PHD THESIS

(SUMMARY)

Induction and relaxation of bacteriochlorophyll fluorescence of purple bacteria

Emese Asztalos

Supervisor: Péter Maróti, PhD, DSc

Graduate School of Physics

University of Szeged

Faculty of Medicine and Faculty of Science and Informatics

Department of Medical Physics and Medical Informatics

Szeged

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Introduction

Pigment molecules excited by photon absorption can return to their ground state by photon emission. The emission is called fluorescence, if the transition occurs between singlet states. Thanks to the (bacterio)chlorophyll pigments, photosynthetic organisms (green plants, algae and bacteria) are able to emit fluorescence, which is very weak (has low efficiency) and invisible for human eyes (it is in the near infrared range). However, it is widely used to determine the photosynthetic capacity of living organisms. The time dependence of the fluorescence intensity shows a characteristic rising and later falling kinetics ("induction") while the cells are moved from dark into light (steady state excitation). Though the basic phenomena is described 80 years ago on green plants [Kautzky és Hirsch 1931], the first step to its right interpretation was taken only in 1963 [Duysens and Sweers, 1963]. This is the so-called „Q-hypothesis", according which the redox state of Q (the primary quinone) determines the fluorescence yield: the yield (and so the observed fluorescence intensity) is small (F_0) if Q is oxidized and high (F_{max}) if Q is reduced. As the initial steps of photosynthesis determine the redox state of Q, therefore the "Q-hypothesis" involves a complementary relationship between fluorescence and photochemistry. Since the early nineties, numerous devices have been constructed to measure fluorescence induction of higher plants and algae (e.g. PAM), and sophisticated theories have been published to connect the measured data with actual photosynthetic processes. In spite of the efforts, the interpretation of fluorescence induction experienced under various conditions has remained obscure and has shown up severe inconsistence in many fields. On the other hand, the literature related to the bacterial fluorescence induction is poor, not many measuring devices are available and the theories used to understand the data are taken formally from the field of higher plants. The specificity of the bacterial system is usually not taken into account. I mention only one work, in which 5-6 closely neighbored phases were presumed in the rise of fluorescence induction measured in the 1-50 ms time range on *Rhodobacter sphaeroides* [Bína et. al 2010]. On the basis of our experiments presented in this thesis, we conclude that the measured fluorescence kinetics can be easily overlapped by artifacts originating from the measuring device and/or from the measuring conditions.

The fluorescence induction monitored during a dark → light transition is followed by relaxation of the fluorescence yield to the initial dark level after turning off the exciting light. Similarly as the fluorescence induction, the kinetics of relaxation is also sensitive to the several key electron transfer processes in early stage of photosynthesis, thus it can be utilized as a diagnostic tool of the photosynthetic capacity of the organism. The prerequisite is the availability of a proper theory behind the observed phenomena. The former results found in the literature and their interpretations are considered as initial attempts only [Kolber et al. 1998]. All of the theories of

relaxation connect the observed kinetics almost exclusively to the re-oxidation of the reduced quinones of the acceptor side. According to our observations, not only the acceptor side and its redox states, but also the donor side reactions can contribute to determinate the rate of opening of the RC, i. e. the decay kinetics of the fluorescence yield. Additionally, in some strains (e.g. *Rsp. rubrum*) the limitation can come entirely from the donor side. We agree that the acceptor side can be made responsible for the fluorescence relaxation in green plants, as the oxygen evolving complex on the donor side reduces the oxidized dimer in the sub-microsecond time scale. However, it is not always the case in bacteria where the re-reduction time of the oxidized dimer can change on a wide time scale (10^{-7} – 10^{-1} s) depending on the bacterial strains.

Although the kinetic behavior of bacterial fluorescence seems simpler, than that of algae or higher plants, much less results and interpretation on this field have been published so far. With the aim to make contribution to this research, we think the construction of a bacteriochlorophyll fluorometer would have priority to obtain reliable fluorescence data. Based on these experimental results, comparative studies can be carried out on fluorescence kinetics of different bacterial strains under various conditions and treatments of the bacteria. Then coherent kinetic models can be elaborated to understand the connection between induction/relaxation of fluorescence and energy/electron transfer processes in/around the RC.

