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Pourbaix diagram, proton-coupled electron transfer and decay kinetics of a protein tryptophan radical: Comparing the redox properties of $W_{32}^{\cdot}$ and $Y_{32}^{\cdot}$ generated inside the structurally characterized α_3W and α_3Y proteins

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Abstract

Protein “hole” hopping typically involves spatially arranged redox-active tryptophan and/or tyrosine residues. Thermodynamic information is scarce for this type of process. The well-structured α_3W model protein was studied by protein film square wave voltammetry and transient absorption spectroscopy to obtain a comprehensive thermodynamic and kinetic description of a buried tryptophan residue. A Pourbaix diagram, correlating thermodynamic potentials (E°) with pH, is reported for W_{32} in α_3W and compared to equivalent data recently presented for Y_{32} in α_3Y (Ravichandran, K. R.; Zong, A. B.; Taguchi, A. T.; Nocera, D. G.; Stubbe, J.; Tommos, C. *J. Am. Chem. Soc.* **2017**, *139*, 2994–3004). The α_3W Pourbaix diagram displays a pK_{OX} of 3.4, a E° ($W_{32}(N^{\cdot+}/NH)$) of 1293 mV and a E° ($W_{32}(N^{\cdot}/NH)$; pH 7.0) of 1095 ± 4 mV versus the normal hydrogen electrode. $W_{32}(N^{\cdot}/NH)$ is 109 ± 4 mV more oxidizing than $Y_{32}(O^{\cdot}/OH)$ at pH 5.4–10. In the voltammetry measurements, W_{32} oxidation-reduction occurs on a timescale of about 4 ms and is coupled to the release/uptake of one full proton to and from bulk. Kinetic analysis further shows that W_{32} oxidation likely involves pre-equilibrium electron transfer followed by proton transfer to a water or small water cluster as the primary acceptor. A well-resolved absorption spectrum of $W_{32}^{\cdot}$ is presented and analysis of decay kinetics show that $W_{32}^{\cdot}$ persists $\sim 10^4$ longer than aqueous $W^{\cdot}$ due to significant stabilization by the protein. The redox characteristics of W_{32} and Y_{32} are discussed relative to global and local protein properties.

Graphical Abstract

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Notes

The authors declare no competing financial interest.

Supporting Information. Protein production; details of volt-ammetry and spectroscopic measurements; description of TA fitting routines; α_3W urea denaturation plots and ^{13}C -HSQC spectra; TA kinetics data for α_3W and W (aq); pK_{OX} , pK_{RED} , E° , G°_{ET} , G°_{PT} and G°_{CEPT} values modeled for W_{32} , Y_{32} , W (aq) and Y (aq) oxidation-reduction.

INTRODUCTION

Tryptophan (W) and tyrosine (Y) can serve as one-electron mediators in redox catalysis and in multistep “hole” hopping or proton coupled electron transfer (PCET) processes.^{1,2} The frequency of functional W/Y hole hopping/PCET chains remains to be established experimentally but may be quite significant.³ The thermodynamic landscape of protein ET is determined by the reduction potentials (E° 's) of the redox couples involved and how the surrounding protein environment influences these potentials. The molecular basis for tuning E° 's of metallo-cofactors has been the focus of much research effort and a range of mechanisms has been identified and characterized.^{e.g.4} In contrast, little is known regarding the thermodynamic properties of the less common amino-acid radical cofactors. This knowledge gap is due to the highly oxidizing potentials involved and the difficulty of probing the potential of a single specific protein residue. The literature contains only a handful of single-point E° or time-dependent “operational” potentials estimated from kinetic data.^{1,5} Few, if any, general trends can be extracted from the scattered and limited information available. This issue is addressed here.

