Tyrosine D Oxidation at Cryogenic Temperature in Photosystem II†

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ABSTRACT: Photosystem II (PSII) contains two redox-active tyrosines (TyrZ and TyrD) possessing very different properties. TyrD is stable in its oxidized form, while TyrZ is kinetically competent in the water oxidizing reaction. The temperature dependence of the formation of light-induced tyrosyl radical was studied as a function of pH in Mn-depleted PS II from cyanobacteria and plants. Tyrosyl radical formation was observed in the majority of the centers at pH 8.5 and 15 K. The use of two *Synechocystis* mutants where either TyrZ or TyrD was replaced by a phenylalanine residue allowed a clear demonstration that only TyrD and not TyrZ was oxidized under these conditions. By lowering the pH, the fraction of centers undergoing TyrD oxidation greatly diminished. This pH dependence could be fitted by assuming a single protonable group with a pK_a of ∼7.6. This pH dependence correlates with the unexpectedly rapid oxidation of TyrD at room temperature [Faller, P., Debus, R. J., Brettel, K., Sugiura, M., Rutherford, A. W., and Boussac, A. (2001) *Proc. Nat. Acad. Sci. U.S.A. 98*, 14368]. The equally unexpectedly ability to undergo oxidation at cryogenic temperatures reported here, puts limitations on the nature of the protonation reactions associated with electron transfer in this system, raising the possibility of proton tunneling along the hydrogen bond or alternatively the presence of deprotonated TyrD (tyrosinate) prior to oxidation.

Amino acid radicals play important roles in several enzymatic mechanisms (reviewed in ref *1*). Tyrosyl radicals appear to be the most frequently encountered case, and this is attributable not only to the fact that tyrosine is relatively easy to oxidize compared to other amino acids but also to the fact that tyrosine redox reactions involve proton coupled electron transfer (*1*).

The pK_a of the phenol group of reduced tyrosine in water is reported to be ∼10.3. For the oxidized tyrosine, a pK_a was calculated of about −2 (*2*, *3*). Thus, upon oxidation of tyrosine, there is a strong driving force to eject the phenolic proton. The coupled transfer of electrons and protons is an important mechanism in charge transport in biological systems. This subject has had a great deal of theoretical and experimental work in recent years (reviewed in refs *4* and *5*).

Photosystem II (PSII)[1] contains two redox-active tyrosines, called tyrosine Z (TyrZ) and tyrosine D (TyrD), that are located in a 2-fold rotationally symmetrical position on the homologuous subunits D1 and D2, respectively (*6*−*8*). This location renders TyrZ and TyrD equally situated relative to the two central chlorophylls, P_{D1} and P_{D2}. These chlorophylls bear the photogenerated cation (P^+) that is considered to be the oxidant for the tyrosines (*8*−*12*, reviewed in ref *13*). Although TyrZ and TyrD of PSII are at symmetric positions in the subunits D1 and D2, respectively, they exhibit different kinetics and redox potentials and play completely different functional roles in the enzyme. TyrZ is involved in the catalytic reaction of PSII. The second tyrosyl radical, TyrD•, is stable during enzyme function. In contrast to TyrZ, TyrD is not essential for survival of photosynthetic organism (*6*, *7*), and its function has been suggested to be in the assembly of the Mn cluster (*7*, *14*, *15*) and/or in redox tuning of P/P^+ (*15*−*17*).

TyrD•, the oxidized state, is thought to be immobilized in a site and to have a well-defined and ordered H-bond interaction between its phenol oxygen and a proton from D2 His-190 (*18*) (in higher plants, His-189 in cyanobacteria). The environment of TyrD• differs in spinach and *Synechocystis* in that TyrD• seems to accept two hydrogen bonds in *Synechocystis* but only one in spinach (*19*). The site is well shielded from the lumen (*19*−*22*) and is thought to be relatively hydrophobic (*23*−*25*). In the reduced state at pH 6.3, TyrD is likely to be protonated and to form a hydrogen bond to D2 His-190 (*26*). TyrD• is neutral because of the loss of its proton upon oxidation (*27*, *18*, *20*, *28*). On the basis of these data, it is generally accepted that upon oxidation of TyrD, its proton is transferred to D2-His190

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[1] Abbreviations: CAPSO, 3-(cyclohexylamino)-2-hydroxypropanesulfonic acid; Car, redox active β-carotene; ChlZ, redox active chorophyll in PSII; EPR, electron paramagnetic resonance; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 2-(N-morpholino) ethanesulfonic acid; P, photooxidisable chlorophyll in PSII, where primary electron hole is stabilized; PPBQ, phenyl-1,4-benzoquinone; PSII, photosystem II; Q_A, Q_B, the two quinones acting in series as electron acceptors in PSII; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; TyrZ, the tyrosine acting as the electron donor to P^+; TyrD, the tyrosine acting as a side path donor to P^+.