Aims

- To design and to construct a set-up to measure the fluorescence induction of different bacterial strains under well-defined conditions and in wide time range with rectangular shape of illumination or a series of flashes. The advantage of measuring with flash series over continuous illumination is that the fluorescence induced by individual flashes is insensitive to the fluctuation of the background.
- To understand the origin of the different kinetic components of the multiphasic fluorescence induction measured under various conditions of bacterial strains *Rba. sphaeroides*, *Rsp. rubrum* and *Rvx. gelatinosus*.
- To use the fluorescence induction as assay for physiological investigations: to determine the photosynthetic capacity of bacteria and to follow the drop the capacity of bacteria exposed to heavy metal contamination.
- To reveal the background of fluorescence relaxation in bacteria: what electron transfer reactions in the RC are the bottle neck of re-opening of the closed RC.

Methods

Cell cultures

Bacterial cells (*Rba. sphaeroides*, *Rsp. rubrum Rvx. gelatinosus*) are cultivated anaerobically in Siström' minimal medium in 1 liter screw top flasks under continuous illumination of about 13 W m^{-2} provided by tungsten lamps (40 W). The cytochrome c_2 deficient mutant of *Rba. sphaeroides* CYCA I was cultivated in the dark on a shaker (1 Hz) in the presence of antibiotic kanamycin and spectinomycin. Using the strain CYCA I, the kinetic contribution of the redox states of the donor side is negligible, because of the absence of the cyt c_2 and long lifetime of P^+.

Most of the measurements were carried out on intact bacterial cells. Optical detection of whole cells is more difficult than that of the chromatophores (isolated membrane fraction) or RC proteins. This is partly due to the much smaller concentration of the pigments in the cells than in purified solutions. For example, 1-2 μM RC concentration can be easily achieved in detergent solution, but the actual RC concentration of the culture of 10^9 cell/ml concentration is 3 orders of magnitude smaller and amounts to about 1 nM only. Additionally, the cells constitute a highly scattering medium for optical investigations.

Fluorescence induction and relaxation

Fluorescence induction can be measured during rectangular shape of illumination or with a series of short and weak flashes. The flashes are delivered by a high power laser diode with peak wavelength at 808 nm, halfbandwidth of 2.5 nm and maximum power of 2 W. It assures close to perfect excitation, because it fits the 800 nm absorption band of the LH2 antenna complex of *Rba. sphaeroides*. Measurements are carried out with bacterial cell cultures in a 3*3 mm prismatic quartz cuvette. The illumination is homogenized by ground glasses and the layer thickness is small enough to avoid secondary effects. For detection of the fluorescence, an avalanche photodiode is applied and is protected by an IR cutoff filter from the scattered laser light. To measure fluorescence induction and relaxation, a new pump-and-probe device was constructed and built in our laboratory. Devices used so far measure the fluorescence induction and subsequent relaxation separately, but in our setup, they can be measured simultaneously. Moreover, the device is capable to detect flash-induced absorption changes.

Delayed fluorescence

Cells are excited by a 532 nm wavelength laser light, produced by a frequency doubled and Q-switched Nd:YAG laser and delayed fluorescence is detected with a cooled photomultiplier (PMT). The PMT is protected from the very intense prompt fluorescence by an electronically controlled

mechanical shutter or electronic gating. In the latter case, the delayed fluorescence is measured using a photon counting card supplied by MCDWIN software.

Flash-induced absorption change

Flash-induced absorption change of whole cells was measured by a home-built setup. A monochromatic, continuous measuring beam, which was obtained from a tungsten lamp and a VIS/NIR monochromator, passed through the sample. To attenuate the scattered light from the actinic flash, a second monochromator was placed between the sample and the detector. The flash excitation changed transiently the absorption of the sample and the kinetics of the absorption change was measured. The detector was a photomultiplier and the excitation was provided by a laser diode or a Xe-flash lamp with appropriate color filters.

Chemicals

The redox state of the cells can be modified by certain chemicals: aqueous solutions of potassium-ferricianide and sodium-dithionite were used to shift the actual redox potential to oxidative or reductive directions, respectively. To mediate the actual redox potential, conventional redox mediators were applied. Terbutryn competes to the Q_B binding site of the RC with the native ubiquinones, thus we used to block the interquinone electron transport ($Q_A^- \rightarrow Q_B$). Myxothiazol and antimycinA were applied to inhibit the function of cytochrome bc_1 complex.

