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Review

Coupling of electron and proton transfer in the photosynthetic water oxidase

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Abstract

According to current estimates, the photosynthetic water oxidase functions with a quite restricted driving force. This emphasizes the importance of the catalytic mechanisms in this enzyme. The general problem of coupling electron and proton transfer is discussed from this viewpoint and it is argued that 'weak coupling' is preferable to 'strong coupling'. Weak coupling can be achieved by facilitating deprotonation either before (proton-first path) or after (electron-first path) the oxidation step. The proton-first path is probably relevant to the oxidation of tyrosine Y_Z by P-680. Histidine D1–190 is believed to play a key role as a proton acceptor facilitating Y_Z deprotonation. The pK_a of an efficient proton acceptor is submitted to conflicting requirements, since a high pK_a favors proton transfer from the donor, but also from the medium. H-bonding between Y_Z and His, together with the Coulombic interaction between negative tyrosinate and positive imidazolium, are suggested to play a decisive role in alleviating these constraints. Current data and concepts on the coupling of electron and proton transfer in the water oxidase are discussed.

Keywords: Proton-coupled electron transfer; Photosystem II; Water oxidation; Redox active tyrosine

1. Introduction

As emphasized in other reviews in this special issue, the water oxidase has to achieve both a fairly complex mechanistic task and a still more complex catalytic challenge. The mechanistic aspects consist of managing the abstraction of four electrons and four protons from two water molecules. The driving force is provided by four turnovers (which can be largely separated in time) of the primary photochemical donor, P-680 (abbreviated P), which is a pure electron carrier. Specific problems are raised by the need for handling and storing the intermediates of very high redox potential required for the efficient oxidation of water. A combination of electron and proton transfer comes into play almost from the start with the reduction of P-680 by tyrosine Y_Z (D1–161).

The catalytic challenge arises from the quite parsimonious ΔG_0 granted by Photosystem II (PS II) for driving the reaction. Current estimates (from Vass and Styring, [1], combining their own data with those of [2–5]) for the midpoint potentials of the redox couples involved are as follow: 1120 mV for P^+/P^1, 950–990 mv for Y_Z^{ox}/Y_Z^{red}, ≤ 740 mV for S_1/S_0, 900–950 mV for $S_{2/}S_1$ and S_3/S_2. State S_4 is in fact $(Y_Z^{ox}S_3)$, so that the midpoint potential for S_4/S_3 is the same as Y_Z^{ox}/Y_Z^{red}. The Vass and Styring compilation was made for pH 6. There are large error bars

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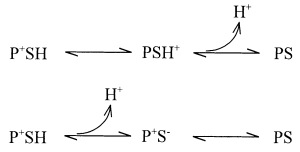
in these estimates and many of them are mutually dependent, but this is presently the best we have. The average value for the four S couples is +890 mV (taking the upper limit for S_1/S_0 and the central value of the ranges estimated for the other couples). The midpoint potential for the O_2/H_2O couple at pH 6 is 870 mV [6]. Therefore, the ΔG_0 available to drive water oxidation is only about −20 mV. This small ΔG_0 has to be reconciled with a particular catalytic difficulty raised by the coupling of protolytic reactions with electron transfer. This is the question addressed from a general standpoint in Section 2.

2. Oxidant-induced deprotonation: strong versus weak coupling

The coupling strength between electron transfer and a protolytic reaction can be quantified by the pK shift resulting from the redox change. Strong coupling (large ΔpK) generally implies that the protonatable group is in direct chemical interaction with the redox center. Weak coupling (small ΔpK) may easily occur in proteins through electrostatic interaction of the redox center with protonatable groups. We first consider a strong coupling case, involving a redox carrier 'S' which is a very weak acid in its reduced state (with $pK_a = pK_r$) and a very strong one in the oxidized state ($pK_a = pK_o$). The dependence of the E_m on pH is shown in Fig. 1 (top), together with the E_m of a redox partner 'P', assumed to be a pure electron carrier. The oxidation of S by P^+ is thermodynamically favorable (ΔG_0 negative) above pH 2. For definiteness, we assume the surrounding pH to be 8. The reaction has to take one of two possible paths:



In addition to the 'electron-first' or 'proton-first' paths, a 'concerted path' may be considered. A theoretical investigation of such a process has been developed by Cukier [7,8]. This author describes the reaction path as a zig-zag where the proton displaces adiabatically along its coordinate to a certain configuration that permits electron tunneling, followed by relaxation to the final state [7]. We thus feel that the concerted path does not differ essentially from the proton-first path, as long as the electron transfer is not rate-limiting, as considered in the following.

Both paths in the above scheme imply an infrequent transition state, PSH^+ or P^+S^-, respectively. More precisely, the logs of the equilibrium constants for the left-hand side reactions are (with notations defined in the legend of Fig. 1):

$$\log(K_{et}) = -\frac{E_m^{SH} - E_m^{P}}{60} = -\left(pH - pK_o + \frac{\Delta G_0}{60}\right) \quad (1)$$

$$\log(K_H) = -(pK_r - pH) \quad (2)$$

equivalent to *minimum* activation energies of $60(pH - pK_o) - \Delta G_0$ and $60(pK_r - pH)$, respectively. The purpose here is to estimate the fastest possible rate for the overall reaction, using the first or second path. We thus make the assumption that the equilibria on the left-hand sides are fast with respect to the second reaction. The effective rate constant for the overall process is then of the form $(K/(1+K))k \approx Kk$, where K is the equilibrium constant for the first reaction and k the rate constant for the second one. We are dealing here with the energy barrier implied by the first factor K.

A refinement can be introduced for the treatment of the electron-first process. The pH corresponding to a mole fraction of 1 for protons (i.e. $[H^+] = 55$ M)

[1] In a recent paper, Tommos and Babcock [29] have emphasized the adequacy of the midpoint potential of P^+/P with regard to thermodynamic efficiency. The optimum potential span which can be delivered from a photochemical converter driven by a 1.8 eV photon is about 1230 mV [86]. The authors then reason that, if the E_m of the 'primary acceptor' is taken as that of Q_A/Q_A^- (around −50 mV), that of the primary donor should be around 1180 mV. This line of thought, although re-emerging from time to time in the photosynthetic literature, makes no real sense. It arises from a confusion between the maximum electron motive force that can be sustained by the photochemical converter at steady-state and the midpoint potential interval between two particular carriers (see [87,88]). This appears obvious when realizing that the selection of the P–Q_A couple is arbitrary. For instance, pheophytin, with a much lower E_m than Q_A is more entitled to be considered as the primary acceptor. If pheophytin is dismissed on the basis of its short lifetime in the reduced state, the same may be objected against P as the relevant primary donor, because of its short lifetime in the oxidized state.