The α_3X model protein system was designed to systematically map and compare the E° 's and PCET characteristics of W and Y radicals formed inside a structured protein. The 65-residue three-helix bundle (α_3) proteins contain a buried radical site (position 32), which is occupied by W_{32} (α_3W),^{6,7} Y_{32} (α_3Y),^{6,8,9} fluoro- Y_{32} (α_3F_nY , $n = 2, 3$)^{10,11} or 2-, 3- or 4-mercaptophenol (MP) ligated via a disulfide bond to C_{32} (2-, 3- or 4MP- α_3C).^{12,13} In this report we used the structurally characterized α_3W protein⁷ and protein film square-wave voltammetry (SWV)¹¹ to measure $E^\circ(W_{32})$ as a function of pH. We show that W is a “hot” protein hole-transfer agent and that there is a significant difference in the E° 's of W_{32} and Y_{32} when compared under highly similar experimental conditions. Additionally, flash-quench transient absorption (TA) methods were used to obtain high-quality spectra of $W_{32}^\cdot$ and to investigate the PCET kinetics of W_{32} oxidation and the stability of the $W_{32}^\cdot$ state. Experimental and modeled redox properties for W_{32} , Y_{32} , and aqueous (aq) W and Y are summarized and discussed relative to structural and dynamic properties of the α_3W and α_3Y proteins.

RESULTS

Protein film SWV characterization of α_3 W

A SWV study of α_3 Y revealed that the Y_{32} redox cycle is fully reversible and that true thermodynamic potentials can be determined for the $Y_{32}(O\cdot/OH)$ redox couple.¹⁴ Similar results were obtained for 2MP- α_3 C and $\alpha_3(3,5)F_2Y$.^{10,13} In subsequent work the α_3X /SWV approach was further refined to involve protein film samples. This method improved the Faradaic response significantly and facilitated studies of several very high potential α_3F_nY proteins.¹¹ Here we apply protein film SWV to α_3 W and, for the first time, obtain thermodynamic E° 's and a Pourbaix diagram for a protein tryptophan residue.

A SWV measurement consists of a train of forward (here oxidative) and reverse (reductive) potential pulses superimposed on a staircase potential ramp. The induced current is sampled at the end of each applied pulse and plotted against the staircase potential. The measurement generates a forward (I_{for} ; Figure 1 shows oxidative currents in orange), a reverse (I_{rev} ; reductive currents in purple) and a net ($I_{net} = I_{for} - I_{rev}$; blue) voltammogram. All voltammograms are displayed in units of μA . When using SWV to investigate electrode mechanisms and to determine thermodynamic and kinetic parameters, it is essential to evaluate the lineshapes and positions of all three components as a function of experimental settings.¹⁵ The Faradaic response of a strongly adsorbed redox pair is at its maximum when the frequency of the applied pulses (f ; s^{-1}) is close to the surface standard ET rate constant (k_{sur} ; s^{-1}).¹⁶ Under these conditions, I_{for} and I_{rev} are approximately equal in width and height, the maxima/minimum of the three individual currents (I_{for} , I_{rev} and I_{net}) are nearly coincident, and the peak potential of I_{net} (E_{net}) equals E° .¹⁷ To find these conditions for α_3 W, protein film SW voltammograms were collected as a function of f . The observed α_3 W response was poor below 120 Hz, reached a maximum in the 200–300 Hz region, and rapidly declined above 300 Hz. These results show that $200\ s^{-1} < k_{sur} < 300\ s^{-1}$ and a more detailed analysis was therefore conducted for this SW frequency range.

Figure 1 displays protein film voltammograms of α_3 W (in color) collected at 210, 240 and 270 Hz at pH 9.9 (panels A–C)), 8.4 (D–F) and 6.9 (G and H). There is no significant change in E_{net} as a function of f . $E_{rev} - E_{for}$ and I_{for}/I_{rev} values of $-8 \pm 4\ mV$ and 1.2 ± 0.2 , respectively, were observed across the whole data set. The average $E_{net}(210\text{--}270\ Hz)$ value shifts by $55 \pm 2\ mV/pH$ unit between $pH\ 6.93 \pm 0.09$ and 9.92 ± 0.06 . (panel I). The pH dependence implies that there is a net of approximately one proton released upon W_{32} oxidation and taken up upon subsequent reduction of the radical. In these experiments, the W_{32} redox cycle occurs within 4.8 (210 Hz) to 3.7 (270 Hz) ms. We conclude that α_3 W is reversibly oxidized and reduced in an overall charge-neutral manner and that $E_{net}(210\text{--}270\ Hz)$ represents the true thermodynamic potential of the $W_{32}(N\cdot/NH)$ redox couple.

