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Review

Two tyrosines that changed the world: Interfacing the oxidizing power of photochemistry to water splitting in photosystem II☆

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ABSTRACT

Photosystem II (PSII), the thylakoid membrane enzyme which uses sunlight to oxidize water to molecular oxygen, holds many organic and inorganic redox cofactors participating in the electron transfer reactions. Among them, two tyrosine residues, Tyr-Z and Tyr-D are found on the oxidizing side of PSII. Both tyrosines demonstrate similar spectroscopic features while their kinetic characteristics are quite different. Tyr-Z, which is bound to the D1 core protein, acts as an intermediate in electron transfer between the primary donor, P_{680} and the $CaMn_4$ cluster. In contrast, Tyr-D, which is bound to the D2 core protein, does not participate in linear electron transfer in PSII and stays fully oxidized during PSII function. The phenolic oxygens on both tyrosines form well-defined hydrogen bonds to nearby histidine residues, His_Z and His_D respectively. These hydrogen bonds allow swift and almost activation less movement of the proton between respective tyrosine and histidine. This proton movement is critical and the phenolic proton from the tyrosine is thought to toggle between the tyrosine and the histidine in the hydrogen bond. It is found towards the tyrosine when this is reduced and towards the histidine when the tyrosine is oxidized. The proton movement occurs at both room temperature and ultra low temperature and is sensitive to the pH. Essentially it has been found that when the pH is below the pK_a for respective histidine the function of the tyrosine is slowed down or, at ultra low temperature, halted. This has important consequences for the function also of the $CaMn_4$ complex and the protonation reactions as the critical Tyr–His hydrogen bond also steer a multitude of reactions at the $CaMn_4$ cluster. This review deals with the discovery and functional assignments of the two tyrosines. The pH dependent phenomena involved in oxidation and reduction of respective tyrosine is covered in detail. This article is part of a Special Issue entitled: Photosystem II.

1. Introduction

There are many proton steered electron transfer reactions in enzymes. The side chain of tyrosine participates in several very important electron transfer reactions of this type. In many cases these are essential steps in the enzyme's catalytic mechanism. Such redox reactions involving a tyrosine side chain occur in, among others, ribonucleotide reductase, prostaglandin H synthase, galactose oxidase, and photosystem II (PSII)[1] [1]. The best studied biological electron transfer reactions are found in the photosynthetic reaction centers where triggering with ultra short light pulses from lasers provides exceptionally good time resolution in kinetic studies.

In this contribution we will cover the reactions involving the two redox active tyrosines in PSII, Tyr-Z (Y_Z) and Tyr-D (Y_D). Tyr-Z is the interface between the oxidizing power of P_{680}^+, the primary donor in PSII and the oxygen evolving $CaMn_4$ cluster. Tyr-D is also in redox contact with both these sites. There would be no photosynthetic oxygen evolution without these tyrosines—when they became functional water oxidation could evolve. The world changed, both geologically and biologically, and we now live in a biosphere dominated by aerobic life. Both tyrosines have remained during evolution for 2.5 million years and there are no oxygen evolving organisms known where they are not present. Both tyrosines are essential to photosynthesis and they are clearly "two tyrosines that changed the world". We will first describe their discovery and identification and thereafter discuss their function and how their redox properties are controlled by unusual hydrogen bonds.

1.1. Discovery and early work

In the 1950s EPR was introduced to biological materials and in 1956 B. Commoner and co-workers [2] put a chloroplast suspension from a tobacco plant in the EPR cavity for the first time. Two EPR

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[1] $CaMn_4$ cluster, the catalytic center consisting of four Mn-ions and one Ca-ion; D1 and D2, the core protein subunits in PSII; EPR, electron paramagnetic resonance; His_D, histidine 189 (cyanobacterial numbering) on the D2 protein that participates in hydrogen bonding to Tyr-D; His_Z, histidine 190 on the D1 protein that participates in hydrogen bonding to Tyr-Z; OEC, oxygen evolving complex consisting if the $CaMn_4$ cluster and surrounding amino acid ligands; P_{680}, primary electron donor chlorophylls in PSII; PSII, photosystem II; Q_A and Q_B, primary and secondary plastoquinone acceptors of PSII; S states, intermediates in the cyclic turnover of the OEC; Tyr-D (Y_D), tyrosine 160 on the D2 protein; Tyr-Z (Y_Z), tyrosine 161 on the D1 protein.

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signals were discovered. One was formed immediately when the suspension was exposed to light. This signal was a simple narrow radical signal and decayed quickly when light was turned off. It was named Signal I and is now known as the radical from P_{700}^{+}, the oxidized form of the primary electron donor in photosystem I [3,4].

A second signal remained in the dark for many hours. It was distinguished from Signal I in that it was ~20G broad and had a higher *g*-value ($g = 2.0046$). The signal was denoted Signal II and it is now known that B. Commoner and co-workers had discovered the Tyr-D• radical in PSII. Of course this could not be known at the time as neither PSII nor the concept of photosynthetic reaction centers was known. It was to take long time before full understanding of Signal II was to occur.

1.2. Signal II_{slow}, Signal II_{fast} and Signal $II_{very\ fast}$

15 years later photosynthesis research went through a remarkable period. The concept of photosynthetic reaction centers was introduced and scientists started to purify photosynthetically active biochemical preparations of increasingly higher purity and increased homogeneity from many different organisms. It was discovered that the oxygen evolution from PSII occurred with a period of four oscillations after single light flashes. This lead to the development of the S state cycle nomenclature and the understanding that PSII contained what is now known as the oxygen evolving complex (OEC). EPR was brought back to photosynthesis research and has been a core technology ever since.

In a series of key papers the EPR group around Melvin Klein and Kenneth Sauer with among others G.T. Babcock, B. Blankenship and J.T. Warden returned to Signal II [5–10]. The radical was soon shown to derive from PSII but more remarkable was that there were three kinetically distinguishable components involved. The dark stable Signal II, discovered by B. Commoner in 1956, was renamed to Signal II_{slow} or Signal II_D reflecting that it decayed very slowly making it easy to detect in the dark ("Dark" gave rise to the index D leading later to the nomenclature Tyr-D).

Lasers and improved EPR spectrometers permitted better kinetic resolution and studies of smaller and transient signals which led to the discovery of a new signal with similar line width (~20G) and high *g*-value as Signal II_D (Fig. 1A, spectra a and b). This radical decayed in the long milliseconds–seconds time regime after the light was turned off and was named Signal II_{fast} (Fig. 1A, spectra c and d; kinetic trace in Fig. 1B, trace f) [7]. It was observed when the OEC was inactivated through for example washing with Tris-buffer (a treatment which removes the $CaMn_4$ cluster and some protein subunits protecting the OEC). Further work established that it was present to the same amount as Signal II_D (compare Fig. 1A, spectra b and d) and that the formation and decay kinetics were sensitive to pH and many other environmental factors. The biochemical behavior and kinetic signature of Signal II_{fast} allowed its assignment to a kinetic component Z in PSII (…X, Y, Z etc.) discovered earlier by optical spectroscopy (this Z later appears in the name Tyr-Z) [11,12].

A third version of Signal II was soon discovered in PSII where the OEC was kept intact. Here Signal II_{fast} was not present. Instead a broad radical that decayed in the few 100 microseconds to 1 millisecond time regime after a flash could be observed [9]. This signal was discovered after a heroic effort and showed a small, broad, high *g*-value radical spectrum with clear similarities to Signal II_D. It was named Signal $II_{very\ fast}$. The fast decay has made this signal almost impossible to study directly at room temperature and there are only a handful EPR studies available in the literature [8–10,13,14].

1.3. Identification of the tyrosines that give rise to the Signal II radicals

The number of redox active species and the molecular origins of Signal II_D, Signal II_{fast} and Signal $II_{very\ fast}$ were not clear and one or several protein bound cationic plastoquinones were long thought to give rise to the radicals. These suggestions became difficult to reconcile after careful analysis of the first PSII core preparations. Here only the quinone from the first acceptor Q_A was found while the analysis did not reveal the presence of any extra quinones [15]. It was also unclear if there was only one redox component that gave rise to all kinetic types of Signal II or if there were several. However it became slowly clear that Signal II_{slow} was always present to 1 spin per PSII center. Signal II_{fast} could be formed to the same amount and at the expense of Signal $II_{very\ fast}$ (but not vice versa). This indicated the presence of, at most, two components per PSII reaction center [8,16].

