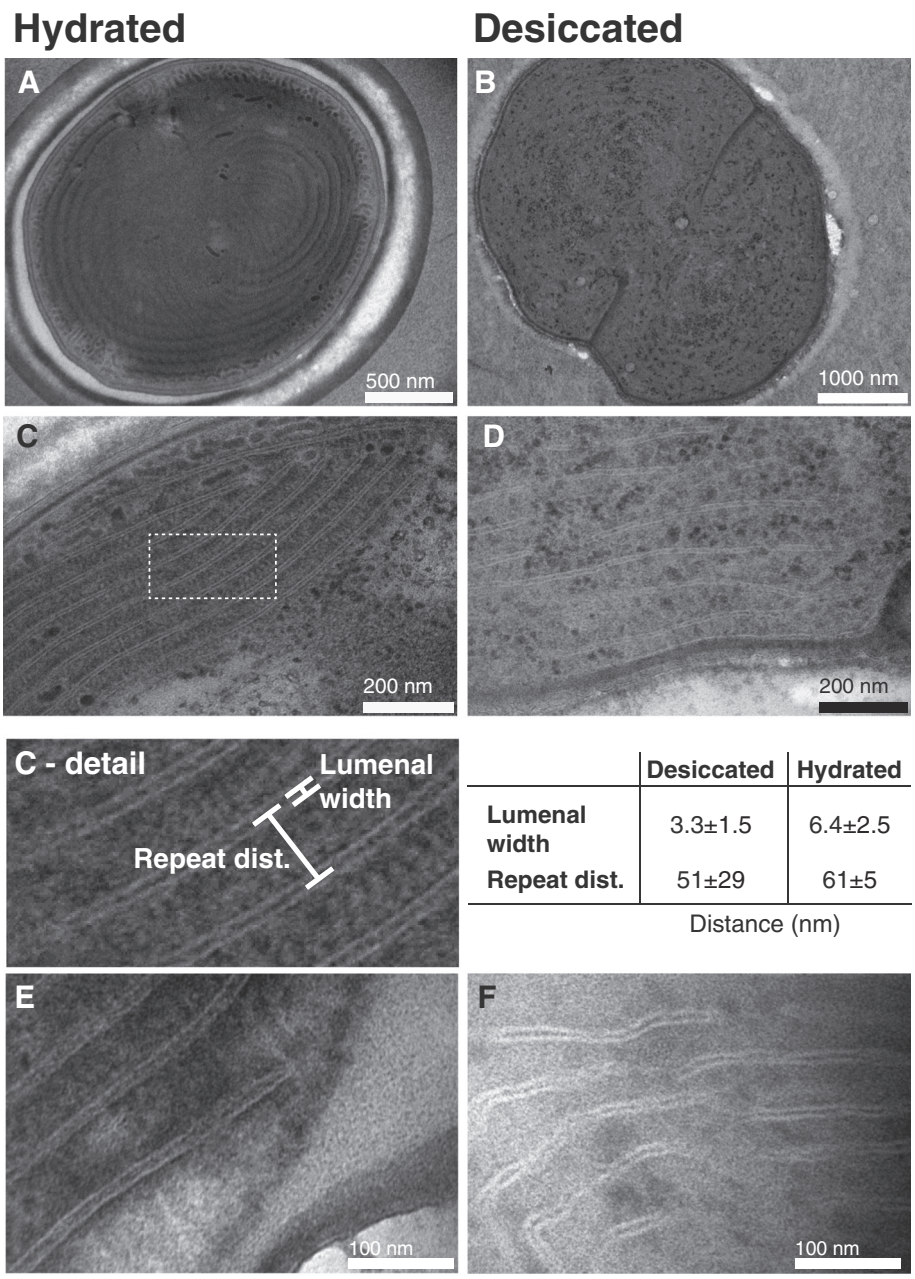

Fig. 3. TEM images of *Leptolyngbya*. The organized structure associated with the thylakoid membranes is apparent in the hydrated sample (A) and missing in the desiccated sample (B). Panels C and D show close up images of hydrated and desiccated samples, accordingly. The square in panel C is magnified in C-detail, below. Repeat distances and lumenal width were calculated from 50–100 images. Close up TEM images of the thylakoid membranes of hydrated and desiccated *Leptolyngbya* cells are shown in (E) and (F), respectively.

of specific components supports dissipation of energy rather than conversion of energy to photochemistry.

In the antenna bed, the same complexes that are ordered in rods to direct energy efficiently to the RCs in the hydrated state, are re-ordered in the desiccated state (Fig. 3). The fluorescence life-time is shortened and a new fluorescence band at 700 nm emerges (Fig. 2). This is done without changing the absorption cross-section (Supplemental Fig. 2). These observations are incompatible with previously published mechanisms for energy dissipation in cyanobacteria, where shortening of the fluorescence decay lifetime occurred but a 700 nm red-shifted component was not detected (reviewed in [33]). It is interesting to compare these results with data we have recently published on *in vitro* analyses of phycocyanin nanowires [21]. Strong excitonic coupling in phycocyanin nanowires generated an energy band structure with a gap that is red shifted compared to the spectra of the individual component, shorter fluorescence decay life-times, higher heat dissipation and energy transfer over large distances — due to efficient energy transfer [21]. Some of these features resemble the results obtained in the desiccated state.

We suggest that the desiccated organization of PBSs favors tight PBS to PBS excitonic coupling over PBS to RC coupling. In the desiccated state this organization will provide two advantages: a) better dissipation of antenna excitation due to the energetic stabilization of the system; and b) a large part of the excitation energy will be transferred parallel to the membrane plane instead of to the RCs [21]. Similar migration of excitation energy to longer wavelengths was indicated in desiccated mosses and chlorolichens [7–9]. However, in those cases the molecular nature of the far red state has not been spectroscopically identified.

In addition to the protection provided by the PBS system, the activity of the RCs is extensively modified. Electron transport is blocked by the

presence of a stable P_{700}^{+}. Beyond blocking electron transport, P_{700}^{+} provides additional excitation energy quenching capacity. The mechanism preventing the reduction of P_{700}^{+} can be a direct result of the constriction of the thylakoid lumen that may impede diffusion of reduced plastocyanin from Cytochrome b_6f to PSI [44,45]. There is evidence to suggest that quenching of PSII by spillover to PSI serves as a protective mechanism in chlorolichens as well [9].

Dissipation of energy *via* the stabilization of oxidized chlorophylls may seem like a radical approach. However, these organisms live under extreme conditions where they are exposed to very high light intensities under which "standard" quenching mechanisms cannot provide sufficient protection. The structural and the functional acclimation identified in this work provides an easily reversible structural alteration that allows desert crust cyanobacteria to transition safely and efficiently between a fully functional hydrated state and a fully protected desiccated state.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbabio.2015.07.008.

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