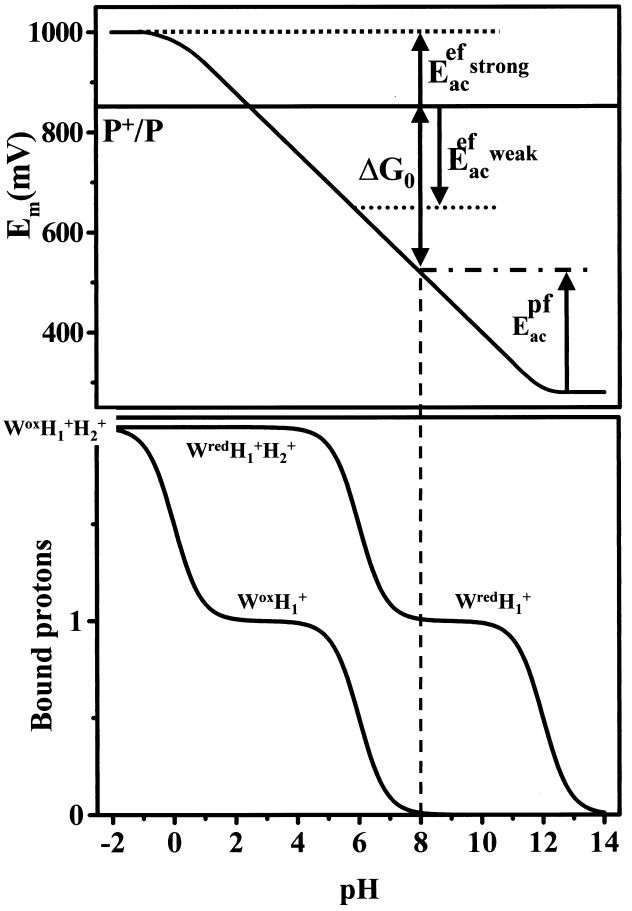


Fig. 1. The top panel shows the dependence on pH of the midpoint potential of a proton/electron carrier S (for 'strong coupling') with $pK_r = 12$ or $pK_o = 0$ in its reduced or oxidized state, respectively. When going oxidized, S releases one proton in (roughly) the pH domain bounded by pK_r and pK_o. The midpoint potential of S varies from $E_m^{SH} = 1000$ mV at low pH to $E_m^S = E_m^{SH} - 60 \times \Delta pK = 280$ mV at high pH. The horizontal line indicates the midpoint potential of a pure electron carrier P ($E_m^P = 850$ mV), which may oxidize S. E_{ac}^{ef} and E_{ac}^{pf} indicate the minimum 'activation energies' required for the reaction at pH 8, through the electron-first or proton-first routes, respectively. The bottom panel shows the pH titration of a di-acid W (for 'weak coupling'), in its reduced and oxidized states. The pKs were chosen so that the E_m and oxidation-induced proton release are the same as for S at any pH ($pK = 6$ for the second protonation of the reduced form and for the first protonation of the oxidized form). The midpoint potential of the one-protonated form (WH_1^+) is $280+60 \times \Delta pK = 280+6 \times 60 = 640$ mV.

is -1.7. Thus, depending on the value of pK_o, two cases arise. If $pK_o < -1.7$, a possible transition state is (S, H^+) where the proton is not any more bound to S, but still confined in its vicinity. One may then replace pK_o by -1.7 in Eq. 1. This is the case ('configuration energy') considered by Krishtalik [6,9]. On the other hand, if $pK_o > -1.7$, SH^+ is an obligate intermediate in the electron-first path and Eq. 1 applies.

The above example illustrates the strong coupling case and its inherent sluggishness. We now consider a weak coupling case, which does not involve a single redox-induced pK shift but rather several (two in this example) smaller shifts. The system, denoted W, behaves as a polyacid with two pKs in both the reduced and oxidized state (see Fig. 1, bottom panel). It may be viewed as being composed of a redox center interacting electrostatically with two protonatable groups. For the sake of illustration, we have chosen the pKs and ΔpKs so that the thermodynamics is the same as in the previous case: same proton release and consequently same dependence of E_m on pH. The catalytic properties are, however, drastically different. For instance, at pH 8, the oxidation using the electron-first path implies state WH_1^+ as an intermediate, with an $E_m = 640$ mV, which is below E_m^P. Thus, a negative 'activation energy', compared with a high barrier in the strong coupling case. For the proton-first path, the weak coupling strategy will be to provide an effective pK_r closer to the working pH: this possibility is discussed in detail for the case of Y_Z in Section 3.

The point that we wish to make is that a basic catalytic strategy for achieving fast electron transfer from a proton/electron carrier to a pure electron carrier can be to split the overall ΔpK into smaller lumps. The protein medium, providing a network of protonatable groups (amino acids or waters), interacting both electrostatically and via H-bonds, is ideally suited for providing weak coupling and even for weakening a molecule with inherent strong coupling properties.

3. Oxidation of Y_Z

3.1. Reaction path

A tyrosine is at first glance a very 'catalytically incorrect' object, with $pK_r \approx 12$ and $pK_o \approx -2$ (in vitro, [10,11]). Nevertheless, Y_Z features among the rapid carriers in photosynthesis, able to reduce P^+ on a sub-μs time scale ($t_{1/2}$ around 30 ns in the S_0 and

S_1 states, see [12] for a review). Rapid electron transfer at this stage is of course desirable to obviate quantum yield failure through $P^+Q_A^-$ recombination. The catalytic tuning exerted by the protein is evidenced by the fact that the disruption of the Mn cluster causes a dramatic slowing of the oxidation kinetics of Y_Z (by a factor of 50–500 depending on pH), showing that presumably small structural modifications have large kinetic consequences. This situation is of course unfortunate on practical grounds because, although the study of Y_Z^{ox} is much easier in Mn-depleted material due to its longer lifetime, transposition of the knowledge thus gained to the intact system is hazardous.