I_{net} obtained from α_3Y protein films was consistently $\sim 2\text{--}4$ times greater than I_{net} obtained from α_3W protein film samples. This observation suggests a difference in the effective surface coverage, i.e. in the number of protein molecules electrically connected to the electrode. Y_{32} is centrally placed in the α_3 scaffold⁹ making the Y_{32} /electrode distance insensitive to the orientation of the protein in the film. In contrast, W_{32} is asymmetrically placed relative to the central axis of the protein.⁷ This is likely to result in a greater

distribution in the W_{32} /electrode distance and making some protein orientations electrochemically inactive.

$E^{\circ'}$ vs pH diagrams of α_3W and α_3Y constructed from protein film voltammetry and solution pH titration data

Figure 2 was made as follows: (i) $E^{\circ'}$ ($W_{32}(N\cdot/NH)$) was determined at pH 6.9, 8.4 and 9.9. Replica voltammograms ($n = 2-4$) were collected at three different SW frequencies (210, 240 and 270 Hz, Figure 1) for each pH point. This entire data set was then reproduced with independently prepared samples. The average $E^{\circ'}$ (1097 ± 4 , 1023 ± 2 and 933 ± 3 mV) vs pH (6.93 ± 0.09 , 8.39 ± 0.09 and 9.92 ± 0.06) values from these two data sets are shown as red circles. (ii) $E^{\circ'}$ s for $Y_{32}(O\cdot/OH)$ were reproduced from ref 11 (blue circles). (iii) All $E^{\circ'}$ s were obtained from protein films prepared from solution samples containing stable and structurally well defined α_3W and α_3Y molecules (Figures S1 and S2).⁶⁻⁹ (iv) The pH dependence of $E^{\circ'}$ (α_3W 55 ± 2 mV/pH unit; α_3Y 55 ± 1 mV/pH unit; Figure 1I) and the pK of oxidized W_{32} (pK_{OX} 3.4; *vide infra*) and reduced Y_{32} (pK_{RED} 11.3)^{6,18} were used to predict $E^{\circ'}$ s (α_3W green lines; α_3Y orange lines) outside the pH range investigated electrochemically (see Table S1 for details). (v) $W(aq)$ has a pK_{OX} of $4.2-4.3 \pm 0.1$.^{19,20} Incorporating a series of F_nY s at site 32 increased their pK_{RED} 's by 0.83 ± 0.06 (presumably by destabilizing the F_nY-O^- state).^{11,18} A protein-induced shift of similar magnitude is likely for α_3W (by destabilizing the W_{32}^{+} state) and a pK_{OX} of $4.25 - 0.83 = 3.4$ was predicted for W_{32} .

Redox kinetics of α_3W

The evolution and decay kinetics of oxidized W_{32} were followed by optical TA spectroscopy. In these experiments, a laser flash was supplied to α_3W samples containing a photosensitizer, $[Ru(L)_3]^{2+}$ ($L = 4,4'-R_2-2,2'$ -bipyridine, $R = H$ or CH_3) and a sacrificial oxidative quencher, $[Co(NH_3)_5Cl]^{2+}$. The laser flash-quench method generates the $[Ru(L)_3]^{3+}$ oxidant in situ, which subsequently oxidizes W_{32} . Figure S3 summarizes the chemical reactions that occur during the flash-quench photolysis process. Details regarding sample preparations and experimental settings are also provided in the SI.

