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REVIEW

of the dissertation for the doctor's degree of the Hungarian Academy of Sciences

titled

"On the role of light-harvesting complex II in regulating the excitation energy flow "

by Petar H. Lambrev Ph.D.

The subject of the dissertation is the light harvesting complex of photosystem II (LHCII) which is the most abundant membrane protein on the planet. It is responsible for harvesting solar power and transferring the excitation energy to the photosynthetic reaction center of plants. The study deals with the structural and functional plasticity of LHCII and with the mapping of the electronic excitation transfer (EET) network of LHCII.

Modern and state-of-the-art methods were applied from broad fields of spectroscopy, biophysics, and biochemistry. The use of 2D electronic spectroscopy and the deep evaluation of the results are especially attractive and novel. The results are sound, well demonstrated and (without any doubts) belong to the frontiers of science.

The formal scientometric data of the candidate (MTA MTMT 2022/02/22) are promising but not outstanding: out of the 57 (total) number of scientific articles, 21 papers indicate the name of the candidate on one of the terminal places in the list of the authors, the cumulative impact factor of the journals amounts to 249, the number of independent references sums 1400 and the Hirsch index is 23.

My comments with critical edge will concentrate on major and general issues selected by topics.

1. *Structure of the LHCII*

- While the common feature of natural light-harnessing systems in photosynthetic bacteria (LHI and LHII) is a ring of any order of magnitude, in plants the organisation is mainly trimeric (or multiples thereof). Do you have a reasonable explanation for that?

- Theses 1 and 2 include the dependence of the structure and mechanism of LHCII on molecular surroundings (environment) concluded mainly from measurements carried out in different model systems where the lipid/protein ratio was one of the essential variables. An important but obvious question arises whether the (rather general and still indirect) declarations can be made more specific to **lipids in the complex**?

- It is well known that the high-resolution structure of plant $C_2S_2M_2$-type PSII–LHCII supercomplex reveals remarkable structural roles of lipid molecules in stabilizing the PSII core complex (Sheng et al.

2018). Moreover, they contribute to oligomerization of PSII core dimer and LHCII trimers and mediate the assembly between PSII and the peripheral antenna complexes including LHCII, CP29, CP26, and CP24. Additionally, they might influence the biological function of the supercomplex by interacting with the neighbouring protein subunits and the function-related cofactors (e.g., chlorophylls and carotenoids). The interesting problem remains open to me: how the interfacial lipid molecules affect the energy transfer, the electron transport kinetics, and the spectroscopic features of the PSII–LHCII supercomplex?

2. *Nonphotochemical quenching (NPQ)*

- Photoprotection by non-photochemical quenching (NPQ) is a very broad term. In most physiological conditions, the main NPQ component is qE, a process in which the energy absorbed in excess is dissipated as heat. Additional components include state transitions [qT], photoinhibition [qI], zeaxanthin-dependent quenching [qZ], and LCNP-dependent quenching [qH]. The process is triggered by the low luminal pH that activates the PsbS protein and the xanthophyll cycle. Due to the complicated nature of the NPQ, it is not yet clear if this process involves **any structural changes** in the PSII. Some data suggest that NPQ involves the association and disassociation of antennae from the core (Holzwarth et al. 2009; Betterle et al. 2009; Johnson et al. 2011), while other experiments indicate that the antenna size of PSII even increases during NPQ (Belgio et al. 2014). Recent results from Bielczynski et al. 2022 indicate that qE rise is not accompanied by a structural disassembly of the PSII supercomplexes. Based on your thesis 6 and 7 and on the general discussion of the dissertation, a firm statement about the structural changes in the PSII upon high light excitation can be deduced. How do you comment the results which seem (in the published form) to contradict this view?

- The nonphotochemical quenching (NPQ) protects photosynthetic organisms from excess irradiation. This process involves the mechanism where the excess excitation energy is dissipated through heat that converts to mechanical vibrations. This is just the subject of the photoacoustic spectroscopy, that was not mentioned in the introduction of the dissertation. The **photoacoustics** has gained broad fields of applications due to the extreme sensitivity and compactness of the method. What do you think, why this method has not been used to study NPQ?