The electron-first route for the oxidation of Y_Z by P^+ appears unlikely because of the very large energetic barrier: the E_m of the $Y_ZO\text{-}H^+/Y_ZOH$ couple is expected (from in vitro data) to be around 1500 mV [11], far above that of P. The proton-first route is a better candidate, especially as it may be facilitated by His D1–190 190 as a proton accepting base. In mutants where this residue was replaced by other amino acids [13–16], the reduction of P^+ was dramatically slowed (compared with Mn-depleted WT, since the mutants fail to assemble the Mn cluster). The system can be 'reconstituted' by adding imidazole or other diffusing small bases which restore a rapid reaction (the effect is modulated by pH depending on the pK of the added substance, in agreement with its involvement as a proton acceptor) [14]. Although the role of His-190 as a proton acceptor for Y_Z seems clear, there is evidence that the tyrosine may not be directly H-bonded to the His nitrogen [17], at least in the Mn-depleted system or in an acetate-treated system where the Mn cluster is still present. On the other hand it is clear that Y_Z^{ox} is H-bonded [18–21]. A chain of water molecules has been hypothesized as a link between Y_Z and His [16]. For simplicity we assume in the following direct H-bonding between both partners, but the possibility of a more complex structure should be kept in mind. Thus, the reaction scheme that is currently favored by most authors [14,16,22–24] is:

$$P^+ Y_Z\text{-}O\text{-}H \cdots N\text{-}His \xleftrightarrow{K_H} P^+ Y_Z\text{-}O^- \cdots H^+\text{-}N\text{-}His \longleftarrow P\, Y_Z\text{-}O^{\bullet} \cdots H^+\text{-}N\text{-}His$$

where the dotted line stands for a H-bond. The right-hand state where the proton released from the tyrosine remains H-bonded to the radical is analogous to what is believed to occur for $Y_D^{\bullet}$ ('proton rocking' model, first proposed by Babcock et al. [25]). As in Section 2, we assume that the left-hand equilibrium is not rate-limiting. The rate constant for proton tunneling through a H-bond can be as high as $6\times10^{12}\times10^{\Delta pK}$ s^{-1} (where ΔpK is the difference between proton acceptor and donor) [26,27]. Thus, as put forward by numerous authors [14,22,28,29], there is no difficulty in obtaining a rate constant of 10^9 s^{-1}, even if the ΔpK is somewhat 'uphill'. Whereas the requirement for a non-limiting proton transfer rate imposes no strong constraint on the ΔpK, the effective reaction rate is controlled by the equilibrium constant K_H, which in turn depends on the ΔpK (see Eq. 5 below). This effective rate constant is:

$$\frac{K_H}{1+K_H}k_{et} \tag{3}$$

where k_{et} is the electron transfer rate constant. For a fast reaction, a sufficiently large K_H is required, which can be obtained if the pK of the proton acceptor is not too low with respect to that of Y_Z. But we immediately have to face another problem: if the accepting base has a high pK, it will be already protonated in the dark at physiological pH and unavailable as a proton acceptor for Y_Z. Let us first examine how large K_H should be to account (using Eq. 3) for a $(40\ ns)^{-1}$ rate for the reduction of P^+.

A rough estimate of k_{et} can be obtained from the Dutton–Moser formula [30,31] derived from the electron transfer theory of Marcus [32] with parameters accommodating the current knowledge relevant to the protein medium:

$$\log(k_{et}) = 13-[(1.2-0.8\,\rho)(R-3.6)]-3.1\frac{(\Delta G_0-\lambda)^2}{\lambda} \tag{4}$$

We adopt the following figures: ρ ('packing density') = 0.76 [31], R (edge to edge distance between P and Y_Z) = 9.5 Å (from Svensson's model structure [33]), $\Delta G_0 = -40$ meV (estimated from the relative amplitude of the rapid phase of P^+ reduction, see below). The activation energy of the reaction was estimated by Eckert and Renger [34] as $E_a \approx 100$ meV. Then, the reorganization energy λ is approximately $2(2E_a-\Delta G_0)\approx 500$ meV [28]. Inserting these guesses into Eq. 4 yields $\log(k_{et})\approx 7.6$, corresponding

to $t_{1/2} \approx 17$ ns. This would imply $K_H \geq 1$. There are of course large error bars in the above estimate for k_{et}, especially since the real structure (R) is not known. Nevertheless, it seems reasonable to conclude that K_H cannot be very small, i.e. we may tentatively adopt 0.1 as a lower limit.

Recognition of this fact may not be unrelated to a trend in recent literature reporting spectroscopic information favoring to various extents a 'tyrosinate character' for Y_Z. Clearly, if Y_Z were a tyrosinate, the whole catalytic problem would be solved. But is it?

3.2. Is Y_Z a tyrosine or tyrosinate?

Candeias et al. [35] studied the optical spectra ($Tyr^{\bullet}$–Tyr) generated by pulse radiolysis in aqueous solution at different pHs. The spectra obtained by these authors differ from those of Bent and Hayon [36] that were previously used as the in vitro reference to discuss the nature of Y_Z or Y_D. They concluded that the spectra obtained in vivo for (Y_Z $^{\bullet}$–Y_Z) are closer to their in vitro spectrum at pH 12 (radical-tyrosinate) than at pH 8 (radical-tyrosine) and suggest that Y_Z is a tyrosinate. There is however a serious problem with the spectra found by Candeias et al. Since the spectrum of the oxidized radical ($Tyr^{\bullet}$) is not expected to depend on pH, the (double) difference of the oxidized minus reduced spectra at pH 12 and 8 should match the tyrosinate–tyrosine difference (easily obtained from the spectra of reduced tyrosine at two pHs, see e.g. [22]). This is not the case below 265 nm where the Candeias spectra are almost superimposed whereas the tyrosinate–tyrosine difference rises very steeply. Thus, at least in the low wavelength region, these spectra appear unreliable.

