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Scientific Report on the STSM

The Short Term Scientific Mission took place at the Freie Universität Fachbereich Physik, Berlin(DE) from the 14/06/2015 to the 17/07/2015. The visit aimed to strengthen the already existing collaboration with Prof Holger DAU allowing discussion and preparation of the future neutron scattering experiments. In addition, I was also able to carry out new spectroscopic studies on the dynamics properties of native and mutated photosynthetic green algae and PSII complexes. Therefore, I investigated correlations among structural, dynamics and functional proprieties natural and mutated photosynthetic D1 reaction centre protein in green algae using time-resolved investigation of *prompt and delayed fluorescence*. These experiments were performed to complete and better understand previous results obtained using elastic neutron scattering experiments, which suggested that point genetic mutations notably affect the Temperature dependence of the overall protein dynamics. In the specific, at physiological temperature, point mutations cells showed a higher flexibility of the whole system.

The activities were scheduled as follow:

1) Discussion, planning and preparation of the forthcoming elastic neutron scattering experiment and proposals for future beam-time applications.

2) Preparation of Chlamydomonas rheinhardtii algae cultures: wild type and 'mutants' with genetic variation of photosystem-II proteins

3) Biophysical experiments: time-resolved investigation of *prompt and delayed fluorescence* chlorophyll fluorescence after laser-flash excitation using PSII and Chlamydomonas rheinhardtii algae cultures wild type as well as mutant samples

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Description of the work carried out during the STSM

1**) Discussion**. During the first days of the STM at the at the Freie Universität Berlin I have been working with Prof. Dau and his coworkers Dr. Ivelina Zaharieva and PhD student Zhiyong Liang on the preparation of the forthcoming neutron scattering experiment planned in Munich at the Sphere backscattering spectrometer (3 days of measurements). In order to optimize the experimental time at the large scale facility and be able to measure a complete set of data it has been necessary to discuss longtime and into details about
-the number of samples to measure,
-the quantity of PSII to purify,
-the percentage of water-glycerol perpetuated mixtures to prepare,
-how to prepare the PSII mixtures,
-the temperatures range to measure,
-the sample holders to use and planning to have them made at the University workshop....
The submitted proposal is reported in Appendix 1.

2) **Preparation of Chlamydomonas rheinhardtii algae cultures for biophysical experiments at the FU.** With the assistance of Dr. Yvonne Ziilinger (DAU group) I have taken care to prepare all the algae cultures necessary to perform the fluorescence experiments. The "plates" to prepare the cultures have been sent from our co-workers Dr. Pina Rea and Maya Lambreva from the Institut of Crystallografy CNR. After inoculation in a Tris-acetate photsphate (TAP) media the cells were grown phototrophically with continuous illumination (50 µmol m^{-2} s^{-1}), flask shaking (150 rpm) and constant temperature of 24 ±1 °C. The culture growth phase was survey by measurements of culture optical density (OD) at 750 nm and total chlorophyll content at OD 652. In all analysis algae cultures in exponential growth phase (optical density OD 750= 0.45-0.5) were used and eventually diluted with TAP media. Set up and growing of the algae takes almost a week and after two weeks from the first preparation we refreshed the sample preparation with new cultures (200-250 ml). Because of lack of time and volume capacity to grow the cells in large scale (all the bio-reactor were busy for other cultures at this time) we were not able to prepare the thylakoid membranes. Therefore, the experiments have been performed only using whole cells.

In total four different samples were grow reference strain IL, and the mutants S264K; I163N and F265T.

IL is an D1 intronless mutants having the wild-type sequence of the D1.

Mutant S264K host an aminoacidic substitution in one of the aa involved in the H-bond formation with plastochinone. It is known to be resistant to atrazine. It has a photosynthetic efficiency and an electron transport chain impared compared to the reference strain IL.

Mutant I163N host an aminoacidic substitution in close proximity to the Tyr161. It has a photosynthetic efficiency an electron transport chain similar to the reference strain IL. It is more stable in High light conditions, and a better performance after 15 days stay onboard the International Space Station.

Mutant F265T host an aminoacidic substitution in the Qb binding pocket. Molecular dynamic simulations studies indicates that this strain is more sensitive to atrazine. Experimental

test confirmed the computational results. It has a photosynthetic efficiency and an electron transport chain strongly impared compared to the reference strain IL, and it grows at lower rate compared to IL and the other strain.