As described above, the W_{32} redox cycle is coupled to proton release and uptake to/from bulk (at $pH > pK_{OX}$) and $W_{32}\cdot$ is stable on the experimental timescale of the SWV measurements (~ 4.8 ms as determined by the SW frequency). TA difference spectra obtained at pH 5.5 and 8.5 (Figures 3A and D) confirm the formation of a long-lived deprotonated $W_{32}\cdot$ state. $W\cdot$ and $W\cdot+$ (where $W\cdot+$ is the protonated form of the radical) display absorption maxima at ~ 510 and 560 nm, respectively.^{19,20} The absorption spectra of oxidized α_3W show a well-resolved peak with a maximum at 510 nm consistent with the formation of deprotonated $W_{32}\cdot$ at both pH 5.5 and 8.5. TA kinetic traces were collected at 450, 510 and 560 nm (Figures 3B and E) to monitor the decay of $[Ru(bpy)_3]^{3+}$, the formation and yield of $W_{32}\cdot$, and the possible formation of W_{32}^{+} , respectively. PCET rate constants (k_{PCET}) associated with $W_{32}\cdot$ formation were extracted from kinetic measurements as described in the SI (pages S3-4).

The amplitude of the positive signal at 510 nm was consistently ~ 4 times greater than at 560 nm in kinetic traces collected at pH 5.5 and 8.5, and in $W_{32}\cdot$ spectra collected at pH 5.5 and 8.5. This observation demonstrates that the positive intensity at 560 nm at short timescales (Figures 3B and E) is due to absorption in the red edge of the $W_{32}\cdot$ band and not from the accumulation of W_{32}^{+} . The positive amplitude at 510 nm was used to calculate $W_{32}\cdot$ and $W\cdot$ (aq) yields, which were ~ 1.0 for both systems. We note that the yields were obtained using an $\epsilon_{510}(W\cdot)$ of 2300^{20} rather than $1850^{19} \text{ M}^{-1} \text{ cm}^{-1}$. The latter value resulted in radical yields that were unrealistically high (i.e. significantly larger than 1.0). We conclude that the $2300 \text{ M}^{-1} \text{ cm}^{-1}$ value reported in ref 20 more accurately describes ϵ_{510} for a deprotonated $W\cdot$ radical.

Decay of the long-lived $W_{32}\cdot$ state was monitored at 510 nm (Figures 3C and F). $W_{32}\cdot$ and $W\cdot$ (aq) decay was dominated by a second-order process attributed to radical-radical dimerization (k_{dim}) at both pH 5.5 and 8.5. The predominance of a radical-radical coupling mechanism for decay was also observed for $Y_{32}\cdot$ and $Y\cdot$ (aq).⁹ A second minor decay pathway was observed for $W_{32}\cdot$, which we assign to $W_{32}\cdot$ reacting with a reduced α_3W substrate molecule (k_{sub}). Details regarding the curve fitting routines to determine rate constants k_{dim} and k_{sub} are given in the SI. Table 1 summarizes rate constants (k_{PCET} , k_{dim} , k_{sub}), $W_{32}\cdot$ yield and kinetic isotope effects (KIE) obtained from kinetic studies of W_{32} oxidation. Reference data were also collected on W (aq) (see Figure S7) and k_{PCET} , k_{dim} and $W\cdot$ yield are reported in Table 1.

Correlating data obtained from protein films and solution samples

The redox properties of α_3W and α_3Y have been characterized by protein film voltammetry and by solution methods including CD, NMR, and steady-state and transient absorption spectroscopy. Protein films are not individual proteins in solution and the structure of the protein in the film may not be identical to the structure of the protein in solution. Nevertheless, earlier work has shown that the α_3X proteins bind to the electrode surface in a reversible manner that is subject to the KCl concentration.^{13,14} Salt-dependent binding is consistent with a three-helix bundle fold⁶ where the apolar residues in the heptad *a* and *d* positions form the main part of the protein interior and the polar/charged residues in the remaining heptad positions form the exterior. The folded α_3 scaffold extends the radical lifetime (evident from the reversible protein film voltammograms and measured directly by TA for α_3W and α_3Y in solution) and shields X_{32} from chemical groups present on the electrode surface. The latter is evident by the fact that $E^\circ(X_{32}\cdot/X_{32})$ is independent of the material from which the working electrode is made.^{11,13,14}