Haumann et al. [37] also compared the in vivo oxidized minus reduced spectra of Y_Z with literature data in vitro and concluded that Y_Z was a H-bonded tyrosinate. Unfortunately, the in vitro reference spectra were misinterpreted in this study. The authors assumed that these were *absolute* spectra of the radical species, whereas all published spectra thus far are oxidized minus reduced differences, with no reliable extinction coefficient that would allow the computation of the radical spectrum (see [36,38–40] cited in [37]). Based on this incorrect assumption, the reconstructed oxidized minus reduced difference spectra for tyrosine and tyrosinate are of course meaningless.

Clearly, the Y_Z community is presently in great need of reliable optical spectra for the radical minus tyrosine or tyrosinate in vitro that would help to decide whether Y_Z looks more like a tyrosine or tyrosinate. There are, however, FTIR results (based on the comparison of in vitro and in vivo infrared spectra) which do suggest that Y_Z is protonated at physiological pH. In O_2-evolving PS II, a band at ≈ 1255 cm^{-1} was interpreted by Noguchi et al. [41] as the ν(CO) mode of a protonated tyrosine. A different interpretation was proposed by Berthomieu et al. [20] who ascribed this band (observed at pH 6, in Mn-containing, Ca^{2+}-depleted material and in Mn-depleted material) to the δ(COH) mode. The definitive assignment of this band awaits, however, a deuteration study (Catherine Berthomieu, private communication).

Kinetic information on what the pK of Y_Z could be has been reported by Hays et al. [16]. In His-190-depleted mutants, the reduction of P^+ is accelerated at high pH. If this is due to the formation of tyrosinate (restoring rapid kinetics in the absence of an efficient proton acceptor), this suggests a pK of around 10 for Y_Z (in this Mn-depleted, His-depleted system). Also based on a study of His-190-depleted mutants, but using a more indirect method, Mamedov et al. [15] estimated a pK of about 8. Hays et al. [16] have discussed Mamedov's data and argued that they would be more consistent with a higher pH (≈ 9) (note that *Chlamydomonas* membranes were used in Mamedov's work, whereas Hays or Diner use particles from *Synechocystis*). A pK around 8.3 (also in Mn-depleted material) has been reported by Diner et al. [22] based on optical spectra and the effect of D/H isotopic substitution on P^+ reduction kinetics. The authors reported a modification of the oxidized minus reduced spectrum of Y_Z with pH. They suggested that the double difference obtained by subtracting the changes measured at pH 9 and 6 was close to that of reduced tyrosinate–tyrosine. In our opinion, this interpretation is not really convincing. There is some resemblance in the shape of the spectra insofar as one accepts to ignore baseline distortions. For instance, the model spectrum (tyrosinate–tyrosine) has a negative peak around 295 nm, whereas the experimental one (oxidized minus re-

duced Y_Z at pH 9 minus pH 6) is flat and close to zero in this region. More important, the steep drop of the model spectrum below 265 nm is not reproduced in the experimental spectrum. We believe that the pH effect found by these authors might be better accommodated with a weaker molecular modification (i.e. a change in the H-bonding pattern) than formation of tyrosinate around pH 8. The isotopic effect on the P^+ reduction kinetics was also investigated by Hays et al. [16] with results similar to those of Diner et al., but a different interpretation that supports a pK of 10. For lack of cogent spectroscopic evidence of a lower pK, we accept this value in the following and discuss on this basis the constraints that bear on the pK of the proton acceptor (assumed to be His-190).

3.3. Constraints on the proton acceptor

Scheme 1 shows the network of equilibria involved for the reduced Y_Z. The pK_as of Y_Z and of the nitrogen acceptor are, respectively, pK_Z and pK_N. These values are assumed to take into account the effect of the protein environment, but not the interactions (H-bond and Coulombic) between both partners, that we wish to examine specifically. Let us denote E_H the energy of the H-bond between Y_Z and the histidine nitrogen and Ψ the absolute value of the electrostatic energy for an elementary dipole with charges located on Y_Z and His. Fictitious states (B and D, with no H-bond) are featured to pinpoint the role of E_H. One has:

$$K_H = 10^{pK_N - pK_Z + \Psi/60} \quad (5)$$

(note that K_H is pH-independent). State (A), where both Y_Z and His are protonated, is kinetically incompetent for reducing P^+ and should be kept to a minimum amount at physiological pH. In fact:

$$[A] = \frac{1}{1 + 10^{pH - pK_N}\,[10^{pH - pK_Z} + 10^{E_H/60}(1 + K_H)]} \quad (6)$$

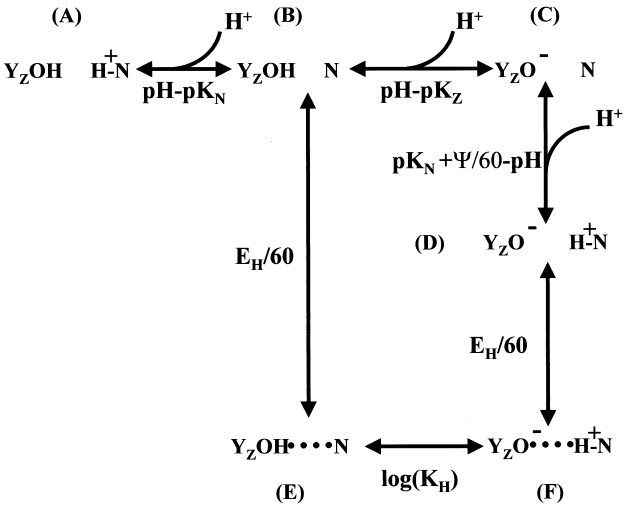
For $pK_Z > pK_N$ (and $K_H < 1$), this behaves as a group with an equivalent pK of:

$$pK_A \approx pK_N - E_H/60 \quad (7)$$

The interactions between Y_Z and His (H-bond and electrostatics) turn out to be decisive in allowing both a sizeable K_H and a low level of [A]. The condition $K_H \geq 0.1$ entails:

$$\Psi/60 \geq pK_Z - pK_N - 1 \quad (8)$$

If $\Psi = 0$ then $K_H > 0.1$ implies $pK_N > 9$ (assuming $pK_Z = 10$). Furthermore, if $E_H = 0$, then $pK_A > 9$ and $[A] \approx 1$ (inactive state) in the whole physiological pH range. On the other hand, assuming $E_H = 180$ meV (a reasonable H-bond strength), one can have $pK_A \leq 4$ (ensuring a low amount of the inactive state in the relevant pH range) for $pK_N \leq 7$ (from Eq. 7). Then, using Eq. 8, $K_H \geq 0.1$ is achieved for $\Psi \geq 180$ mV. For a dielectric constant $\varepsilon = 10$, this would correspond to a distance ≤ 8 Å, which is not unreasonable. The length of an H-bond is of course much shorter (≈ 2.9 Å), but the distance between charges must be larger than the bond length. On the other hand a Coulombic interaction ≥ 180 mV is probably still compatible with a distance between Y_Z and its proton acceptor greater than one H-bond, as may be the case if Y_Z and His are connected through a small water chain. Fig. 2 shows the role of E_H and Ψ on the pH titration of the inactive (A) and active (F) states for fixed values of pK_N (6) and pK_Z (10).



Scheme 1. Equilibria between the reduced Y_Z and a proton acceptor N. pK_Z and pK_N are the pKs of of Y_Z and N, respectively, in the absence of H-bond between them and in the absence of Coulombic interaction. E_H is the energy of the H-bond (assumed identical for states E and F), Ψ is the electrostatic interaction in state F. The log(K_{eq}) of the equilibrium constant for each reaction is indicated with the convention K_{eq} = [right-hand state]/[left-hand state] or [bottom state]/[top state].

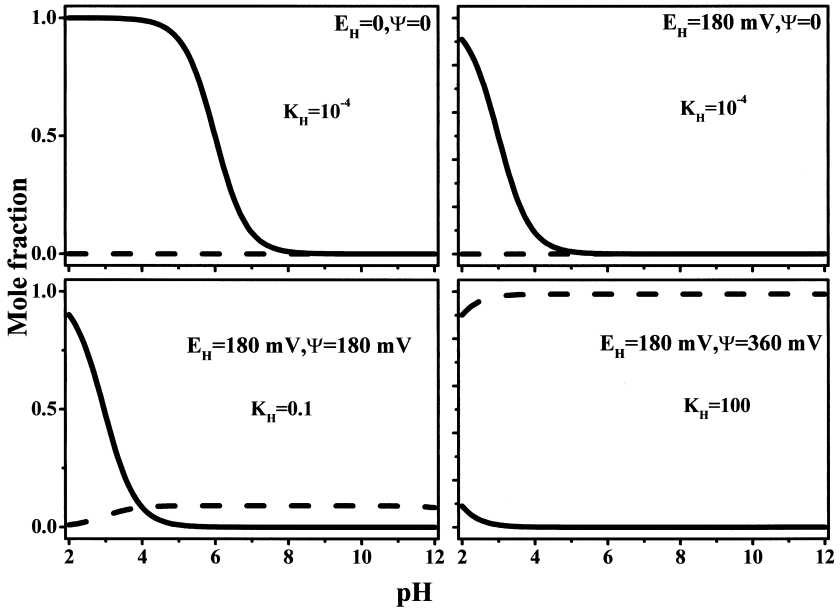


Fig. 2. Theoretical pH titrations for various values of E_H and Ψ of the fraction of: (solid line) the kinetically incompetent, doubly protonated state (A); (dashed line) the kinetically competent tyrosinate state (F). The proton acceptor has $pK_N = 6$ and Y_Z has $pK_Z = 10$ throughout.

An additional factor which facilitates the proton-first pathway in the oxidation of Y_Z is the field generated by P^+, which is expected to lower the ΔpK (provided Y_Z is located closer to P than His). In the Mn-depleted system, we estimated the magnitude of this effect around 70 mV [44]. Its role is thus smaller than that of E_H and Ψ and will be neglected in the present discussion.

3.4. pH dependence of the kinetics

Information on the pK of His-190 can be derived from the pH dependence of P^+ reduction kinetics or from the equilibrium constant between P and Y_Z (K_{ZP}), estimated (in the Mn-depleted system) through the kinetics of recombination with Q_A^-. Concerning the effect of pH on kinetics, one should distinguish two possibilities. The pH may modify the reaction rate constant (or several rate constants if the process is multiphasic). If such is the case, the proton equilibria involved must be fast with respect to the reaction rate (the various protonation states are averaged out). The second case is when the proton equilibria are slow with respect to the observed kinetics. One should then observe several phases corresponding to the various protonation states present, and the effect of pH should be to modify the relative weights of these phases rather than their rates. The pKs derived from the pH dependence have different significance in either case. If the proton equilibrium is rapid, the effective pK is that present during the reaction (e.g. the pKs of tyrosine and histidine are expected to be electrostatically shifted in the presence of P^+). In the second case, the relevant pKs are those of the dark-adapted system.

A problem is that it may not be obvious to distinguish experimentally between these two cases. For example, for the P^+ reduction kinetics in the Mn-depleted system, the former interpretation (pH dependent rate constant) has been retained by Conjeaud and Mathis [42] and more recently by Diner et al. [22]. The second one (constant rates, pH-dependent weights) has been preferred by Ahlbrink et al. [43] and Hays et al.[16]. In the latter studies, both groups found a modulation by a pK of 7–7.5 and ascribed it to His-190.

In Mn-depleted material, we investigated [44] the dependence on pH of the proton release and of the recombination rate of $Y_Z^{ox}Q_A^-$ (reflecting the equilibrium constant K_{ZP}). For this slow reaction (10–100 ms range) rapid proton equilibration is obvious. A pK of 6 was observed to control both the recombi-

nation rate and the proton release (interpreted as a pK shift from p$K \geq 9$ in the dark to 6 in the presence of Y_Z^{ox}). A very similar pH dependence of the recombination rate was found by Mamedov et al. [15] (the authors actually reported a higher pK value, 7.5, but this pK was obtained by plotting the kinetics *half-time* rather than the rates, see Fig. 3B in their paper; when plotting rate versus pH, the pK is close to 6). We proposed (as also did Mamedov et al.) that this group could be the distal nitrogen of His-190. From the extent of the change of K_{ZP} with pH, we estimated that the group with p$K \approx 6$ in the presence of PY_Z^{ox} was shifted to p$K \approx 7.2$ in the presence of P^+. This pK would fit nicely with that (7–7.5) found to modulate the P^+ reduction rate (see above), but only if proton equilibration is fast compared with P^+ reduction kinetics. If the kinetics are controlled by the protonation states prevailing in the dark, then another group with p$K \approx 7$–7.5 in the dark-adapted state must be involved, because our model requires a high pK (≥ 9) in the dark for the group which is shifted to p$K \approx 7.2$ in the presence of P^+ and to 6 in the presence of Y_Z^{ox}.