With the exception of I163N, all the mutants have a reduced content of chla, and carotenoids.

3) **Biophysical experiments.** At the end of the second week once the algae cultures were ready to be used the following measurement were performed:

- Detection of time courses of the chlorophyll fluorescence yield in the time domain ranging from 100 µs to 1000 ms after excitation by nanosecond laser flashes (*prompt fluorescence)*
- Detection of time courses of the delayed chlorophyll fluorescence (recombination fluorescence) in the time domain ranging from 10 µs to 100 ms after excitation by nanosecond laser flashes (*delayed fluorescence*)

The decay in the *prompt* chlorophyll fluorescence after Laser-flash excitation is an experiment that reflects the rate constants of Qa=>Qb electron transfer. The prompt fluorescence decays will also allow the Qa correction of the delay fluorescence data.

The *delay* fluorescence results from repopulation of chlorophyll state of the PSII antenna system by recombination of radical –pair states. Delay fluorescence time courses gives complementary insight in the kinetic (constant rate) and energetics (free energy differences) of the processes at the donor side of the PSII.

Prompt (PF) and delayed fluorescence (DF) spectra were collected using a commercial fluorometer. Time course of delay and prompt fluorescence, after laser flash excitation of 5mJ/cm2 (pulses of approximately 5 ns full width and lambda=532nm) were measured using a home build set up for the DF and a commercial instrument for the PF.
Both experiments worked quite well on intact cells, however we had to spend few days to optimize the samples conditions (dark adaptation time, concentration), instrument and laser set up. The delayed fluorescence was more difficult on intact cells because of a high level of light-scattering, but after several test we achieved to measure good spectra.
In order to perform time-resolved investigation of *prompt and delayed fluorescence* 50ml of cultures were harvested, for each experiment, using a clean bench. At the end of the day of the measurements all left samples were wasted. For both types of measurements 1.5 ml of fresh sample was used and wasted after laser exposure.The wild type and mutants were measured at 3-4 distinct concentrations and each measurement at given concentration was repeated minimum 4 times up to 8 times. All samples were dark-adapted (4 hours) and all measurements were made in the dark.
I perform the time-resolve experiments with the help of the Phd Zhiyong Liang who help me for the alignment and general laser set up.

Prompt fluorescence. I first started the measurement using the prompt fluorescence set up. Once the laser alignment has been optimized, I spent several days repeating the measurements at room temperature for all samples. The samples were excited, via the top side of the cuvette, by a saturating ns Laser pulse. The number of recorded flashes was 32, which corresponds to the flashes in the delay fluorescence experiments. The time course in the yield of the prompt fluorescence were measured using a pump-probe. Figure 1 reports an example of the recorded measurement for each sample and at various concentration. Spectra with a similar profile intensity show the reproducibility of the measurement at the same concentration and will be all used to improve the statistics of the profile. It is possible to remark different time dependence of the decay, between the four samples, which suggests diverse constant rate. A detailed data analysis will be performed in collaboration with Prof. Dau and his coworkers. The prompt fluorescence decays will be also used for the Qa^- decay correction of the delay fluorescence decay.

In the *Delayed fluorescence experiments* the dark adapted samples were excited by a saturating Laser flash, which was widened by lens and shaped by an aperture to yield approximately homogeneous illumination matching the width of the used optical cuvette. The intensity of the delayed fluorescence decreases by about five orders of magnitude in the time range from10 µs to 60 ms after the laser flash. Therefore, the final decay intensity are a combination of the signal of two amplifiers. The first provide data points for t< 500 µs and the second the data points for t > 500 µs. For details on the used set up please refer to Prof. Holger Dau website. Figure 2 represents an example of the collected data. It reports the change in the yield of delayed fluorescence decay for wt (WT) and all mutants (F, I, S) after the third laser flash whitout corrections and including the best fit (straight line). The third flash time course is directly related to the kinetics and energetics of dioxygen formation in the S3=>S4=>S0 transition. Figure 3 shows the flash number dependence of the ampliture of the O_2-formaton component in the DF transients measured for all samples.for each flash the DF intensities measured in the time interval up to 2ms (figure 3a) and 200 µs (figure 3b) were summed up. The intensities of the wild type and I163N profile presents a pronounced peak at the third flash caractheristic of a correct O_2-formation. On the other hands the integrated intensities associated to the S264K and F265T mutants show a lack of the third-flash peak suggesting a miss functioning of the OEC. A detailed data analysis and interpretation will be performed in collaboration with Prof. Dau and his coworkers. A quantitative analysis of the DF transients will facilitate the determination of the free energy changes and rate constants in the dioxygen formation. The data analyses aimed to verify whether and to a what extent wild-type and mutant strains are characterized by a different dynamics behavior.