In 1986 the first PSII reaction center preparation appeared and the core of PSII was shown to be a dimer of the D1 and D2 proteins [17]. This made the assignment of Signal II to two components, homologously placed in the D1 and D2 proteins easy to reconcile but there was still a lack of quinones in the preparations to account for two components. The quinone assignment was changed and Signal II was shown to originate from tyrosine residues in PSII. This was proposed from radical specific iodination of the D1 and D2 subunits [18]. In this experiment, a reaction with iodine that was known to be quite tyrosine radical specific was found to label the D2 protein in the dark. The labeling kinetics was found to correlate with the decay (probably reduction) of Signal II_{slow} measured with EPR. In the light, the procedure also labeled the D1 protein suggesting that there was one

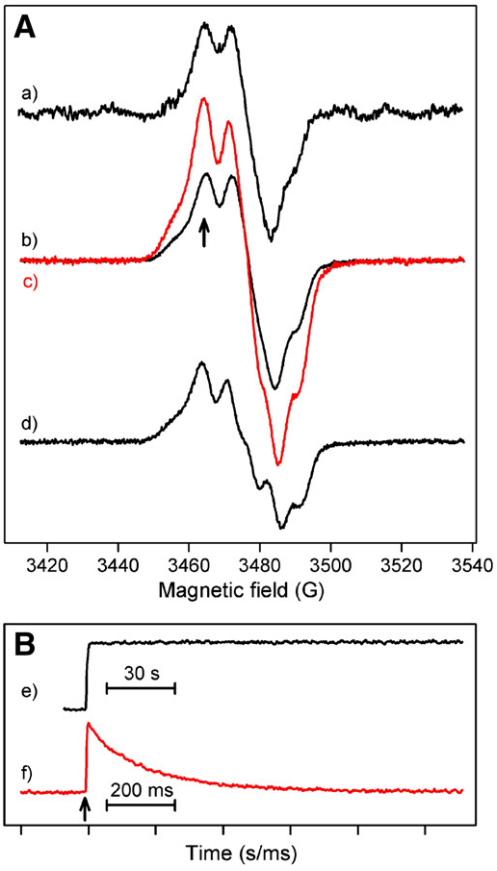

Fig. 1. EPR spectra of the tyrosine radicals Tyr-D and Tyr-Z in PSII recorded at room temperature. (A) The figure shows the X-band EPR spectra of Tyr-D from an intact spinach leaf (a), tyrosines from Mn-depleted PSII enriched membranes in dark (b, Tyr-D) and during continuous illumination (c, Tyr-D plus Tyr-Z), and the pure Tyr-Z difference spectrum (d, spectrum b-*minus*-spectrum c). (B). Kinetic traces of Tyr-D (e) and Tyr-Z (f) in Mn-depleted PSII enriched membranes. Tyr-D was chemically reduced before induction. The induction was done with a 532 nm laser flash (indicated with an arrow). The measurements were done at the field position indicted by an arrow in panel A. Note the split time scale in B used to show the extended life time of $Tyr\text{-}D^{ox}$ while $Tyr\text{-}Z^{ox}$ decays in <1 s. in Mn-depleted PSII.

dark stable tyrosine radical on the D2 protein (giving rise to Signal II_{slow}) and one light induced radical on the D1 protein (giving rise to the fast decaying components of Signal II). Simultaneously and very conclusively it was shown that the radicals originated from tyrosines using EPR spectroscopy on PSII prepared from *Synechocystis* grown on isotopically labeled tyrosine [19]. Interestingly the proposal that two homologous tyrosines in the sequences of the D1 and D2 proteins gave rise to Signal II_{slow} (Tyr160 on the D2 protein) and Signal II_{fast} components (Tyr161 on the D1 protein) was first made in the iodination study (cyanobacterial numbering) [18].

This suggestion was proven correct through site directed mutagenesis in *Synechocystis* 6803. Signal II_{slow} (Fig. 1, spectra a and b) was identified as the radical from Tyr-160 on the D2 protein while Signal II_{fast} (Fig. 1A, spectra c and d) could be shown to originate from Tyr-161 on the D1 protein [20]. This lead to new nomenclature and Signal II_{slow} is from then called Y_D (or Tyr-D) while Signal II_{fast} components are called Y_Z (or Tyr-Z).

The high *g*-value (2.0046–48) indicated that the radicals should originate from the deprotonated neutral radical states of respective tyrosyl residue, most likely from deprotonation of the phenolic proton upon oxidation of the tyrosyl side chain. This assignment was supported by the available redox potential on the primary donor in PSII, P_{680}^{+} (ca 1.1–1.2 V). If the radicals were present in the protonated state of the tyrosyls this redox potential would not be sufficient to oxidize the tyrosyl side chain. It was also known that the $CaMn_4$ cluster was much closer to Tyr-Z than to Tyr-D (see Svensson et al. 1990 and discussion therein [21]) although both radicals could participate in electron transfer reactions to and from the $CaMn_4$ cluster. These reactions were all pH dependent. Another important factor is that, at this time (ca 1990), neither tyrosine radical was thought to be possible to oxidize at very low temperatures.

2. Overview of the photochemistry and electron/proton transfer in PSII

Before we can discuss the structure and function of the tyrosines in detail it is necessary to briefly describe PSII and the function of this multi-tasking enzyme. Fig. 2 presents the many redox components in PSII and their relative place in the center. The first step in photosynthetic oxygen evolution in PSII is light excitation of the primary electron donor, P_{680}. This results in ultrafast electron transfer to the acceptor side of PSII via the first electron acceptor pheophytin (Fig. 2, reaction 1) to the quinone acceptors Q_A (Fig. 2, reaction 2) and Q_B (Fig. 2, reaction 4). Q_B is reduced by two electrons concomitantly with its double protonation. Thereafter it dissociates from PSII (Fig. 2, upper right) into the thylakoid membrane and an oxidized quinone takes its place in the Q_B binding pocket.

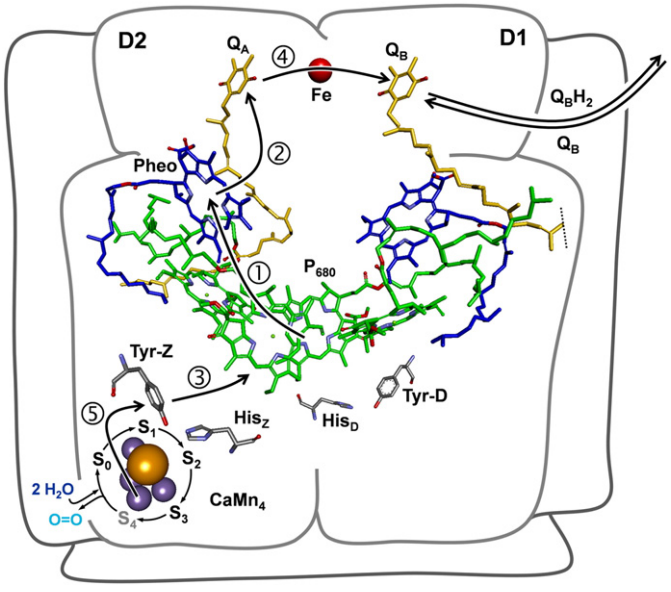

Fig. 2. The redox cofactors in PSII. The arrows indicate the different electron transfer reactions. The more than 25 protein subunits are not shown in detail. The lower part of the figure shows the oxygen evolving cycle (the S state cycle) in the OEC. O_2 is released in the $S_3 \rightarrow [S_4] \rightarrow S_0$ transition.

On the oxidizing side of PSII, P_{680}^{+} has a redox potential high enough (ca +1.2 V) to extract electrons that ultimately come from water. The catalytic site for water oxidation is the $CaMn_4$ cluster which is mainly bound to the D1 reaction center protein. The mechanism for water oxidation is described by the S-cycle [22] where the S_n-states represent intermediate redox states in the oxygen evolving cycle (S_n state; n = 0–4; Fig. 2, lower left). After accumulating four oxidizing equivalents (forming the transient S_4 state in the OEC) two water molecules are oxidized, O_2 is released and the $CaMn_4$ cluster returns to the S_0 state.

Tyr-Z and Tyr-D on respectively the D1 and D2 proteins are located to the oxidizing side of PSII in symmetrical positions around P_{680}. Tyr-Z is an intermediate in steady state oxygen evolution and shuttles electrons (Fig. 2, reactions 3 and 5) from the $CaMn_4$ cluster to P_{680}^{+} (the oxidized primary donor). When Tyr-Z is oxidized it forms a neutral radical by coupled deprotonation of the phenolic proton. Tyr-D is a side path electron donor in PSII. When PSII is kept in the dark for prolonged time Tyr-D is slowly reduced. This reaction takes several hours and one can wonder if this is at all important in PSII chemistry. However, it is a natural reaction and occurs every evening when the night sets in. Immediately when PSII is again exposed to light (during exposure to the very first few photons when sun rises or, in the laboratory, following a short light flash) Tyr-D is oxidized to its neutral radical. Also in this case oxidation of the tyrosine is coupled to deprotonation. Tyr-D then stays in the oxidized form and it does not participate in steady state electron transfer.

