Water Collective Dynamics in Whole Photosynthetic Green Algae as Affected by Protein Single Mutation

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Matherial and Methods

The biological material

In this work, wild-type and single point mutant of the unicellular green algae *Chlamydomonas reinhardtii* have been used. *C. reinhardtii* has long been used as a powerful model system for studying photosynthesis and cell motility,[1] and more recently for bio-fuel and bio-hydrogen production.[2,3] Furthermore, the distinctive fluorescence and electrochemical properties of *C. reinhardtii* photosynthetic apparatus have been also exploited for the construction of biosensors,[4] and biophotovoltaic devices.[5] In **Figure S1**, an overview of the cell morphology is provided, along with a brief description of the light phases of photosynthesis, and a 3D model of the D1-D2 photosynthetic reaction center proteins hosting an aminoacidic substitution in a region close to Mn complex (mutant I163N).[6]

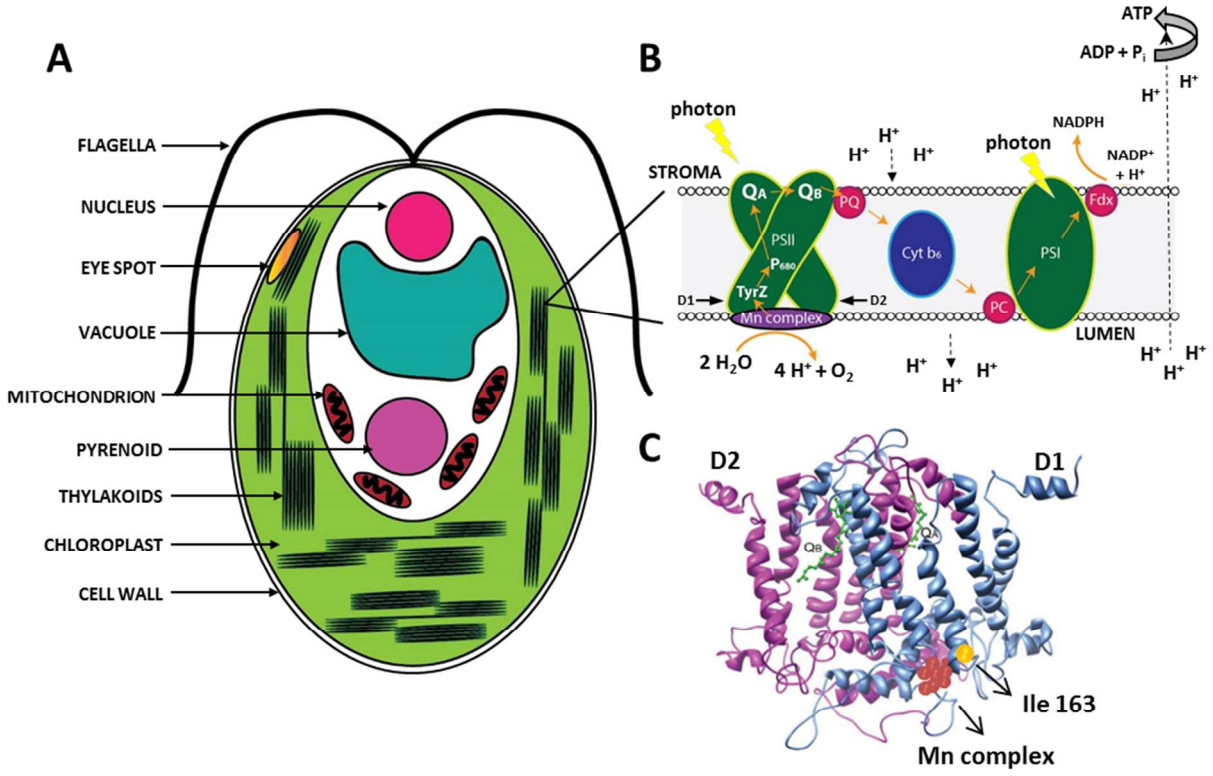

Figure S1.

A.) A sketch of *C. reinhardtii* cell's morphology. *C. reinhardtii* is a unicellular green alga of about 10 micrometres in diameter. The cell is enclosed in an extracellular cell wall and contains two large flagella for swimming and reproduction. A single large cup-shaped chloroplast occupies most of the cellular volume, and surrounds the nucleus, some mitochondria, a contractile vacuole, and a pyrenoid for carbon dioxide fixation. Inside the chloroplast, thylakoids, a membrane-made compartments, host the pigment-protein complexes and all the redox-cofactors necessary to perform the photochemical reactions of the photosynthesis. The eye spot, a light-sensing apparatus, is also found inside the chloroplast anchored on the thylakoid membranes.