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and transferred back upon reduction of $TyrD^{\bullet}$ (for reviews, see refs *13* and *29*−*31*):

$$Tyr-OH\cdots N-His \Leftrightarrow Tyr-O^{\bullet}\cdots {}^{+}HN-His$$

It was generally assumed that the proton transfer reactions associated with redox chemistry of the tyrosines in PSII are inhibited at low temperature and that this is the reason for the loss of TyrD and TyrZ oxidation as the temperature is lowered. TyrZ oxidation in Mn depleted PSII is completely inhibited around 180 K at pH 6.5 (*32*). In Mn-containing PSII, the temperature dependence of each step in charge accumulation cycle has been measured, and it is assumed that any step where this occurs must involve TyrZ oxidation. The first step (known as S1-to-S2 transition) occurs down to ∼150 K (*33*−*35*), while all the other steps are inhibited at temperatures ∼220 K. Recently, Nugent et al. (*36*) reported indications that in a fraction of centers, TyrZ can be oxidized at cryogenic temperatures (down to 6 K) in the S1 state; however, the chemical identification of the state formed as a tyrosine was hampered by a magnetic interaction that obscured its spectroscopic signature (i.e., a "split" signal was observed apparently from a radical formed in the presence of the Mn cluster in the S1 redox state). If tyrosine oxidation does occur at this temperature, then it would imply important limitations on the nature of the proton coupled electron-transfer reactions.

Less is known about TyrD oxidation, and although no detailed study on the temperature dependence of the TyrD oxidation has been reported, there are several reports in which reduced TyrD is unable to donate electrons at low temperature (see ref *37* for a recent example, see discussion for details of experiments in which TyrD oxidation takes place under special conditions; *38*, *39*). It is worth noting, however, that some early reports (*40*, *41*) did show increases in the EPR signal of the tyrosine radical (signal II), indicating some tyrosine oxidation at low temperature in intact PSII at about pH 7.5 in thylakoids and chloroplasts, respectively.

Recently, we reported a pH dependence (apparent pK_a ∼7.7) for the TyrD oxidation rate at room temperature ($t_{1/2}$ ∼ 190 ns at high pH, >150 μs at low pH) (*17*). This was a more drastic pH dependence than that seen for TyrZ ($t_{1/2}$ ∼ 190 ns at high pH; ∼20 μs at low pH) (*42*, *19*, *43*). The difference in the pH dependence of the oxidation rate of the two tyrosines was attributed to the differences in the deprotonation reactions, with the TyrD oxidation at low pH being shut down because of pre-protonation of its more limited proton accepting system (*17*).

A situation that was discussed in the context of rapid TyrD oxidation at higher pH was the possibility that TyrD is already deprotonated in its reduced state (i.e., a tyrosinate) and thus its oxidation would not be limited by proton-transfer reactions (*17*). If this were the case, then the pure electron transfer reaction might be expected to occur at cryogenic temperatures. In this work we report a study of the temperature dependence of Tyr D oxidation as a function of pH in Mn-depleted PSII. We report Tyr D oxidation at cryogenic temperatures in nearly all centers when incubated above the pK_a of pH ∼7.6.

