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Minireview

Discovery of pheophytin function in the photosynthetic energy conversion as the primary electron acceptor of Photosystem II

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Abstract

This minireview describes the discovery of participation of pheophytin, a metal-free derivative of chlorophyll, in the early steps of photosynthetic solar energy conversion as the primary electron acceptor of Photosystem II.

Abbreviations: Chl – chlorophyll; PS II – Photosystem II; RC – reaction center; P_{680} (or P680) – the primary electron donor of PS II; Pheo – pheophytin, the primary electron acceptor of PS II; F – chlorophyll fluorescence yield; ΔF – photoinduced change of Chl fluorescence; PQ – plastoquinone; Q (also known as Q_A) – the 'primary' (plastoquinone) electron acceptor of PS II; E_m – midpoint redox potential

Discovery of reversible photoreduction of pheophytin in the primary light reaction of Photosystem II

Until the late 1970s, it was widely accepted that the primary electron acceptor of Photosystem II (PS II) taking electrons from the excited primary electron donor, chlorophyll P_{680}, is a special form of bound plastoquinone (referred to as Q; also known as Q_A) with redox potential of -130 mV; its one-electron reduction is accompanied by characteristic absorbance changes (ΔA) in the UV spectral region as well as by a shift of absorption bands in the visible region (for review, see Knaff 1977). The transition of PS II reaction centers (RC) to the state $P_{680}Q^{-\cdot}$ due to photochemical or dark reduction of Q results in a 3–4-fold increase in chlorophyll (Chl) fluorescence yield (ΔF) (from the level F_0, corresponding to so-called 'constant F', to the maximum level F_{max}) related to the inability of the RC in the state $P_{680}Q^{-\cdot}$ to use the excitation energy for the primary charge separation according to the hypothesis proposed by L.N.M. Duysens and H.E. Sweers (1963). (A photograph of Duysens can be seen in Delosme and Joliot 2002.)

However, in the early 1970s, Karapetyan et al. (1971), Karapetyan and Klimov (1973) and Klimov (1973) demonstrated that PS II reaction centers evidently remain photochemically active even after transition to the 'closed' state $P_{680}Q^{-\cdot}$ as a result of dithionite-induced reduction of Q (monitored by corresponding increase of Chl fluorescence, F, to the level F_{max}). The photochemical activity was revealed by the effect of photoinduced 3–4 fold decrease of F (i.e., practically back to the level F_0) in both pea chloroplasts and PS II preparations. The fluorescence decrease was accompanied by ΔA revealing photobleaching of nearly 1% of total Chl. However, the effects were ascribed at that time to a 'reductive photoinactivation' of PS II (rather than to the primary PS II photochemistry as it was done later) since both the negative ΔF and ΔA were completely or partially irreversible and, besides, the quantum yield of the new photoreaction was found to be very low (close to 0.03). Four years later, in our joint work with Alexandr Klevanik, Vladimir Shuvalov and academician A.A.

Figure 1. Left to right: Vyacheslav Klimov, Vladimir Shuvalov, Alexandr Klevanik and the late Alexandr Krasnovsky. The photographs of the first three were taken at the Institute of Photosynthesis, USSR Academy of Sciences (Pushchino) in 1976 (when the reversible photoreduction of pheophytin in PS II was discovered). The photograph of Krasnovsky was taken at the A.N. Bakh Institute of Biochemistry, USSR Academy of Sciences, in Moscow.