For each transition $S_n \rightarrow S_{n+1}$, one electron is removed from the $CaMn_4$ cluster to Tyr-Z (Fig. 2, reaction 5). Early work by C.F. Fowler [23] showed that the oxidations of the Mn-ions are coupled to a release of protons that oscillates with a period of 4. The nearest integer values for the release was 0:1:2:1 (the actual values were non integer) for the consecutive S state transitions, $S_1 \rightarrow S_2 \rightarrow S_3 \rightarrow S_0 \rightarrow S_1$. Since then the experimental efforts made to clarify the pattern of protons liberated during water oxidation have presented a large variety of results where the observed release greatly depends on preparation type and pH (reviewed by J. Lavergne and W. Junge 1993 [24]).

The efficiency of the individual transitions in the S cycle depends to a large extent on the proton concentration in the bulk. Each transition shows a characteristic pH dependent behavior, first resolved by G. Bernát et al. using EPR spectroscopy [25]. A prerequisite for the analysis was the earlier characterization of the pH effects of the $CaMn_4$ cluster itself (e.g. protonation/deprotonation of the μ-oxo bonds changing the Mn coupling), decreasing the multiline signal amplitudes in the S_2 and S_0 states at high and low pH [26]. The highest efficiencies for the transitions are found at pH ~ 6.5. At low pH, all S state transitions are inhibited except the $S_1 \rightarrow S_2$ transition [25,27]. The effect was attributed to protonation of one or several amino acid residues in the proton exit pathway from the $CaMn_4$ cluster and/or Tyr-Z [25].

The four protons generated for each completed reaction cycle are to be expelled into the thylakoid lumen, increasing the proton gradient. The release is likely to occur via specific pathways directed to the luminal side of the membrane. Defined channels has been suggested to facilitate the proton transfer within the protein (see F. Ho this issue) as well as specific amino acids that are involved in the expulsion of the protons from the catalytic site (e.g. Arg357 of the CP43 protein, [28,29]).

In recent years the strong connection between electron and proton transfer in the OEC has become increasingly clear. This has led H. Dau and coworkers to introduce the "extended" S-cycle with eight discrete steps where electron transfer and proton release strictly alternate [29,30]. The suggested extended S-cycle with an alternate electron and proton removal is in fact well compatible with the proton release pattern 0:1:2:1 first presented by C.F. Fowler. The "extended" S-cycle highlights the importance of controlled electron and proton movements on the donor side of PSII to keep the $CaMn_4$ cluster (and Tyr-Z) in charge balance to enable di-oxygen formation (see H. Dau this issue).

During catalysis in PSII both the release of protons from the water oxidation reaction and take up protons during the reduction of Q_B are directly coupled to electron transfer steps. In many cases the protonation or deprotonation reactions steer the efficiency of the electron transfer. This also holds for the redox chemistry involving the tyrosines as it is known that both radicals originate from the deprotonated neutral form of the oxidized radical state [19,31–35]. Thus, both tyrosines are deprotonated during oxidation. Consequently, they must reprotonate during reduction. This has prompted many studies of pH dependent kinetics and equilibria and of deuterium isotope effects on kinetics from various steps involving respective tyrosine. The results from many of these investigations were at the time difficult to interpret all the way but with the present knowledge that the hydrogen bonds between respective tyrosine and histidine (Fig. 3) exist, they are easier to reconcile. Here we will use the knowledge of the hydrogen bonds to examine much of the existing pH dependent data to understand how these hydrogen bonds steer the oxidation/reduction chemistry of the tyrosine radicals in PSII.

3. The hydrogen bond between $Tyr_{Z,D}$-OH and $Histidine_{Z,D}$

With the identification of the two Signal II radicals to Tyr-D and Tyr-Z it became possible to address their properties at molecular level. This was done through a combination of computer modeling, site directed mutagenesis and EPR spectroscopy. The first detailed prediction of the molecular structure around Tyr-D and Tyr-Z was obtained by homology based computer modeling using the core of the 3-dimensional structure from the purple bacterium *Rhodopseudomonas viridis* as framework [36]. An important basis for the computer modeling was that all spectroscopic knowledge about Tyr-D (in particular) and Tyr-Z (to the extent known) was used to orient respective tyrosine residue in the model. This was critical since the bacterial reaction center did not have tyrosines in these positions. Thus they had to be modeled in. It was clear from the model that both tyrosyl side chains could be inserted in the protein structure to fit with all available spectroscopic data. It was then found that they both formed hydrogen bonds to homologous histidine counter partners, Tyr-D-OH to His_D (His189) on the D2 protein and Tyr-Z-OH to His_Z (His190) on the D1 protein (cyanobacterial numbering).

It was clear early on that these hydrogen bonds were important. They were both probed by site-directed mutagenesis of respective hydrogen bonding pair and by various spectroscopic measurements. In essence it was found that when His_Z or His_D was removed, the function and molecular/electronic structure of respective tyrosine was severely altered or the tyrosine often became non-functional. In the case of His_Z, its function could be replaced by a buffer [37] or high pH [38] rendering Tyr-Z functional. In the case of His_D such replacement studies were less conclusive in part reflecting the problems connected to study Tyr-D oxidation (which occurs only one time in a particular PSII center) in part that the environment of Tyr-D is very shielded from the external surroundings. All studies however, were taken as evidence for the existence and importance of the Tyr–His hydrogen bonds [39].

The computer model [36,40] served long as the structural basis for much analysis involving the protonation and deprotonation reactions of Tyr-D and Tyr-Z but it was not until the better resolved X-ray structures of PSII appeared that the two hydrogen bonds were finally ascertained [41–43]. The very recent structure of PSII to 1.9 Å resolution [44] reveals further information about the hydrogen bond pattern around respective tyrosine. Fig. 3 shows the hydrogen bonds between Tyr-D and His189 on the D2 protein and the corresponding pair on the D1 protein between Tyr-Z and His190. The tyrosine and the histidine residues in both pairs are close enough and oriented well to form strong, well defined hydrogen bonds. The hydrogen bond between Tyr-D and His189 on the D2 protein is 2.78 Å when measured from the oxygen in Tyr-D to the participating N-atom in D2-His189 while the corresponding distance for Tyr-Z on the D1 protein is 2.49 Å. The distances vary slightly from the distances obtained in the earlier structures (Table 1) but in most cases Tyr-Z has been found closer to His_Z than Tyr-D to His_D.

Interestingly, the new 1.9 Å structure [44] reveals that both tyrosines also interact with close lying water molecules (Fig. 3). Tyr-Z is connected to a water molecule (2.46 Å away) which in turn is connected to another water molecule. Tyr-D is also clearly in contact with a water molecule but this is not fully occupied in the structure and seemingly can take two positions (2.65 Å or further away). The exact angles for the water interactions and how these affect the hydrogen bond properties of respective tyrosine is not known at present since these waters were discovered only very recently [44].

The hydrogen bonds from the tyrosines define much of the function of these critical redox components in PSII and this

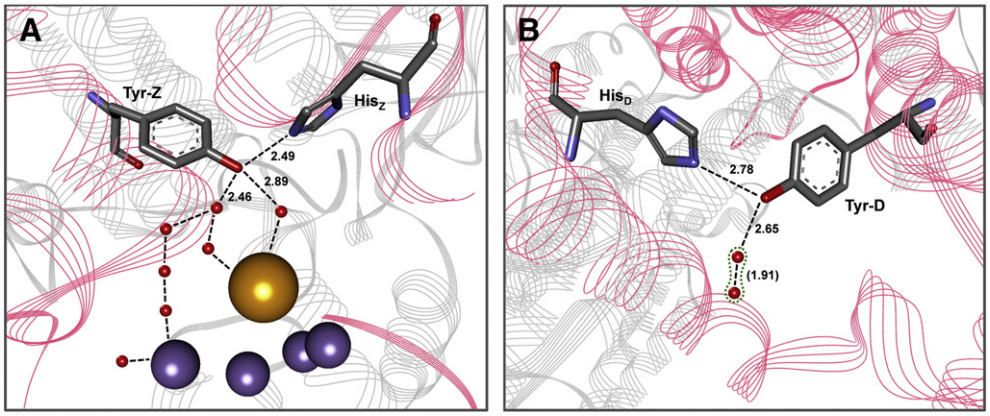

Fig. 3. The molecular structure around Tyr-Z (A) and Tyr-D (B). The structure (including the $CaMn_4$ cluster) was obtained from the 2.9 Å resolution structure (Protein Data Bank ID: 3BZ1) [43]. The indicated distances were presented at the 15th International Photosynthesis Congress and are from the 1.9 Å resolution X-ray structure [44]. The water molecules are inserted by us at the approximate position in the 1.9 Å structure.