DISCUSSION

Global and local protein effects on E° of W and Y

Figure 2 presents the absolute and relative E° 's of W_{32} and Y_{32} across a pH range that covers most biological redox processes. This data set provides a unique guide to predict how the thermodynamic profile of a radical/hole transfer chain will change when replacing a W with a Y , or *vice versa*. This statement is based on the following results: The Pourbaix diagrams are based on true thermodynamic potentials. This conclusion is evident from the

SWV responses of α_3W (Figure 1) and α_3Y ^{11,14} and consistent with the TA-monitored $X_{32}\cdot$ decay kinetics presented here (Table 1) and in ref 9. A major effect of the protein matrix is to effectively suppress radical side reactions and extend the lifetime of the $X_{32}\cdot$ state (seconds) well beyond the experimental timescale of the SWV measurements (ms). In contrast, there are large uncertainties in the potentials reported for the aqueous systems over the past few decades since irreversible radical side reactions compromise both pulse radiolysis and voltammetry measurements. Importantly, all SWV and TA studies are supported by extensive structural characterization. α_3W (RCSB PDB ID 1LQ7)⁷ and α_3Y (2MI7)⁹ form well-defined three-helix bundles at pH 5.5, as shown by solution NMR spectroscopy. W_{32} and Y_{32} are both buried (see Figure 4A for details).^{7,9} The two proteins remain stable (global stabilities of -3.7 to -4.0 kcal mol⁻¹ at pH 5–8.5 and -3.2 to -3.5 kcal mol⁻¹ at pH 8.5–10; Figure S1),^{8–10} helical ($51\text{--}52 \pm 1$ residues of 65 in total; pH 5–10)^{6–9} and well-folded (Figure S2)⁸ across a broad pH range. While there are minor structural differences between the W_{32} and Y_{32} sites (*vide infra*), the overall environments of W_{32} and Y_{32} are highly similar.

Table S1 summarizes experimental and modeled pK_{OX} , pK_{RED} , $E^\circ(X\cdot+/X)$ and E° (pH 7.0) values for W_{32} , Y_{32} , W (aq) and Y (aq). E° (pH 7.0) of $W_{32}(N\cdot/NH)$ and $Y_{32}(O\cdot/OH)$ equal 1095 ± 4 mV and 986 ± 3 mV, respectively. $E^\circ(W_{32}(N\cdot/NH) - Y_{32}(O\cdot/OH))$ is constant at $109 \text{ mV} \pm 4 \text{ mV}$ between pH 5.4 and 10 (Figure 2). Table S1 lists recent literature consensus E° (pH 7.0) values for aqueous W and Y .^{11,22} Comparing the E° (pH 7.0) values of α_3Y and α_3W to those of aqueous Y and W gives a difference of $\sim 45\text{--}65 \text{ mV}$ ¹¹ and $\sim 65 \text{ mV}$, respectively. Similarly, comparing the 1293 mV potential of $W_{32}(N\cdot+/NH)$ with the 1190 mV potential estimated for $W(N\cdot+/NH)$ (aq) yields a difference of $\sim 100 \text{ mV}$. Despite the uncertainties in the aqueous potentials, it appears clear that the protein milieu increases E° for both the neutral ($X\cdot/X$) and the cation ($X\cdot+/X$) redox pair. The latter result was expected due the energetic penalty of solvating a charge in the low dielectric protein medium. The protein-induced increase in $E^\circ(X_{32}\cdot/X_{32})$ for the overall charge-neutral α_3X redox proteins represents a more surprising result.