In O_2-evolving material, Meyer et al. [45] reported that, in the presence of S_1, the reduction of P_{680}^+ is slowed only by a factor of two when decreasing the pH from 7 to 4 ($t_{1/2} \sim 20$ ns at pH 7.0, 40 ns at pH 4.5). This was analyzed in terms of a pH-dependent rate constant (first case above) although this implies unexpectedly fast proton equilibrium. On the other hand, Christen et al. reported that the relative amplitude of the sub-μs phases decreases drastically at pH lower than 6 [46]. The interpretation proposed by these authors is that protonation of His190 occurs at low pH, forming the kinetically incompetent state (A) of Scheme 1. On this basis they estimated a pK (pK_A in our notation, see Eq. 5) of about 5.0. This value is significantly lower than the pK of about 7.0–7.5 proposed in [16,43] for this residue in the absence of Mn cluster, which may be one clue to the slower kinetics in the Mn-depleted system.

4. Stabilization and reduction of $Y_Z^\bullet$

4.1. Stabilization phase

In a matter of a few tens of ns, we have reached a state $Y_Z^\bullet$–O...H^+-His, that we denote $Y_Z^\bullet NH^+$ for short. The equilibrium constant with P is probably rather small at this stage: $[PY_Z^\bullet NH^+]/[P^+Y_Z^{red}] \approx 4$ in the S_0 or S_1 states. This estimate is derived from the extent of P^+ reduction in the sub-μs domain (about 80%, [28,34,47–50]). Thus, if the E_m of P is 1120 mV, that of the $Y_Z^\bullet NH^+/Y_Z^{red}$ couple is ≈ 1085 mV. We accept here the assumption that the multiphasic reduction of P^+ is a homogeneous process, i.e. that the 20% slow phases concern all centers. However, we previously proposed [51] a different, heterogeneous model, where the slow phases would occur in a particular conformation of the center ('bad stabilizer'). Equilibration between this conformation and the more efficient one ('good stabilizer') was estimated to occur in the 10 ms range. The best resolved evidence for this conformational equilibrium was, however, obtained in Mn-depleted material. Whether the slow phases of P^+ reduction in intact material are partly due to a heterogeneous conformation of the center remains, in our opinion, an open possibility. The model proposed by Tommos and Babcock [29] also assigns the slow phases to a particular conformation of the center (see below the discussion of the H-abstraction model).

In the framework of the homogeneous interpretation, the 1–100 μs phases of P^+ reduction reflect a stabilization of Y_Z^{ox}. The persistence of a relatively large fraction of P^+ in this time range must entail significant recombination and diminished quantum yield (contributing the photochemical misses responsible for the damping of O_2 evolution sequences). How large is the energetic stabilization occurring during these phases? The ΔE_m for the equilibrium of relaxed Y_Z^{ox} with the Mn cluster in states S_1 or S_2 was estimated by Vos et al. [5] around -50 mV. If the midpoint potential of these S states is 900–950 mV (see Section 1) then Y_Z^{ox} lies around 950–1000 mV and the stabilization from the 1085 mV starting point is 85–135 meV.

What is the pK of state $Y_Z^\bullet NH^+$? Since the $Y_Z^\bullet NH^+$ proton is involved in a H-bond, one has:

$$pK(Y_Z^\bullet NH^+) = pK_N + E_H/60 \quad (9)$$

To keep the blocked state (A) to a negligible amount before charge separation, we must have p$K_A \leq 5$,

thus (from Eq. 7) $pK_N \leq 5 + E_H/60$. This implies:

$$pK(Y_Z^{\bullet}NH^+) \leq 5 + 2E_H/60 \quad (10)$$

Thus, for $E_H = 180$ meV, $pK(Y_Z^{\bullet}NH^+) \leq 11$. This means that the H-bond may provide sufficient free energy to stabilize the $Y_Z^{\bullet}NH^+$ state at physiological pH.

The stabilization phases may reflect essentially an internal structural relaxation involving rearrangement of the H-bond network or, alternatively, proton release to the bulk. Let us first examine the latter possibility (proton release to the lumen is an essential feature of the H-abstraction model discussed below, but for Babcock and coworkers [29] diffusion of the proton away from the Y_Z region is initiated during the sub-μs phase). Releasing a proton from state $Y_Z^{\bullet}NH^+$ (with pK expressed in Eq. 9) to the bulk at pH 6 (standard condition for the Vass and Styring estimates [1]) implies a $\Delta G_{dep} = 60(pH - pK_N) - E_H$. Then, if proton release into the bulk accounts for the stabilization energy of 85–135 mV, pK_N must be as low as 1–2 (for $E_H/60 = 3$). Furthermore, the condition $K_H \geq 0.1$ requires (from Eq. 8) that Ψ is greater than 420 mV.

Because of these two strong constraints specifically imposed by the dumping of the H^+ into the bulk and for other reasons explained in Section 4.2, our preferred picture for the relaxation and reduction of Y_Z^{ox} is the following. The 1–100 μs stabilization phases are due to rearrangements caused by the charged $Y_Z^{\bullet}NH^+$ species, which include proton movements (as suggested by the effect of H/D isotopic substitution on these phases [46,49,50]) and, possibly, marginal 'domino' proton release to the bulk. These rearrangements may be triggered by the pK shift of the distal histidine nitrogen when the proximal one becomes protonated [15,22,44]. These rearrangements, rather than the release of the $Y_Z^{\bullet}NH^+$ proton, provide the 85–135 mV stabilization and increase the pK of the $Y_Z^{\bullet}NH^+$ state. The reduction of $Y_Z^{\bullet}NH^+$ would then involve the transfer of an electron from Mn and restoration of the initial proton equilibrium between Y_Z^{red} and His.