B.) Schematic draw of the electron transfer chain in oxygenic photosynthesis, the flow of the linear electron transport is indicated by arrows. The pigment protein complex Photosystem I (PSI) and Photosystem II (PSII) are embedded in thylakoid membranes and work synchronously for the conversion of the light energy into chemical energy. In the reaction center of PSII, a heterodimer composed by the proteins D1 and D2, the energy captured by antenna molecules is funneled to the primary chlorophyll P680. An electron of P680 is excited and transferred through a series of acceptor molecules reducing first the plastoquinone Q_A (bound to D2), and then Q_B (bound to D1). A redox-active residue in the D1 protein, the Tyrosine 161 (TyrZ) reduces the $P680^+$, by extracting electrons from a manganese cluster (Mn complex), which in turn withdraws electrons from water, leading to water oxidation with formation of molecular oxygen. The fully reduced Q_B (PQ) leaves D1 and transport the electrons to a proton pump called cytochrome b6 complex (Cyt b_6). From here the electrons are transferred to PSI by another mobile electron carrier called plastocyanin (PC). Under light excitation, PSI uses the electrons donated from PSII to reduce NADP+ to NADPH. The movement of electrons in the electron transfer chain is

coupled with a transfer of hydrogen ions thought the thylakoid membrane, and the generated proton gradient is the driving force for the synthesis of ATP.

C.) Ribbon-diagram overview of the *C. reinhardtii* PSII reaction center D1-D2 heterodimer. The modeled three-dimensional structure displays the D1 (cyan) and D2 (magenta) proteins. In ball-and-stick representation the plastoquinone electron acceptors Q_A (bound to D2) and Q_B (bound to D1) are colored in green. The manganese complex and the modified amino acid I163 (which is located near to the redox-active TyrZ) have been hand-drawn and displayed as dark-red and orange spheres, respectively. Adapted from ref 7.

The I163N mutant was obtained by combining a directed evolution strategy followed by site-directed mutagenesis experiment. In detail, a random mutagenesis approach was used to produce a pool of mutants hosting aminoacidic substitutions in a specific region of the PSII-D1 protein. Following, the pool was exposed to different types of ionizing radiation, or their combination with high-light intensities in order to isolate new strains with stress-tolerant phenotypes.[8] The I163N strain was among the survivors to high energy neutrons exposure (dose rate 0.23 mSv h-1 for 24 h).[8] In order to rule out the introduction of additional mutations possibly induced by exposure to neutrons, this strain was also produced by site-directed mutagenesis and further characterized in comparison with the wild type (Table S1 and Table S2).

Table S1. Oxygen production capacity (data from ref. [9] and content of chlorophyll *a* (Chl *a*) per cell (data from ref. [6]) of *Chlamydomonas* strains. The rate of oxygen evolution of I163N mutant is higher, especially under high light intensity, and the content of Chl *a* per cell is half compared to the wild type. The strains were grown on Tris-Acetate Phosphate (TAP) medium under continuous illumination of 50 μmol m^{-2} s^{-1} white light, temperature of 24±1 °C and 150 rpm agitation. The oxygen measurements were performed at 24 °C (Chlorolab 2, Hansatech,

Instr. Ltd, Norfolk, UK) on samples containing equal amount of chlorophyll (15 μg mL^{-1}) and in presence of 10 mM $NaHCO_3$, as additional carbon source. [4] Average values from at least 3 independent experiment (n=6-9) ±SE are presented.

Strains	O_2 evolution rate (μmol mg chl^{-1} h^{-1})		Chl *a* (pg/cell)
	200 μmol m^{-2} s^{-1}	600 μmol m^{-2} s^{-1}	
wild type	65±2	68±1	0.0086±0.0019
I163N	82±1	164±3	0.0045±0.0003

Table S2. Response of the Chlamydomonas strains to the harsh environment of low earth orbit space missions (data from ref. [6]). The F_v/F_m fluorescence parameter was recorded by *ad hoc* designed multicell fluorescence sensor during the space-flight and after landing. The F_v/F_m ratio represents the PSII photochemical efficiency of the *Chlamydomonas* strains immobilized on TAP medium and placed in the biocells of the fluorimeter (for more details see ref. [6]). In flight, the F_v/F_m of the wild type declined daily, while I163N mutant maintained high PSII activity in flight and showed a slight increase in F_v/F_m value toward the end of the flight. The F_v/F_m ratio of mutants continued to remain higher even after landing, being higher than even the ground controls. Following the space-flight and landing on earth, the algal cultures were transferred to liquid TAP medium and re-growth under standard growing conditions (see Table S1) and re-growing capacity of the strains was evaluated.