MATERIAL AND METHODS

Construction of D1-Tyr161Phe and D2-Tyr160Phe Mutants and Isolation of PSII Particles from Synechocystis Sp. The D1-Y161F and D2-Y160F mutations were constructed in the psbA-2 and psbD-1 genes, respectively, of the cyanobacterium *Synechocystis* sp. strain PCC 6803 (*44*, *6*). The plasmid bearing the D1-Y161F mutation was transformed into a host strain of *Synechocystis* that lacks all three psbA genes (*45*) and contains a hexahistidine tag (His tag) fused to the C-terminus of CP47 (*46*). The plasmid bearing the D2-Y160F mutation was transformed into a host strain of *Synechocystis* that lacks both psbD genes (*16*) and contains a hexahistidine tag (His tag) fused to the C-terminus of CP47 (*46*). Single colonies were selected for ability to grow on solid media containing 5 μg/mL kanamycin monosulfate (*44*, *16*). The control wild-type* strain was constructed in identical fashion as the D1-Y161F mutant, except that the transforming plasmid carried no site-directed mutation. The designation wild-type* differentiates this strain from the native wild-type strain that contains all three psbA genes and is sensitive to antibiotics. Cells were propagated, and PSII particles were isolated as described previously (*46*). The PSII particles were isolated in a buffer containing 50 mM MES−NaOH (pH 6.0), 20 mM $CaCl_2$, 5 mM $MgCl_2$, 0.03% (w/v) n-dodecyl β-D-maltoside, 25% (v/v) glycerol, and concentrated to ∼1 mg of Chl/mL (*46*). Manganese-depleted wild-type* and D2-Y160F PSII particles were prepared by treatment with NH_2OH and EDTA, as described previously (*47*). (The D1-Y161F PSII particles contain no Mn cluster (*44*), so they were not treated with NH_2OH.) The concentration of MES in the PSII particles was decreased to 5 mM by 10-fold dilution, followed by reconcentration. The final solution contained 5 mM MES−NaOH (pH 6.0), 20 mM $CaCl_2$, 5 mM $MgCl_2$, 0.03% (w/v) n-dodecyl β-D-maltoside, 25% (v/v) glycerol. The mutant and wild-type* PSII paticles were stored at 77 K (at ∼1.5 mg Chl/ml).

PSII-Enriched Membranes from Spinach. PSII-enriched membranes from spinach were prepared essentially by the method in (*48*) with the modifications described in ref *49*. Mn was depleted from PSII by treating with NH_2OH (*37*). Before use, the pH of the PSII samples from spinach and *Synechocystis* was adjusted by adding one of the following buffers (stock solution of 0.4 M) to a final concentration of 50 mM: pH 9.5 CAPSO/NaOH; pH 8.0−9.0 Tris/HCl; pH 7.0 and 7.5 HEPES/NaOH; pH 6.0 and 6.5 Mes/NaOH. The final Chl concentration for EPR measurements was ∼0.1 mg Chl/ml for *Synechocystis* sp. and 0.5 mg Chl/ml for spinach PSII.

EPR Spectroscopy. X-band EPR spectra were recorded with a Bruker ESP 300 spectrometer in an Oxford Instruments cryostat cooled with liquid helium. Illumination at 5−15 K was performed in the cavity using a 800 W tungsten lamp filtered through 5 cm water and three infrared cutoff filters.

Quantification of the EPR signals from $TyrD^{\bullet}$ and $Car^{+\bullet}$/$ChlZ^{+\bullet}$ (carotenoid$^{+\bullet}$/chlorophyll $Z^{+\bullet}$) was performed by double integration of the signal. The quantification of these signals at different values of pH were calculated relative to the stoichiometric $TyrD^{\bullet}$ signal at pH 6.5 (taken as 100%) (*50*). The error in the quantification was estimated to be less than 10% (relative to stoichiometric $TyrD^{\bullet}$). Stoichiometric $TyrD^{\bullet}$ was obtained by illumination of the PSII membranes for 2 min at pH 6.5, 273 K in the presence of 200 μM PPBQ, 15 s dark incubation, and subsequent freezing in a 200 K bath (ethanol/solidCO_2). The sample at pH 6.5 was chosen

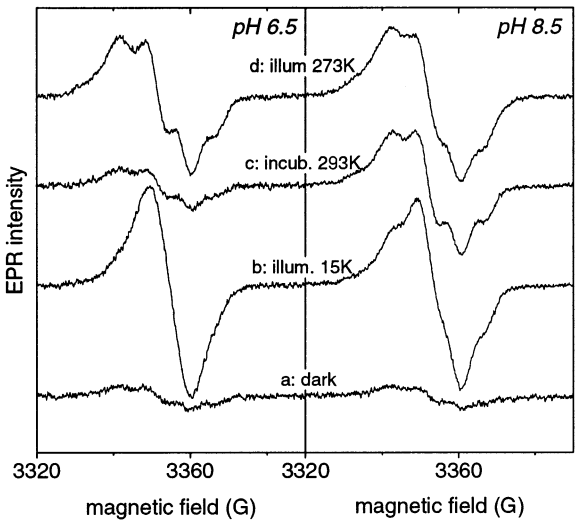


FIGURE 1: EPR spectra of Mn-depleted PSII particles from wild-type* *Synechocystis* sp. PCC 6805. The left and the right panels show the experiments at pH 6.5 and 8.5, respectively. Both panels show a series of experiments, depicted from bottom up. (a) After NH_2OH treatment. (b) After illumination for 10 min at 15 K. (c) After incubation for 40s at 293 K. (d) After illumination at 273 K in the presence of 0.1mM PPBQ. EPR conditions, 15 K, 5 μW, and 9.5 GHz; modulation amplitude, 4 G; modulation frequency, 100 kHz; conversion time, 83 ms.