Mathematical methods

In some limiting cases, the set of differential equations could be solved exactly. In vast majority of cases, however, analytical solution could not be expected but numeric methods had to be applied. It included partly (Marquardt-) fit of the parameters to the measured kinetics, and partly computer simulation. The primary role of the applied computer methods was not the precise determination of the parameters, but to recognize the tendencies only. This kind of approach provided a qualitative rather than a quantitative interpretation, and helped to decide whether the applied kinetic models are appropriate and should be discarded.

Results

1) A new bacteriochlorofill fluorometer was constructed: it is portable due to its compact design, cheap, easy to handle, computer controlled and suitable for measuring fluorescence of intact bacterial cells

The constructed bacteriochlorophyll fluorometer is a portable device for determining properties of photosynthetic bacteria by measuring flash-induced changes (Figure 1.). We can monitor the induction and relaxation of bacteriochlorophyll fluorescence during and after the excitation laser

flash, respectively and the absorption change due to the oxidized dimer after a flash. The advantage of this device is that all three optical assays are integrated into one setup. Exciting (808 nm) and monitoring (785 nm for absorption change and 808 nm for fluorescence) flashes are produced by high power laser diodes. The low sample volume (200 µl), the fast time resolution (5 µs) and sensibility (16 bit), stability and reproducibility of the signal from biological sample are attributed to the compact design.

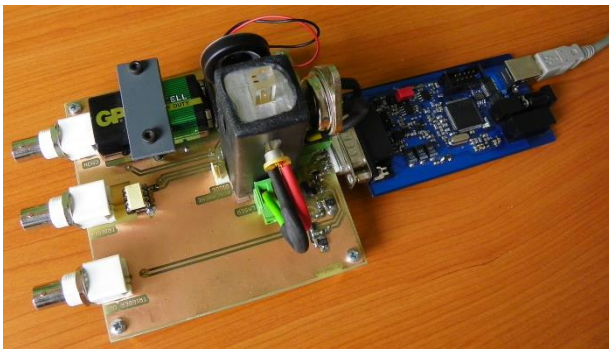

Figure 1. The bacteriochlorophyll fluorometer

The well-known pump-and-probe method is used. A strong flash with variable duration induces photochemical and photophysical reactions in the sample and the kinetics of relaxation (decay) is monitored by a series of weak and short flashes. Changes of the actinic flash intensity and/or duration (10 µs – 1 ms) will affect the (redox) state of the photosynthetic apparatus characterized by the actual levels of the yield of fluorescence (F) and absorption change (ΔA) of the bacterium measured by the series of weak testing flashes.

Kocsis P, Asztalos E, Gingl Z, Maróti P (2010) Kinetic bacteriochlorophyll fluorimeter. Photosynth. Res, 105: 73-82.

2) Two precursor states of delayed fluorescence were identified (cyt $c_2^{3+}PQ_A^-$ és $P^+Q_A^-$) in *Rba. sphaeroides* intact cells, whose lifetimes are controlled by the cyt bc_1 complex (cyt $c_2^{3+} \rightarrow$ cyt c_2^{2+}) and charge recombination.

Under physiological conditions in intact cells, the flash induced charges in the RC are stabilized due to the fast forward and the much (2-3 order of magnitude) slower backward electron transfer reactions. The stored free-energy can be used later by energy consuming reactions. While backward reactions usually have minor role ($k_{r1} << k_2$ vagy k_3 és $k_{r2} << k_1$), they could become significant, if the transport of charges is inhibited by a variety of chemical treatments (e.g. inhibitors) and/or by modification of the free-energy levels of the components. Measuring delayed fluorescence is an excellent tool for measuring backward reactions. In addition, we carried out prompt fluorescence and flash-induced absorption change measurements to study the role of charge recombination.

In intact cells, DL comes from thermal repopulation of antenna bacteriochlorophylls from the $P^+Q_A^-$ or cyt $c_2^{3+}PQ_A^-$ precursor states through P*. The enthalpy change accompanying the backward reactions was determined using van't Hoff plot: $\Delta H_{P^*Q} = 340$ meV (pH 7.8) for the $P^+QA^- \leftrightarrow$ P* reaction. The free-energy change in intact cells at room temperature is $\Delta G^0_{P^*Q} = 870$ meV, so the contribution of entropy change ($-T \cdot \Delta S^0 = 530$ meV) is bigger than the enthalpy change.