There is a small difference in the pH dependence of the α_3X potentials, $55 \pm 1\text{--}2 \text{ mV/pH}$ unit, relative to the theoretically predicted value of $\ln(10)RT/F = 59 \text{ mV/pH}$ unit at 25 °C. This observation was originally made for α_3Y and assigned to redox Bohr effects.¹⁴ Alternatively, it reflects a more global effect. The isoelectric point was determined to be ~ 8.0 for both α_3W and α_3Y by isoelectric gel electrophoresis (not shown). The minor deviation in the pH dependence relative to theory may result from a weak electrostatic influence on the $W_{32}(N\cdot/NH)$ and $Y_{32}(O\cdot/OH)$ redox pairs as the net charge of the α_3 scaffold transition from positive in the acid-neutral range of the Pourbaix diagram to negative in the alkaline region.

Global and local protein effects on the stability of $W\cdot$ and $Y\cdot$

The use of a sacrificial quencher in the TA measurements permitted the observation of $W_{32}\cdot$ and $W\cdot$ decay kinetics. Kinetic analysis shows that $W_{32}\cdot$ decays with intermolecular radical-radical and radical-substrate reactions as the major and minor pathways, respectively. Similarly, $Y_{32}\cdot$ decays via radical-radical dimerization.⁹ No evidence of intramolecular $W_{32}\cdot$

or $Y_{32}\cdot$ decay was observed. These observations show that the native state of the protein protects $X_{32}\cdot$ from reacting (Figure 4B and Figure 6 in ref 9). Furthermore, it is clear that protein dynamics, i.e. local, sub-global or global unfolding events, are involved in the decay of $W_{32}\cdot$ and $Y_{32}\cdot$. In other words, the protein transiently opens and exposes $W_{32}\cdot$, which then reacts irreversibly with a second transiently exposed $W_{32}\cdot/\alpha_3$ species (major decay pathway) or with a reduced α_3W molecule (minor decay pathway). For α_3Y , $Y_{32}\cdot-Y_{32}\cdot$ dimerization was the only detected radical decay pathway.⁹ These equilibrium structural fluctuations of the protein result in an apparent reduction in k_{dim} of $W_{32}\cdot$ relative to $W\cdot$ (aq) and is similar to that for $Y_{32}\cdot$ relative to $Y\cdot$ (aq) (both on the order of $10^4 \text{ M}^{-1} \text{ s}^{-1}$). Additionally, $k_{\text{dim}}(W_{32}\cdot)/k_{\text{dim}}(Y_{32}\cdot)$ and $k_{\text{dim}}(W\cdot)/k_{\text{dim}}(Y\cdot)$ both equal 1 and 0.4 at the pH 5.5 and 8.5, respectively (Table 1). These results suggest that the decays of $W_{32}\cdot$ and $Y_{32}\cdot$ are both subject to the same dynamic event(s) in the protein. NMR studies to investigate these issues further are in progress (Glover, Valentine, Liang, Wand, Tommos, on-going).

Radical formation in solvated α_3W

W_{32} is oxidized to a neutral radical with water as the primary proton acceptor. This conclusion is based on the constant k_{PCET} between pH 5.5 and 8.5 (i.e. OH^- or phosphate buffer ions are not significant) and on the structural features of the W_{32} site. The very minor solvent exposure of W_{32} (with an average solvent accessible surface area (SASA) of $< 4\%$) is localized to the indole edge of the aromatic ring and centered at the indole hydrogen (white labels display atom-level SASAs in Figure 4A). There are no basic side chains in close vicinity to the W_{32} indole hydrogen. Nonetheless, the SWV data described above show that redox-driven proton release and uptake occur within a few ms. Likewise, formation of the neutral $W_{32}\cdot$ radical was observed by TA spectroscopy to occur on a ms time scale (Figure S5). Thus, the indole proton is most likely connected to the bulk phase either by water penetration or by protein fluctuations.