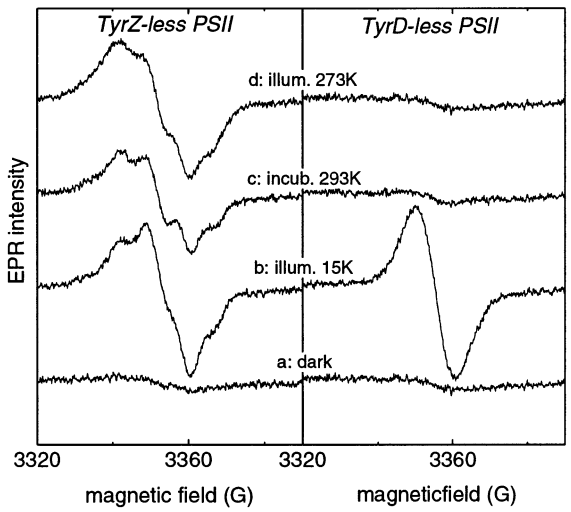


FIGURE 2: EPR spectra of Mn-depleted mutant PSII particles from *Synechocystis* sp. PCC 6803 at pH 8.5. The left and the right panel shows the TyrZ-less (D1-Tyr161Phe) and TyrD-less (D2-Tyr160Phe) PSII, respectively. Both panels show a series of experiments (bottom up). (a) After NH_2OH treatment. (b) After illumination for 10min at 15 K. (c) After incubation for 40 s at 293 K. (d) After illumination at 273 K in the presence of 0.1 mM PPBQ. For EPR conditions, see Figure 1.

because at higher pH, illumination at 273 K induced not only the $TyrD^{\bullet}$ but also a fraction of an unresolved organic radical (see, for instance, at pH 8.5 in Figure 1). The spectra were recorded at 15 K and two different conditions of power (i.e., 1 and 5 μW) and the quantification was performed for both conditions, yielding no significant difference

RESULTS

Tyrosine D Oxidation. Figure 1 shows EPR experiments with NH_2OH-treated wild-type* PSII particles from *Synechocystis* sp. PCC 6803 (see Material and Methods). The left and right panel show the same series of experiments done at pH 6.5 and 8.5, respectively. Starting at the bottom of the figure, a series of spectra were all recorded at 15 K (a) prior to illumination, (b) after illumination at 15 K, (c) after thawing for a few seconds, and (d) then after thawing, addition of electron acceptor (PPBQ) and continuous illumination at 273 K (2min).

At both values of pH, the $TyrD^{\bullet}$ signal is small (5−15% of the centers) prior to illumination (Figure 1, "a: dark") The virtual absence of signal is due to the prereduction of TyrD by the NH_2OH treatment and dark incubation. Illumination at cryogenic temperature (15 K) at pH 6.5 induced an unresolved signal (g-value ∼2.0025; line width ∼10.5 gauss) assigned to a β-carotene radical cation ($Car^{+\bullet}$) or a mixture of β-carotene/chlorophyllZ radical cations ($Car^{+\bullet}$/$ChlZ^{+\bullet}$) (*51*, *52*). In contrast, at pH 8.5 a different signal is induced after illumination at 15 K. This signal showed features typical of a mixture of the signals from the tyrosine radical and the $Car^{+\bullet}$/$ChlZ^{+\bullet}$.

The subsequent incubation for 40 s at 293 K resulted in the disappearance of the $Car^{+\bullet}$/$ChlZ^{+\bullet}$ signal (Figure 1, "c: incub 293 K"). This is expected, since $Car^{+\bullet}$ and $ChlZ^{+\bullet}$ are relatively unstable and hence rapidly reduced at higher temperature. After the warming procedure, the sample at pH 6.5 exhibited almost no radical signal, in contrast, at pH 8.5 a signal typical for $TyrD^{\bullet}$ remained (g-value 2.0046, line width 19−20 G). $TyrD^{\bullet}$ is known to be relatively stable with a reduction half-time of minutes to hours, and hence it is not expected to decrease after a short incubation at 293 K (*14*, *29*, *30*).

