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Review of PhD thesis

Title: „Photosynthetic electron transport in purple bacteria: an *in vivo* spectroscopic study"

Author: Mgr. David Bína

The thesis focuses on correlation between observed light-induced absorption changes and fluorescence yields of bacteriochlorophylls in whole cells of photosynthetic bacteria. Due to the light scattering, the detection and identification of different pigment forms in the absorption change spectra is not straightforward. However, the author could identify the absorption changes attributed to the electrochromic shifts of the carotenoids and to the redox changes of the dimer of the RC and of the water soluble electron carrier cytochrome c_2. He was able to track the kinetics of theses species on wide time range. One of the most spectacle results of the thesis is the demonstration of the influence of the membrane potential on the yield of bacteriochlorophyll fluorescence. Although the effect has not been proven too large (the yield of fluorescence is quenched by about 10%), its discovery after systematic research is a significant achievement. Several additional interesting findings were described which may draw the attention of the scientific community.

The thesis (80 pages, written in English) is presented in form of three papers (chapters II-IV) published in reviewed journals of Photosynthesis Research (2006 and 2009) and Photochemical and Photobiological Sciences (2005) with detailed introduction (chapter I), not yet published and preliminary results (chapter V) and short summary (chapter VI). It is well written and easily digestible.

Based on the high quality of the PhD thesis, it can be declared, that the author has accommodated a broad spectrum of research methods, was introduced into research work of international reputation, and made the first definite steps to establish a highly ranked scientific career.

To a work that covers large and overlapping fields of various disciplines, several remarks and questions can be addressed. Although they do not jeopardize the essence of the work, the initiated discussion will probably help to get deeper understanding of the basic problems. Taking into account this principle, I collected from each chapter one fundamental subject around which remarks and questions will be listed.

Comments and questions

I. Although the title of the thesis concentrates definitely on purple bacteria, large part is devoted to oxygenic photosynthesis (chapters III and partly I and II). Chapter I (Introduction) is oversized (31 pages out of 80 pages) and the temptation to demonstrate here nice (color) figures (bacteria strains, molecular organizations, atomic structures, etc.) was not utilized. Frequently, not the original source of the discovery is cited but a (text)book (e.g. Bacon Ke (2001)).

II. „*Chlorophyll triplet relaxation and T-S spectroscopy*" (pages 37 and 38)
As the detection of (bacterio)chlorophyll triplet after single flash excitation a) in solution, b) at room temperature and c) in the presence of oxygen is really a great challenge, some concerns may arise about the conclusions drawn from e.g. Figure II. 4.
The absorption band around 600 nm has been characterized as the oxidized state of the RC dimer (P^+) all over the thesis. Now, „carotenoid triplet state relaxation" appears in the first line of the figure legend. In the best case scenario, it can be considered as an indirect consequence of the actually measured P^+ decay. The absorbance change disappears with a time constant of 8.4 μs that would mean that all of the flash induced P^+ converts to carotenoid triplet: $P^+Q_A^- \rightarrow$ car T.

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Questions: 1) What does the natural electron donor to P^+ (cyt c_2^{2+}) do? 2) What is the oxygen-dependence of the lifetime (that would be a clear indication of triplet detection)? 3) How many repetitions were needed to get the excellent decay curve from the noisy spectra at 600 vs. 700 nm (inset)?

III. *Fluorescence quenchers in PSII.*
Q, P^+ and carotenoid triplets are usually considered as fluorescence quenchers in PSII. To my best knowledge, the reduced Pheo does not belong to this list. Can you offer evidences for the fluorescence quenching properties of $Pheo^-$?
Changes of fluorescence intensity do not mean necessarily appearance of fluorescence quencher because (among others) the absorbed and emitted light by the sample can also change by lowering the temperature (among others, the spectra become narrower). We have good reasons to assume that the absorption/emission spectra of room- and low temperature PSIIRC are different. How did you determine the "yield" from the "intensity" of the fluorescence at 77 K?
The experiment in Fig. III.4. is similar to the "light bulb effect" done by David Kleinfeld (San Diego 1984) in isolated bacterial RC. According to his interpretation, the "mystic" conformational change is accompanied by proton uptake. Can it be applied to fluorescence experiments in PSIIRC?