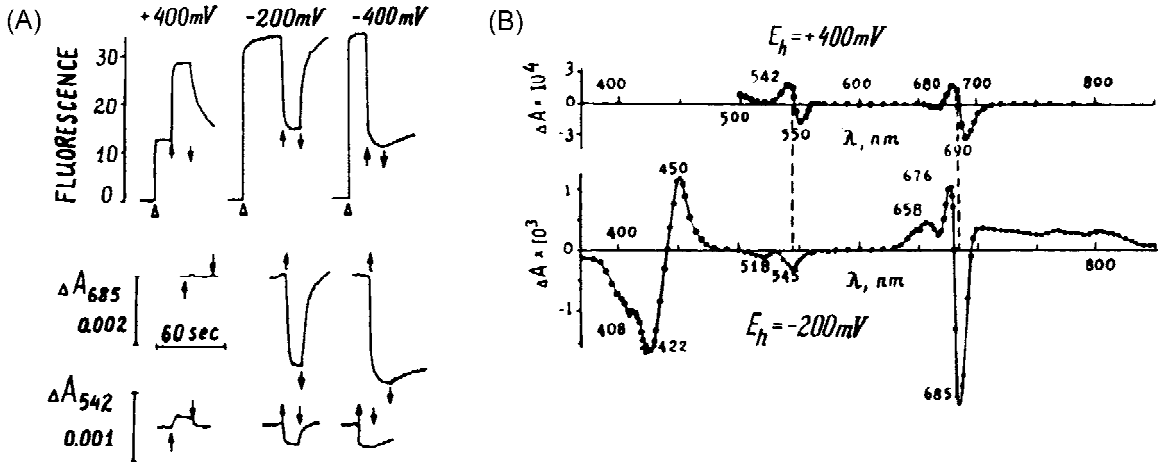

Figure 2. (A) Kinetics of photoinduced changes of Chl fluorescence yield (ΔF) and absorbance changes (ΔA) related to both photoreduction of Q (at $E_h \approx +400$ mV) and photoreduction of Pheo (at $E_h \approx -200$ mV and –400 mV) in PS II particles DT-20. Δ – measuring light exciting the Chl fluorescence, on; ↑ and ↓-actinic light on and off, respectively (reproduced from Klimov et al. 1977). (B) Light minus dark difference absorption spectra related to both photoreduction of Q (at $E_h \approx +400$ mV) and photoreduction of Pheo (at $E_h \approx -200$ mV) in DT-20 particles. Reproduced from Klimov et al. (1977).

Krasnovsky (see Figure 1) performed in Pushchino, in the Institute of Photosynthesis, the conditions were found (E_h of –200 mV) when the photoinduced decrease of F from the level F_m in the presence of dithionite was completely reversible, and the photoinduced ΔA coincided kinetically with the negative ΔF. Dark relaxation of these spectral changes was considerably slowed down upon lowering E_h from –200 mV – 400 mV (Figure 2) indicating the reductive nature of the photoprocess. The difference (light minus dark) absorption spectrum under these conditions (measured point - by point from the kinetics experiments) corresponded to reversible photoreduction of pheophytin (Pheo) 'a' (Klimov et al. 1977; Klevanik et al. 1977). Formation of anion-radical $Pheo^{-\cdot}$ in this photoreaction was confirmed by the observation of the corresponding Electron Paramagnetic Resonance (EPR) signal (Klimov et al. 1979b). Thus, these findings suggested that Pheo may act as the primary electron acceptor in PS II reaction center (like bacteriopheophytin in bacterial RCs). However, the first response to our reports on the new (and very important) role of the metal-free derivative of Chl in plant photosynthesis met with serious objections since traditionally Pheo was considered as a product of Chl degradation in plant cells, and the content of Pheo in isolated chloroplasts was even used as a test on their non-intactness. So, we had to present reliable experimental data proving that the photoreducible Pheo is really the natural primary electron acceptor of PS II rather than a product of Chl destruction involved in a photoreaction under reducing conditions. The following results have convinced us that the photoreduction of Pheo occurs

in PS II reaction centers and that Pheo is the primary electron acceptor of PS II acting between P_{680} and Q.

- (1) Photoreduction of Pheo is observed in various preparations containing active PS II reaction centers: fresh-isolated chloroplasts, 'heavy' subchloroplast fragments and Chl-protein complexes of PS II from pea (*Pisum sativum*) as well as from mutants of *Chlamydomonas reinhardtii* lacking Photosystem I (PS I); and it is not seen in PS I preparations from pea, in *Chlamydomonas* lacking PS II, and in light harvesting complexes (Klimov et al. 1980a).
- (2) Concentration of the 'photoactive' Pheo in different preparations is in the ratio of 1:1 with the concentration of the PS II reaction centers; this photoreaction is inhibited (along with other characteristic PS II photoreactions) in the temperature region of 40–45 °C and it is activated with Mn^{2+} (a specific electron donor to PS II) in Mn-depleted preparations (Klimov et al. 1980a).
- (3) Photoreduction of Pheo occurs at temperatures as low as 100 K; it is observed only after prior reduction of Q, it is accompanied by a 3–4-fold change of Chl fluorescence yield, and it is completely reversible in the dark (Klimov et al. 1977).

All these above properties are characteristic of photoconversions of RC components.