Strains	$\Delta F_v/F_m$ [a]	Growth[b]
wild type	-0.200±0.004	0.4±0.07
I163N	+0.005±0.003	1.6±0.05

[a]Values represent the difference between F_v/F_m on the day after landing and those registered in space on the last day of the flight, using the equation: $\Delta F_v/F_m = (F_v/F_{m\ after\ landing} - F_v/F_{m\ in\ flight})$.

[b]Algal growth is evaluated by culture optical density (OD) at 750 nm. The growth is represented as the ratio of OD_{750nm} values measured after 3 days of re-growth of the space-flown samples and the corresponding ground controls. Growth = $OD_{750Flight}/OD_{750Control}$.

Neutron scattering sample preparation.

The experiments on the collective dynamics of cellular waters have been carried out in deuterated cells (for details refer to ref 10). To this purpose, the conditions for culturing *Chlamydomonas* strains in D_2O were set-up at the ILL deuteration Laboratory (Grenoble, France). The cells were grown mixotrophically in deuterated medium with continuous illumination (50 μmol m^{-2} s^{-1}), flask shaking (150 rpm) and constant temperature of 24 ±1 °C. Chlamydomonas strains were not capable to grow in 100% deuterated medium and the maximum deuterium concentration allowing normal growth without inducing cell death or chlorophyll loss was 70% and was used for the final experiment. The culture growth phase was survey by measurements of culture optical density (OD) at 750 nm. Algae cells grown in 70% deuterated TAP (Tris-Acetate-Phosphate) medium were harvested at OD_{750}=1 by weak centrifugation, and the cell pellet re-suspended and cultured in 100% D_2O medium for 48h in order to increase the intracellular and *de novo* incorporated D_2O. Later on, the cells were collected and a controlled amount of cell pellet layered into the sample holder.

In order to collect data with a high statistic to achieve a good separation of the contributions deriving from water and cell components, 3.5 g of cell pellet were necessary for each

measurement. In order to not stress the photosynthetic cell, the pellet has been refreshed every 2 days of measurements. Storage of the deuterated algal cell paste at room T for approx. 48 hours did not lead to any significant cell death. The sample water content and sample water loss during the experiment was surveyed by measuring sample fresh and dry weight on an analytical balance. Water constitutes approx. 80% of the total cell weight (in a sample of 3.5g fresh weight we have 2.8 g of H2O) and 0.7 g of protein; h=4). To gain further insights into the contributions deriving from water and cell components, a measurement on slightly dehydrated algae was necessary. A new set of samples was hence prepared as described above and dehydrated of an approx. 60% of the bulk water. The remaining approx. 20% water corresponds to the macromolecular hydration water. The dehydration was performed gently heating the sample at 65 C for few hours. Completely dry cell (380 mg) and re-hydrated cells (we added 1 g of water; h= 2.6) of the wild type algae were also measured to compare the contributions of biomolecules to the collective dynamics and the difference of protein hydrated powder versus whole hydrated cell. Given the quality of the data, the analysis of the completely dry cells was not conclusive. Therefore for a completely dry protein we will referred to published results by Paciaroni and co-workers.[11] Finally, four living samples and one in powder form were analyzed as a function of water content.

Neutron Brillouin Scattering

Measurements of the collective dynamics in our samples were realized with the BRISP spectrometer installed at the high flux reactor (HFR) of the Institut Laue Langevin (ILL, Grenoble, France) [12]. BRISP is a hybrid direct geometry spectrometer exploiting a crystal monochromator and a time of flight analysis of the scattered neutrons. The spectrometer was

operated at 83.6 meV (corresponding to an incident neutron wavelength of 1 Å) providing an energy resolution of about 3 meV and a Q range of $0.1<Q<1.7$ Å^{-1} (corresponding to a distance detector-sample equal to 4m). Given the cinematic range, only spectra between $0.2<Q<1.2$ were analyzed. The cell pellets were loaded into slab containers of 2mm tick and measured at ambient pressure and room temperature. The raw spectra were subtracted of empty cell and environmental background contributions, corrected for self-absorption, multiple scattering and normalized to a vanadium standard.

The choice of the model adopted to interpret the $S(Q,\omega)$ depends on physical considerations about the system under investigation. Therefore, the collected spectra, of biomolecules hydration water, are often analyzed using damped harmonic oscillators (DHOs) in order to characterize the *water collective excitations* propagating throughout the system. More complex models can be considered but at the expense of employing a larger number of free parameters.