IV. *Light scattering*
The light scattering during strong continuous illumination becomes very large and makes the determination of P^+ and cyt c^{3+} (Fig. IV.3a) and probably of electrochromic shift (Fig. IV.3b) difficult (if not impossible) at $t > 10$ s which is, however, the characteristic time domain of the effect of membrane potential on fluorescence yield and absorption changes (Figs. IV. 4-7).
Although I do not know how the change of the optical density (ΔA) was measured in relative units and how can it be negative at $t < 1$ s (Fig. IV.3a), I conclude that the scattering increased at least one order of magnitude between 1 and 80 s.
The author argues for structural (conformational) changes of the plasma membrane caused by reorganization of pigment-protein complexes. If this is the case,
- the increase of scattering should be reversible,
- the structural changes should be seen even microscopically because the change of scattering is so large and
- the spectrum of (Mie) scattering should be selective (it is attached to absorption bands and determined by the Kramers-Kronig relationship) and not „distorted by an upward baseline shift apparent over the whole spectrum" (page 52).
Did you apply any special techniques (integrating sphere, near position of observation etc.) to reduce or to correct for the large light scattering?

V. *Stationary behaviour of fluorescence and absorption change*
After a transient phase ("induction") of the photosynthetic machinery of the whole cells of bacteria, one can expect stationary operation of the apparatus under continuous illumination ($t > 10$ s). The author demonstrated that the membrane potential did have only minor effect on the fluorescence yield (~10 % quenching) and the carotenoids fulfilled their photoprotective role. What processes have to be assumed to explain 1) the significant drop of the fluorescence yield (see inset of Fig. V.1 but compare it to Figs. IV.4 and 7) and 2) the remarkable oscillatory behaviour of P^+ (Fig. V.4.)?
The long-lived P^+ is exceptionally interesting as there are several routes for fast re-reduction by its natural donor (cyt c^{2+}), by Q_A^- (charge recombination) or by unidentified reducing agents in the solution (the actual redox potential should be definitely much lower than the midpoint potential of the P/P^+ redox couple (~500 mV)). All these reactions are ready to compete with light excitation that generates P^+.

The submitted work fills the requirements of the PhD thesis at the University of South Bohemia, therefore I suggest the oral presentation and defense of the thesis in front of the elected PhD committee. In case of adequate answers to the questions, I recommend the award of PhD scientific degree to the author.

Péter Maróti.

Péter Maróti.

Report on PhD. thesis submitted by Mgr. David Bína

Title: Photosynthetic electron transport in purple bacteria: an in vivo spectroscopic study

Supervisor: doc. RNDr. František Vácha, PhD.

PhD. thesis of David Bína deals with studies of electron transport chain in photosynthetic reaction centers of plants and purple bacteria. The core of the thesis consist of four original papers, three of them already published, one is presented as an unpublished work. The thesis can be viewed as application of a home-built kinetic spectrometer (described in detail in Chapter II) to study influence of protein conformational changes on electron transport chain in reaction centers of PSII (Chapter III) and purple bacteria (Chapters IV and V). The material covered in the thesis clearly demonstrates David Bína's abilities to deal with technical (building and fine-tuning the spectrometer described in Chapter II), experimental (experiments described in Chapters III-V), but also theoretical (modeling fluorescence induction curves in Chapter IV) aspects of research. Although this PhD. thesis may seem short, it has to be taken into account that the work on the thesis has included the building and tuning of the spectrometer. Since this aspect of scientific work is rarely covered in PhD. theses nowadays, the shorter length is certainly counter-balanced by the content.