The 'variable fluorescence' of PS II as nanosecond Chl luminescence resulting from charge recombination in [$P_{680}^{+\cdot}$ $Pheo^{-\cdot}$]

The experiments revealing that Pheo functions in PS II reaction centers have modified our view on the origin of the 'variable Chl fluorescence' (ΔF) in this photosystem. In fact, if Pheo, not Q, is the primary electron acceptor in PS II then reduction of Q would not be accompanied by increase in the fluorescence yield since RC remains in the photochemically active form [P_{680}Pheo] $Q^{-\cdot}$. Experimental evidence has been obtained (Klimov et al. 1978) that the increase in Chl fluorescence accompanying reduction of Q is actually the appearance of luminescence resulting from charge recombination in [$P_{680}^{+\cdot}$ $Pheo^{-\cdot}$] after charge separation in the primary photoreaction. Unlike the ordinary Chl fluorescence, this emission decreases upon lowering the temperature from 0 to –100 °C (activation energy is about 0.04– 0.08 eV), and it disappears completely as the result of photoreduction of Pheo, indicating that Pheo takes part in the formation of an ion-radical pair with a Chl (probably with P_{680}) (Klimov et al. 1978). These data also show that the electron return from $Pheo^{-\cdot}$ to the excited level of P_{680} ($^1P_{680}$) requires an activation energy equal just to 0.04–0.08 eV. The low activation energy can account for a high quantum yield of this recombination luminescence, comparable with that of fluorescence (Klimov et al. 1978). Luminescence life-time measurements showed that the [$P_{680}^{+\cdot}Pheo^{-\cdot}$] $Q^{-\cdot}$ state decays to [P_{680}Pheo] $Q^{-\cdot}$ during 2–4 ns (Klimov et al. 1978) or 4.3 ns according to a more accurate later study (Shuvalov et al. 1980).

The 'recombination' origin of the ΔF is clearly shown for the case of double reduction of Q (Klevanik et al. 1991) or during the absence of Q (for instance, in isolated D_1/D_2/cyt b_{559}-complex (Govindjee et al. 1990)). In the case of single reduction of Q, both the recombination luminescence (Klimov et al. 1978) and a decrease of the rate of electron transfer from $^1P_{680}$to Pheo in the presence of negatively charged Q may contribute to the photoinduced increase of Chl fluorescence (Klevanik et al. 1991). It is necessary to note, however, that the 'recombination' origin of variable Chl fluorescence is not yet accepted by all in the field.

Direct detection of charge separation between P_{680} and Pheo by means of time resolved spectroscopy

As follows from the measurements of the recombination luminescence (Klimov et al. 1978; Shuvalov et al. 1980), the photoreaction [P_{680}Pheo]$Q^{-\cdot}$ $\rightarrow$ [$P_{680}^{+\cdot}Pheo^{-\cdot}$] $Q^{-\cdot}$ should be accompanied by ΔA with a life-time of 4 ns and with a spectrum including ΔA of both P_{680} and Pheo (Klimov et al. 1980a). Indeed, Shuvalov et al. (1980) found that irradiation of PS II (with Q pre-reduced in the dark) with a nanosecond laser pulse (λ = 694.3 nm) induces ΔA in the region 400–600 nm which appear during $\leq$1 ns and decay during $\sim$4 ns. These ΔA, as well as the nanosecond recombination luminescence, are not seen after preliminary photoreduction of Pheo or after oxidation of Q. The spectrum of the ΔA is very close to the expected spectrum calculated as the sum of the differential absorption spectra for photooxidation of P_{680} and photoreduction of Pheo.

These data allow us to conclude that, in fact, the primary photoreaction of PS II results in electron transfer from $^1P_{680}$ to Pheo with the formation of the ion-radical pair [$P_{680}^{+\cdot}$ $Pheo^{-\cdot}$] which decays during

~4 ns if Q is pre-reduced. When Q is oxidized, the electron from $Pheo^{-\cdot}$ is transferred to Q at least 10 times faster than back to $P_{680}{}^{+\cdot}$ i.e. in < 400 ps. The conclusion follows from a 10-fold decrease of both nanosecond recombination luminescence and corresponding ΔA, if Q is pre-oxidized (Klimov et al. 1978; Shuvalov et al. 1980). Later the formation of $[P_{680}{}^{+\cdot}$ $Pheo^{-\cdot}]$ was confirmed by the experiments in subnanosecond time domain (though the time of charge separation was not resolved) (Nujis et al. 1986).

Wasielewski et al. (1989) using isolated D_1/D_2/cyt b_{559} complex were the first who determined the time of charge separation between $^1P_{680}$ and Pheo with picosecond time resolution (see Seibert and Wasielewski, this issue). According to their data, this time is equal to 3 ± 0.6 ps. However other groups found a 21 ps time for the charge separation (Durrant et al. 1992). The controversy is still under debate. However, Greenfield et al. (1997), after some key corrections, found that the measured charge separation and charge equilibration was about 8 ps. Diner and Rappaport (2002) have recently reviewed the structure and function of PS II and concluded that the actual charge separation time may even be close to just one ps.

