The mechanism for proton-coupled electron transfer from tyrosine in a model complex and comparisons with Y_Z oxidation in photosystem II

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In the water-oxidizing reactions of photosystem II (PSII), a tyrosine residue plays a key part as an intermediate electron-transfer reactant between the primary donor chlorophylls (the pigment P_{680}) and the water-oxidizing Mn cluster. The tyrosine is deprotonated upon oxidation, and the coupling between the proton reaction and electron transfer is of great mechanistic importance for the understanding of the water-oxidation mechanism. Within a programme on artificial photosynthesis, we have made and studied the proton-coupled tyrosine oxidation in a model system and been able to draw mechanistic conclusions that we use to interpret the analogous reactions in PSII.

Keywords: photosystem II; proton-coupled electron transfer; tyrosine; ruthenium; photochemistry

1. INTRODUCTION

Electron transfer in proteins is often coupled to the transfer of protons. In PSII of oxygenic photosynthesis, the chlorophyll pigment P_{680} is initially photo-oxidized to P_{680}^+ and then reduced by electron transfer from a particular amino acid, Y_Z . The Y_Z radical generated oxidizes the oxygen-evolving Mn cluster in a subsequent step. Without deprotonation, the oxidized Y_Z would have a positive charge. The high redox potential of the $\text{TyrOH}^+/\text{TyrOH}$ couple makes the pure electron transfer to P_{680}^+ endoergonic. However, tyrosine deprotonates readily upon oxidation, which increases the driving force of the reaction. The total reaction free energy depends on the free energy associated with deprotonation, and in oxygen-evolving centres the overall reaction is slightly exoergonic. In addition, the subsequent reduction of the oxidized Y_Z by the Mn cluster is coupled to its protonation.

It has recently been debated how the proton- and electron-transfer reactions of Y_Z are coupled and if one is a prerequisite for the other. There is also the question whether Y_Z is hydrogen bonded to one or more bases under all conditions (pH, redox state and presence or absence of Mn) and where the Y_Z proton goes upon oxidation and from where it comes back, etc. (for recent examples, see Ahlbrink *et al.* 1998; Diner *et al.* 1998; Hays *et al.* 1998, 1999; Tommos & Babcock 2000; Diner 2001; Rappaport & Lavergne 2001; Renger 2001 and references therein). This discussion was fuelled by a proposal that

Y_Z is not just an electron-transfer intermediate, but is intimately involved in the water-oxidation mechanism by abstracting hydrogen atoms from water bound to the Mn cluster (Tommos *et al.* 1995; Hogansson & Babcock 1997). In this model, the maintenance of charge neutrality was emphasized (Tommos & Babcock 2000), so as to avoid the energy penalty for creating charges in a protein. The hydrogen-atom abstraction mechanism has implications also for the oxidation mechanism of Y_Z .

Mechanistically the protonic motion can be coupled to the electronic motion in two limiting ways in consecutive reactions (figure 1). In one case, the proton has to dissociate completely prior to electron transfer so that a consecutive proton-transfer–electron-transfer (PTET) mechanism can occur. In the other limiting case the electron-transfer event can occur without the aid of a proton movement and the proton dissociates only as a consequence of the electron transfer, which gives a consecutive ETPT. In neither of these cases is the proton motion involved in the actual electron-transfer event, and the reorganization energy is unaffected by the proton motion. In between these two extremes lies the concerted electron/proton transfer (CEP) mechanism, where electron transfer and deprotonation are simultaneous, with a single transition state.

In electron-transfer reactions, the positions of the nuclei have to distort to the transition-state configuration, which allows electron transfer to occur. The stronger the bonds, and the longer distance the nuclei have to distort from the equilibrium position of the reactants, the larger the inner reorganization energy, λ_{in} . When dissociative nuclear motions are coupled to the electron transfer, λ_{in} is therefore often large. The reorganization energy for a CEP reaction would thus be highly dependent on the force con-

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Figure 1. Possible reaction paths for proton-coupled electron-transfer reactions from tyrosine (TyrOH) to the photo-oxidized Ru(III) sensitizer of figure 2. Reactions I and II are both consecutive pathways where in II deprotonation is a pre-requisite for electron transfer and the tyrosinate ion is the electron donor (PTET) and in I the tyrosine radical deprotonates as a consequence of electron transfer (ETPT). In reaction III the electron transfer and the deprotonation occur simultaneously in one concerted reaction step (CEP).

stant for the phenolic O–H bond and the length that it has to distort to reach the transition state. The reorganization energy is consequently an important parameter in proton-coupled electron-transfer reactions that can give aid in elucidating the mechanism, and in the case of CEP, indirectly may give insight into the transition-state configuration. Biologically productive electron transfers are usually only slightly exoergonic. CEP is a charge neutral reaction that avoids the high-energy intermediates created in the consecutive PTET or ETPT mechanisms. CEP thus represents what could be called an energy-conservative mechanism, which allows for efficient reactions with a low driving force, but the penalty paid is a relatively large reorganization energy.