To quantify the induced signals, an electron acceptor (PPBQ) was added, and the samples were illuminated for 2 min at 273 K, resulting in a stoichiometric oxidation of TyrD at pH 6.5 (*50*) (Figure 1, left panel, labeled "d: illum. 273 K"). By comparing the light-induced $TyrD^{\bullet}$ signal at 15 K and pH 8.5 with the stoichiometric $TyrD^{\bullet}$, the relative amount of TyrD oxidized was deduced to be ∼75% at pH 8.5 and ∼8% at pH 6.5. The quantification of $Car^{+\bullet}$/$ChlZ^{+\bullet}$ after subtraction of the $TyrD^{\bullet}$ was ∼83% at pH 6.5 and ∼23% at pH 8.5.

It is clear from the experiments shown in Figure 1 that a tyrosine residue undergoes oxidation at cryogenic temperatures at pH 8.5. Although $TyrD^{\bullet}$ is generated upon thawing, it is not clear from this experiment whether the tyrosine generated at cryogenic temperatures is TyrZ or TyrD, since TyrD is capable of donating to $TyrZ^{\bullet}$ and could conceivably do so when the temperature is raised. Furthermore, the small spectroscopic differences between $TyrZ^{\bullet}$ and $TyrD^{\bullet}$ that may help to identify the low temperature donor are obscured by the $Car^{+\bullet}$/$ChlZ^{+\bullet}$ signal. To determine whether $TyrD^{\bullet}$ is generated at cryogenic temperatures, we examined tyrosine oxidation in two mutants from *Synechocystis* sp. PCC 6803, one that lacks TyrZ (D1-Tyr161Phe) and one that lacks TyrD (D2-Tyr160Phe).

Figure 2 shows a series of experiments similar to those shown in Figure 1, but in this case using the TyrZ-less and Mn-depleted TyrD-less PSII particles and both at pH 8.5. The left panel shows that the data obtained with the TyrZ-less PSII particles were almost identical to the data obtained with the Mn-depleted, wild-type* PSII particles (Figure 1, right side). After illumination at 15K, a signal was induced that is a mixture of $Tyr^{\bullet}$ and $Car^{+\bullet}$/$ChlZ^{+\bullet}$. Because the

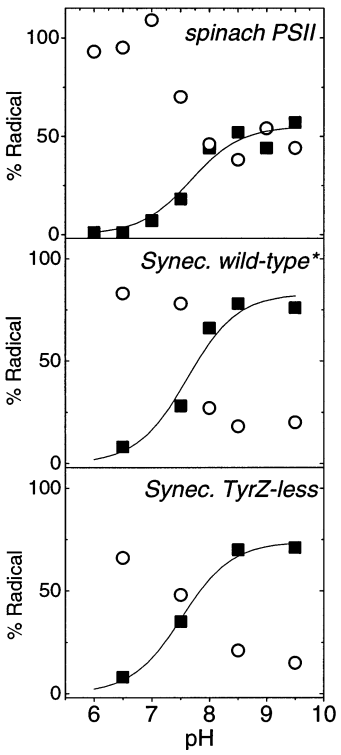


FIGURE 3: pH dependence of the TyrD oxidation at 15 K in Mn-depleted PSII from spinach (top panel), Mn-depleted wild-type* (middle panel), and TyrZ-less mutant *Synechocystis* sp. PCC 6803 (D1-Tyr161Phe) (bottom panel). The relative amounts of TyrD (solid squares) and Car/ChlZ (hollow circles) oxidized during illumination for 10min at 15 K were shown. Stoichiometric oxidized TyrD was taken as 100% (for details see Materials and Methods). The solid line shows a single proton titration fit of the data points for TyrD oxidation (solid line).

mutant possesses no TyrZ, the signal can be attributed to $TyrD^{\bullet}$.

In the right panel, illumination at 15 K of the TyrD-less PSII (D2-Tyr160Phe) induced only a signal attributed to $Car^{+\bullet}/ChlZ^{+\bullet}$, with no apparent contribution from tyrosyl radical. The results of Figure 2 are strong evidence that the TyrD undergoes oxidation at 15 K while TyrZ does not. It seems most likely that this is also the case for the Mn-depleted, wild type* PSII (Figure 1).