Setting aside the precise mechanism by which the indole group contacts water, the oxidation mechanism is either (a) pre-equilibrium ET followed by proton transfer (ETPT) or (b) pre-equilibrium concerted electron-proton transfer (CEPT) followed by escape of the proton from the primary water cluster to the bulk. A pre-equilibrium model predicts, for both case (a) and (b), a tenfold increase in k_{PCET} for each 59 mV increase in oxidant strength. This prediction is in very good agreement with the observed 660 times increase in k_{PCET} when using $[\text{Ru}(\text{bpy})_3]^{3+}$ relative to $[\text{Ru}(\text{dmb})_3]^{3+}$ as the flash-quench oxidant (160 meV difference in driving force; Table S1). Additionally, G_{ET}° is predicted to be 35 and 195 meV uphill for $\alpha_3W/[\text{Ru}(\text{bpy})_3]^{3+}$ and $\alpha_3W/[\text{Ru}(\text{dmb})_3]^{3+}$, respectively (Table S1). The observed KIE (Table 1) arises from the PT step in case (a) or from the CEPT step itself in case (b).

We favor case (a) the ETPT mechanism. Following Krishtalik's argument,²⁶ CEPT to a small water cluster is much more uphill than ET, because of the unfavorable transfer of a proton from $W_{32}\cdot$ ($\text{p}K_{\text{OX}} = 3.4$) to a small water cluster ($\text{p}K_{\text{a}}(\text{H}_3\text{O}^+/\text{H}_2\text{O}) = 0$). With "small water cluster" we are referring to the molecular description of a proton released into bulk water. Here the primary PT is always to a small water cluster and is followed by thermal transfer to new water clusters. These clusters break up and reform on a 10's of fs

time scale.²⁷ Modeling predicts a G°_{ET} of 35 meV, G°_{PT} of 200 meV and a G°_{CEPT} of 235 meV for the $\alpha_3\text{W}/[\text{Ru}(\text{bpy})_3]^{3+}$ system (Table S1). One caveat to this argument is results obtained on linked $\text{Ru}(\text{bpy})_3$ -tryptophan model systems that show that CEPT nevertheless can be competitive.²⁸ The moderate KIE's observed for W_{32} oxidation are not evidence for a CEPT reaction. They may be due to an isotope effect on the position of the initial ET equilibrium or the follow-up PT step from the $\text{W}_{32}\cdot^-$ (small water cluster) intermediate. A study on $\text{Ru}(\text{bpy})_3$ -linked bromo-W reported KIE of 2 for a reaction attributed to ETPT.²⁹

Most likely, W_{32} and W (aq) oxidation occur via ETPT while Y_{32} and Y (aq) oxidation occur via a CEPT or PTET mechanism.⁹ The $k_{\text{PCET}}(\text{W}/\text{W}_{32})$ ratio is much larger than $k_{\text{PCET}}(\text{Y}/\text{Y}_{32})$: 3000 vs 200 at pH 5.5 (Table 1). The protein has a more pronounced effect on the kinetics of W oxidation relative to the kinetics of Y oxidation. Modeling suggests significant differences in the G°_{ET} step of W_{32} and W (aq) oxidation (30 vs -65 meV; Table S1) in contrast to the G°_{CEPT} step of Y_{32} and Y (aq) oxidation (100 vs 85 meV; Table S1). The decrease in $\text{p}K_{\text{OX}}$ and increase in $E^{\circ}(\text{W}(\text{N}\cdot+/\text{NH}))$ induced by the low dielectric protein milieu slows down ETPT. Nevertheless, $E^{\circ}(\text{W}_{32}(\text{N}\cdot+/\text{NH}))$ is still much lower than $E^{\circ}(\text{Y}_{32}(\text{O}\cdot+/\text{OH}))$ (1293 vs 1510 mV; Table S1). This situation favors an ETPT mechanism via the protonated radical intermediate in $\alpha_3\text{W}$, instead of CEPT or PTET as in $\alpha_3\text{Y}$. Thus, while E° (pH 7.0) is clearly greater for $\alpha_3\text{W}$ than $\alpha_3\text{Y}$, the much greater $\text{p}K_{\text{OX}}$ values of W_{32} (3.4 vs -2.5; Table S1) stabilizes the $\text{W}_{32}\cdot+$ intermediate relative to $\text{Y}_{32}\cdot+$, and enables a different oxidation mechanism.