Let us summarize our analysis of the constraints bearing on Y_Z and its proton acceptor. To a large extent this does not rely on a specific model: the proton acceptor need not be a histidine, the H-bond link between the partners may be indirect, provided there is some energetic cost E_H for the doubly protonated state.

1. Rapid electron transfer to P^+ implies a facilitated proton transfer to an accepting base. To be efficient this base must be a good acceptor (high pK), but a vacant one (low pK).
2. These conflicting requirements can be reconciled thanks to two contributions: the H-bond between Y_Z and the acceptor (which disfavors the doubly protonated state) and the Coulombic energy between tyrosinate and the protonated acceptor (which increases the K_H).
3. If the proton is dumped to the bulk during the relaxation phase (H-abstraction model), the energy dissipation at this stage implies a pK_N for the acceptor in the 1–2 range (depending on the H-bond strength) and a fairly large electrostatic interaction, $\Psi > 420$ mV.

4.2. The H-atom abstraction model

In the H-atom abstraction model proposed by the Babcock and Britt groups [23,24,29,52,53], the proton from $Y_Z^{\bullet}NH^+$ is released into the lumen, leaving the neutral oxidized radical $Y_Z^{\bullet}$. The reduction of $Y_Z^{\bullet}$ would then proceed by abstracting, on each S-state transition, one H atom from the water substrates ligated to Mn.

In the above discussion, we have made the assumption that the 30 ns phase of P^+ reduction corresponds to the overall reaction $P^+Y_ZO–H...N \leftrightarrow PY_ZO^{\bullet}...H^+N$. However, according to Babcock and coworkers [29], the presence of a charged species such as $Y_Z^{\bullet}NH^+$ in a medium with low dielectric constant would entail a prohibitively high energetic cost (Born energy term) so that the reduction of P^+ would require the release of the proton to the bulk or at least its dilution in the protein medium over a 20 Å radius sphere. This argument is hard to accept for two reasons. Firstly, it is difficult to see how a sub-μs reaction could be at all feasible if a prohibitive energy barrier is present until the proton has been diluted away at a large distance. If both the cationic radical form and the form $Y_Z^{\bullet}NH^+$ were very high energy states, there is just no reaction path left for a rapid reaction. Secondly, it is well known [54–57] that the Born energy is just one among several

(five) terms that have to be taken into account for estimating the energetics of a charged species in a protein. The modulation range allowed by the sum of these terms is very large and there is no difficulty in (over-) compensating the Born term. For example, Blomberg et al. [58] have performed ab initio calculations showing that, whereas the formation in a medium with $\varepsilon = 3$ of a cationic tyrosine radical at the expense of P_{680}^+ is energetically most unfavorable, the reduction of P_{680}^+ by a Tyr-His-Glu$^-$ chain becomes exothermic, illustrating the effect of a single anion on the stabilization of the cationic tyrosine radical. A striking demonstration of the large range of energetic control exerted by the protein medium towards the introduction of a net charge is provided by the tetraheme subunit of *Rhodopseudomonas viridis* reaction center. The midpoint potential of the heme of lowest redox potential is similar to its value in solution (−60 mV versus −70 mV) [59,60] and the midpoint potentials of the four hemes span 450 mV despite the similar value of the Born energy term. According to the calculations of Gunner and Honig [60], a single arginine residue accounts for the increase by about 370 mV of the midpoint potential of the heme of highest potential.

The H-abstraction model does not necessarily require a high energy barrier for the $Y_Z^{\bullet}NH^+$ state and ultrafast proton diffusion. The proton release could occur, as considered in Section 4.1, during the 1–100 μs phases. Nevertheless, in our opinion, the H-abstraction model is difficult to reconcile with a number of experimental findings: (1) As further discussed below, in materials which present the best guarantees for intactness and oxygen-evolution performances (including crystallizable particles [61]), the extent of proton release depends on the S-state. It is not 1 on each transition as required by the H-abstraction model, but, for instance, much less than 1 upon formation of S_2 and much more than 1 upon formation of S_4 (see below). (2) During the whole lifetime of Y_Z^{ox} a chlorophyll bandshift is observed [44,62–67]. From experiments with mutants (Diner and Lavergne, unpublished; see [66]) the chlorophyll undergoing the shift was identified as that ligated to His D1–198 (named P_A and considered as belonging to the special pair constituting P-680). Although other explanations cannot be excluded [24,29], the simplest one is an electrochromic or, possibly, structural effect due to the charged $Y_Z^{\bullet}NH^+$ state. (3) The only evidence for 'stoichiometric' (or close to that) rapid proton release to the lumen, occurring before Y_Z^{ox} is re-reduced has been obtained using very high concentrations of the dye neutral red (an amphiphilic proton acceptor) [68], or in particles with somewhat degraded water oxidation characteristics (slower reactions, increased photochemical misses, loss of the proton oscillation pattern) (see [69]). It has been demonstrated by Hays et al. [14] that small exogenous soluble bases such as imidazole can accelerate Y_Z oxidation (in a Mn-depleted system, with or without His-190 present), presumably by picking up the Y_Z proton. We consider likely that neutral red may act in a similar manner and that the rapid release is induced rather than just monitored by the dye. Based on a kinetic study of the chlorophyll bandshift, we concluded that fast (tens of μs) proton release was only occurring upon formation of state S_4 (and at low pH upon formation of S_1) [70]. (4) According to the nice mass spectrometry experiments of Messinger et al. [71], the water substrates are still exchangeable in the S_3 state (with one fast and one slow exchanging water). In the H-abstraction model, three H atoms have been removed from the Mn–H_2O complex at this stage. We doubt that the strengths of the Mn–O bonds at this step could be weak enough to allow exchange with oxygen from bulk water, especially for the case of the fast exchange phase. This point has been recently emphasized by Hillier and Wydrzynski [72] who discuss the available data for the exhange rate of water on metal ligands. Their (perhaps extreme) conclusion is that the accumulation of oxidizing equivalents up to S_3 occurs on Mn ligands which are not direct ligands to the substrate water molecules.