- The model based on radical pair mechanism (Fig. 5.14) seems to be oversimplified, therefore the results of the numerical simulation (Table 5.1) should be taken with caution. I see the weakness of the model based on which the NPQ and PPE (photoprotective effect) were calculated in the following points:

- The RC becomes closed if either the donor (via P^+) or the acceptor (via $Pheo_A^-$) sites (or both) will be blocked (for bacterial systems see Kis et al. Nature Sci. Rep. 12:14298, 2022). However, due to their different redox states, not all closed RCs are capable to form radical pairs. The model in Figure 5.14 is insensitive to this fact.

- The conditions of dark adaptation and closed PSII RCs of Arabidobsis are hard to fulfil simultaneously. Do you think that these conditions are achieved in the F_m state of the fluorescence induction kinetics? In which redox state is the RC closed?

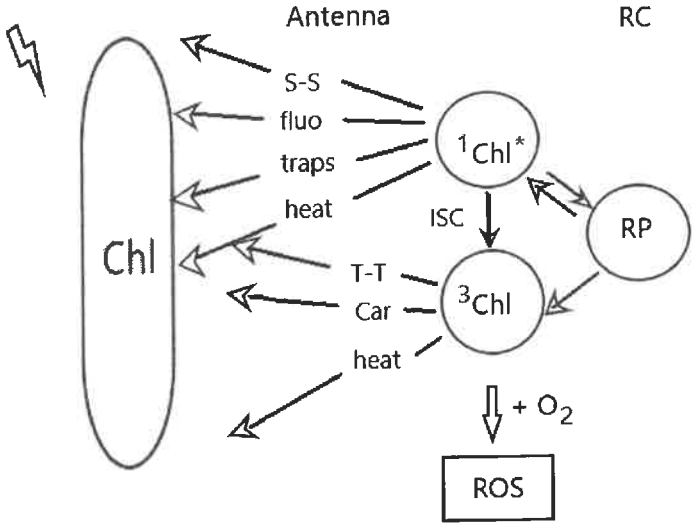
- What are the states and functions of the two radical pairs connected in series in the model (RP_1 and RP_2)?

- No traps of the excited bulk Chls (excitons) are considered (carotenoids, singlet-triplet transition, exciton-exciton annihilation, etc.) which can compete with the radical formation in the RC.

- ^{3}Chl should not be taken as final product in the model (the photodamage is attributed to ROS) but a pool of Chl triplets with inflow (from antenna and radical pair mechanism) and outflow (spontaneous deactivation, triplet-triplet annihilation, quenching by carotenoids and/or by triplet oxygen) should be involved.

- The antenna detachment inherently modifies the organization and the circumstances of energy migration. Consequently, the model should be changed, as well (probably from "lake" to "domain" and/or to "separate" units, see below). The highly qualitative treatment (page 70-71) is obviously not satisfactory. Furthermore, the concepts of reaction rates and reaction rate constants are not properly handled (they are commuted).

Most of these objections can be avoided by a minimum model to derive NPQ and photoprotection (photoprotective effect). Due to an extensive interaction between singlet and triplet excitons in the RC and in the antenna, it is worth to consider several additional pathways (see Maróti and Lavorel, *Photochem. Photobiol.* 29, 1147-1151 (1979); for bacteria Sipka and Maróti, *Photosynth. Res.* 136, 17–30 (2018)).



3. *"Far red" chlorophylls in LHCII*

- According to thesis 2b, the "far red" chlorophylls of long fluorescence decay (large lifetime of excited state) are permanently present in PSII and are part of the photoprotective mechanism by deceleration of the migration and therefore of the funnelling of the excitation energy from the bulk to the RC via uphill transfer. They are considered as signature of NPQ. However, this mode of action is against the effective light harvesting function of LHCII and the concept of RC beeing a deep (effective) trap for the excitons. By what stoichiometry and spatial arrangement of the "red" chlorophylls in LHCII can be achieved the delicate balance between these contradictory functions *in vivo*? How can we understand the nature of low-energy excited states in efficient light collection of photosynthesis?