Photoaccumulation of the long-lived state [P_{680} $Pheo^{-\cdot}$]$Q^{-\cdot}$

Nanosecond spectroscopy shows that Pheo remains in the reduced state during a time less than 400 ps after the charge separation if Q is oxidized, and during about 4 ns if Q is reduced. On the other hand, under continuous irradiation at $E_h \leq$ –200 mV the spectral effects related to Pheo photoreduction have a life-time longer than 1 s (Figure 2). This is related to the transition of RC from the state $[P^{+\cdot}{}_{680}Pheo^{-\cdot}]$ $Q^{-\cdot}$ (~4 ns) to the long-lived state $[P_{680}Pheo^{-\cdot}]$ $Q^{-\cdot}$ due to fast electron transfer from Y_Z to $P_{680}{}^{+\cdot}$ which competes with the charge recombination in $[P_{680}{}^{+\cdot}Pheo^{-\cdot}]$ (Klimov et al. 1977, 1980a). Requirement of the fast electron donation to $P_{680}{}^{+\cdot}$ for photoaccumulation of long-lived $Pheo^{-\cdot}$ is confirmed by the data on activation of this photo-process upon addition of Mn^{2+}, a specific electron donor to PS II.

Since the electron donation to $P_{680}{}^{+\cdot}$ occurs in ≤ 1 μs (Gläser et al. 1976; Van Best and Mathis 1978) and the charge recombination needs ≈4 ns (Klimov et al. 1978; Shuvalov et al. 1980), then the quantum yield for photoaccumulation of the state $[P_{680}Pheo^{-\cdot}]Q^{-\cdot}$ is expected to be equal to ≥ 0.004. An experimental estimation of the quantum yield, based on the comparison of rates of photoinduced ΔF related to photoreduction of both Pheo and Q (Klimov et al. 1977, 1980a), has given a value (0.002–0.005) close to the expected.

EPR properties of PS II reaction centers with reduced Pheo indicated that the state $[P_{680}Pheo^{-\cdot}]Q^{-\cdot}$ is accumulated only under irradiation at 200–220 K (Klimov et al. 1980b, c). Continuous irradiation at room temperature probably results in electron transfer from $Pheo^{-\cdot}$ to $Q^{-\cdot}$ with the formation of $[P_{680}Pheo]QH_2$, and the state $[P_{680}Pheo^{-\cdot}]QH_2$ is accumulated as a result of a following cycle of Pheo photoreduction.

Klimov et al. (1985, 1986) found that Pheo photoreduction can be observed in both thylakoids and PS II preparations under anaerobic conditions, without the addition of dithionite or other reductants. The reaction of photoaccumulation of the long-lived state $[P_{680}Pheo^{-\cdot}]$ is used for characterization of the photoactive Pheo and for the determination of PS II reaction center concentration.

Interaction of Pheo with PS II reaction center components

Interaction of $Pheo^{-\cdot}$ with $Q^{-\cdot}$ is revealed by the appearance of the doublet EPR signal (jointly with the free-radical EPR signal of $Pheo^{-\cdot}$) when the [P680 $Pheo^{-\cdot}$] $Q^{-\cdot}$ state is accumulated in PS II (Klimov et al. 1980b, c). Properties of the doublet signal (its line-shape and microwave saturation, disappearance upon extraction of PQ or non-heme iron and recovery upon subsequent addition of exogenic PQ or Fe^{2+}, respectively, as well as observation of the EPR signal of anion-radical $PQ^{-\cdot}$ (after extraction of iron) show that Q is a complex of PQ with Fe and that the doublet signal results from an exchange interaction of $Pheo^{-\cdot}$ and $Q^{-\cdot}$ (Klimov et al. 1980b, c). A rough estimate of the distance between $Pheo^{-\cdot}$ and $Q^{-\cdot}$ from the splitting of the signal (~52 gauss) yields the value of 9–11 Å (Klimov and Krasnovsky 1981). (For the structure of PS II reaction center, see Zouni et al. (2001).) Analysis of characteristics of the EPR doublet in PS II has shown that reduced Q (which is $PQ^{-\cdot}Fe^{2+}$) should have an EPR signal with a g-value of 1.8 (Klimov et al. 1980c). Later this signal was indeed found to exist (Nugent et al. 1981).

Estimation of the midpoint redox potential, E_m, for Pheo/Pheo$^{-\cdot}$ and $P_{680}^{+\cdot}$ /P_{680}

From redox titration of reversible photoinduced ΔA related to photoreduction of Pheo in PS II, Klimov et al (1979a) showed that the value of E_m for Pheo/Pheo$^{-\cdot}$ is equal to –610 ± –30 mV. The data showing that Pheo functions in PS II reaction centers has considerably changed our view on the thermodynamic properties of PS II since the redox potential of -610 mV is low enough to expect photoreduction of electron acceptors typical for PS I (methylviologen, ferredoxin, $NADP^+$).