Determination of the enthalpy change of cyt $c_2^{3+}PQ_A^-$ ↔ cyt $c_2^{2+}PQ_A$ transition from the observed DL is not so easy, because of the short lifetime of the precursor, thanks to the steep temperature dependence of cyt c_2^{3+} re-reduction rate. At higher temperatures, the repopulation of P* can be higher, but also smaller because of the short lifetime of the precursor. Considering the two antagonistic effects, 1020 meV enthalpy change can be rated for the cyt $c_2^{3+}PQ_A^-$ ↔ cyt $c_2^{2+}PQ_A$ reaction.

The free-energy gap between P* and $P^+Q_A^-$ has minor but, definite pH dependence, that refers to flash-induced protonation steps around the quinone binding site. Since we fitted the moderate pH dependence of the free-energy gap with the sum of two Henderson-Hasselbalch curves, very small p*K* shift was obtained for the acidic ($pK_1' - pK_1 = 0.75$) and alkaline ($pK_2' - pK_2 = 0.4$) groups, which indicates that the protonatable groups are in weak interaction with flash-induced charges on Q_A^- and P^+. The pH independence of the free-energy gap of the cyt $c_2^{3+}PQ_A^-$ ↔ cyt $c2^{2+}PQ_A$ reaction was observed, which refers to the participation of P^+.

Back reactions result in delayed fluorescence and are waste processes with respect to light energy utilization. Their contribution is controlled by addition of redox chemicals and inhibitors and by modification of the duration of the excitation. Our results can be the basis of a monitoring system for studying the light utilization of photosynthetic bacteria.

Asztalos E, Maróti P (2009) Export or recombination of charges in reaction centers of intact cells of photosynthetic bacteria. Biochim. Biophys. Acta 1787, 1444-1450

3) The fluorescence induction of photosynthetic bacteria is multiphasic: it is determined by photochemistry, connectivity between the photosynthetic units (PSU), formation of triplet states, donor and acceptor side electron transfer reactions and cyclic electron transfer.

The phases can be divided according to their kinetics into the following groups:

If the excitation light intensity is relatively small, the photochemical phase will dominate. This process reflects the closure of the RC ($PQ_A \rightarrow P^+Q_A^-$). The photochemical rising phase is modified on the faster and slower time scales, because other energy and electron transfer pathways and reactions may be coupled to the charge separation reaction between P and Q_A. This can easily happen in living systems: after charge separation, the charges will be removed from the donor and acceptor sides by electron transfer reactions and new hit (excitation) can also occur. The measurement of the pure photochemical rise in living system is difficult. Moving towards slower time scales (smaller exciting light intensities), the electron transfer reaction will have more time to transport the charges from the RC, therefore larger fraction of the RC will be open and the level of fluorescence will drop. The smaller is the excitation intensity, the smaller will be the level of fluorescence (Figure 2.). The donor side electron transfer as part of the cyclic electron transfer

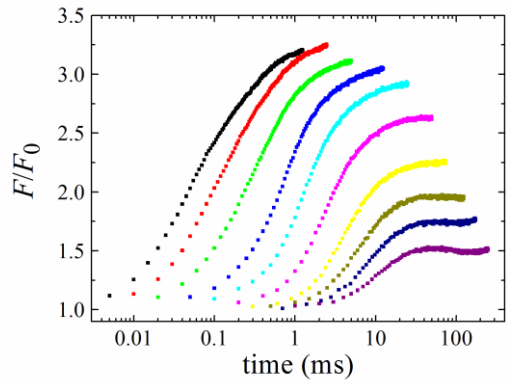

Figure 2. Fluorescence induction of bacterial cells, measured with series of flashes.

competes with the photochemistry. Measuring fluorescence induction in the slow time scale, allows us to determine the quasi-photochemical quenching, which can be related to the photochemical capacity and to the rate of cyclic electron transfer in this system. The yield of fluorescence will reach lower or higher values depending on whether the cyclic electron transfer through the complexes is faster or slower, thus it provides assay to monitor the physiological conditions of the cells.