In this article, we present results for a proton-coupled electron transfer from tyrosine in a simple model compound, for which we showed that tyrosine oxidation is a CEP (Sjödin *et al.* 2000). This is developed as a part of a project on mimicking PSII reactions in artificial photosynthesis (Sun *et al.* 2001). We include new data on reaction entropy and kinetic isotope effects (Sjödin *et al.* 2002), and extend our earlier discussion of Y_Z oxidation in PSII, and the effect of hydrogen bonds on the protonation–deprotonation reactions. The structure and properties of the model system are well known, which allows us to draw more certain conclusions. Based on similarities to the kinetic data for PSII, we then make mechanistic suggestions for the much more complex related reaction of PSII.

2. DATA AND KINETIC MODEL FOR THE Ru–TyrOH MODEL SYSTEM

In figure 2, the structure of the complex Ru–TyrOH is shown, in which a tyrosine moiety is linked to a photosensitizer of the Ru(II)–polypyridine type. The Ru(II) complex was photo-oxidized to Ru(III) by an external acceptor on the nanosecond time-scale. In aqueous media, an intramolecular electron transfer from the tyrosine followed on the microsecond time-scale, regenerating the Ru(II) sensi-

Figure 2. The Ru–tris-bipyridine part acts as a sensitizer that absorbs light at 450 nm and when excited it transfers an electron to the external electron acceptor (methyl viologen). Tyrosine donates an electron to the photo-oxidized Ru(III) and is deprotonated. Ru–TyrOH, Ru(II)(bpy)₃(4'-Me-4'CONH-L-tyrosine ethyl ester-2,2bpy)²⁺.

tizer (Magnusson *et al.* 1997). Simultaneously, a tyrosine radical appeared, as seen in both the optical and EPR experiments. Because the pK_a value for tyrosine shifts from 10 to -2 upon oxidation, the electron transfer is coupled to deprotonation of the tyrosine. We have earlier presented kinetic data for the proton-coupled electron transfer from tyrosine to the photo-oxidized Ru(III) (Sjödin *et al.* 2000), which was obtained by following the bleaching recovery of the Ru(II) absorption *ca.* 450 nm. The rate constant for this reaction is highly dependent on pH (figure 3a). At a pH value below the pK_a value of the phenolic tyrosine proton (*ca.* 10) the rate constant increases monotonously with the pH. Above the pK_a the rate constant was much larger and independent of the pH. Around the pK_a value both kinetic phases were found. At low pH values, the activation energy was high ($E_a = 0.32$ eV at a pH of 7), but at a pH of more than 10 it was much lower ($E_a = 0.05$ eV).

We have shown (Sjödin *et al.* 2000) that the data cannot be explained by any of the consecutive mechanisms, that is electron transfer followed by deprotonation of the oxidized tyrosine (ETPT, figure 1, mechanism I) or deprotonation of tyrosine followed by electron transfer from the tyrosinate anion (PTET, figure 1, mechanism II). In the ETPT mechanism none of the reaction steps is pH dependent, so the overall reaction would not show the experimentally found pH dependence. In the PTET mechanism the reaction rate will be limited by the rate constant for tyrosine deprotonation, k_d . For tyrosine the value of $pK_a = 10$ implies that the deprotonation is very slow: the rate constant $k_d \leq 10 \text{ s}^{-1}$.¹ This is several orders of magnitude too low to account for the observed rates.²

Instead the electron transfer from tyrosine to Ru(bpy)₃, in Ru(bpy)₃–TyrOH must occur as a CEP reaction (figure 1, mechanism III). That is, electron transfer and deprotonation of the tyrosine phenol group to bulk water solution occur simultaneously in one reaction step, with a common transition state. For the reaction to occur, the system must be in a reaction coordinate region in which the free-energy surfaces for the reactant and product state cross. The phenolic OH bond of tyrosine is broken in the reaction, so that the product's free-energy surface is repulsive in the coordinate reflecting O–H motion (see figure 4).³ A concerted reaction means that the O–H bond has to stretch in order to reach the transition state, where the

Figure 3. The pH dependence of the electron-transfer rate constant (a) from tyrosine to the photo-oxidized Ru part in Ru-TyrOH and (b) from Tyrosine_Z to P₆₈₀⁺ in Mn-depleted PSII particles (re-plotted from Ahlbrink *et al.* 1998), in an aqueous solution.

Figure 4. Schematic picture of the free-energy surfaces for the reactant and product states in CEP. The product state is repulsive in the proton coordinate, that includes the reorganization of all inner and solvent coordinates associated with the proton movement. All other contributions to the reaction coordinate are perpendicular to the figure plane. The free energy of the product state decreases with the pH value, due to an increased mixing entropy of the released proton. The concomitant decrease in the activation energy gives the pH dependence of the reaction rate.