In hydrated biological systems, the collective excitations were found to be characterized by the propagation of two modes in analogy with the findings in bulk water: a high-energy linearly dispersing mode, and a low-energy mode of rather constant energy. In this picture, the hydration water dynamic structure factor can be expressed as follows:

$$S(Q,\omega) = \mathrm{a(Q)}\delta(\mathrm{Q}) + \left[\mathrm{n}(\omega)+1\right]\left\{\frac{\mathrm{a}_{\mathrm{H}}(\mathrm{Q})\Gamma_{\mathrm{H}}(\mathrm{Q})\omega}{(\omega^2 - \Omega_{\mathrm{H}}^2(\mathrm{Q}))^2 + (\Gamma_H(\mathrm{Q})\omega)^2} + \frac{\mathrm{a}_{\mathrm{L}}(\mathrm{Q})\Gamma_{\mathrm{L}}(\mathrm{Q})\omega}{(\omega^2 - \Omega_{\mathrm{L}}^2(\mathrm{Q}))^2 + (\Gamma_{\mathrm{L}}(\mathrm{Q})\omega)^2}\right\}$$

The first term is a Dirac delta function $\delta(w)$ of intensity $a(Q$, which represents both the elastic and the quasi-elastic response of the bio-system. The quasi-elastic component is assumed to have a negligible width as compared to the instrument energy resolution. When this is not the case a

finite width function is employed instead of the delta function, (e.g. a Lorentzian function being often fairly appropriate). The term *n(E)* is the Bose factor, while the term between curly brackets is the sum of two DHO response functions: a high-energy one, whose parameters are identified by the subscript *H,* and a low-energy one, identified by the subscript *L*. Each DHO function is characterized by three *Q*-dependent parameters, that is the proper frequency $\Omega(Q)$, the damping factor $\Gamma(Q)$, and the intensity *a(Q)*. The resulting data were corrected and analyzed using the ILL LAMP programs

Figure S2 shows experimental data set measured ,at two wave vector values, and different hydration degree of wild type Chlamydomonas (Fig. 1a, 1c, 1e, 1g), I163N mutant (Fig. 1b, 1d, 1f, 1h), highly hydrated powders (Fig. 1i, 1l) together with the best fit using a two component damped harmonic oscillators.

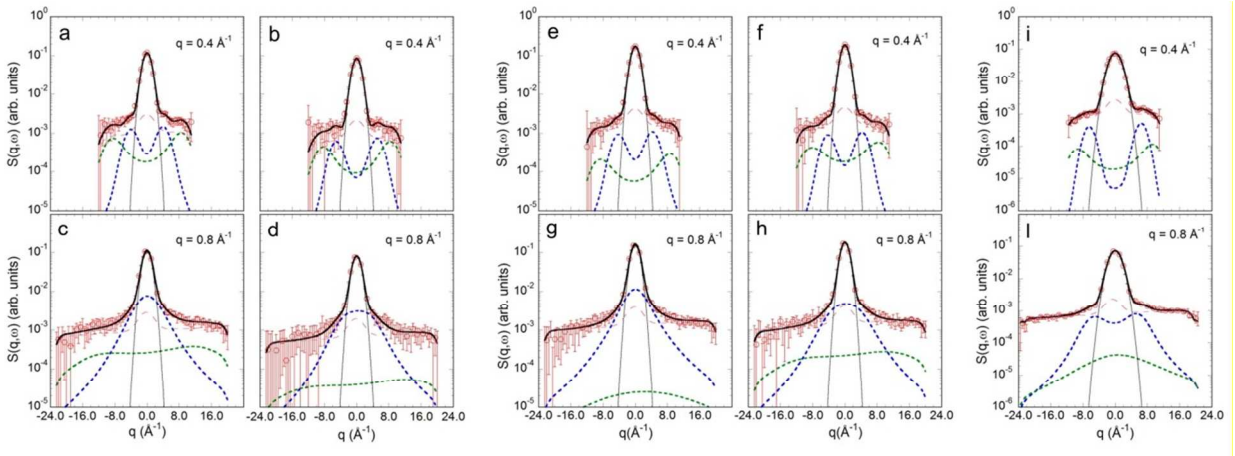

Figure S2 Coherent inelastic spectra of (a, c) intact wild type Chlamydomonas and (b, d) mutant I163N intracellular water at selected q-values. Coherent inelastic spectra of (e, g) dehydrated wild type Chlamydomonas and (f, h) dehydrated mutant I163N intracellular water at selected q-values. In (i, l) we show also the spectra of highly hydrated powder. The black thick line shows fits of the data with two DHOs model.The black thin line shows the

experimental resolution function. The blue and green dotted lines represent respectively the resulting low- and high-frequency DHO. Finally, also the calculated multiple scattering is shown (dashed magenta line).

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