As shown in Figure 1, the oxidation of TyrD at cryogenic temperatures is pH-dependent, i.e., in a minority of centers at pH 6.5 and in the majority of centers at pH 8.5. To determine the apparent pK_a of this phenomenon, the light-induced signals of $TyrD^{\bullet}$ and $Car^{+\bullet}/ChlZ^{+\bullet}$ were measured and quantified at different pH values in TyrZ-less and Mn-depleted wild-type* PSII particles from *Synechocystis* sp. PCC 6803, as well as in Mn-depleted PSII membranes from spinach (Figure 3) (for details see materials and methods). The overall features of the data look very similar. At low pH, only small amounts of TyrD were induced upon illumination at 15 K, but at higher pH, TyrD oxidation occurred in the majority of centers. The oxidation of Car/ChlZ showed exactly the opposite behavior: dominant at lower pH, minor at high pH. The sum of $TyrD^{\bullet}$ and $Car^{+\bullet}/ChlZ^{+\bullet}$ induced upon illumination at 15 K was at each pH about 100% (see Figure 3). This is in line with the fact that PSII centers undergo only one turn-over at cryogenic temperatures because of the low temperature-induced block in electron transfer between Q_A and Q_B (*53*). The amount of

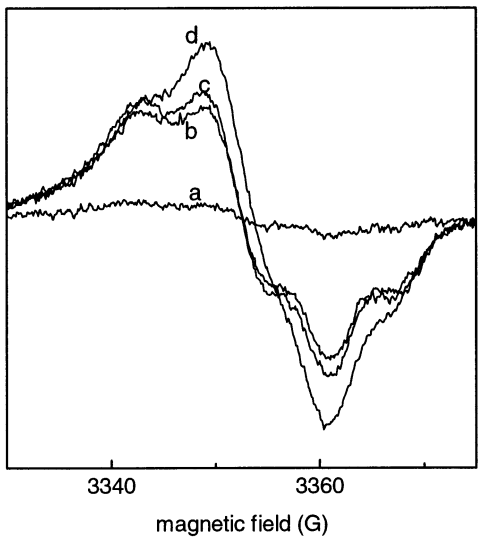


FIGURE 4: Illumination of Mn-depleted PSII particles from wild-type* *Synechocystis* sp. PCC 6803 at 15 K and pH 8.5. (a) After NH_2OH treatment. (b) 0.5s illumination at low intensity. (c) 2 min illumination at low intensity. (d) 8 min illumination at strong intensity.

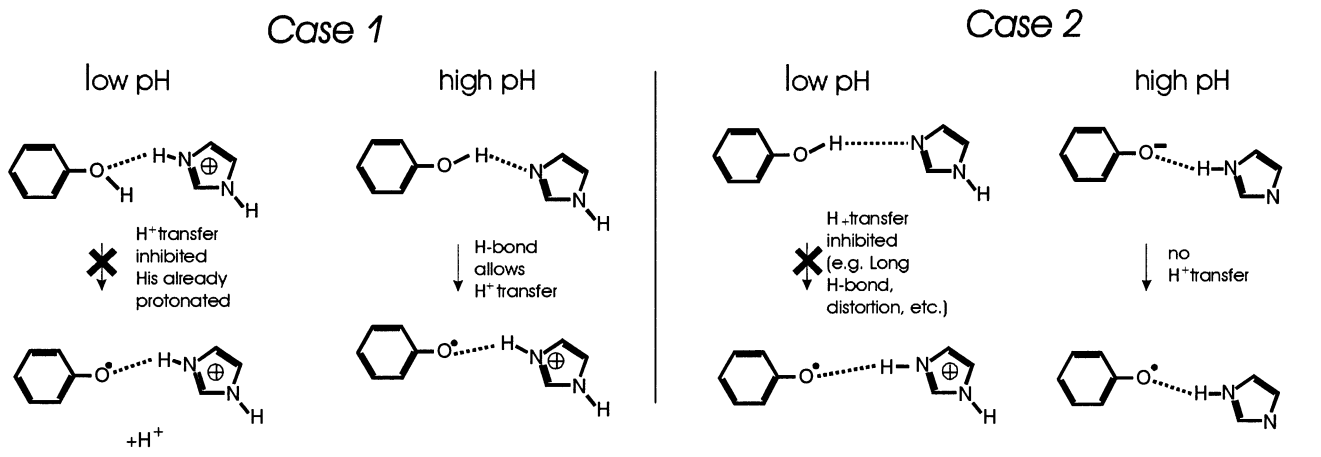
$TyrD^{\bullet}$ induced at cryogenic temperatures were each fitted to a single proton titration curve, which led to pK_a s of 7.7, 7.6, and 7.5 for Mn-depleted PSII from spinach, Mn-depleted wild-type* PSII, and TyrZ-less PSII from *Synechocystis* sp. PCC 6803, respectively. Differences between spinach and *Synechocystis* sp. were observed, not in the overall behavior but in the quantification. The fraction of centers undergoing TyrD oxidation at higher pH were ~75% in PSII particles from *Synechocystis* sp. PCC 6803 but only ~55% in PSII from spinach.