system may go from reactant to product state by shifting the electronic distribution, and the proton dissociates along the repulsive product surface. The elongation of the O–H bond, and the concomitant reorganization of the solvent in response to that movement, will give a significant contribution to the total reorganization energy of the reaction. This explains the large activation energy we determined at a pH of more than 10. From the standard Marcus equation (equation (2.1)) (Marcus & Sutin 1985)

$$k_{\text{ET}} = (2\pi/\hbar)H_{\text{rp}}^2(4\pi\lambda k_{\text{B}}T)^{-1/2}\exp[-(\Delta G^0 + \lambda)^2/4\lambda k_{\text{B}}T], \quad (2.1)$$

$$\Delta G^0 = -F(E_{\text{Ru}}^0 - E_{\text{TyrOH}}^0 + (RT/F)\ln 10 \times \text{pH}), \quad (2.2)$$

we determined a reorganization energy λ for the CEP

reaction as $\lambda = 2.0$ eV, at neutral pH. When correction is made for the significant reaction ΔS^0 the value becomes somewhat lower, $\lambda = 1.4$ eV, see below. This is still much higher than the value we obtained for the simple electron transfer (that is not coupled to deprotonation) from the tyrosinate at a pH of more than 10: $\lambda = 0.9$ eV. A value of $\lambda \approx 1.0$ eV is typical for electron transfer in polar solvent, when no large bond length changes are involved. The much higher value we obtain at a pH of less than 10 is indicative of a CEP. We also found a significant kinetic deuterium isotope effect (Sjödin *et al.* 2002), $k_{\text{H}}/k_{\text{D}} = 2.0\text{--}2.5$ at a pH of less than 10, consistent with a direct involvement of the proton movement in the transition state. Obviously, this case of proton-coupled electron transfer proceeds faster if the endoergic steps of the consecutive reactions can be avoided, and instead the reaction proceeds directly to the product state in a CEP reaction, even if this implies a high reorganization energy.

With a CEP mechanism, one can also explain the fact that the reaction rate is pH dependent. The redox potential for tyrosine decreases with pH, by 59 mV per pH unit, until the pK_a value is reached at a pH of 10, above which the potential is constant at *ca.* -0.7 V (versus NHE). Thus, the free energy of the product state decreases with pH. Therefore, the overall proton-coupled electron transfer becomes increasingly exoergic as pH increases, because tyrosine is deprotonated in the reaction. As illustrated in figure 4, this may affect the position of the transition state along the O–H coordinate, leading to a decrease in activation energy with pH and a faster reaction. If the shape of the product's free-energy surface is close to parabolic in the region around the transition state, one would predict that the rate depends on driving force just as for a simple electron-transfer reaction (equation (2.1)). Indeed, we could fit the data ($\log k_{\text{CEP}} = f(\text{pH})$) for Ru-TyrOH at a pH of less than 10 in figure 3a to the standard Marcus equation ($\log k_{\text{ET}} = f(\Delta G^0)$, equation (2.1)) by incorporating a pH-dependent driving force due to the pH-dependent redox potential for tyrosine, i.e. by changing the reaction ΔG^0 by -59 meV for each pH unit increase (equation (2.2)).⁴ In the fit, the reorganization energy was fixed according to the temperature-dependent

measurements, so that only the pre-exponential factor was allowed to vary. The sloping line at a pH of less than 10 in figure 3a is the result of that fit. The curvature of the line is very small because the data lie far from the maximum in the bell-shaped Marcus curve predicted by equation (2.1) (Marcus & Sutin 1985), i.e. it is in the region where $-\Delta G^0 \ll \lambda$. Note that it is only with a CEP mechanism that one can explain why the observed reaction rate directly follows the change in driving force for the overall reaction. In consecutive reactions, with real intermediates, the pH dependence for the observed reaction is instead given by that of the individual steps.

The calculation of the reorganization energy λ above based on the experimental activation energies ignores the entropy increase in the CEP reaction that is due to the mixing entropy of the released proton. This entropy increase is pH dependent, and is the cause of the pH dependence of the tyrosine redox potential. We have estimated an entropy change for the CEP reaction of $1.4 \times 10^{-3} \text{ J mol}^{-1} \text{ K}^{-1}$ at a pH of 7 (Sjödin *et al.* 2002). Since $\Delta S^0 > 0$, the driving force increases with temperature giving a more pronounced increase in rate constant as temperature is increased. At 25 °C and a pH of 7, the entropic contribution $-T\Delta S^0$ would decrease ΔG^0 by 0.41 eV². In a determination of the reorganization energy, the effect of a temperature-dependent ΔG^0 must be included when entropy changes are as large as in the present case. When this correction is made in the evaluation of the temperature-dependent rate data from equations (1.1) and (1.2), the calculated reorganization energy is much lower, $\lambda = 1.4 \text{ eV}$ compared with 2.0 eV when entropy effects were neglected. Still, the reorganization energy is higher than for the simple electron-transfer reaction from deprotonated tyrosine in Ru-TyrO⁻ at a pH of more than 10: $\lambda = 0.9 \text{ eV}$. We ascribe the difference, *ca.* 0.5 eV, to the reorganization accompanying the stretch of the O-H bond.⁵

We emphasize that a large entropy change in the general case may lead to large errors in the calculation of reorganization energies from activation energy data, if the entropy change is not taken into account. In biological systems, it is usually difficult to evaluate the reaction entropy. We hope that our data may serve to illustrate to what extent the entropy changes that occur when a proton is released to bulk water affects the evaluation of the reorganization energy.