The submitted text matches all requirements for a PhD. thesis. A short, but concise introduction precedes the four papers; division into chapters and sub-chapters is clear and helps to navigate in text. The quality of English is reasonable, though I have found places where I cannot avoid feeling of a "Czenglish" style, and a different choice of wording would be appropriate. Some words, though they can be found in various online dictionaries, are not commonly used in scientific text (e.g. monochromic, polychromic on page 18; the correct words are monochromatic and polychromatic). Yet, since English is not my native tongue, I do not feel competent to judge details of English grammar used in the thesis. The text is readable and understandable. My main objection to the formal aspects of the thesis is the choice of the title. Only two chapters (Chapters IV and V) deal with the electron transport in purple bacteria. The rest of the thesis is either technical (Chapter II) or focuses on electron transport in plants (Chapter III). In fact, I could not find description of PSII RC isolation in the thesis, except a reference, thus it is not clear from which organism the PSII RC described in Chapter III were actually isolated. In any case, using 'purple bacteria' in the title seems rather inappropriate.

There are some minor mistakes in the introductory Chapter I:

1. In description of the LH2 complex of purple bacteria the author correctly points out that there are either 8 or 9 carotenoids, depending on the species, but wrongly states that the carotenoid is rhodopin glucoside. This carotenoid occurs only in specific LH2's, it is never found in those having 8-fold symmetry.
2. Similar mistake is made in sub-chapter I.2, where one specific measurement of energy transfer between carotenoid and BChl-a, resulting in a rate constant of 1/61 fs, is generalized to all LH2's. This time constant varies dramatically between different LH2's, its actual value depends on carotenoid embedded in a particular LH2.
3. In part dealing with optical spectroscopy (page 17), the author states that "...when electron moves from higher to lower energy level with accompanying emission..." This statement is incorrect, because electron moves from orbital with higher energy to an orbital with lower energy. Also, luminescence is not the only process that results from such a process. There is another mechanism called stimulated emission.
4. Transition between vibrational energy levels is not called internal conversion. The internal conversion is a non-radiative transition between two electronic levels. The transition between vibrational levels is generally called vibrational relaxation.

Besides these minor mistakes that in no way decrease the quality of the thesis, I would like to hear author's opinion on the following points:

In Chapter II, and also at a few places later, the specified spectral resolution of the apparatus is given by the used grating. However, I suppose the other factor affecting spectral resolution is the output aperture of the optical fiber that in this case plays a role of the entrance slit. Another factor affecting the spectral resolution is the number of detector elements. This aspect is obviously negligible when CCD is used as a detector, but when the diode array is used, the 38 elements must significantly affect the spectral resolution. Thus, I would like the author to comment on the spectral resolution and discuss the effect of various elements. Also, it is not clear which data presented in the thesis were measured with CCD detectors, and which were collected using the diode-array. For example, how many experimental points have the spectra shown in Fig. II.2?

In Chapter II, page 35, the author writes that the time-resolution of the instrument is is determined by the width of the measuring pulse. I would like to hear author's explanation why. In my ultrafast world, the time resolution is given by the convolution of the excitation and measuring pulses, because both inevitably contribute to time resolution. I do not understand why it should be different at a time scale of microsecond. If the author's statement is correct, does it mean that is I use 1 μs measuring pulse and 100 μs excitation pulse, the time resolution is 1 μs?

In Chapter V, page 58, the author notes that the fluorescence induction curves in purple bacteria are remarkably similar to those measured for organisms utilizing oxygenic photosynthesis. Among many differences between these organisms, the non-photochemical quenching (NPQ), which is characteristic of oxygenic photosynthesis, should affect the fluorescence induction curves. Is the similarity of the purple-bacterial and oxygenic fluorescence induction curves caused by specific conditions that prevent NPQ or there is another explanation why these curves are so similar.

The PhD. thesis of David Bína matches all requirements necessary for the doctoral thesis at the University of South Bohemia. Therefore, providing that the author answers the comments outlined above, I recommend acceptance of this thesis and awarding the PhD. degree.

2.11.2009

prof. RNDr. Tomáš Polívka, PhD.