- In Fig. 5.10, the far-red-emitting forms have much more different spectra than the Chls: very broad and complex. Do they correspond to single species but with extremely complex energetic structure?

- It is confusing to plot the normalized and not the relative amplitudes of the components.

- The sequential model is essentially a branched model with elements coupled in series and parallel. Why the deactivation processes of Chl* different from those CHl*s which transfer the electron excitation energy to far-red-Chls were not considered? They may compete with reactions to and within the CTs.

- "*...the sequential model...explicitly defines the CT states...*" Rather the experiments than a model can prove the CT states.

4. *State transitions*

As photosystem I and photosystem II work in series to drive the electron transport, a balanced excitation pressure is required for optimal photosynthetic performance. When the light conditions favour the excitation of one photosystem over the other, a mobile pool of trimeric LHCII moves between both photosystems thus tuning their antenna cross-section. This process is called "state transition".

- Naively thinking, the balance of the excitation energy between the two photosystems will remain unchanged unless the spectral composition of the excitation persists. However, state transition does take place, when the intensity (low ↔ high) and not the spectrum of the illumination changes. Why?

- When PSII is overexcited then multiple LHCIIs can associate with PSI forming a well-characterized PSI–LHCI–LHCII supercomplex. Based on your model-experiment, can you identify the predicted multiple binding sites of the "additional" trimeric LHCII (thesis 7)? What was the role of LHCI in the excitation energy transfer from LHCII to PSI RC in your (model) experiments?

- Table 6.8: How were you able to calculate the quantum yield of photochemistry based on the expression given in the legend?

- Kinetic scheme (Figure 6.30 A) and fluorescence lifetimes (Table 6.7): According to the model, the weakly bound LHCII (W) transfers excitation energy to the trap (PSI-LHCI core) with rate of $(300\ ps)^{-1}$, although no corresponding component of the fluorescence decay can be seen. The major components appear with lifetimes of 20 ps and 80 ps.

- Thesis 7 says that "*LHCII is an efficient antenna for both photosystems*" because of state 1-state 2 transitions which regulate the balance of excitation energy distribution between both photosystems via lateral diffusion of a mobile fraction of LHCII. This is particularly true under low light conditions. However, state transitions tend to be suppressed under high-light conditions in plants (Oxborough et al.1987). The regulatory function of LHCII seems to contradict the photoprotective role which is one of the most important functions of the LHCII. Can you comment on that?

5. *Energy transfer in LHCII and size of the PSU*

- "*In 1932 Emerson and Arnold proposed that a large number of Chls - as many as 2500 molecules – cooperate to carry out* ***one photochemical reaction****, leading to the concept of the photosynthetic unit.*" This photochemical reaction was the evolution of oxygen, and the reported number of Chl molecules should be related to 1 molecule of evolved oxygen. Furthermore, the evolution of oxygen is followed by not a single photochemical event but a series of reactions.

- There are several models of antenna organization (for bacteria, see de Rivoyre et al. *BBA* 1797, 1780–1794 (2010)): (a) "lake model", characterized by perfect connectivity where energy moves freely between constituent units. This model is often used for PSII. (b) "puddle model" (or model of separate units), an extreme case in which excitation energy absorbed by antenna chromophores is always transferred to the same reaction centers. (c) "domain model", where two reaction centers are

close to each other but the groups of two are not connected to each other. This model is well suited for scenarios in which dimeric aggregation of reaction centers exist. Which model do you use?

- The essential feature of the light harvesting in the antenna is the energy funnel to the reaction center biased by the random walk of the excitation. They contribute to the irreversibility of the trapping of the energy at the reaction center. Here, however, the major photochemical step, the charge separation in the RC was omitted. In lack of the efficient trap by the RC, the excitons can visit much more sites during its long lifetime than in the presence of photochemistry. This will lead to overestimation of the size of the PSU in lack of suited corrections.

- Our understanding of the structure and function of domains and PSUs has become highly sophisticated by recent results from experiments (e.g., high-resolution crystallography) and theory (e.g., statistical models) (see for bacteria Maróti et al. *Nature Sci. Rep.* 10:14012 (2020)). What do you mean structural and functional domain sizes? How can the functional domain size be larger than the structural domain size (page 120)?