3. COMPARISON BETWEEN THE KINETIC DATA FOR Ru-TyrOH AND MANGANESE-DEPLETED PHOTOSYSTEM II

We have shown that the proton-coupled electron-transfer reaction from tyrosine in Ru-TyrOH at a pH of less than 10 is a concerted reaction (CEP), that gives a large reorganization energy ($\lambda = 1.4 \text{ eV}$), and a kinetic isotope effect $k_{\text{H}}/k_{\text{D}} = 2.0\text{--}2.5$. The monotonous increase in rate with pH is a signature of a proton release to the bulk and can be explained by the increase in driving force for CEP. At a pH of more than 10 the reaction is much faster, with a pH-independent rate corresponding to electron transfer from the already deprotonated tyrosine in Ru^{III}-TyrO⁻. The activation energy is much lower when the pH is more than 10 ($E_{\text{a}} = 0.05 \text{ eV}$) than at a pH of less than 10

($E_{\text{a}} = 0.30 \text{ eV}$ at a pH of 7). The reorganization energy $\lambda = 0.9 \text{ eV}$ at a pH of more than 10 is typical for a simple electron transfer in water, when no large inner reorganization of the reactant molecules is involved. Around the $\text{p}K_{\text{a}}$ value of reduced tyrosine ($\text{p}K_{\text{a}} = 10$) tyrosine oxidation is biexponential, with one slow phase ($\tau \approx 4 \mu\text{s}$) and a hundred times faster phase ($\tau \approx 40 \text{ ns}$), corresponding to reactions in the Ru^{III}-TyrOH and Ru^{III}-TyrO⁻ species, respectively.

As shown in figure 3a,b, there are strong similarities in the kinetic behaviour for electron transfer from tyrosine to Ru(III) in Ru-TyrOH, and from Y_{Z} to P_{680}^{+} in Mn-depleted PSII (Ahlbrink *et al.* 1998). At lower pH (pH < 7.6), the data for PSII show a pH dependence similar to that in Ru-TyrOH. The activation energy ($E_{\text{a}} = 0.30 \text{ eV}$ at neutral pH) and the isotope effect ($k_{\text{H}}/k_{\text{D}} \approx 2.2$) are also both very similar to those in Ru-TyrOH. At a pH of more than 7.6 in PSII, the rate was much higher and pH independent, and the activation energy was lower ($E_{\text{a}} = 0.15 \text{ eV}$). At pH values of *ca.* 7.6 both kinetic phases were present, differing in rate by one order of magnitude.

We have argued that, because of the similarity in the kinetics at low pH, Y_{Z} oxidation at a pH of less than 7.6 in Mn-depleted PSII is also a CEP, with a concerted proton release to the bulk. In this way the pH dependence of the rate can be understood.⁶ The CEP reaction has the consequences of high activation energy, and a proton release directly to bulk water. The reorganization energy at neutral pH can be estimated to $\lambda = 1.4 \text{ eV}$ (not corrected for entropy changes) from the data in Ahlbrink *et al.* (1998), consistent with a polar environment and a significant reorganization from the tyrosine O-H bond.

There are thus strong similarities in the kinetics for Ru-TyrOH and PSII at lower pH values. The kinetics at higher pH values are qualitatively also very similar, but show important quantitative differences. The activation energy in PSII at high pH values is $E_{\text{a}} = 0.15 \text{ eV}$, which is lower than at low pH values, but not as low as the 0.05 eV for high pH values in Ru-TyrOH. Also, the apparent $\text{p}K_{\text{a}}$ value where the change to faster kinetics occurs is at a pH of 10 in Ru-TyrOH but at a pH of 7.6 for PSII. The difference in rate between the slower and faster phases around the $\text{p}K_{\text{a}}$ is one order of magnitude in PSII, while it was two orders of magnitude in Ru-TyrOH. In one of the mechanistic models proposed, Ahlbrink *et al.* (1998) explained the results by hydrogen bonding of Y_{Z} to a base (presumably His190) that was titrated with an apparent $\text{p}K_{\text{a}}$ of 7.6. Below a pH of 7.6, the base is protonated and the system would be very similar to our Ru-TyrOH with a proton release to the bulk. Above a pH of 7.6, the proton would instead be transferred to His190. The hydrogen bond thus increases the rate by a factor of 10, and decreases the activation energy from 0.30 to 0.15 eV (Ahlbrink *et al.* 1998). If the reaction still follows a CEP mechanism, as outlined above, with the hydrogen-bonded Y_{Z} , this explains that the rate becomes pH independent, as the proton is no longer released to the bulk, and the reaction driving force is no longer dependent on the proton activity of the water. In § 4 we will discuss possible reasons for the higher rate in the hydrogen-bonded system.

Figure 5. Thermodynamic cycle for calculation of the expected ΔG^0 for CEP when tyrosine is hydrogen bonded to a histidine. The data for the electron-transfer steps are for Ru-TyrOH. For PSII, the corresponding values for ΔG^0 are *ca.* 0.14 eV less negative (see § 4a).

4. IN WHAT WAY DOES HYDROGEN BONDING INCREASE THE RATE OF TYROSINE OXIDATION?

There are a number of different factors that may be responsible for the observed rate enhancement when tyrosine is hydrogen bonded. In a CEP mechanism, as in any single-step electron-transfer reaction, the rate constant (equation (2.1)) is determined by the electronic coupling (H_{rp}), the reaction driving force ($-\Delta G^0$) and the reorganization energy (λ). We will discuss which of these factors that may explain the effects of the hydrogen bond on the reaction kinetics. We will also discuss the possibility of a change of mechanism as the tyrosine becomes hydrogen bonded.