Review of the doctoral thesis of Mgr. David Bína

Photosynthetic electron transport in Purple bacteria: an in vivo spectroscopic study

The work of Mgr. David Bína deals with application of kinetic spectroscopic and fluorescence technique (microsecond and miliscecond time scale) in research of basic photosynthetic events in bacterial reaction centers.
The submitted thesis contains 80 pages, consists of an introductory chapter, five research papers and final summary of the experimental results. Paper I (Bína et al. Photosynt Res 88: 351-356) describes the new multichannel kinetic spectrophotometer-fluorometer. This instrument was originally designed by Dr. Šiffel, former advisor of Mgr. Bína, and it operates at microsecond to second time scale. This instrument has been used in all the following studies and the chapter represent a nice technical and methodical introduction to the rest of the thesis. Paper II (Litvín et al. Photochem Photobiol Sci 4:999-1002) describes a study on conformational changes of PSII reaction center and their role in non-radiative energy dissipation. Paper III (Bína et al. Photosynt Res 99:115-125) presents the kinetic measurements of in vivo bacteriochlorophyll changes in *Rhodobacter sphaeroides*. This paper is followed by not yet published Paper IV describing absorbance changes accompanying the fast fluorescence induction in Rhodobacter sphaeroides. These two studies on *Rhodobacter* I consider as the main part of the presented thesis.

In brief, the thesis is a high quality work, which fully meets the international standards. The major part of the results was published in respected international journals. The last paper still awaits the publication. The strong part of the thesis is the use of novel instrumentation combining both fluorescence and absorption measurements, which makes it possible to get better information about the basic photochemistry and subsequent electron thransfer proceses occuring in the bacterial reaction centers.

In spite of that I am obliged to raise some comments and questions:

The numbering system of chapter I. Introduction is not clear.

The introduction chapter is not really up to date. For instance at page 9 the author recapitulated the main phyla of phototrophic bacteria. Unfortunatelly he failed to report

newly discovered group of thermophilic aerobic phototrophs belonging into *Acidobacteria* (Bryant et al., Science 317: 523-526, 2007). At page 11 the author mentioned that "several full genomes of non-sulfur bacter have been sequenced…". In fact there are several tens of finished full genome projects. The author failed to mention the first fully sequenced anoxygenic phototrophis organism *Rba. capsulatus*. Actually, in this project participated also the laboratory of Prof. Václav Pačes from the Inst. of Molecular Genetics in Prague. Similarly there was recently an enormous progress in structural (mostly AFM) studies of puple photosynthetic membrane architecture, which has not been mentioned.

At page 18 the author writes: "…only the chlorophyll of the primary donor of the reaction center is oxidized by light while to each reaction center there may be thousands of pigment molecules in the light harvesting complexes.". This seems to be exagerated. Does the author know what is the typical size of the bacterial photosynthetic units?

The second presented paper (Litvín et al., chapter III) describes the study with photosystem II. This is little out of the topic of the presented thesis which is dedicated to purple bacteria.

The main part of the experimental work has been performed with *Rhodobacter sphaeroides*. Only in one place the author reports that "similar changes were observed also with *Rsp. rubrum* and *Rubrivivax gelatinosus*". How similar-different were the changes in those different species? Do they exhibit the same behaviour? Also the physiology of non-sulfur purple bacteria changes upon cultivation conditions. Did the author tried to perform the same measurements with *Rba. sphaeroides* cultures grown photoheterotrophically (under semiaerobic conditions)?

At page 58 the author talks about $P870^+$ state. Does it mean $P870^+Q_A$ state or it is possible to obtain also $P870^+Q_A^-$ state?

Did the author try to use selective inhibitors such as terbutrine or KCN to see their effect on absorbtion and fluorescence kinetics?

The author presents several figures with linear regression of the experimental data (Fig. IV.5, IV.6 and V.2). Unfortunately he fails to report equations or R^2 values of those analyses.

The last paper (chapter V) is refered as unpublished. What is its current status?

In summary, the work of Mgr. David Bína presents important and new results and proves his ability to conduct productive scientific research. The submitted doctoral thesis meets international standards. I have no doubt that Mgr. David Bína fully deserves the title *Doctor of Philosophy* Ph.D.

Třeboň, November 5th, 2009

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