Table 1
Hydrogen bond distances between Tyr-Z and His_Z on the D1 protein and Tyr-D and His_D on the D2 protein, measured in the available X-ray structures of PSII. The distances are between the phenolic oxygen and the imidazole N_ε in the respective histidine.

Tyr-Z–His_Z (Å)[a, b]	Tyr-D–His_D (Å)[a, b]	Reference
2.74/2.35	2.99/3.07	[41]
2.78/2.75	2.59/2.68	[42]
2.66/2.63	2.92/2.95	[43]
2.49/–	2.78/–	[44]

[a] Distances for the two different monomers are given.
[b] The hydrogen bonds in the Tyr–His pairs vary in the different PSII structures and it is not entirely certain that these differences reflect errors in the structural determination. Instead they could for example reflect varying degree of protonation in the water structures close to respective tyrosine, or even (in the case of Tyr-D) that the tyrosine is reduced in one case and oxidized in another structure. Note that the distances vary between the PSII monomers in the X-ray structures which are from the dimeric PSII. In the most recent structure this measurement has yet to be done, the values reported are from the public presentation by J-R. Shen [44].

contribution will deal with how they tune the pH dependent reactions involving Tyr-D and Tyr-Z. Throughout the text the focus is on the Tyr–His pair and how the proton moves in this hydrogen bond. This is however not the entire truth and as shown in Fig. 3 there are water molecules involved making this a more extended hydrogen bond network than considered before. The function is consequently more complex and this water offers for example a second base close to Tyr-Z (Fig. 4, panel II). It is interesting that such a base has been proposed from pH dependent results on the so called split EPR signals in PSII (see below) [45,46]. At present it is premature to discuss how these waters affect the hydrogen bonding properties of the two tyrosines. It is now clear that there are waters there (Fig. 3). This fact is very likely to drive new experimental design and create new thinking. This starts now and this review reflects much of the thinking up to date.

4. The function of Tyr-D, pH dependent reactions

Tyr-D• is a very oxidizing species (E_m Tyr-D•/Tyr-D 700–800 mV) [47]. Despite this the radical is remarkably stable and decays only over many hours. This stability is useful and much is known about the redox and protonation chemistry involving Tyr-D. Tyr-D•/Tyr-D is known to participate in several different reactions that are pH dependent and these will be discussed here. Tyr-D also participates in other redox equilibria in PSII that might be pH dependent although this has not been well described. This includes a very slow electron transfer to oxidized Cyt b_{559} [48,49]. These reactions are not covered here.

4.1. Electron transfer between the $CaMn_4$ cluster and Tyr-D/Tyr-D•

Tyr-D•/Tyr-D participates in redox equilibria with the different intermediates in the S-cycle. When PSII samples where Tyr-D is reduced are exposed to varying number of flashes Tyr-D becomes oxidized in a few seconds after 1 and 2 flashes but not after 3 or 4 flashes [5]. The reason is that Tyr-D can be oxidized by the OEC in both of the S_2 and S_3 states but not when the OEC is in the S_0 or S_1 states. It has also been conclusively shown that the flash first results in formation of the S_2 or S_3 state (after 1 or 2 flashes respectively) and that these states thereafter oxidizes Tyr-D [47,50,51]. Electron transfer in the opposite direction also occurs. Tyr-D• can oxidize the S_0 state to the S_1 state in a reaction that takes hours to complete at room temperature [47,50]. Although slow, this latter reaction is important in real life where night follows day; i.e. PSII is incubated in darkness for 12 h every 12 h, leading to Tyr-D reduction and S_0 oxidation in 25% of PSII.

All these reactions are pH dependent. The time constant for the electron transfer from Tyr-D to the S_2 or S_3 states is pH dependent and varies from ca 1 s at pH 8.0 to ca 20 s at pH 5.0. The electron transfer from the S_0 state to Tyr-D also depends on pH, the half time for the reaction is ca 5 min at pH 8.0 and 40 min at pH 5.0. These reactions are steered by two different protonation events. One protonation occurs with a pK_a of 7.3–7.5 (Table 2) [47] and retards the electron transfer from Tyr-D to the S_2 (or S_3) states nearly by a factor of 20. Already in the original publication [47] it was proposed that protonation of His_D controlled this electron transfer. When His_D is deprotonated this allows faster oxidation of Tyr-D. This is due to the hydrogen bond between the tyrosine and the histidine which is well defined when the histidine is deprotonated. Thus, the oxidation of the tyrosine and the concomitant deprotonation occur without any restrictions and with low activation energy. In contrast, when the histidine is protonated the hydrogen bond is less strong, or maybe even broken, and electron transfer from Tyr-D is much slowed down.

The other protonation was proposed to involve a group close to the OEC. It has a pK_a of 5.8–6.0 and steers electron transfer towards Tyr-D•

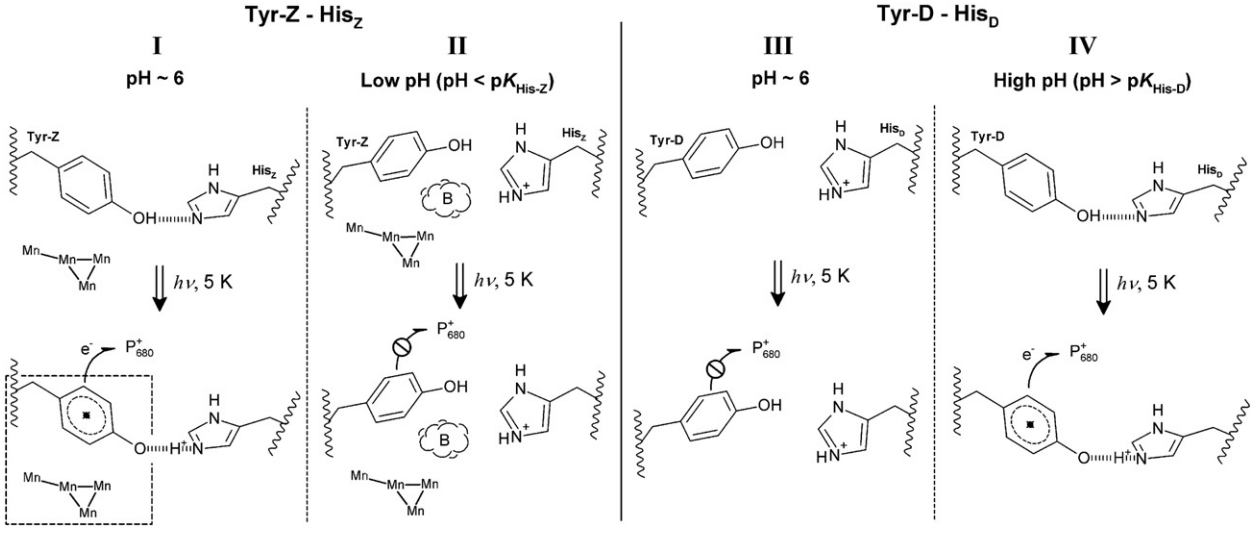

Fig. 4. Schematic representation of the effect of forming and breaking of the essential hydrogen bonds between Tyr-Z and His_Z (left panels) and Tyr-D and His_D (right panels). At pH values above the pK_a of His_Z (I) and His_D (IV), low temperature oxidation of Tyr-Z and Tyr-D is possible. At pH values below respective pK_a (II; III), low temperature oxidation of the tyrosines is not possible (indicated by a stop-sign). A second base (B, in panel II) in close proximity to Tyr-Z has been suggested to accept the proton from Tyr-Z when His_Z is protonated [45,46]. However deprotonation to this base is only available at room temperature and not at 5 K.

Table 2
pK_a values measured in Mn-depleted or intact PSII preparations that can be assigned to Tyr-D–His_D and Tyr-Z–His_Z.

		pK_a	Reference
Tyr-D–His_D	Mn-depleted	7.7	[52]
	Mn-depleted[a]	7.6	[53]
	Intact	7.3–7.5	[47]
	Intact[a]	8.0	[45]
Tyr-Z–His_Z	Mn-depleted	6.9–8.3	[54–57][b]
	Dark grown[c]	7.6	[38]
	Intact	4.5–5.3	[54,58–61][b]
	Intact[a]	4.1–4.9	[45,46,62,63]

[a] Measured by means of split EPR signal induction at cryogenic temperature (5 K).
[b] Observed in early studies when the identity of Tyr-Z and His_Z was unknown.
[c] Measured in dark grown *Chlamydomonas reinhardtii* where PSII lacks the $CaMn_4$ cluster.

when the OEC is in its least oxidizing state S_0 [47]. When the pH is below the pK_a, reduction of Tyr-D is very slow. The same pK_a also accelerates electron transfer from reduced Tyr-D to the S_2 state. In the original publication the protonating group was not identified but it was proposed to reflect a protonation observed to retard electron transfer from Tyr-Z to P_{680}^+. It now seems that the pK_a ~5.8–6.0 might involve protonation of His_Z. When this is protonated, electron transfer from Tyr-Z to P_{680}^+ is indeed slowed down (see below Section 5.2). It is likely that this protonation brakes or weakens the hydrogen bond enough to slow down, but not completely inhibit, Tyr-Z oxidation.