- I have concerns about the numerical evaluation of the data. According to my calculations, the numerical values of the first, fourth and seventh rows (different environments) in Table 6.6 do not obey the simple expression $k_q = K_{SV}/\tau_0$ given in the dissertation. Assuming that the measured Stern-Volmer constants, K_{SV} and the fluorescence lifetimes, τ_0 data are correct, then I will obtain k_q values different from those given in Table 6.6:

Molecular environment	Lifetime τ_0 (ns)	Stern-Volmer constant K_{SV} (M^{-1})	Bimolecular rate constant k_q ($M^{-1}\cdot s^{-1}$)	
			Dissertation	Control calculation
LHCII trimers	3.5	$1.4\cdot10^4$	$3.6\cdot10^{12}$	$4.0\cdot10^{12}$
LHCII membranes L/P 300:1	2.1	$9.7\cdot10^4$	$3.4\cdot10^{13}$	$4.6\cdot10^{13}$
LHCII-enriched membranes	1.6	$4.6\cdot10^4$	$5.1\cdot10^{13}$	$2.9\cdot10^{13}$

Therefore, the functional domain sizes given in the table could be wrong. The deviations are not negligible. Did I miss something in the "control-calculations"?

- The functional domain sizes of LHCII in Table 6.6 were calculated based on the assumption of identical light harvesting antenna systems of the species with only difference of the interunit connectivity. Do you think that this condition was fulfilled?

- What is the type of quenching: static (association) or dynamic (diffusion controlled)? The Stern-Volmer analysis of the fluorescence quenching in different molecular environments clearly indicates the lack of diffusional limitation. You obtained k_q (the bimolecular quenching rate constant) values as high as $1.1\cdot10^{14}$ $M^{-1}s^{-1}$ (Table 6.6). For diffusion-limited quenching (i.e., quenching in which the time for quencher PPQ to diffuse toward and collide with excited Chl in the LHCII is the limiting factor, and almost all such collisions are effective), the bimolecular quenching rate coefficient, derived from the Stokes–Einstein relation, is given by $k_q=8RT/(3\eta)$, where R is the ideal gas constant, T is temperature and η is the viscosity of the solution. In water (η = 1 mPa·s, T= 300 K), $k_q = 7\cdot10^9$ $M^{-1}s^{-1}$. This is orders

of magnitude less, than the observed bimolecular rate constant. What mechanism (different from diffusion) can assure the observed very high rate of the quenching? Perhaps, a different and more complex view should be applied (see the next point)?

- The central idea of the determination of the functional domain size in the dissertation is the linear proportionality of the bimolecular rate constant of the fluorescence quenching to the domain size. Can you offer me evidence of that? I do not see how the fluorescence quenching in the presented form relates to the energy transfer in LHCII and additionally, to the domain size of the LHCII. I guess that the quenching of the excited state of the pigment can occur through combined diffusion processes of the quencher, the LHCII particle and the exciton. Unfortunately, the migration of the exciton is not diffusion limited as those of the quencher and the LHCII particle. Formally, a mixture of static and dynamic quenching processes may take place.

- The drop of anisotropy of Chl fluorescence is a sensitive indicator of the transfer of electron excitation energy within the pigment bed. The method is particularly suited to estimate the orientation of the Chls in the aggregates and the diffusion length of the exciton. Do you have any data (either from your lab or from the literature) supporting your results on excitation energy transfer in LHCII?

- May I ask you to estimate (or to measure) the rate constants of processes (photochemical trapping and losses in forms of fluorescence, heat, etc.) competing with the energy transfer in the antenna of any of your systems performing photochemistry (see the model-calculated values of a truncated system in Table 5.1)?

6. *Exciton dynamics*

- All the absorption changes spectra used to construct the 2D plots are normalized and amazingly smooth probably due to the very high light intensity of the measuring beam. How large are the intensities of the pumping and probing pulses?

I'm wondering whether the bright detection beam can cause any disturbing effects like singlet-singlet annihilation or other ways of (e.g., triplet) quenching of the excitons? How low the laser pulse energy should be kept to avoid the exciton-exciton annihilation? Can you offer me the actual absorption change not in relative units but in absolute units (mOD)?

