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An electrogenic reaction associated with the re-reduction of P680 by Tyr Z in Photosystem II

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The location of tyrosine Z (D1 Tyr-161), the immediate donor to photooxidized P680, has been probed using a fast photovoltage technique. We found an electrogenic reaction with an amplitude of 16% of the charge separated state $P680^+Q_A^-$ and a time constant of 29 ns which is attributed to the electron transfer between Tyr Z and P680. The result is discussed in the light of current structural models.

Introduction

The Photosystem II (PS II) reaction center (RC) consists of two homologous polypeptides, D1 and D2 [1–3]. Together they constitute the RC-core which binds the following cofactors required for electron transfer: the primary donor, P680, presumably a chlorophyll *a* (Chl *a*) dimer, a pheophytin molecule, ($Pheo_A$), and two plastoquinones, Q_A and Q_B. Two histidine residues that are thought to provide ligands to the central Mg atoms of the primary donor are each located on one of the subunits [1,2,4]. Likewise, the binding site of the plastoquinones is established by both subunits.

Absorption of light energy drives the primary charge separation between P680 and Q_A via the intermediary pheophytin ($Pheo_A$). The re-reduction of P680 occurs through a tyrosine residue, Tyr Z (or Y_Z), that has been identified as Tyr-161 on the D1 subunit [5–8]. Another tyrosine residue, Tyr-161, located on the D2 subunit (Tyr D or Y_D) may form a very long lived radical which renders it accessible for EPR measurements [9–12]. The electrons which re-reduce Tyr Z originate from the oxygen evolving complex (OEC) which cycles through so-called S-states, thereby extracting four electrons from two molecules of water [13–17].

The fact that both subunits are needed to bind the primary donor and the acceptor quinones in addition to sequence homologies in those regions which are thought to bind the redox cofactors, has led to the proposal that the D1/D2 heterodimer of PS II is homologous to the L/M heterodimer of the purple bacterium *Rps. viridis* [1–4], the crystal structure of which is known at 2.3 Å resolution [18]. The L and M subunits, each containing five transmembrane helices, are arranged in approximately twofold rotational symmetry with respect to each other. The redox components necessary for electron transport are located along two nearly symmetrical, membrane-spanning branches.

An equivalent high resolution X-ray crystallographic structure of the RC of PS II is not yet available. Its 3-dimensional structure is, therefore, currently deduced from the one of *Rps. viridis*. Although the degree of identity between the D1 and L subunit and D2 and M is only about 10–20% [4,19,20], the folding pattern for the D1/D2 subunits is thought to be the same [1,2,4,21]. Similarly, the arrangement of the redox cofactors seems to parallel the one in *Rps. viridis* [18,22].

The re-reduction of the primary donor in the two types of RCs, however, occurs in different ways. In *Rps. viridis* a tightly bound cytochrome *c* subunit serves to re-reduce the primary donor. In PS II the OEC with Tyr Z as the intermediary electron carrier delivers electrons to P680. The different functions of the re-

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Abbreviations: Chl *a*, chlorophyll *a*; DCBQ, dichloro-*p*-benzoquinone; OEC, oxygen evolving complex; $Pheo_A$, intermediary pheophytin acceptor of PS II; PS II, Photosystem II; P680, primary donor of PS II; Q_A, primary quinone acceptor in PS II; Q_B, secondary quinone acceptor in PS II; RC, reaction center; Tyr Z, tyrosine electron donor in PS II.

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spective donor sides go in line with a number of amino acids at the oxidizing side of the RC not being conserved between purple bacteria and higher plants [19]. In particular, the primary amino acid sequence of the L subunit contains an arginine (Arg-135) instead of the tyrosine residue (Tyr Z) present in PS II. These differences lead to considerable uncertainties in the construction of three-dimensional structures of the reaction center of PS II using the one of *Rps. viridis* as a template.

In addition to structural information obtained through sequence comparisons between the RC of PS II and that of *Rps. viridis*, time-resolved EPR measurements yield information on absolute distances between prosthetic groups in PS II. For instance, a distance of 14 Å between the primary donor of PS II and Tyr D has been proposed [12]. The complementary information on Tyr Z is not available, since the phenoxy radical formed upon oxidation of Tyr Z under in vivo conditions is too short-lived to be observed directly by EPR. Since Tyr Z and Tyr D are presumably symmetrically situated in the photosynthetic membrane, a corresponding distance is assumed for Tyr Z [12,23].