An important feature is that both the reduction of Tyr-D• from the S_0 state and the oxidation of reduced Tyr-D from the higher S states are very temperature sensitive and the reactions essentially freeze out already at ca 250 K [49]. As we shall see below this makes these reactions very different from the direct oxidation of Tyr-Z and Tyr-D from P_{680}^+ which can occur at ultralow temperatures (<5 K) at optimal pH values.

4.2. Direct oxidation of Tyr-D by P_{680}^+

It was long thought that Tyr-D could not be oxidized at very low temperature. This is also true at most of the pH values studied ("normal" pH range for PSII is pH 5.5–7). The notion proved wrong when A.W. Rutherford and coworkers could demonstrate very efficient oxidation of Tyr-D at elevated pH [52,53] and EPR studies revealed that Tyr-D could indeed be oxidized at ultralow temperature (<10 K). The oxidation increased as pH increased with a pK_a ~7.6 (Table 2) [53]. No intermediates were observed indicating that the reaction involved direct electron transfer from Tyr-D to P_{680}^+. At room temperature the same electron donation from Tyr-D to P_{680}^+ could be followed by optical spectroscopy and it was found that Tyr-D oxidation at elevated pH occurred with very fast kinetics in the nanosecond time regime [52]. At pH values below the pK_a, other processes not involving Tyr-D became dominating in the reduction of P_{680}^+. Which process that dominates depends on the system studied but in intact PSII electron transfer from Tyr-Z is always much faster.

It is interesting to analyze the very fast oxidation of Tyr-D that occurs at elevated pH and which is also possible at ultra low temperature. It is known that Tyr-D is always protonated in the reduced state but deprotonated in the oxidized state [64]. However, at very low temperature displacements of larger entities like amino acid side-chains or even smaller species like protons are very restricted, deprotonation of the tyrosine should be difficult to achieve. Thus, observation of efficient oxidation of the tyrosine at very low temperature must then indicate that there is a special effect involved in the deprotonation. The origin for this is found in the hydrogen bond between Tyr-D and His_D (Fig. 4), maybe also involving the recently discovered close lying water molecule (Fig. 3), and indicates that the hydrogen bond takes a perfect geometry to allow the proton movement towards the nitrogen in the histidine. That this indeed occurs at ultralow temperature indicates that the movement is much favored and demands very small energy. In case the hydrogen bond is less favorable, or even broken, deprotonation cannot occur at temperatures in the 5–10 K region (Fig. 4, panels III and IV).

The observed pK_a of ~7.6 [53] for the onset of the low-temperature oxidation of Tyr-D is interesting and two possibilities have been considered. The pK_a could reflect deprotonation of Tyr-D itself. However, this is unlikely since FTIR studies indicate that Tyr-D is protonated in the entire pH interval discussed [64]. Instead the pK_a is probably best assigned to titration of His_D. When the histidine is deprotonated this promotes close interaction between Tyr-D and His_D, setting a very well defined hydrogen bond (Figs. 3 and 4, panel IV). At ultra low temperature, this allows proton reshuffling in the hydrogen bond from the tyrosine towards the histidine which is a prerequisite for electron transfer from the tyrosine. This low temperature deprotonation of Tyr-D involves an observable (with high frequency EPR) intermediate state where the proton is closer to its original position than in the final state [53]. The relaxation to the final state cannot occur at this temperature where large protein motions are unlikely but proton movement in a well tuned hydrogen bond will pose little problem. When His_D instead is protonated this perturbs the hydrogen bond to Tyr-D which now becomes less well defined. The hydrogen bond might well still exist but does not anymore allow low-temperature oxidation of the tyrosine (Fig. 4, panel III).

The first experiments demonstrating low-temperature oxidation of Tyr-D were carried out in PSII lacking the $CaMn_4$ cluster [52,53]. Here Tyr-Z is less efficient which allows competition from Tyr-D. The measurements were later taken further to intact, oxygen evolving PSII [45] where Tyr-Z normally outcompetes Tyr-D. Interestingly, it was found that oxidation of Tyr-D outcompeted oxidation of Tyr-Z at elevated pH at 5 K. The pH dependence of this efficient oxidation of Tyr-D was steered by a pK_a ~8.0 (Fig. 5D; Table 2) [45], which also was assigned to protonation of His_D. It was also found that Tyr-Z was also efficient at the high pH values provided Tyr-D was already oxidized. Thus both tyrosines can be oxidized at ultralow temperature at high pH (see Section 5.2.2 for discussion of oxidation of Tyr-Z at ultralow temperature).

It is in this context useful to note that His_D protonation is almost insensitive to the presence or absence of the $CaMn_4$ cluster. The pK_a for its oxidation is ~7.6 in the absence of the cluster and ~8.0 in the presence (Table 2). This is not surprising given the ca 40 Å long distance between Tyr-D and the $CaMn_4$ cluster. In the case of Tyr-Z, the presence or absence of the $CaMn_4$ complex has very large effect on the pK_a of the corresponding His_Z (see Section 5.2.2).

4.3. Possible function of Tyr-D in PSII

Earlier studies with site-directed mutants where Tyr-D was replaced by other redox inactive residues lead to the conclusion that it is not required for the water oxidation process [65,66]. Tyr-D is nevertheless completely conserved in the D2 protein in all natural species. It is thus a very important redox cofactor and two roles have been suggested for Tyr-D in order to improve and maintain efficient PSII turnover [67]. The first role is electrostatic where oxidized and deprotonated Tyr-D results in presence of a positive charge (H^+) in the vicinity of His_D. Consequently, via columbic interaction, this leads to the increase of the potential energy of the primary donor, P_{680}^+ [67]. This can have several important consequences such as accelerating the Tyr-Z oxidation, facilitating the photoactivation process and localization of the highly oxidizing species only on the D1 protein side of the PSII complex [67,68]. The second role is the participation of Tyr-D in the redox reactions within PSII. Tyr-D oxidizes the S_0 state to the S_1 state in the dark, thus moving the S cycle one step forward without charge separation [47,50]. It also works in the opposite direction and

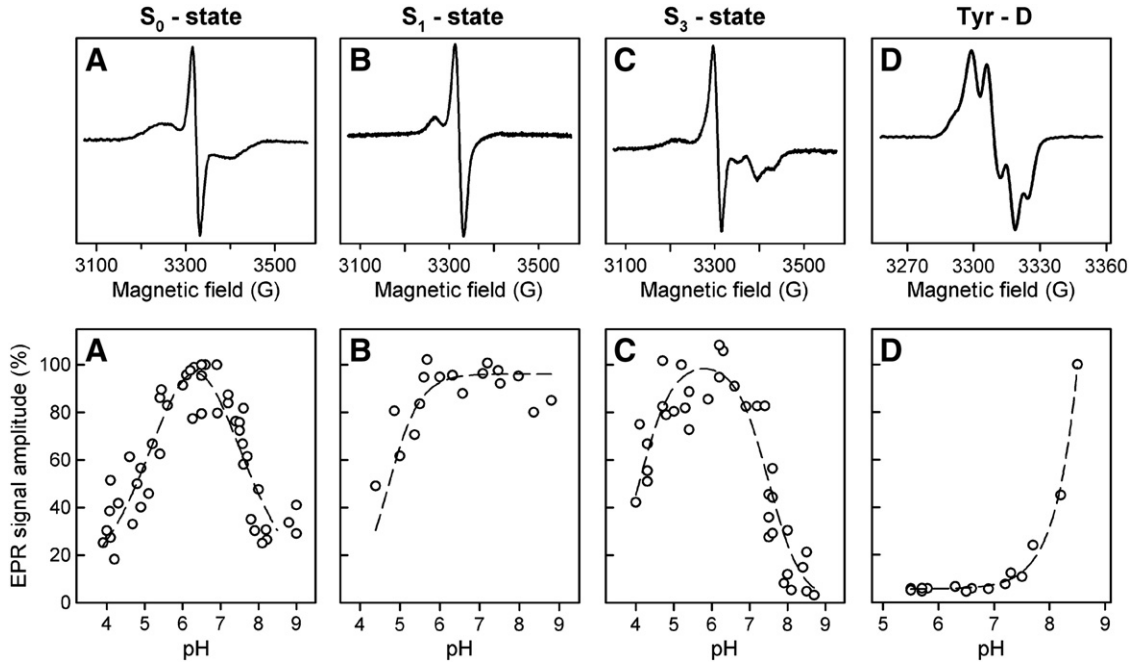

Fig. 5. pH dependence of the formation of the split S_0, (A), split S_1 (B), split S_3 signals (C) and the pH dependence for the oxidation of Tyr-D in intact PSII (D). The top part of the figure show the corresponding EPR signal recorded at 5 K.