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Figure 1. Prompt fluorescence data – 3rd flash

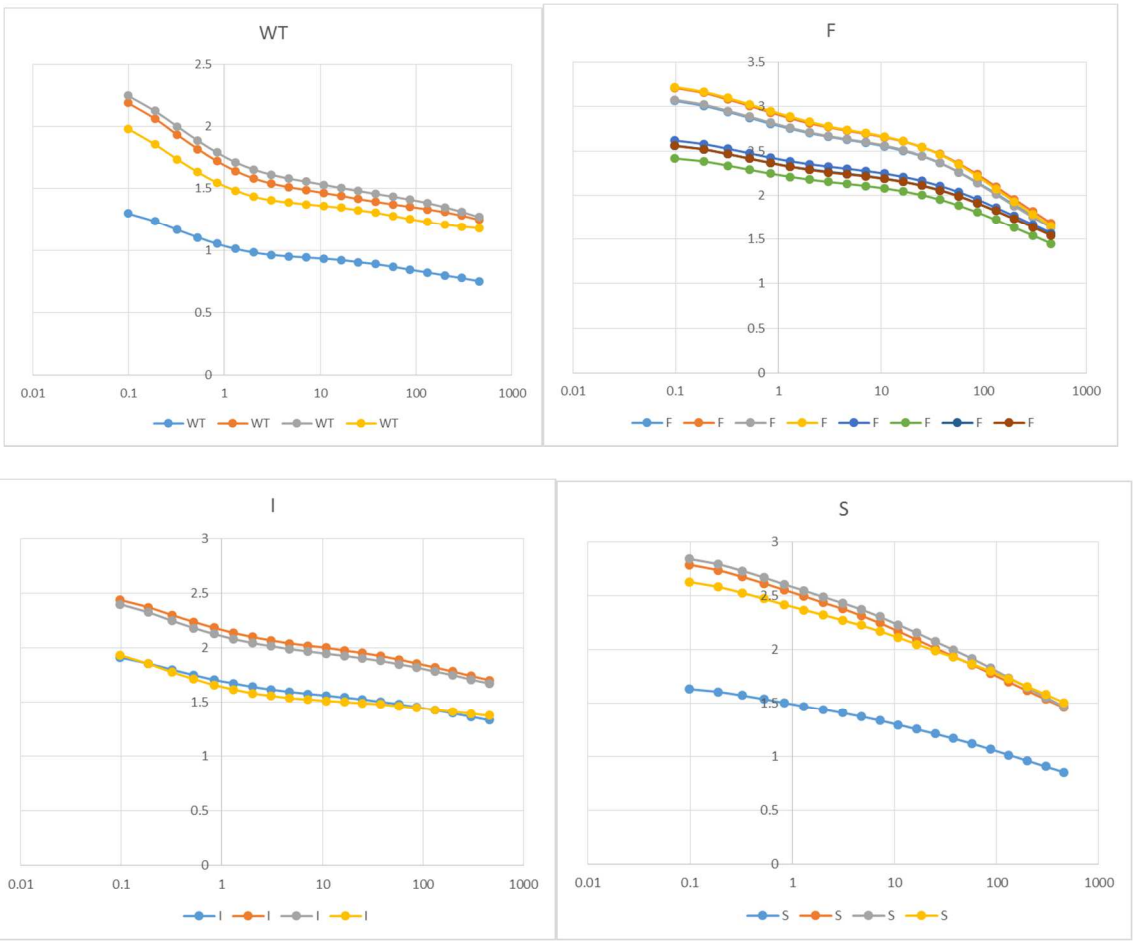

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Figure 2. Delayed fluorescence data – 3rd flash