We have considered two possible explanations for the phenomena associated with photogeneration of both $TyrD^{\bullet}$ and $Car^{+\bullet}/ChlZ^{+\bullet}$ at low temperature: (i) the centers are heterogeneous, with one population of centers in which only TyrD is oxidized and another in which only Car/ChlZ is oxidized; (ii) the centers are homogeneous, but the distribution of oxidized TyrD and Car/ChlZ is caused by competing electron donations. In the case of pH 8.5 (75% TyrD, 25% Car/ChlZ oxidation), TyrD would have to donate its electron ~3 times faster than Car/ChlZ. To distinguish between the two possibilities, the time course of the low-temperature oxidation was studied. Figure 4b shows that all TyrD was already oxidized after a short period of time ($^1/_2$ s) with weak light. In contrast only a small amount of $Car^{+\bullet}/ChlZ^{+\bullet}$ was present. Only after prolonged illumination with strong light was Car/ChlZ completely oxidized (Figure 4d). This result suggests that the centers are heterogeneous: in about 75% of the centers, the TyrD donates much faster than Car/ChlZ and is therefore oxidized. In the other 25% of the centers, the TyrD electron donation does not occur (or at least it is much slower than Car/ChlZ donation), allowing the much lower quantum yield oxidation of Car/ChlZ to occur.

DISCUSSION

The present studies provide strong evidence that TyrD is oxidized at cryogenic temperatures. At pH 6.5, this TyrD oxidation occurred in a minor fraction of centers. At higher pH (8−9.5), TyrD was oxidized in the majority of the

Scheme 1



centers. This pH dependency could be fitted with a single proton titration exhibiting an apparent pK_a of about 7.6 ± 0.1 for Mn-depleted PSII preparations from spinach and *Synechocystis* (wild-type* and TyrZ-less mutant) (see Figure 3).

This pK_a is almost identical to the pK_a of 7.7 reported for the amplitude of the submicrosecond TyrD oxidation (*17*) (with TyrD being oxidized with a submicrosecond rate ($t_{1/2}$ ~190ns) at higher pH but at a rate much slower than charge recombination (>150 μs) at lower pH). Moreover, the fraction of centers able to undergo this submicrosecond TyrD oxidation at high pH was about 67% in the TyrZ-less mutant (D1-Tyr161Phe), virtually the same value (70%) as for TyrD oxidation at cryogenic temperatures (see Figure 3). Furthermore, the proportion of centers showing the submicrosecond oxidation at room temperature and the yield of TyrD oxidation at cryogenic temperature showed the same species dependence. In Mn-depleted PSII membranes from spinach both values were ~55% compared to ~70% in PSII particles from *Synechocystis* (see Figure 3 and ref *17*).

These data indicate strongly that the submicrosecond kinetics and the ability to undergo low temperature oxidation are closely related and probably represent the same centers and the same redox and deprotonation chemistry. The model put forward to explain the pH dependence of the submicrosecond kinetics can thus be applied to the low-temperature redox reactions. The most straightforward candidates as the groups exhibiting this pK_a of 7.6 are either the TyrD itself (modulated by the adjacent His) or the adjacent His (D2-His189) (*17*).

The origin of this pK_a is not clear yet. It could arise from a single amino acid or a complex network of several amino acids including other components (e.g., water). In Scheme 1 we consider only what we consider to be the two simplest explanations for the data based on only two components: TyrD and its likely H-bonding partner, D2-His189. It is useful perhaps to consider these amino acids as one species. Below we briefly outline the two options that we consider to explain low-temperature oxidation of TyrD.