By increasing the excitation intensity, closing of the RC takes place in shorter time. The rise of fluorescence kinetics shifts towards shorter time scale. In a relatively narrow time period, the reciprocity relationship of light intensity and rise time (their product is constant) can be measured: the higher is the excitation the faster is the rising. In the range of very high excitation light intensities, fluorescence quenchers (mainly triplets) appear. Triplet quenching can originate from carotenoid (Car) or bacteriochlorophyll (BChl) triplets, when the RC is already closed. Their lifetime is about 2-10 μs (Car) and 60 μs (BChl) depending on the strain of the bacterium.

In all bacterial cultures we used, the rise of the fluorescence induction is biphasic. Both phases depend on the excitation light intensity. Terbutryn, the potent inhibitor of the interquinone electron transfer, reduces the amplitude of the second phase. This suggests that the occurrence of this phase needs multiple turnovers of the RC, i.e. multiple photon hits. In CYCA I strain, where only one photon hit (one charge separation) is possible in the observed time range because of the lack of cyt c_2, the amplitude of second phase is much smaller than that of the first phase. From these observations, we conclude that no matter on how intense or long is the excitation, the maximum level of fluorescence can be reached, if the RC is closed from the donor and acceptor sides simultaneously, and the ubiquinone pool is also reduced. The $P^+Q_A^-QH_{2,\,pool}$ state gives the true F_{max} level. Although the RC is trapped in PQ_A^- (wt) or $P^+Q_A^-$ (in CYCA I) states in the presence of terbutryn, another additional rise still remains in the kinetics after the photochemical phase, which smaller in amplitude and could not be linked to photochemistry. This phase can be qualitatively explained by connectivity between PSUs, that includes the excitation energy migration from closed RC to an open RC. This phenomenon is responsible for the deviation of the fluorescence induction from strictly exponential rise. Fluorescence starts with lag phase (CYCA I cells) or straight line (2.4.1. wild time) and thereafter rises monotonously to F_{max} similarly as an exponential rise.

4) The kinetics of fluorescence relaxation is limited by donor and/or acceptor side reactions in the different strains: *Rvx. gelatinosus* is acceptor side limited, *Rsp. rubrum* is donor side limited and *Rba. sphaeroides* is limited by both sides but the donor side is dominant.

In bacteria, the kinetics of fluorescence relaxation is complex and the role of re-reduction of the oxidized dimer is not negligible. In green plants, the donor side does not play any role in the relaxation, because it is very fast. Therefore, exclusively the redox properties and the re-oxidation kinetics of the acceptor side determine the observed relaxation of fluorescence. In bacteria, the case is more sophisticated: the kinetics of relaxation reflects the contribution of all donor and acceptor side reactions that opens the RC (Figure 3.).

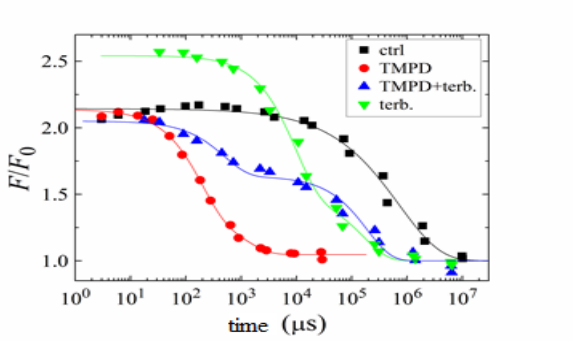

Figure 3. Induction of fluorescence in intacts cells of CYCA I. Conditions: untreated (■), + 100 μM terbutryn (●), + 5 mM TMPD (▲), + terbutryn and TMPD (▼) and duration of exciting laser flash 40 μs.