Our objections against the H-abstraction model only relate to this model as a unique route occurring on each S-state transition. More flexible views are conceivable, however: the H-abstraction mechanism could occur on some of the transitions (possibly depending on pH). A rapid phase (30 μs) of proton release has been observed upon formation of the Y_Z^{ox} S_3 (S_4) state [70,69,73], concomitant with an initial lag in the reduction kinetics of Y_Z^{ox}. This was interpreted as a priming step preceding the water oxidation reaction. An attractive possibility is that on this particular step, the Y_Z proton is released to

the bulk and that H-abstraction from water is involved in the final, rate-limiting reaction of the water oxidase.

4.3. Proton release during the S-cycle

Different patterns have been reported for different materials, with two main cases. *Oscillating patterns* have been found for PS II in its native membrane (thylakoids or membrane preparations) [68,74] and recently for a preparation of core particles [61]. 'Oscillating' means that the release is not the same on each transition in the S-cycle. Although there are differences between the published results, there are also, fortunately, some common features. Firstly, the amount of uncompensated charge (electrons removed minus protons released) remains smaller than 1 throughout the S-cycle (there may be one positive charge present but never more). Secondly, the release on $S_1 \rightarrow S_2$ is smaller than 1, that on $S_2 \rightarrow S_3$ is close to 1. Also, the oscillating patterns all depend on pH. Beyond that, the discrepancies are significant, especially at low pH. They cannot be accounted for by moderate pK shifts in the various materials. It cannot be excluded that the differences (or some of them) are not real, but stem from the methods used for extracting the individual contribution of each S-transition from the damped experimental sequence (see the discussion in [75]). A *non-oscillating* pattern (one H^+ released on each transition, irrespective of pH) has been found in various preparations of core particles [76,77]. Obviously, the loss of peripheral polypeptides or structural modifications occurring in such preparations is responsible for the modified pattern. From this startpoint, two attitudes have been defended. For the supporters of the H-abstraction model, the non-oscillating pattern reveals the intimacy of the catalytic mechanism, rid from incidental perturbations due to the protein environment in more intact material. We [74] and others [61] have argued in the opposite direction. The occurrence of an uncompensated charge (as in the $S_1 \rightarrow S_2$ transition) can be understood if, in the region where the electrostatic influence of the charge is strong, there is no protonated group able to release its proton. This may occur either because there is no outlet channel or, as shown in Section 3.3, because a H-bond locks the proton in place. If the protein–water interface comes closer to the charge, or if a looser structure emerges, deprotonation may occur. An illustration of what we have in mind is the study of Shifman et al. [78] of electron/proton coupling in heme–protein maquettes. The oxidation of the heme causes a release of protons distributed over a number of residues that tends to compensate the charge and this does not crucially depend on a particular residue. Thus, the natural response of a protein (unless appropriately shielded) is to smooth out uncompensated charge. Conversely, it is more difficult to see how neutral changes (like H-atom abstraction from the catalytic center) could induce a modulation of proton release without resorting to ad hoc structural changes (Bohr effects).

Two observations support the view that the smoothing out of the oscillating pattern in PS II core particles is due to a loosened structure. The addition of glycerol, which is able to cause protein refolding [27], restores an oscillating pattern [79]. Furthermore, Schlodder and Witt [61] have recently reported an oscillating pattern in an improved preparation of PS II particles, which are crystallizable. This property indicates a stable and well defined structure.

As indicated above, a common feature found in proton oscillations is a lower release upon the $S_1 \rightarrow S_2$ transition, indicating an increment of the net charge of the system at this stage. Several phenomena showing different behavior of states S_0,S_1 with respect to S_2,S_3 are generally ascribed to the positive charge present on the latter states (although the additional involvement of a conformational change cannot be excluded). The sub-μs phases of P^+ reduction by Y_Z are 5–10 times slower in the presence of S_2 or S_3 than for S_0 or S_1 [47]. The extent of the μs phases is also increased, reflecting a lower equilibrium constant K_{ZP} in the charged states [43,48,49,80]. The yield of chlorophyll fluorescence (F_0 level, with oxidized Q_A) is higher in the S_2,S_3 states [81]. This correlates with a decreased photochemical efficiency: the charge separation (measured from the field-indicating carotenoid change) induced by a weak flash in algae lacking PS I is 8% smaller in states S_2,S_3 (J. Lavergne, unpublished results).

There is a trend among specialists of the water oxidase towards favoring integer, pH-independent proton release patterns as reflecting the true nature

of the oxidized intermediates. The fact that the observed oscillating patterns are non-integer and vary depending on pH is ascribed to non-specific interference of the protein that are of little interest. This is the viewpoint of supporters of the H-abstraction model (which requires the 1,1,1,1 pattern), but also that of Witt's group (who favors a 1,0,1,2 pattern). There is a cultural difference in this respect with specialists of the bacterial acceptor side who consider the (non-integer, pH-dependent) proton uptake induced by the semiquinone state Q_B^- as the key to its stabilization and attach great importance to the molecular details involved [82,83]. We would like to advocate a similar attitude towards the water oxidase. Firstly, the actual proton release pattern controls the thermodynamics of oxidant accumulation. The potentials of the S-states at a given pH depend on the protolytic reactions, regardless of whether they are direct or not. Secondly, a non-integer, pH-dependent pattern indicates the involvement of groups with p*K*s in the physiological domain. This means a 'weak coupling' mechanism occurring on some of the S-states, which as illustrated in Section 2, may play an important catalytic role.

5. Conclusion

It may be hoped that in the reasonably near future two major pieces of information concerning the water oxidase will become available. Progress in PS II crystallization [84,85] opens the prospect of unraveling the three dimensional structure at atomic scale. Progress in spectroscopic investigations (e.g. low frequency FTIR) may be expected to provide information on an essential question: at what steps and how does water oxidation chemistry come into play?

Progress on these fronts will undoubtedly boost our understanding in this field. Undoubtedly also, in our opinion, it will not put an end to the confrontation of models and concepts because of the inherent complexity of this fascinating enzyme.

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