(a) Factors affecting the rate of a concerted electron-transfer deprotonation

(i) Electron coupling

The pre-exponential factor, which is proportional to H_{rp}^2 , was even somewhat lower when tyrosine was deprotonated in Ru-TyrO⁻ compared with the protonated Ru-TyrOH (Sjödin *et al.* 2000). It seems unlikely that a hydrogen bond would give a large difference in the opposite direction than a complete deprotonation, so a higher value of H_{rp} is probably not the correct explanation for the higher rate in the hydrogen-bonded system.

(ii) Driving force

When tyrosine is hydrogen bonded to a base, the driving force for CEP is not pH dependent because the proton moves to the base instead of being released to the water. A sufficient increase in driving force could explain the higher rate in the hydrogen-bonded system. The driving force for CEP with tyrosine hydrogen bonded to a histidine can be estimated from the thermodynamic cycle in figure 5, in which data for Ru-TyrOH are used for the electron-transfer steps. The ΔG^0 for the two proton-transfer reactions from tyrosine to histidine is calculated from the corresponding equilibrium constants: $\Delta G^0 = 0.059 \times \Delta pK_a$ (eV) ($\Delta pK_a = pK_a(\text{tyrosine}) - pK_a(\text{histidine})$), which gives $\Delta G^0 = -0.48$ eV and $+0.24$ eV for the oxidized and reduced tyrosine, respectively. The difference between these values is 0.72 eV. The difference in ΔG^0 for the pure electron-transfer steps without deprotonation must then

be equal to that value, in order to close the cycle. For the non-hydrogen-bonded Ru-TyrOH system, the electrochemical data give $\Delta G^0 = +0.26$ with the proton on tyrosine and $\Delta G^0 = -0.56$ eV when tyrosine is deprotonated (Sjödin *et al.* 2000). The difference between these values is 0.82 eV, i.e. 0.10 eV too large. This small discrepancy can be attributed to the effect of the hydrogen bond: the TyrOH state is somewhat easier to oxidize to TyrOH⁺ in the presence of a hydrogen bond, giving $\Delta G^0 < +0.26$ eV, while the TyrO⁻ is somewhat more difficult to oxidize when hydrogen bonded, giving $\Delta G^0 > -0.56$ eV. With these limits, and the requirement of a 0.72 eV difference between the two values, we obtain $\Delta G^0 = -0.51 \pm 0.05$ eV and $\Delta G^0 = +0.21 \pm 0.05$ eV for non-proton-coupled oxidation of TyrO⁻ and TyrOH in the Ru-TyrOH molecule, respectively. The thermodynamic cycle then closes to give $\Delta G^0(\text{CEP}) = -0.27 \pm 0.05$ eV for the concerted reaction. In PSII, the $P_{680}^{+/0}$ potential has been estimated to *ca.* +1.12 V (versus SCE) (see Tommos & Babcock 2000; Diner (2001) and references therein), that is *ca.* 0.14 V below that for our Ru(bpy)₃³⁺ oxidant. We thus predict that the driving force for a CEP in PSII, when tyrosine is hydrogen bonded to a histidine, is $\Delta G^0 \approx -0.13$ eV. For both Ru-TyrOH and Mn-depleted PSII these values correspond to the driving force at a pH of *ca.* 7 in the absence of hydrogen bonds. For Mn-depleted PSII the rate constant with hydrogen bonds is about ten times higher than the rate at a pH of 7, without hydrogen bonds (figure 3b). This indicates that the hydrogen bond does not induce a sufficient change in ΔG^0 to explain the higher rate. In order to do so, a stronger base with a pK_a value two units higher than histidine (i.e. two units closer to the tyrosine pK_a value of 10), has to be assumed.

The pK_a values used for tyrosine and histidine in the estimates above are those in pure water, which gives a true measure of the difference in proton affinity of the bases. In a protein, the *apparent* pK_a value may shift due to electric fields and changes in dielectric properties. However, the pK_a shift for two neighbouring amino acid residues, that share a hydrogen bond and that also have the same charge (positive acid-neutral base) will probably be similar in most cases, because they experience a similar environment. Thus, the ΔpK_a will not be very different from that in pure water in most cases.⁷

(iii) Reorganization energy

Neither changes in electronic coupling nor in the driving force seems to explain the rate increase upon hydrogen bonding of the tyrosine. Instead, we suggest that the increase in rate can be explained by a decrease in the reorganization energy λ of the tyrosine O-H bond, including that of the polarization of the environment associated with that movement. Hydrogen bonding to a base would facilitate the distortion along the proton coordinate. The activation energy reported for PSII at a pH of more than 7.6 (figure 3b) is 0.15 eV (Ahlbrink *et al.* 1998), and with the estimated $\Delta G^0 = -0.13$ eV above, a value of $\lambda = 0.85$ eV may be calculated, which is much lower than for the non-hydrogen bonded reaction. This is within experimental uncertainty equal to the reorganization energy we derived for Ru-TyrOH at a pH of more than 10, when tyrosine is deprotonated: $\lambda = 0.90$ eV. The identical values do not mean that the hydrogen bond

Figure 6. 'Rate ladder', showing the predicted pH dependence and relative rates for CEP involving a non-bonded tyrosine, a hydrogen-bonded tyrosine and a deprotonated tyrosine. We suggest that the main reason for the different rates between these situations is the successive decrease in reorganization energy when tyrosine is first hydrogen bonded and then deprotonated. The pH-dependent rate for the non-bonded tyrosine is a signature of proton release to the bulk.

removes all the reorganization of the O–H coordinate. Instead, it may reflect experimental uncertainties. Alternatively the O–H reorganization of Y_Z (λ_{in}) may be compensated for by a somewhat lower polarity (reflected in a lower λ_{out}) of the hydrogen-bonded Y_Z in Mn-depleted PSII as compared with Ru–TyrOH.