deactivates higher S states by reducing the S_2 and S_3 state to the S_1 state and preventing the reduction of the $CaMn_4$ cluster from the outside of the PSII complex [47,49]. The oxidation and reduction of Tyr-D are also important during the repair cycle of PSII and the photoactivation process where it is acting as a sink for an electron hole. It was shown that Tyr-D can reduce P_{680}^{+} and Tyr-Z• (via an equilibrium) when the electrons from the $CaMn_4$ cluster are not available [69,70]. Possibly, it can also help to relieve the electron pressure from the acceptor side via recombination with Q_A^- when the electron flow is too high [67].

5. The function of Tyr-Z, pH dependent reactions

Tyr-Z• is a very oxidizing species (E_m Tyr-Z•/Tyr-Z>900–1000 mV) [30,47]. This makes the radical very reactive and in the presence of the $CaMn_4$ cluster the oxidized tyrosine is reduced with fast, S state dependent kinetics. This fast kinetics has made the Tyr-Z• radical difficult to study directly at room temperature and there exist only very few studies using EPR spectroscopic detection of the radical [9,13]. Instead the kinetics of Tyr-Z oxidation is often followed as reduction of P_{680}^{+}, which is possible to measure by time resolved absorption changes [71–73]. In recent years however, S state dependent oxidation of Tyr-Z has been observed with EPR to occur at ultralow temperatures (~5 K). This has allowed many studies of the function of Tyr-Z which were not possible before. Both methods reveal that Tyr-Z•/Tyr-Z participate in electron transfer reactions that are pH dependent.

The electron transfer from the $CaMn_4$ cluster to P_{680}^{+} involves two discrete steps. First P_{680}^{+} oxidizes Tyr-Z to the neutral radical Tyr-Z• in the nanoseconds to microseconds time regime (Fig. 2, reaction 3) [71,73]. Second, Tyr-Z• is reduced by the $CaMn_4$ cluster in the microseconds to milliseconds time scale (Fig. 2, reaction 5) [13,74]. The details of this coupled electron–proton transfer reaction are critical for the function of Tyr-Z and variations of electron–proton transfer reactions play central roles in several steps associated with the light driven oxidation of water.

The formation of the neutral radical Tyr-Z• implies that the oxidation of Tyr-Z-OH is associated with a deprotonation. Tyr-Z has also been suggested to be a tyrosinate when reduced [75] in which case a deprotonation would not be needed. However, it is likely that also the tyrosinate would be hydrogen bonded. In this case the phenolic proton would be shifted more towards its base (His_Z) already in the reduced state [76]. The exact nature of the initial state (tyrosine or tyosinate) has been addressed with optical and FTIR difference spectroscopy [75–78]. Although not conclusively proven, the FTIR experiments indicate that Tyr-Z is protonated at physiological pH both in Mn-depleted and intact PSII [77,78].

Thus, since the oxidized form of Tyr-Z is a neutral radical (see Section 1) it is generally accepted that the phenolic proton of Tyr-Z moves away in the hydrogen bond to His_Z (Fig. 4, panel I) when Tyr-Z is oxidized. Upon reduction from the $CaMn_4$ cluster Tyr-Z is reprotonated. This alternating proton movement occurs during steady state oxygen evolution. It is thus very different and physiologically much more important than the proton movement involved in the oxidation and deprotonation of Tyr-D which occurs only once, where after the Tyr-D• radical stays in the oxidized state the entire day (until several hours after sunset).

The deprotonation/reprotonation of Tyr-Z has been discussed in the context of a hydrogen abstraction event where a hydrogen atom would be extracted from the $CaMn_4$ cluster by Tyr-Z• upon each S state transition [33,79,80]. This pathway demands a proton expulsion pathway from His_Z and it is worth pointing out that the new 1.9 Å X-ray structure for PSII at first glance does not rule out the existence of such a pathway [44]. An alternative, and presently preferred, mechanism involves a proton rocking model where the proton moves back and forth between Tyr-Z and His_Z in the hydrogen bond, i.e. the proton never leaves the immediate vicinity of Tyr-Z/His_Z [54,81,82]. It has been suggested that the positive charge in the Tyr-Z/His_Z moiety, instantaneously formed upon Tyr-Z oxidation, triggers deprotonation reactions in the $CaMn_4$ cluster through electrostatic interactions. Several models for such a mechanism have been proposed [29,30,74,83,84]. Both mechanisms involve His_Z to function as an efficient proton acceptor for the proton leaving Tyr-Z. However, the proton movements associated with Tyr-Z oxidation and reduction are not necessarily identical in each S state and will certainly be dependent on pH.

5.1. Tyr-Z in the absence of the $CaMn_4$ cluster

Tyr-Z is difficult to study in intact, oxygen evolving PSII due to the fast kinetics. When the $CaMn_4$ cluster is removed (or absent for some reason), electron transfer from and to Tyr-Z can still occur but Tyr-Z is oxidized and reduced much slower. However, the absence of the $CaMn_4$ cluster opens up the structure around Tyr-Z which makes Tyr-Z• more accessible for external reagents. In addition, the hydrogen bond involving the Tyr-Z-OH proton is disordered and it is likely that the Tyr-Z-OH interacts with several more hydrogen bond acceptors instead of only one very well defined to His_Z (and potentially the water molecule discovered in the 1.9 Å structure [44]; Fig. 3). Therefore results on the function of Tyr-Z in the absence of the $CaMn_4$ cluster must be interpreted with care as they will not immediately apply to the fully functional system.

In the absence of the $CaMn_4$ cluster electron donation from Tyr-Z to P_{680}^+ is strongly pH dependent [57] and occurs in a few 100 ns (at high pH) to the 30 μs (at low pH) time regime. The rate increases with the pH with a pK_a ~6.9–8.3 (Table 2) [54–56]. A similar pK_a for efficient Tyr-Z oxidation (pK_a ~7.6) was found in PSII from dark grown *Chlamydomonas reinhardtii* where the $CaMn_4$ cluster is not yet assembled (Table 2) [38]. It is clear that these pK_a values reflect the same process, protonation of His_Z. At high pH (pH > pK_a of His_Z) the oxidation of Tyr-Z occurs very fast and is coupled to deprotonation in the well-defined hydrogen bond also in PSII where the $CaMn_4$ cluster is absent. At pH < pK_a of His_Z, the oxidation is much slower due to the disordered hydrogen bond involving Tyr-Z-OH (observed by EPR spectroscopy [34]). The deprotonation of Tyr-Z can occur, but is not as favorable as when the His_Z is available for perfect hydrogen bond formation.

5.2. Tyr-Z in the presence of the $CaMn_4$ cluster

5.2.1. Oxidation of Tyr-Z at room temperature, pH dependent reactions

At room temperature Tyr-Z• formation and decay is best studied indirectly via the reduction of P_{680}^+ (formation of Tyr-Z•) or oxidation of the $CaMn_4$ cluster (reduction of Tyr-Z•) while direct EPR observation of Tyr-Z• is difficult due to the fast kinetics.

Oxidation of Tyr-Z is multiphasic, S state dependent and very fast [59,71,73,85,86]. It occurs dominantly with nanosecond kinetics but there is also always a microsecond component. The oxidation is fastest in the S_0 and S_1 states where the dominating kinetic phase has a half time of ca 20 ns. This kinetics has small activation energy [72] and is strongly pH dependent. Between pH 5.5 and 8.0 this kinetic phase is in essence pH independent [58,87]. A very useful observation is that this fast kinetics does not show any significant deuterium isotope effect [88–90], suggesting that proton exchange with the bulk is not essential in this reaction. The most straight forward explanation is that this fast Tyr-Z oxidation can occur only when the phenolic proton from Tyr-Z is tightly held in the well set hydrogen bond to His_Z. When the hydrogen bond is in place, Tyr-Z is oxidized very quickly but the proton is never expelled to the medium. Analysis within the framework of the Marcus theory has led to the conclusion that this fast kinetics is limited by electron transfer [91]. Consequently the proton has to shift within the hydrogen bond in <20 ns. It is not unlikely that this is the fastest proton coupled electron transfer that occurs in nature, clearly it is the fastest in photosynthesis.