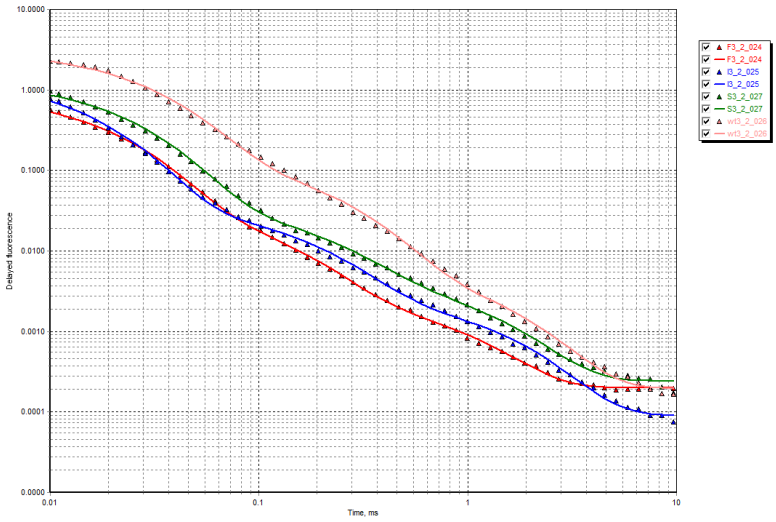
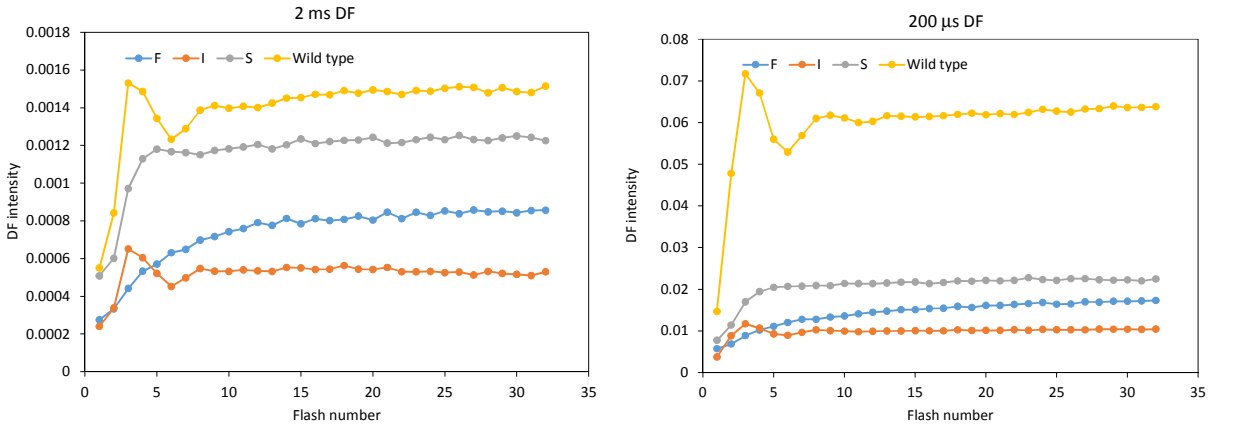


Figure 3. Flash numbers dependence of the amplitude



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Appendix 1

The impact of solvent viscosity on reaction kinetics: light-driven catalysis in Photosystem II (PSII) investigated in various glycerol-water matrices

ABSTRACT

Photosynthetic water oxidation takes place in photosystem II (PSII), a cofactor-protein complex embedded in the thylakoid membrane of plants, algae and cyanobacteria (*1-2*). The process of light-driven water oxidation facilitates the use of water as a raw material for formation of biomass in general and of energy-rich carbohydrates specifically. Light-driven water oxidation also has shaped the atmosphere by production of molecular oxygen (O_2). In this context, it is of great relevance to study the structure/function/dynamics relationships of proteins of photosynthetic organisms, in order to identify the parameters underlying performance in terms of charge separation efficiency, catalysis, protein stability, and functional reliability. We believe that the clue for the key towards understanding of the kinetic efficiency of the reactions in PSII is the coupling of functional processes to protein dynamics. The present study addresses this problem comparing the neutron scattering experiments of PSII complex in various solvent viscosity matrices.