We suggest that the reorganization energy goes from high to intermediate to low when the tyrosine goes from non-hydrogen bonded to hydrogen bonded to deprotonated. This would be reflected in the experimental activation energies that go from 0.30 eV at low pH in PSII or Ru–TyrOH, to 0.15 eV in PSII at a pH of more than 7.6, to 0.05 eV for Ru–TyrOH at a pH of more than 10. This is schematically illustrated in the 'Rate ladder' of figure 6, in which the pH dependence for the rate constant is given for a general system. At low pH values, tyrosine is deprotonated to the bulk, which gives a pH-dependent rate due to an increasing reaction entropy, and also a high value for λ . At intermediate pH values, in the presence of a hydrogen bond, the proton is no longer transferred to the bulk. The rate is then independent of pH, and λ is at intermediate values. Finally, at a pH of more than 10 when the reduced tyrosine is initially deprotonated, the rate is pH independent and λ is small.

(b) *A change to a proton-transfer–electron-transfer mechanism?*

Other mechanisms for tyrosine oxidation in Mn-depleted PSII at high pH have been suggested, e.g. a consecutive PTET (Ahlbrink *et al.* 1998; Diner *et al.* 1998; Haumann *et al.* 1999; Rappaport & Lavergne 2001). We showed in § 2¹ that when tyrosine is not hydrogen bonded to a base, deprotonation is too slow to explain the observed microsecond rates. However, even though the mechanism is CEP in the absence of a hydrogen bond,

the mechanism could in principle change to a consecutive PTET upon hydrogen bonding. The electron transfer for PTET would then be driven from the small fraction of tyrosine that is deprotonated even at a pH of less than 10. Without hydrogen bonding to a base, the deprotonation of tyrosine in water is very slow ($k < 10 \text{ s}^{-1}$). With a better base than H_2O , the deprotonation rate constant for reduced tyrosine can be many orders of magnitude faster. From absolute rate theory and the fact that the ratio of forward and reverse reaction is given by the equilibrium constant, the deprotonation rate constant can be estimated to $6 \times 10^{12} \times 10^{-\Delta pK_a} \text{ s}^{-1}$ (Diner *et al.* 1998). This gives a rate constant of $6 \times 10^8 \text{ s}^{-1}$ for deprotonation of tyrosine that is hydrogen bonded to histidine ($\Delta pK_a = 4$). Since the rate constant for electron transfer from deprotonated tyrosine, k_{ET} , is much smaller than the rate constant for the reverse proton transfer back to tyrosine ($\approx 6 \times 10^{12} \text{ s}^{-1}$), the predicted rate constant for PTET is given by

$$k_{PTET} = 10^{-\Delta pK_a} \times k_{ET}, \quad (4.1)$$

where $10^{-\Delta pK_a}$ is the fraction of deprotonated tyrosine molecules. This equation is valid when the fraction of tyrosinate is small, e.g. when $10^{-\Delta pK_a} \ll 1$. In this kinetic model, the activation energy would be given by the sum of ΔG^0 for the proton equilibrium $\text{TyrOH} \leftrightarrow \text{TyrO}^- + \text{H}^+$ ($0.059 \times \Delta pK_a$) and the activation energy for the electron-transfer step from TyrO^- , resulting in $E_a(\text{predicted}) = 0.059 \times \Delta pK_a + E_a(k_{ET})$. The latter is probably similar to, or somewhat higher than, that for Ru–TyrO[−] at high pH ($E_a = 0.05 \text{ eV}$), as the driving force is somewhat lower for the weaker oxidant P_{680}^+ . The experimental activation energy in PSII at a pH of more than 7.6 is $E_a = 0.15 \text{ eV}$ (see § 3). With $E_a(k_{ET}) \geq 0.05 \text{ eV}$ this gives $0.059 \times \Delta pK_a \leq 0.10 \text{ eV}$ in this model, which would indicate that $\Delta pK_a \leq 2$. This seems too small for a tyrosine–histidine couple, and indicates that either the mechanism is not a consecutive PTET but rather CEP also with a hydrogen-bonded tyrosine, or alternatively that the hydrogen bond is not to histidine but to another base with a pK_a closer to that of tyrosine. However, irrespective of the identity of the base, the apparent pK_a value is 7.6, and if ΔpK_a would be ≤ 2 , then the apparent pK_a of Y_Z would be ≤ 9.6 . This seems inconsistent with the data of figure 3*b*, where the kinetics has been followed up to a pH of 10.5 without any sign of Y_Z deprotonation. Instead, we tend to believe that the mechanism is not PTET, but CEP also at higher pH. Equation (4.1) predicts a very strong dependence of the rate on the pK_a value for the base for a PTET mechanism. If histidine would be replaced by another base, the observed rate would increase by a factor of ten for each pK_a unit difference. This dependence is much stronger than that predicted for the CEP mechanism. Also, the activation energy for PTET would change by 59 meV per pK_a unit difference. With kinetic experiments on D1-His190 mutants, these predictions may be of value for the determination of the oxidation mechanism of hydrogen-bonded tyrosine.