In fact, using the approach of photoreduction of Pheo under anaerobic conditions, it has been shown that the mentioned electron acceptors accelerate reoxidation of Pheo$^{-\cdot}$ in PS II (Klimov et al. 1985, 1986). Since the energy of a quantum which excites P_{680} equals 1.8–1.82 eV, the energy barrier between the [$P_{680}^{+\cdot}$ Pheo$^{-\cdot}$] and [$^1P_{680}$ Pheo] levels is about 0.04–0.08 eV, E_m for $P_{680}^{+\cdot}$/ P_{680} was estimated as +1.12 ± 0.05 V (Klimov et al. 1979a). A similar (1.0–1.3 V range) value of E_m for $P_{680}^{+\cdot}$/P_{680} was calculated earlier from data on temperature dependence of delayed light emission of the pair [$P_{680}^{+\cdot}$ $Q^{-\cdot}$] (Jursinic and Govindjee 1977). The value of 1.12 V for redox potential of P_{680} gives us an idea on possible mechanism of water oxidation in PS II: the water oxidation can not occur by the one-electron mechanism (requiring a potential of 2.3 V); the most probable (and widely accepted now) mechanism is the simultaneous ('concerted') four-electron oxidation (which needs a potential of 0.81 V), while the two-electron mechanism (with production of H_2O_2 as an intermediary product) requiring a potential of 1.3 V can not be completely excluded. The value of 1.12 V is widely used now for E_m of $P_{680}^{+\cdot}$ / P_{680}, although as mentioned above, it has not been determined directly (just estimated) and needs additional experimental confirmation.

A scheme of charge separation and stabilization in PS II reaction centers

From available data, the sequence, kinetic and energetic characteristics of early reactions in PS II reaction centers were summarized 22 years ago by Klimov and Krasnovskii (1981) in the following scheme (supported in many details by subsequent work; see also discussions in Ke 2001).

Figure 3. A photograph of the author (VVK) (center) at a conference in the US with Professor Gunnar Öquist (Umea University, Sweden) (right). Photo by Govindjee.

Singlet excitation of P_{680} ($E_m \approx 1.12$ V) leads to electron transfer from $^1P_{680}$ to Pheo ($E_m \approx -0.61$ V) in less than 1 ns. The process is accompanied by an energy loss of $\sim$ 0.06 eV and results in the formation of the ion-radical pair [$P_{680}^{+\cdot}$Pheo$^{-\cdot}$]. Charge recombination in this pair (which could occur during 4 ns) is prevented by faster (<400 ps) electron transfer from Pheo$^{-\cdot}$ to the 'stable' electron acceptor Q ($E_m \approx$ –130 mV), which appears to be a PQ-Fe^{2+} complex. This electron transfer is accompanied by a loss of $\sim$ 0.5 eV leading to the formation of the state [$P_{680}^{+\cdot}$ Pheo]$Q^{-\cdot}$ which recombines much slower ($\sim$150 μs) than the state [$P_{680}^{+\cdot}$Pheo$^{-\cdot}$]$Q^{-\cdot}$. Recombination of the state [$P_{680}^{+\cdot}$Pheo]$Q^{-\cdot}$ is prevented by faster ($\leq$ 1 μs) reduction of $P_{680}^{+\cdot}$ with electrons from a secondary donor Y_Z. The latter reaction leads to further stabilization of the separated charges. Reduction of Y_Z^+ leads ultimately to oxidation of H_2O and electron from PQ$^{-\cdot}$-Fe^{2+} goes to the electron transport chain between two photosystems. (See Renger, this issue, and Joliot, also this issue, for discussions on oxygen evolution.)

The scheme demonstrated that PS II reaction centers, especially their electron acceptor system, have a number of properties which are similar to those of RC of purple photosynthetic bacteria, and suggestion for the common origin of PS II and photosystem of purple photosynthetic bacteria throughout the evolution of photosynthesis has been made from this comparison (Klimov and Krasnovskii 1981) while before that, PS I was considered as an analogue of bacterial reaction centers. (See Blankenship, 2002, for a full discussion on this topic.)

Acknowledgments

I am very thankful to Govindjee for critical editing of this manuscript. I have been fortunate to visit several laboratories around the world, and to attend and participate regularly at many international conferences dealing with photosynthesis research. As an example, I show here a photograph of myself at a conference in USA having lunch with Professor Gunnar Öquist of Umea University, Sweden (Figure 3).

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