Introduction

Today, water oxidation by PSII is of special interest (i) because of its employment in biotechnological systems for fuel production (e.g. photobiohydrogen) and (ii) as a blueprint for synthetic systems mimicking the functionality of the biological process (artificial photosynthesis).(*3-4*) Moreover, the PSII is of interest for application in semi-artificial hybrid systems aiming at novel analytical or sensory devices (5-6).

In spite of recent progress in crystallographic characterization (*1*) and advanced biophysical investigation of PSII function (e.g., **7**, *8*), a crucial aspect of the PSII function has remained enigmatic. The reaction kinetics of the processes in PSII that proceed in the microsecond and millisecond domain (*4*) have been discussed either in terms of electron-transfer theory or in the framework of transition-state theory. However these conceptual approaches largely have failed. They can explain neither the often surprisingly low values of experimentally determined activation enthalpy nor the enigmatically high value of the entropy of activation. We believe that the key to understand the kinetic efficiency of the reactions in PSII is the coupling of functional processes to protein dynamics.

Results of preliminary work.

We have tracked the reactions at the electron donor side of PSII (water oxidation) and at its acceptor side (quinone reduction) observable after laser-flash excitation in the time domain of 10 µs to several hundred milliseconds. By variation of the glycerol content in the otherwise aqueous buffer (from 0% to 50%), we detected a pronounced influence of glycerol on electron and proton transfer step in water oxidation and quinone reduction (Yong et al, unpublished). Moreover, we have detected first indications that activation enthalpies are strongly modified on increasing glycerol concentrations. These findings have led us to the working hypothesis that the detected change on PSII function results from an influence of the increased solvent viscosity on the protein dynamics. This is in line with earlier investigation on (clearly smaller) biological macromolecules, for which a strongly reduction of the extent of the protein structural fluctuations amplitude on increasing the glycerol concentration has been revealed by neutron scattering experiments (9, 10).

Now we intend to correlate the PSII ns-ps dynamics, as measured by NS experiments, and its electron and proton transfer performance. The dynamics of PSII have been investigated by NS experiments before (11). Yet the combined investigation of solvent viscosity and temperature influence on reaction kinetics and protein dynamics represents a novel line of research, we consider as especially promising.

Proposed experiment.

We aim at probing the effect of solvent viscosity on the ns-ps dynamics of PSII, and eventually relate this behaviour to its biological functionality. To this purpose, we will measure the mean square displacement (MSD) and internal protein dynamics in various water-glycerol perdeuterated mixtures ranging from 0 up to 50% of glycerol content. The PSII samples prepared from spinach leaves protocols well established in the applicant's labs. The PSII particles will be purified and washed using deuterated buffers and will be provided in form of suspensions of PSII membrane particles. These particles consists of sheets of PSII-rich membranes (the lipid component is comparatively small); their extension exceeds 100 nm within the membrane plane. The signal from these systems comes mainly from hydrogen incoherent scattering. As the protein component is dominant in these PSII particles, and the hydrogen percentage is higher in proteins compared with every other type of macromolecule, it is reasonable to assume that protein motions dominate the macromolecular scattering signal from PSII. We will measure the PSII system in the solution at a concentration suitable to obtain a good signal. Complementary measurements will be performed also on the system in the sticky paste form: a) 80% D2O, 5% glycerol (D), 15% PSII particles b) 35% D2O, 50% glycerol (D), 15% PSII particles.

We propose to investigate the temperature and solvent viscosity dependence of the PSII MSD on the long time scale using the high resolution and high flux Sphere backscattering spectrometer. Considering the size of the particles, the global translational motion can be safely neglected. We will use Sphere in the standard elastic scan configuration, to acquire spectra between 50 K and 320 K. Measurements from 280 K to 320 K will be first performed before cooling, in order to probe and compare the complex flexibility before and after freezing. Working in a ramp mode (heating the sample in with different rate as a function of T, while measuring elastic scans), we estimate that **3 days** of beam time would be enough to measure 6 samples in solution (0, 10, 20, 30, 40, 50, % in glycerol) (8h/sample), 2 (6h/sample) in the *paste* configuration, calibration, vanadium and empty cell