(c) *A gated mechanism?*

A conceivable alternative is a gated mechanism (Diner *et al.* 1998; Diner 2001). Y_Z oxidation would then occur from a hydrogen-bonded situation at all pH values. The

pH dependence of the reaction rate would have to be explained by a pH-dependent setting of the hydrogen bond. The bond can only be formed when the base is deprotonated

When $\text{pH} > \text{p}K_a$ for the base the hydrogen bond is already set in this mechanism, and the rate is maximized. At lower pH values, the reaction is gated by a transient deprotonation of the base, followed by formation of the hydrogen bond in competition with reprotonation. The jump to much higher rates as pH goes above 7.6 shows that the reaction must be rapid once the hydrogen bond is set (if the gated mechanism is valid). Furthermore, it means that the rate at lower pH must be limited by the formation of the hydrogen bond. Thus, with a steady-state approximation for the intermediates, the rate constant k_{gated} is given by $k_{\text{gated}} = k_d k_{\text{HB}} / (k_p [\text{H}^+] + k_{\text{HB}})$, where k_d and k_p are the rate constants for deprotonation and protonation of the base, respectively, and k_{HB} is the rate constant for formation of the hydrogen bond. If $k_p [\text{H}^+] \ll k_{\text{HB}}$, then $k_{\text{gated}} = k_d$, and the rate would not be pH dependent even below the $\text{p}K_a$ of the base. If instead $k_p [\text{H}^+] \gg k_{\text{HB}}$, then $k_{\text{gated}} = k_d k_{\text{HB}} / (k_p [\text{H}^+])$, and the rate would increase by a factor of ten for each pH unit increase. None of these two cases agree with the observed pH dependence at a pH of less than 7.6. The intermediate situation where $k_p [\text{H}^+] \approx k_{\text{HB}}$ could only hold for about one pH unit. Thus, we believe that this gated mechanism cannot explain the kinetics in the whole observed pH range.

5. CONCLUSIONS

The kinetic data indicate that oxidation and deprotonation of tyrosine follows a concerted (CEP) mechanism, both in Ru-TyrOH and in Mn-depleted PSII. The proton is released to bulk water. The CEP explains the observed increase in rate with pH, and the high activation energy, that are signatures of proton release to the bulk. The large reaction ΔS° for proton release gives an additional increase in the experimentally observed activation energy. Failure to correct for the entropy change results in significant overestimation of the calculated reorganization energy (λ). Still, λ is relatively large for the CEP reaction. We suggest that the increase in the rate of Y_Z oxidation as the pH is raised above the apparent $\text{p}K_a$ in Mn-depleted PSII can be explained by a decrease in reorganization energy associated with the phenolic O-H bond stretch of tyrosine. This would be due to hydrogen bonding to a nearby base, possibly His190, as proposed by several authors.

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ENDNOTES

¹ The deprotonation rate constant k_d is given by $k_d = k_r K_a$, where K_a is the acid dissociation constant ($\approx 1 \times 10^{-10}$ M for reduced tyrosine) and k_r

is the second-order rate constant for protonation of the corresponding base, which is not larger than diffusion controlled, $1 \times 10^{11} \text{ M}^{-1} \text{ s}^{-1}$. This puts the limit for deprotonation at $k_d \leq 10 \text{ s}^{-1}$.

² Our results were identical in a variety of buffers at different concentrations, with additional salt (to test for ionic strength effects), and at a pH of 7 neat water gave the same result as buffer. This shows that the tyrosine oxidation rate was only dependent on pH, i.e. the proton activity, and that there were no specific proton-acceptor effects.

³ In Cukier (1999), a model for a dissociative proton-coupled electron transfer was presented, on a more solid, quantum mechanical footing. Our model instead focuses on the entropy changes, that are needed to explain the pH dependence of the rate. These aspects were not treated by Cukier, but there is no contradiction between our models. Instead, we view them as entirely complementary.

⁴ ΔG° and ΔS° refer to values at standard states, except that the proton activity is that of the pH of interest.

⁵ The fact that the product state is not bound may make the interpretation of the reorganization energy somewhat different from the usual, but it is only the shape of the repulsive part of the product surface that is important in determining the activation energy and thus the rate for these moderately exoergic reactions.

⁶ The somewhat weaker dependence than in Ru-TyrOH may be due to one or both of the following reasons. (i) The PSII reaction is close to equilibrium *ca.* $\text{pH} = 5\text{--}6$ ($\Delta G^\circ \approx 0$), so that the observed rate constant is given by the sum of the rate constants for the forward and backward reactions (standard reversible first-order kinetics). Since the pH dependence of the rates in the two directions most probably opposes each other, the pH dependence of the resulting sum is weaker than for the rate for the forward reaction. In Ru-TyrOH, the driving force is always high enough that the reverse reaction is negligible. (ii) From the data in Ahlbrink *et al.* (1998), it seems that the pH dependence of ΔG° (as calculated from the amplitudes of the equilibrium reaction) is somewhat weaker than in Ru-TyrOH. The reason may be weak electrostatic differences as pH varies. Thus, it seems that the dependence of the rate constant on ΔG° is in near quantitative agreement between Ru-TyrOH and Mn-depleted PSII, which is consistent with the CEP model.