Structural information may also be obtained directly from photovoltage experiments, since the photovoltage amplitude of a given reaction is a measure for the dielectrically weighted distance between the electron carriers projected onto the membrane normal.

Electrogenic reactions on the donor side of photosynthetic RCs have been investigated by photovoltage measurements in the case of *Rps. viridis*, but not in the case of PS II. For the former system the re-reduction of the primary donor by cytochrome *c*, which occurs in the 100 ns time regime, has been analyzed by employing a high-ohmic collodion film technique [24]. Since this experimental approach has not been established for PS II membranes, we have applied a different method based on an electrical orientation of membranes on Pt-electrodes [25].

Fast photovoltage measurements using oriented planar PS II membranes have so far been restricted to the primary photochemistry occurring in the picosecond time regime [25]. Here we report photovoltage measurements on the donor side of PS II in the 10–100 ns time range. The re-reduction kinetics of Tyr Z lie in the same time range and depend on the number of positive charges accumulated in the OEC (S-states) [26–28]. If the photosynthetic system is kept in the dark, the water oxidase relaxes to the S_1 state in which the re-reduction of P680 is 20–30 ns. With the OEC in a higher oxidation state, either S_2 or S_3, the reaction kinetics slow down considerably and become largely biphasic with $\tau_1 = 40$ ns and $\tau_2 = 280$ ns [28]. This difference in kinetics is used in the photoelectric experiments described here to quantify the electrogenicity connected with the electron transfer from Tyr Z to P680. We found an electrogenicity for the reaction Tyr Z $P680^+ \rightarrow$ Tyr Z^+ P680 that amounts to 16% of the charge separated state $P680^+Q_A^-$.

Materials and Methods

Oxygen-evolving PS II particles (BBY-membranes) were prepared from peas (*Pisum sativum*) according to the procedure described by Berthold et al. [29] with the modifications described by Ono and Inoue [30]. For trypsination the fragments were diluted in low salt resuspension medium to a chlorophyll concentration of 100 μM and incubated at room temperature with 2 μg/ml trypsin from bovine pancreas (Sigma). After 5 min the proteolytic treatment was stopped by addition of trypsin inhibitor (Sigma) in 20-fold excess and the preparation was concentrated by centrifugation. For photovoltage experiments, the chlorophyll concentration was adjusted to 1.5 mM. This mild trypsinization leads to a destacking of the BBY membranes so that they may be oriented in an electric field [25]. To ensure intactness of the OEC, controls were routinely used to verify that the trypsination did not affect oxygen-evolution rates.

Orientation of the PS II membranes was carried out in a micro-coaxial cell described elsewhere [31]. To achieve a stable orientation for at least 30 min, the membranes were irreversibly sedimented on one electrode by a 150 ms DC-voltage pulse of 600 V/cm. Following orientation, the sample was dark-adapted for 30 min and excited with a single laser flash of 30 ps duration at 532 nm generated by a Nd-YAG laser. After 500 ms a preflash was given with a custom-built Xenon flashlamp (FWHM $\approx$ 10 μs). A second laser flash of 30 ps duration was applied 500 ms after the preflash. The photovoltage signals were first fed into a high impedance converter (custom-built; input resistance 100 kΩ, output resistance 50 kΩ) and then amplified by a broad-band amplifier (model IV 75, Hahn-Meitner Institut, Berlin). The rise time was limited to 2 ns. Single shots were recorded on a digital 7 GHz oscilloscope (Tektronix 7250) and stored on a personal computer for averaging and data processing.

Results

A typical single-shot photovoltage signal from dark-adapted, irreversibly oriented BBY-membranes is shown in Fig. 1A. The initial fast rise reflects the charge separation between $P680^+$ and Q_A^- [25,32] which is not time-resolved with the experimental set-up used. After the initial rise the signal decays in a complex manner. The decaying phase is a composite of an exponential discharge of the Pt-electrode/electrolyte system overlaid with some ringing due to frequency-dependent variations of the impedance of the high-im-