We observed, that after longer excitation, the half time of fluorescence relaxation amounted 10- 100 ms, and after a short flash it became shorter and the kinetics more complex. The kinetics is determined by the redox states of pool quinones and pool cytochromes. In the strain CYCA I, the relaxation half time is about 100 ms- 1 s, and the duration of excitation does not modify this value. Terbutryn does not have large impact on relaxation. Therefore, the donor side is dominant under these conditions and strain. For acceptor side limitation, another strain, *Rvx. gelatinosus* provides an example: here, the RC has a cytochrome subunit, so the re-reduction of P^+ is fast. We determined the interquinone electron transfer rate $(350\ \mu s)^{-1}$ based on double-flash experiment of electrochromic absorption change (ΔA_{530}), which correlates with the fluorescence relaxation in this strain. From redox titration of whole *Rhodobacter sphaeroides* cells we obtained redox midpoint potential values of 347 mV for cyt c_2^{2+}/cyt c_2^{3+} and 412 mV redox midpoint potential for P/P^+.

Results were submitted for publication to Photosynthesis Research, reference number: PRES-D-14-00118.

5) Hg^{2+} ions have larger destructive effects on *Rba. sphaeroides* cells in the exponential phase of growth and kept in the light, than cells kept in the dark and in the stationary phase. Hg^{2+} ions primarily impair the RC protein, which results in the inhibition of growth of *Rba. sphaeroides* cells.

The photosynthetic bacterium *Rhodobacter sphaeroides* is most sensitive to Hg^{2+} ions out of the investigated heavy metals ion. The mercury ions added in very low (~ 1-10 μM) concentration to the bacterium culture inhibit the cell growth, while other heavy metal ions (Co^{2+}, MoO_4^{2-} and

CrO_4^{2-}) need much higher (1-10 mM) concentration to evoke similar effects. Hg^{2+} -ions inhibit cell proliferation completely for a definite period of time of growth (lag phase of growth), but later (after this lag phase), the cells start to grow at a rate close to that of the untreated culture. During the lag phase, the cells manage to make the mercury ions harmless by physical segregation, chelation, or chemical modifications. The effect of Hg^{2+} was monitored by fluorescence induction, which proved to be a very sensitive tool, so it could be the base of a biomonitoring system.

According to our experiences, the Hg^{2+} ion is very harmful to the photosynthetic apparatus particularly to the RC. The RC protein is among the primary targets of Hg^{2+} destruction. The rise time of fluorescence induction is halved as immediate effect of addition of Hg^{2+} ($t_{1/2}$ ~ 20 s) and recovering this condition will starts only hours later (Figure 4.). Other parameters of fluorescence induction are also modified during mercury treatment. The initial value of the fluorescence (F_0) remains constant, because cells are not dividing, and not producing new pigments. The maximum fluorescence (F_{max}) is continuously decreasing together with the variable fluorescence ($F_v = F_{max} - F_0$). The photosynthetic capacity of Hg^{2+} treated cells measured by the F_v/F_{max} ratio is monotonously decreasing during the mercury exposure, while that of the untreated cells remains constant. The rate and magnitude of decrease of the F_{max} level depend on the physiological state (phase of growth) of the culture and on the light/dark condition of the incubation with Hg^{2+}. Young and prolifering cultures are more sensitive to Hg^{2+} exposure than old cultures in the stationary phase of growth. Additionally, the destruction effect is more pronounced in the light than in the dark. Based on the results obtained from fluorescence induction and supplementary methods, we conclude that Hg^{2+} ions is cell cultures decrease the number of photoactive RCs by eliminating the connectivity between PSUs and by blocking the donor and acceptor side electron transfer reactions

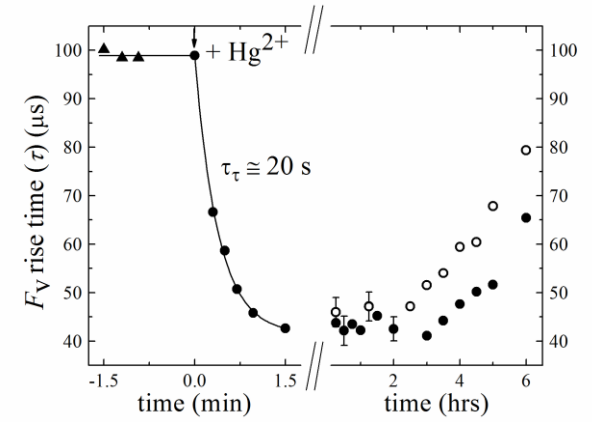

Figure 4. Effect of Hg^{2+} on fluorescence rise time of *Rba. sphaeroides* cells.

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IF: 3.24

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