Case 1 (Scheme 1, Left), Proton Tunneling. At high pH the phenolic hydrogen of the tyrosine group is hydrogen bonded to the histidine in a conformation that allows proton tunneling coupled to electron transfer even at low temperature. This electron transfer is relatively rapid with a measured $t_{1/2}$ of ~190 ns at room temperature (*17*). At low pH, the histidine becomes protonated, the phenolic hydrogen of TyrD is no longer H-bonded to its base, and it is thus unable to leave. TyrD oxidation at all temperatures is thus inhibited. In the literature, tunneling of H-bonded protons is considered to occur in proton-coupled electron transfer and is thought to be possible at cryogenic temperature (for review see, e.g., *54*−*56*, *4*).

Case 2 (Scheme 1, Right), Pure Electron Transfer. At high pH, the tyrosine is deprotonated but H-bonded to the adjacent His. The deprotonated Tyr (tyrosinate) group undergoes electron transfer without the necessity for deprotonation. Consequently, TyrD oxidation occurs at low temperature and has rapid kinetics. At low pH, the protonated TyrD cannot be oxidized, i.e., it is assumed that the H-bond between TyrD and His is inappropriate (*55*) (e.g., lacks sufficient strength, is distorted, etc.) to allow low temperature (and submicrosecond) electron transfer. In this case, we assume that the titration of the phenolic proton is modulated by the protonation state of the distal nitrogen of the His.

At the level of the H-bonding between the tyrosine and histidine, these two models (Scheme 1) represent two extremes and the two simplest cases: the pure H-bonded tyrosine on one hand and, on the other, the fully deprotonated tyrosine acting as an H-bond acceptor from D2-His189. Situations representing cases intermediate between this two extremes might occur. The two states may also be in equilibrium resulting in a statistical distribution or may be affected by other factors (e.g., additional hydrogen bonds). At a proton level, the pure electron transfer case 2 represents a system that differs from case 1 by having one fewer proton overall. It should be possible to test further aspects of these models experimentally in order to determine which of them may be the more valid. Kinetic studies at low-temperature are planned.

Low-temperature TyrD oxidation has been reported previously (*38*, *39*), but under special conditions: PSII samples were frozen under illumination (trapping both $TyrD^{\bullet}$ and presumably Q_A^-) at pH 6.3. The samples were subsequently incubated for many hours or days at 77 K, leading to a partial reduction of TyrD by recombination with Q_A^-. After illumination at 77 K, the TyrD signal was restored. It is assumed then that this low-temperature oxidation of TyrD reflects the fact that the TyrD reduced during the long incubation at 77 K remains in its oxidized (deprotonated?) conformation and environment. Related phenomena are also

known from other electron-transfer reactions (see, e.g., *57*). It is possible that this "oxidized environment" trapped around TyrD reduced at 77 K is similar to the state generated here by raising the pH above 7.7.

Another report related to TyrD oxidation is provided by Sanakis et al. (*58*). These authors showed that the nitroso TyrD is able to undergo oxidation at cryogenic temperature, producing the iminoxyl radical. The nitroso group is expected to change the properties of the tyrosine and perhaps change the protonation state of the TyrD site in an analogous way to the high pH.

In the present work, we also show that while TyrD is able to undergo oxidation at cryogenic temperatures, TyrZ is not. This points to structural differences between their sites (in Mn-depleted PSII). This is in line with several studies on the structure of TyrD and TyrZ and their environments (reviewed in refs *12*, *13*, *30*, and *31*). Many of the features of the TyrZ deduced from previous work could contribute to the lack of low-temperature oxidation: the H bond with a distributed strength, the flexibility of the phenol group, the more aqueous environment, and the higher potential, resulting in what may be a slower electron donation rate from Tyr Z than Tyr D under comparable conditions at high pH in Mn-depleted PSII (*17*).

The unequivocal demonstration here that tyrosine can undergo oxidation at cryogenic temperatures is of special interest in the context of the recent reports that TyrZ oxidation can occur at low temperature in the functional enzyme (*36*, *59*; Zhang, C., and Styring S. personal communication). Because of the lack of a tyrosine-specific spectroscopic signature, the assignments of the cryogenically light-induced "split" EPR signals to TyrZ$^{\bullet}$ interacting with the Mn cluster were only tentative (*36*) and need to be proven by further experiments. The present work might be taken as support for this assignment since it constitutes precedence for tyrosine oxidation at low temperature. At the same time, the present work provides new insight and an experimental system that may allow a better understanding of the proton-coupled electron transfer reactions occurring during tyrosine oxidation not only in PSII but also elsewhere in biology.

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