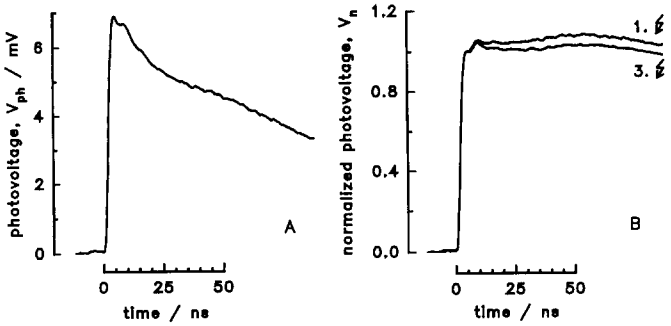

Fig. 1. Typical single-shot photovoltage signals. (A) Original photovoltage signal obtained from a dark-adapted sample. (B) Photovoltage signals evoked by the first and third flash that were normalized with the peak photovoltage set to 1 and deconvoluted. DCBQ was added to a final concentration of 60 μM.

pedance electronics. Repetition of the experiment with newly prepared samples yielded amplitude fluctuations of approximately 20% which can be ascribed to laser instabilities and variations in the sample preparations. All signals were thus normalized with the peak voltage set to 1 prior to further data analysis.

The following experiments which were aimed at resolving electrogenic reactions at the donor side were designed as difference experiments. As mentioned in the Introduction, a flash applied to a sample in the dark-stable S_1-state results in a re-reduction of P680 with a time constant of 20–30 ns. After one more saturating preflash the sample is predominately in the S_3-state from which the re-reduction of P680 is considerably slower and biphasic. We therefore sampled photovoltages evoked by the first and third flash and looked for any differences between the two signals.

The photovoltage signal from the third flash displays a very similar overall shape as compared to the first one but shows an apparently faster decay. This slightly faster decay can only be ascribed to altered electrogenic reactions, since both flashes were given to one and the same sample and therefore the apparatus response function must be identical.

The electric discharge tends to damp electrogenic reactions taking place at later times more than those occurring at earlier times. Amplitudes of reactions taking place in the 50–100 ns time-regime will thus be underestimated. In practice, the decay due to the self-discharge of the measuring cell can be described by a function containing two exponential terms. For further analysis, the original signals were deconvoluted with this decay function, thus avoiding an unequal weighting of electrogenic reactions occurring along the time base. Disturbances due to the slight ringing are still apparent in both traces of Fig. 1B but are suppressed by subsequent subtraction. Two of such normalized and deconvoluted signals, originating from a first and a third laser flash, are shown in Fig. 1B.

Subtraction of these signals yields a trace as shown in Fig. 2A which displays a rising phase with a time constant of about 25 ns, the expected time domain for the reduction of $P680^+$, and a slight decaying phase. To analyze this trace we used an equation which is derived in the following.

According to the literature [26–28] the re-reduction after the first flash is purely monoexponential with $\tau_1 = 20$–30 ns (Fig. 2C, upper dashed line). That of the third flash is bi-exponential ($\tau_2 = 40$ ns, $\tau_3 = 280$ ns) with a relative amplitude of the faster phase of $n = 0.6$ (Fig. 2C, lower dashed line). Accordingly, the expected shape of the difference trace is described by the function:

$$f(t) = a[(1-\exp-(t/\tau_1)) - n(1-\exp-(t/\tau_2)) - (1-n)(1-\exp-(t/\tau_3))] \quad (1)$$

which is also depicted in Fig. 2C, solid line.

The above function was fitted to the data in Fig. 2A by adjustment of the amplitude of the electrogenic phase, a, and its time constant, τ_1. The point where the flash was applied was taken as $t = 0$ for the fit. Data