The nanosecond kinetics decreases both in rate and amplitude in the acidic region. This slow down is steered by a pK_a 4.5–5.3, below the pK_a the kinetics is slower (Table 2) [54,58–61]. Most likely this reflects titration of His_Z which leads to breaking or disordering of the vital hydrogen bond. The result is that Tyr-Z is oxidized in the microsecond time regime instead. This oxidation of Tyr-Z in the microseconds time regime shows a significant deuterium isotope effect, indicating that the phenolic proton from Tyr-Z is in contact with, maybe even directly exchanging with the bulk. Thus, the well tuned hydrogen bond is now broken. As we shall see in the next section, the same pK_a (~4.5) also controls the oxidation of Tyr-Z at ultra low temperature. When the hydrogen bond is well tuned, Tyr-Z can be oxidized at 5–10 K, when the hydrogen bond is less well-defined, low temperature oxidation is not possible (Fig. 4).

Also at neutral pH, there is always a fraction of PSII centers (~20%) where Tyr-Z oxidation occurs in the microseconds time domain. It was long thought that this kinetics reflected damaged centers but there is now consensus that also this is a natural part of PSII electron transfer [73,86,91]. Different from the dominating nanosecond kinetics in the same pH-interval (see above) the microsecond kinetics varies with the S state and shows a significant deuterium isotope effect. Again, the latter indicates that the oxidation of Tyr-Z is limited by extensive movement of one or more protons. This most likely reflects that the tyrosine is not involved in the well set hydrogen bond to His_Z. Instead the phenolic proton probably participates in less defined interactions not permitting very fast deprotonation. The necessary deprotonation accompanying oxidation of Tyr-Z would then have to occur over longer distance and presumably in less favorable geometric orientations, all factors that will slow down the coupled electron transfer.

5.2.2. Oxidation of Tyr-Z at ultralow temperature

When pH dependent effects on the turnover of the OEC are studied at room temperature it is very difficult to discriminate between phenomena occurring at the level of Tyr-Z or somewhere else in the complex chemistry in the $CaMn_4$ cluster. This can be overcome by studies at very low temperature.

The temperature dependence of the S state transitions is not sufficiently studied but available data indicate that all transitions, except $S_1 \rightarrow S_2$, cannot proceed below ca 230 K [92]. $S_1 \rightarrow S_2$ is very different and is inhibited with a half temperature for its inhibition of 140 K. It has been shown to be partially functional even at 77 K [92]. The difference between $S_1 \rightarrow S_2$ and the other steps were immediately connected to the proton release steps and large structural rearrangements in the $CaMn_4$ cluster occurring in all other steps while $S_1 \rightarrow S_2$ is connected to electron transfer only.

One consequence of the functionality of the $S_1 \rightarrow S_2$ transition at 77 K (albeit small) is that Tyr-Z also must be operational at this temperature. This was long over looked but it is now clear that Tyr-Z, similar to Tyr-D, can be oxidized efficiently and to high degree by illumination of intact PSII even at ultralow temperatures (5–10 K).[2]

Starting around the beginning of the new millennium a series of split EPR signals of metallo-radical character were discovered when PSII was illuminated at cryogenic temperature [93–97]. The signals were found to oscillate with the S states [93,98] and have with time been shown to originate (with high probability) from Tyr-Z• in magnetic interaction with the $CaMn_4$ cluster. They can all be formed in high yields and most reports indicate their maximum induction to involve 30–50% of the PSII centers. Since the magnetic and electronic properties of the $CaMn_4$ cluster varies with the S state, the split signals vary in shape and magnetic properties. The interesting properties and the assignment history of these split signals is not the scope for the present discussion (this was recently reviewed in [99]). Instead we will focus our discussion on the pH dependent characteristics of the split signals and use these (Fig. 5A–C) to cast further light on the coupled electron and proton transfer reactions involving Tyr-Z.

It is first useful to briefly describe the photochemistry in PSII at very low temperature (for example 5–10 K). After charge separation, the electron is transferred to Q_A (Fig. 2, reaction 2) but further transfer

[2] Functional Tyr-Z oxidation is a prerequisite for any turnover of the $CaMn_4$ cluster to occur. If Tyr-Z is oxidized at a certain temperature and pH, the possibility exists that Tyr-Z• can oxidize the $CaMn_4$ cluster. If on the other hand Tyr-Z cannot be oxidized, the $CaMn_4$ cluster can never turn over even if there is nothing wrong with the cluster itself. The opposite is not true, the $CaMn_4$ cluster can be inhibited although Tyr-Z can be oxidized, in this case the strongly oxidizing radical on Tyr-Z will oxidize any other available electron donors in PSII.

to Q_B (Fig. 2, reaction 4) is blocked due to the low temperature. Thus, transfer of only one electron needs to be considered. P_{680}^+ can oxidize one of several species, Tyr-Z, Tyr-D (if this is reduced, vide infra) or a close lying chlorophyll (if nothing else is available). Tyr-Z is the most efficient donor at room temperature (Fig. 2, reaction 3) but for this to prevail at low temperature the phenolic proton on Tyr-Z must be in a "tunneling ready" configuration in its hydrogen bond to His_Z (Figs. 3 and 4). In case the hydrogen bond is disordered or broken, low temperature oxidation of Tyr-Z is unlikely to occur. The situation is analogous to that for Tyr-D.

In case Tyr-Z• is formed it cannot be reduced by the $CaMn_4$ cluster (Fig. 2, reaction 5) since all oxidation steps here are frozen out [92]. Instead a quite stable Tyr-Z• radical is formed (it recombines with the electron on Q_A^- in a few minutes at 5–10 K, [98]). The radical is located ca 5 Å from the $CaMn_4$ cluster and is therefore revealed as a magnetic interaction EPR signal recognizable as a split radical EPR signal (indicated by a "box" in Fig. 4, panel I). However, only electron and proton transfers at Tyr-Z are involved in its formation. The split signals are recent, and as yet relatively unexplored, direct spectroscopic probes to the chemistry of Tyr-Z.

Very recently the split signals have been used to investigate the pH dependence for the formation of Tyr-Z• at 5–10 K in the S_0, S_1 and S_3 states (Fig. 5A–C) [45,46,62]. The split signal formed in the S_1 state was also used to investigate the competition between Tyr-Z and Tyr-D oxidation at cryogenic temperatures [45]. The relevance of the low temperature measurements to address functionality at ambient temperature has occasionally been questioned. The proton equilibrium around Tyr-Z and Tyr-D is set at ambient temperature by changing the pH. Thereafter, the sample is frozen to ultra low temperature. If the particular pH allows tunneling of the hydrogen bonded Tyr-OH proton once the system is set at cryogenic temperature it will allow Tyr-Z/D oxidation. This has provided a so far unique opportunity to follow Tyr-Z• formation and its pH dependence in intact PSII centers in the discrete S states. As discussed in Section 5.2.2.1 the low temperature experiments show good agreement with the pH dependence of the kinetic measurements done at room temperature. It thus seems likely that it is useful to use low temperature measurements to address reactions at room temperature, at least in the case of Tyr-Z/D oxidation.

5.2.2.1. Acidic region. At low pH, the split S_3 EPR signal induced at 5 K decreases with pK_a ~4.1 [62]. This is similar to the decrease of the split S_0 (~4.7–4.8) [46,63] and split S_1 (~4.7–4.9) [45,63] EPR signals induced at 5 K (Fig. 5; Table 2). Thus, the onset of the cryogenic Tyr-Z oxidation occurs in the same region (pK_a 4–5, Fig. 5) independent of S state. Below the pK_a, the split signal could not be induced, above the pK_a the formation of respective split signal was efficient. This similarity suggests that the reason for the decrease is the same in all S states. When lowering the pH, the essential Tyr-Z–His_Z hydrogen bond motif is disrupted (Fig. 4, panel II). The most straight forward explanation is direct protonation of the nitrogen in His_Z that is the hydrogen bond acceptor from Tyr-Z (Figs. 3A and 4, panel II). This titration will disrupt the hydrogen bond to Tyr-Z before freezing the sample. In this situation no other proton acceptor is available at this temperature. When the sample is then illuminated at the very low temperature, the hydrogen bond cannot be re-established since protein movements cannot occur and Tyr-Z oxidation is prevented. This is observed as a loss of split EPR signal formation (Fig. 5) [45,46,62]. At room temperature, the possibility of an additional base to become available for Tyr-Z deprotonation at low pH has been proposed (Fig. 4, panel II) [45,46]. The phenolic proton of Tyr-Z has in this situation the possibility to move in a hydrogen bond at room temperature although His_Z is unavailable, observed e.g. as a pH independent $S_1 \rightarrow S_2$ transition [45] or a slower reduction of P_{680}^+ (see below). Although there is no experimental evidence at present it is tempting to speculate that this additional base could reflect the water molecule found to hydrogen bond the phenolic oxygen of Tyr-Z in the recent crystal structure [44].