- In your recent paper (Do et al., EPJ Web of Conferences **205**, 09038, 2019), you estimated that the excitation laser spectrum covered nine excitonic states in LHCII. In the dissertation, you considered eight instead of nine excitonic states in your model. What could be the reason to omit the state centred at 659 nm between the states of 655 nm and 664 nm?

- You argue that *"Model A has more states decaying in 0.1-1 ps and provides better fit"* than model B. However, the data in Table 6.3 do not coincide with EET rates for T_w = 200 fs given in Fig 2 of Do et al. (2019). Can it be said that the model used in your previous publication was not appropriate enough?

- I see contradicting conclusions in the two works when the results are compared with the predicted slow exchange rates of Renger et al. (2011). In the dissertation, you argue that *"the EET times are in line"* with those of Renger's calculations, although the slowest energy transfer was 13 ps. In paper of Do et al. (2019), however, similar (9 and 25 ps) or much slower (>100 ps) transfer times were given but the conclusion (despite the slower transfers) was opposite: *"the rates that we obtain are faster than structure based theoretical model by Renger et al."* The 100 ps transfer time was not slow enough to match Renger's expectation?

- The local pigment excitation energies are tuned by the electrostatic interactions with the protein and solvent environment. The interaction between the energetically varying local states results in a ladder of excitonic states, where the higher energy states are localized toward the peripheral antenna complexes, while lower energy excitons are close to the photosynthetic reaction center. The two-dimensional electronic spectroscopy (2DES) is a suited tool to determine the spatial and temporal structure of the exciton states in the pigment bed (see also the FMO protein of green sulfur bacteria). I'm wondering whether the candidate was able to map out the excitonic level-to-level transfer in LHCII? What are the strongly coupled 8 states used in the dissertation?

- I don't see any sub-picosecond kinetics from which the fascinating 2DES maps could be constructed. Why isn't a single kinetics presented in the dissertation? This would be interesting to me to know whether an oscillation at the very beginning of the trace (up to about 100 fs) is observable, which was discovered by Vos back in the early nineties (and were not referred to in the dissertation (page 82)): Vos et al. Direct observation of vibrational coherence in bacterial reaction centers using femtosecond absorption spectroscopy, PNAS 88, 8885–8889 (1991) and Vos et al. Visualization of coherent nuclear motion in a membrane-protein by femtosecond spectroscopy, Nature 363, 320–325 (1993). These and related phenomena initiated heavy discussion among theoreticians. Is there a longer-lived quantum coherence between excitons in photosynthesis or the energy transfer in the pigment bed is controlled exclusively by promptly excited vibrations? What is your opinion about the possible role of interexciton coherence in excitation energy transfer in photosynthesis?

- The Chl fluorescence decay kinetics were routinely decomposed into the sum of several (up to four or even more) **mono-exponential** components ("global lifetime analysis"). The amplitudes and lifetimes obtained from the formal decomposition of the complex kinetics served as basis of extended kinetic models including energy migration steps where the excitation energy is shared by energy transfer among donors and acceptors. However, even in the simplest case of a single donor-acceptor pair coupled by Förster-type of energy transfer (very weak interaction), the decay of the donor fluorescence **does not follow a mono-exponential decay** kinetics but is modified by an exponential function with term of $\sqrt{t}$ in the exponent:

$$I(t) = I_0 \cdot \exp[-t/\tau_0 - 2\gamma \cdot (t/\tau_0)^{1/2}].$$

Here $\gamma = A/c_A$ and A is the actual and c_A is the critical acceptor concentration. The larger is the actual acceptor concentration, the larger is the deviation of the kinetics of the donor fluorescence from the mono-exponential character. Similarly, the fluorescence of the acceptor also shows a complex (non-mono-exponential) kinetics due to the Förster energy transfer from the donor. In more closely connected and extended system, the deviation from the mono-exponential function could be more pronounced. The complex (deviation from the mono-exponential) kinetics is the natural consequence of the energy transfer. Consequently, the peeling into mono-exponential components and their direct mapping to species and reaction rates in simulated systems does not guarantee the validity of the model.