⁷ Note that the situation is different in aprotic solvents. In these media the oxidized tyrosine cannot deprotonate, and the redox potential is high. With the addition of a base as proton acceptor, oxidized tyrosine can deprotonate and its redox potential is lower. In aprotic solvents, the addition of a base may therefore increase the driving force for proton-coupled electron transfer much more than in aqueous media.

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Discussion

A. Melis (*Department of Plant and Microbial Biology, University of California, Berkeley, CA, USA*). Could you say a few things about the absorbance properties of the Mn complex, i.e. absorbance coefficient and the band width of the complex in solution.

L. Hammarström. The manganese dimers in the lower oxidation states have very little absorption in the visible (a few hundreds of M⁻¹ cm⁻¹), one to two orders of magnitude lower than the absorption maximum of the ruthenium sensitizer. The absorption from the monomeric manganese(II) complexes is even weaker. Thus, direct photochemistry of the manganese is negligible.

C. Dismukes (*Department of Chemistry, Princeton University, Princeton, NJ, USA*). Have you done any tests to check that you are sensing the proton concentration in the medium?

L. Hammarström. Yes, we have checked that. Specifically, we tested different buffers and buffer concentrations, and addition of 0.05 M salt (KCl) to test for ionic strength effects. We also compared neat water with buffer at a pH of 7. The reaction rate was only dependent on

the pH, and not the identity or concentration of the buffer (up to at least 10 mM). Thus, initial deprotonation of TyrOH is far too slow to explain our data. Instead, the concerted electron-transfer-deprotonation mechanism explains the results, and the rate is dictated by the proton activity of the bulk (the pH).

P. L. Dutton (*Department of Biochemistry/Biophysics, University of Philadelphia, Philadelphia, PA, USA—visitor at Inorganic Chemistry Laboratory, Oxford, UK*). Following on from your answer to Professor Dismukes, have you looked at the work with photolyase where there is a pH dependence on the direct release of a proton from a tyrosine?

L. Hammarström. Aubert *et al.* (2000) followed the deprotonation of a light-generated tryptophan radical. The deprotonation rate was independent of pH, but they saw a faster deprotonation when the buffer concentration was increased (TRIS), with significant effects in the 20–100 mM region. Their conclusion was that the deprotonated TRIS became the dominating proton acceptor. The faster deprotonation would then be due to a (transient) formation of a Trp–Tris H-bond, in which proton transfer is faster than to water. This case is clearly different from ours; we do not see this effect.

M. Aukauloo (*Laboratoire de Chimie Inorganique, University of Paris-Sud, Paris, France*). Concerning your ruthenium(II)–dimanganese cluster, is it always feasible to photo-oxidize the manganese ions if electrochemically you actually see the oxidation processes of your manganese complex? An example from K. Wieghardt's laboratory on a ruthenium(II)–dimanganese complex, shows that contrary to electrochemical properties they couldn't photo-oxidize the manganese complex by more than one electron.

L. Hammarström. In the experiments they published, they used a reversible, external electron acceptor (methylviologen). This gives the electron back to the oxidized manganese on the 100 μs time-scale, so there is never any net oxidation at times longer than 1 ms after a flash. That is, each flash starts from scratch. In our experiments we use an irreversible Co(III) electron acceptor, so that we can accumulate oxidative equivalents on the manganese dimer.

R. D. Britt (*Department of Chemistry, University of California, Davis, CA, USA*). Do you see any sign of photoaccumulated Mn^{II}Mn^{III} in your system (by EPR)?

L. Hammarström. No, but we have made controls with the pre-oxidized Mn₂^{II,III}. In neat acetonitrile we see a well-resolved EPR spectrum, but with addition of the aqueous buffer (MES) it disappears. It seems that the effect is mainly due to MES. Experiments are just now being performed in Styring's lab to show that without MES we can see Mn₂^{II,III} being formed in the flash experiments. [This was confirmed over the telephone in the following discussion (authors note).]

M. Iwaki (*Department of Biology, University College London, London, UK*). Do we really need tyrosine in an artificial system you study or could you use other species?

L. Hammarström. We most probably need an intermediate between the photosensitizer and the manganese, for reasons that I discussed (see also our papers, e.g. Sun *et al.* (2001)). This does not necessarily have to be a tyrosine. However, we did choose that because it plays an

important role in photosystem II—maybe even in the actual catalytic steps of water oxidation, as was discussed yesterday—and we are also interested in modelling these reactions.

Additional reference

Aubert, C., Vos, M. H., Mathis, P., Eker, A. P. & Brettel, K. 2000 Intraprotein radical transfer during photoactivation of DNA photolyase. *Nature* **405**, 586–590.

GLOSSARY

CEP: concerted electron/proton transfer
ETPT: electron transfer followed by proton transfer
NHE: normal hydrogen electrode
PSII: photosystem II
PTET: proton transfer followed by electron transfer
Ru–TyrOH: Ru(bpy)₃²⁺–tyrosine complex
SCE: standard calomel electrode