Although, not dissected for the separate S states, similar pK_a values (~4.5–5.3, Table 2) are found for the decrease of the nanoseconds kinetics in the reduction of P_{680}^+ by Tyr-Z, measured at ambient temperature (see Section 5.2.1). The nanoseconds kinetics is thought to reflect an electron transfer not rate limited by a subsequent proton transfer. This could represent oxidation of Tyr-Z coupled to the proton shifting in a well-set hydrogen bond to the nearby His_Z. Thus, both the nanoseconds kinetics at ambient temperature and the split EPR signal formation at 5 K decrease concertedly with similar pK_a values, presumably by protonation of the Tyr-Z–His_Z motif. It seems that this is not S state dependent. Breaking the hydrogen bond involving Tyr-Z-OH always slows down or inhibits Tyr-Z oxidation (depending on temperature).

The pK_a for oxidation of Tyr-Z in the S_3 state (~4.1) [62] is considerably lower than for the induction of Tyr-Z• in the S_0-state (~4.7–4.8) [46,63] and in the S_1-state (~4.7–4.9) [45,63] (Fig. 5; Table 2). The lower pK_a probably reflects the overall charge situation in OEC in the S_3 state where the $CaMn_4$ cluster carries an extra charge when compared to the S_1 and S_2 states. This charge will most likely cause a downshift in the pK_a(s) of a nearby amino acid(s). It could directly affect the pK_a of the Tyr-Z–His_Z motif or alter its pK_a via an interconnected H-bond network.

Furthermore, although the exact pK_a is very different (pK_a ~5 vs pK_a ~8) the observation is the same as for Tyr-D (Fig. 4). The ability to oxidize the tyrosine at ultralow temperature is correlated to nanoseconds kinetics at room temperature. When the hydrogen bond is disrupted or broken, tyrosine oxidation cannot occur at cryogenic temperature while the oxidation kinetics at room temperature are slowed down very much.

5.2.2.2. Alkaline region. In the alkaline region, the split signals behave differently and it is useful to analyze this together with the function of Tyr-Z and the OEC at room temperature. With the exception of the $S_3 \rightarrow (S_4) \rightarrow S_0$ transition there is no significant decrease at ambient temperature in any of the S state transitions at alkaline pH (below pH ~9) [25,27]. This indicates that there are no pH constraints on the transitions between $S_0 \rightarrow S_1 \rightarrow S_2 \rightarrow S_3$ above pH ~6. It also shows that Tyr-Z oxidation is possible at high pH at ambient temperature.

At low temperature this is different and, for the present discussion, the most pertinent finding is that the induction of the split S_1 signal was high and pH independent between pH 5.5–9.0 (Fig. 5) [45]. This directly shows that Tyr-Z-OH is involved in the well tuned hydrogen bond to His_Z above the pK_a of the histidine allowing cryogenic oxidation. At room temperature, the presence of the hydrogen bond reveals itself as the fast nanosecond kinetics in P_{680}^+ reduction that are pH independent above pH 5.5 (Section 5.2.1).

In contrast, by increasing the pH the split S_3 and S_0 EPR signals decrease with pK_a ~7.5 and pK_a ~7.9 respectively (Fig. 5) [46,62]. Interestingly this decrease has been assigned to delicate molecular properties in the $CaMn_4$ cluster and not to decreased ability to oxidize Tyr-Z and the low temperature. The complete analysis of their behavior at high pH is beyond the scope of this article but it is worthwhile to mention a few aspects. In the S_0 state, the decrease of the split signal above pH ~7.7 was suggested to be caused by a pH induced change of the $CaMn_4$ cluster, affecting the magnetic interaction to Tyr-Z•, and not to an inhibition of Tyr-Z oxidation by high pH [46]. The decrease in the induction of the split S_3 signal at high pH is different. In the S_3 state, at pH above ~7.5, a 125G wide split EPR signal is formed already in the dark, i.e. without any inducing illumination. The signal has been characterized and is suggested to arise from a state denoted S_2''Tyr-Z• [100]. It is thought to originate from backwards electron transfer from Tyr-Z to the S_3 state, reducing it to a modified proton deficient S_2 state (indicated by S_2''). Since Tyr-Z• cannot be further oxidized, the induction of the split S_3 EPR signal at

5 K will be prevented by the presence of any S_2''Tyr-Z• formed already before the sample is illuminated [62]. Nevertheless, the existence of oxidized Tyr-Z already in the dark shows that it is indeed possible to oxidize Tyr-Z also in the S_3 state at high pH. To conclude this discussion, the available data suggest that Tyr-Z is possible to oxidize at elevated pH, probably also at low temperature, in all S states.

5.3. Mechanistic role of Tyr-Z in water oxidation

The function of Tyr-Z in PSII reactions is much clearer when compared to the function of Tyr-D. It acts as an intermediate electron transfer component between the $CaMn_4$ cluster and P_{680}^+ and now it is more or less established that together with His_Z and the $CaMn_4$ cluster it is an integral part of OEC, the catalytic core where water oxidation takes place. With respect to deprotonation reactions and/or possible hydrogen atom transfer the detailed mechanism is less resolved. Recent high resolution crystal structure of PSII revealed in much more detail the surroundings of the OEC [44]. It is clear that the protein void between the $CaMn_4$ cluster and Tyr-Z is filled with several water molecules structurally oriented and hydrogen bonded between the Ca ion of the cluster and the phenolic oxygen of the tyrosine residue (Fig. 3). At least one of these water molecules is probably oxidized during the S cycle. It is likely that the close proximity of the Tyr-Z–His_Z hydrogen bond can have unexpected and interesting consequences in the deprotonation events during PSII turnover.

6. Concluding remarks

The existence of the two tyrosine cofactors with different redox properties, but similar spectroscopic characteristics, is a peculiar property of PSII. It reflects the evolutionary steps in the development of the oxygen evolving reaction center from its bacterial ancestors. The tyrosines have different protein surroundings and different accessibility to the luminal part of the thylakoid membrane. There are similarities but also unique and profound differences in the functional properties of Tyr-D and Tyr-Z. They are both conserved over eons of time. The reason is evident for Tyr-Z and mutants in this residue have no $CaMn_4$ cluster [101,102]. Also Tyr-D is completely conserved [21] although site directed mutants in this residue can grow photoheterotrophically with water as electron source [65,66]. Seemingly, the presence of Tyr-D is critical to maintain a functional organism in nature although it is not entirely necessary for the assembly of a functional $CaMn_4$ cluster in a laboratory grown cyanobacterium or green algae.

Water-splitting in PSII is often described as the “holy grail” of biophysics. Nearly always this alludes to the structure and function of the $CaMn_4$ cluster which is seen as the most important redox center to understand, the most important catalytic activity to copy. However, photosynthetic water oxidation is not only about the $CaMn_4$ chemistry. The function in PSII is just as original in the high redox potential achieved by P_{680}^+ which is a prerequisite for the oxidation of water. If P_{680} had been just an ordinary chlorophyll ensemble there had been no water oxidation, and no $CaMn_4$ cluster. This might hold even more for the function of the tyrosines.

Oxidation of two water molecules is a four electron and four proton reaction. It is fascinating how these four electron holes, finally hot enough to oxidize water, can be stored in the $CaMn_4$ cluster and its environment and protected against futile recombinations for minute long periods. If the electron holes shall be stable enough they must be protected against back reactions from the reduced quinone acceptors Q_A and Q_B. This can indeed occur but is very slow. To large extent this reflects the rectifying properties of the electron transfer through Tyr-Z. The electron goes essentially only one way, from the Tyr-Z to P_{680}^+. It does not go easy from Q_A^- or reduced P680 to Tyr-Z and further to the electron hole on the $CaMn_4$ cluster, even if this hole is very prone to take an electron—if it could. Tyr-Z works as a rectifier of the electron transfer. A critical factor in mechanism is the handling of the proton from the tyrosine.

The importance of the tyrosines cannot be over-estimated. The tyrosines earn their place among critical amino acids residues in the history of the development of the biosphere. They were crucial for photosynthetic water oxidation to evolve. This led to oxygen formation in the atmosphere and development of eukaryotic life. Every electron that is extracted from water flows through Tyr-Z. The tyrosines in PSII are “Two tyrosines that changed the world”.

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