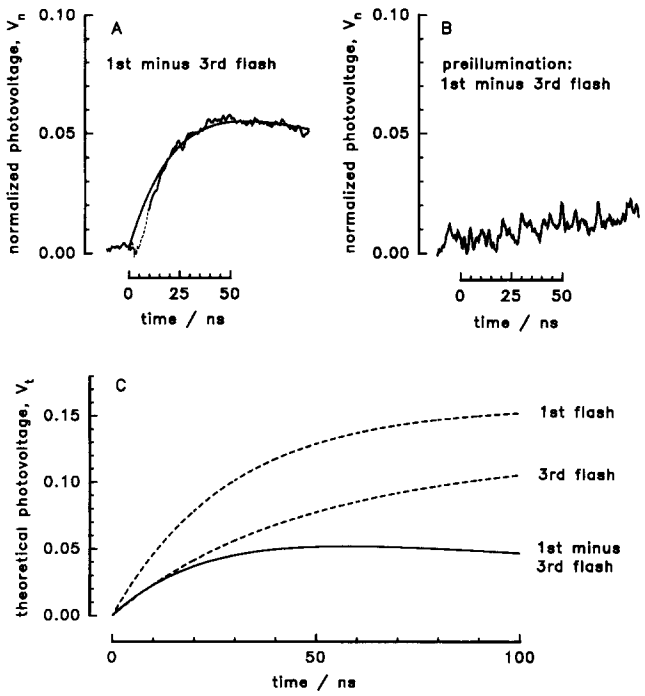

Fig. 2. Difference traces obtained from subtracting the third from the first flash. (A) The first flash was given to a dark-adapted sample. The average of 13 difference traces is shown. (B) The sample had been illuminated with white light prior to the flash sequence. The first flash was given 1 s after the preillumination. 15 difference traces were averaged. (C) Upper dashed line: expected mono-exponential kinetics from the first flash with $a = 0.157$ and $\tau = 29$ ns. Lower dashed line: expected bi-exponential kinetics observed in the third flash with $a_1 = 0.6 * a$, $\tau_1 = 40$ ns and $a_2 = 0.4 * a$, $\tau_2 = 280$ ns. Solid line: difference between the two dashed lines.

points contained in the rising phase of the original signal are not time-resolved and therefore excluded from the fit (as indicated by the dashed line in Fig. 2A). The best fit to the average of 13 independent experiments yielded $a = 15.7 \pm 3.4\%$ and $\tau_1 = 29.0 \pm 5.6$ ns.

As a control of our experimental procedure we followed the same experimental protocol, but using samples preilluminated by a tungsten lamp. In this case, the S-states of the OEC cannot be synchronized by the flash patterns used and consequently no difference photovoltage should appear. The average of 15 such experiments is shown in Fig. 2B.

Discussion

Earlier attempts to resolve the electrogenicity of the reaction Tyr Z $P680^+ \rightarrow$ Tyr Z^+ P680 either by electrochromism [33] or light-gradient photovoltage measurements [32] have not been successful because of an insufficient signal-to-noise ratio. We succeeded in increasing the signal to noise ratio in our photovoltage experiments by about a factor of 10 using oriented PS II membranes.

The photovoltage amplitude connected with the above reaction amounted to 16% of that associated with the formation of $P680^+Q_A^-$. This amplitude is a measure for the relative, dielectrically weighted distance between the electron carriers projected onto the membrane normal (the relative geometric distances are then proportional to the product of photovoltage and dielectric constant).

A possible critisicm that may be raised against our analysis is that the 16% amplitude difference is partially due to contributions of so-called 'inactive centers' [34] to the signal evoked by the first flash. A strong argument against this possibility is the absence of a difference photovoltage in the control experiment in which the S-states have been desynchronized during the preillumination preceding the ps-flash sequence by 1 s (Fig. 2B). If inactive centers would interfere with our photovoltage measurements they should be detectable in our control, since they are known to recover with a half-time of 1–2 s [34,35]. Furthermore, the time constant of 29.0 ± 5.6 ns we observe for the reaction Tyr Z $P680^+ \rightarrow$ Tyr Z^+ P680 coincides remarkably well with the reaction kinetics as determined from spectroscopic measurements [26–28].

If we assume a homogeneous dielectric within the core of the RC and the distance between P680 and Q_A to be 27 Å [12,36], then the projected distance between Tyr Z and P680 corresponds to approximately 4 Å. In order to correlate our result with other distance information a vectorial view of the components involved is required. From EPR measurements the absolute distance between Try D and P680 is estimated to be 14 Å