- A general question to the end: the focus of the treatment of exciton dynamics in the antenna was set on the trade-off between increased absorption cross-section and quantum efficiency of PSII (see the adaptation of green alga *Bryopsis corticulans*). Is this question so essential to the alga indeed or we like to increase its importance? The overall yield of the photosynthesis, namely, is very low (a couple of % only) and the bottleneck is certainly not the high yield of photo-utilization in LHCII.

Minor remarks

- I would not use the phrase "*on the role of...*" in the title as it expresses the feeling that your mind drifted back and forth, and the dissertation might be less concentrated, unfocused, and undirected. To avoid that, a more definite statement in the title would have been chosen.

- The formal classification of each thesis statements is divided into paragraphs. However, statements 5 and 7 include a single point only with indication to additional statements.

- About the shot history of the Förster theory: the referred monograph from 1965 was a late consequence of a much earlier published paper in *Naturwissenschaften* in 1946 outlining the quantum-mechanical behaviour of the transfer of electronic excitation energy between two molecules in a solution. This breakthrough work in spectroscopy explained the transfer of electron excitation energy between two molecules non-radiatively.

- According to the original classification of the strength of coupling (very weak, weak, and strong, Förster 1965), the very weak (dipol-dipol) interaction corresponds to the weak coupling you mentioned in your dissertation. If I'm not wrong, I see some deviations of the definitions between you and Th. Förster.

- $f_D(\nu)$ denotes not the fluorescence quantum yield of the donor in the absence of acceptor, but the normalized fluorescence spectrum of the donor.

- $\varepsilon_A(\nu)$ denotes the absorption spectrum of the acceptor and not the fluorescence of A as could be deduced from the not clearly worded sentence.

- What is C in the expression of the Förster radius (Eq. 2.1.10)?

- The sentence on page 28 is obviously not correct: "*On the acceptor side of PSII, electrons are transferred from Pheo to Q_A in 200-300 ps and then to the two quinones Q_A and Q_B....*"

- I do not dare to judge the English of the dissertation except of two (very frequently used) phrases: According to my view:

- the fluorescence decay can be either short or long, but the fluorescence lifetime can be either small or large (and not oppositely, the fluorescence lifetime cannot be short or long). This why the quantity "lifetime" was introduced to measure the decay.

- I would say "the lifetime of the excited state" instead of "excitation lifetime" which could be confused easily and erroneously with "the duration of the excitation".

Conclusions

The new results of the dissertation are summarized in 7 statements. They are partly overlapping and are different from the point of view of novelty, depth of investigations and significance. Without any doubts, identification of excitonic transitions via anisotropic CD spectroscopy (thesis 3) and reveal of the dynamics of energy transfer via two-dimensional electronic spectroscopy (thesis 4) are the landmarks of the present work. These statements are sound and have the greatest impact. They deserve the attention of the scientific community. The candidate managed to line up to the frontier of photosynthesis research of very fast processes. Using high thoughts, he is at the gate of "quantum biology" (as Hannibal was at the gates of the Roman empire ("*Hannibal ante portas*")). This is not an easy task as it requires hard and long work, fruitful international cooperation and state-of the art

instrumentation. Recently, dr. Lambrev makes effort to arrange a lab in Szeged, comparable to those in leading international centers. I highly appreciate his efforts and results.

Compared to the promising results on 2DES, theses 1 and 2 include modest statements. They are based on mainly routine investigations which were carried out with care and require skill and expertise. The outcome of most of the experiments is not very much surprising and fit to the general expectations. E.g., the statement about the dependence of the structure, self-regulation, and the excitation properties of the LHCII on the environment is not exceptionally new and unexpected (the opposite statement would be surprising), although it should be worked out properly with tiresome efforts.

Most of my questions with stronger criticism are related to theses 5-7. I have the greatest concerns about thesis 6. According to my opinion, they are the weakest points of the dissertation. I hope my worries and uncertainty will disappear after the answer of the candidate and after the open discussion.

There is no question of worth of the open discussion of the dissertation.

Szeged, 6th of October 2022.

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