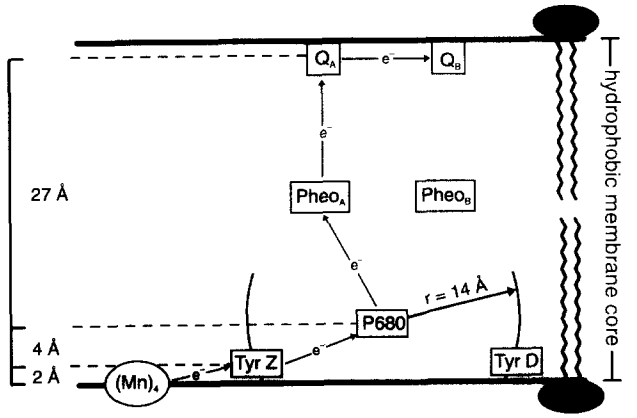

Fig. 3. Scheme of distances projected onto the membrane normal in PS II. Assuming a distance of 27 Å between Q_A and P680 [12,36], and a homogeneous dielectric, the present work gives a dielectrically weighted distance between Tyr Z and P680 of 4 Å. The e^--transfer from the Mn-cluster to Tyr Z spans 5% of the hydrophobic membrane core [39] which would correspond to a distance of 2 Å, again assuming a homogeneous dielectric. For the sake of completeness, we have also depicted the absolute (not the projected) distance of 14 Å between P680 and Tyr D as inferred from time-resolved EPR measurements [12], which is indicated by the circle around P680.

[12]. If the distance between Tyr Z and P680 is taken to be equal to that between Tyr D and P680, one would obtain a transmembrane location of Tyr Z as depicted in Fig. 3.

The distance relations shown in Fig. 3 are essentially compatible with the 3-dimensional RC-structures obtained by molecular modelling procedures [20,37,38], thus supporting the validity of the concepts on which these procedures are based.

The model put forward by Svensson et al. [20,37] predicts a projected distance of 5 Å between Tyr Z and P680. The distance of 4 Å derived from our data is based on the assumption of a homogeneous dielectric within the RC-core. This, however, may not be the case, since the distribution of hydrophilic amino acids may be non-homogeneous. Taking the more polar environment in the immediate surrounding of Tyr Z into consideration, as has been proposed by Svensson and Styring [20], our result would agree with the proposed 5 Å, if the value of the effective dielectric constant between Tyr Z and P680 is about 16% higher than the one between P680 and Q_A.

Distance information on the donor side of PS II have also been obtained through the observation of luminescence changes induced by field-pulses [39]. These indicate an electrogenic reaction associated with the electron transfer from the Mn-atoms of the OEC to Tyr Z that spans 5% of the membrane core. Assuming a thickness of 35 Å of the hydrophobic membrane core this would correspond to a distance of less than 2 Å. Hence, a combination of this result with ours would point towards the Mn-cluster being situated very close

to the membrane–water interface or even slightly buried within the membrane.

One could suspect electrogenic reactions associated with the charge transfer from the OEC to Tyr Z to contribute to the difference trace. This, however, can be excluded since on a time-scale of 10–100 ns the contribution of these reactions to the total photovoltage amplitude can be estimated to be negligible. If one assumes for such a reaction step an electrogenicity of 5% of $P680^+Q_A^-$ [39] and a time constant of 100 μs [40], the contribution of this reaction at 50 ns amounts to 10^{-3}%. Therefore, the experiments presented in this work do not yield any information regarding the former reactions.

The distance between Tyr D and the inner membrane surface has been estimated to be 20 Å [10] or 27 Å [9] by EPR spin interaction techniques. A comparison of these distances with our data turns out to be difficult due to the poorly defined membrane–water interface. Our distance calculation is based upon the thickness given by the hydrophopic membrane interior (length of the transmembrane α-helices or 2 C-18 hydrocarbon chains), whereas the EPR work refers to the entire membrane, including lipid headgroups and surface exposed polypeptides. These numbers may therefore be compatible with our results and the scheme in Fig. 3.

Based on EPR spectra it has been suggested that upon oxidation of Tyr D, the phenol hydrogen is hydrogen bonded to another residue [41–43], presumably a histidine [15,20,44]. The phenoxy radical would thus be neutral in its oxidized form with the positive charge located on the imidazole ring of the histidine. That the oxidized Tyr Z may be neutral as well has been suggested by Eckert and Renger who have postulated that the oxidation of Tyr Z by $P680^+$ may be associated with the shifting of a hydrogen bond [27]. Similarities with respect to the binding pockets in which Tyr Z and Tyr D are located have also been taken as evidence that Tyr Z forms a hydrogen bond to His 190 on the D1 subunit, even though this distance is longer than expected for a hydrogen bond [20]. If this was the case, the center of charge of the oxidized Tyr Z would be at a much larger distance from P680, projected onto the membrane normal, than if the positive charge was located on the tyrosine itself. The small electrogenicity of 16% found in our photovoltage measurements is clearly incompatible with this possibility. However, the data do not exclude a hydrogen-bond to another residue which would then have to be located on the same level as Tyr Z.

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