Summary and future perspectives

The $\alpha_3\text{X}$ model protein system was made to gain a better understanding of how natural proteins effectively manage amino-acid based redox reactions and long-range radical/hole transfer. These types of reactions are integral to many important processes in biology.¹⁻³ The $\alpha_3\text{W}$ data presented here, combined with other recent $\alpha_3\text{X}$ studies,^{9,11,14} allowed us to start formulating a detailed description of key similarities and differences in the redox properties of W and Y when residing in a highly similar protein milieu. These are the main observations: $\alpha_3\text{W}$ and $\alpha_3\text{Y}$ each contain a redox-active aromatic residue at position 32. W_{32} and Y_{32} exhibit an average SASA of <4 and 0% and a residue depth of 5.9 ± 0.3 and 7.7 ± 0.3 Å, respectively. SWV studies showed that W_{32} and Y_{32} can be oxidized and reduced in a reversible manner. Their redox cycles are charge neutral with ET (between X_{32} and the working electrode) and coupled protonic reactions (between $\alpha_3\text{X}$ and the bulk phase) occurring on the time-scale of 2–4 ms. The combination of redox reversibility and broad structural pH stability allowed Pourbaix diagrams to be obtained for W_{32} and Y_{32} . This unique data set revealed that the protein matrix increases E° 's for the ($\text{X}_{32}\cdot^-/\text{X}_{32}$) and ($\text{X}_{32}\cdot+/\text{X}_{32}$) redox couples by about 45–65 and 100 mV, respectively, relative to the corresponding redox couples of aqueous W and Y . A small but statistically significant change in the slope of the $\alpha_3\text{X}$ Pourbaix plots relative to theory indicates that the net charge of the $\alpha_3\text{X}$ proteins has a weak influence on the E° 's of the ($\text{X}_{32}\cdot^-/\text{X}_{32}$) redox couples. Overall, W_{32} and Y_{32} are very hot protein oxidants with E° (pH 7.0) of 1095 ± 4 mV and 986 ± 3 mV, respectively. $\text{W}_{32}(\text{N}\cdot+/\text{NH})$ is 109 ± 4 mV more oxidizing than $\text{Y}_{32}(\text{O}\cdot+/\text{OH})$ at pH 5.4–10. This difference gradually decreases as the pH approaches either the $\text{p}K_{\text{OX}}$ of

W_{32} (3.4) or the pK_{RED} of Y_{32} (11.3). TA spectroscopy, using a flash-quench generated $[\text{Ru}(\text{bpy})_3]^{3+}$ oxidant, was employed to monitor the kinetics of $W_{32}^\cdot$ and $Y_{32}^\cdot$ formation resulting in radical yields of ~ 1.0 and $0.3\text{--}0.6$, respectively. High-quality absorption spectra of $W_{32}^\cdot$ and $Y_{32}^\cdot$ have been obtained. Kinetic analyses suggested that W_{32} is oxidized via a pre-equilibrium ETPT path while Y_{32} oxidation follows a CEPT, or possibly PTET, mechanism. Water or a small water cluster serves as the primary proton acceptor for $W_{32}^\cdot$. Water and/or buffer serve as the primary proton acceptor for $Y_{32}^\cdot$. In light of these latter results, it is interesting that the predicted distances between the indole N and the phenol O to the closest bulk water are 4.1 ± 0.4 and 6.3 ± 0.4 Å, respectively. The protein matrix stabilizes the $X_{32}^\cdot$ state by effectively blocking radical migration (E° of $X_{32} \ll E^\circ$ of all other residues in the α_3 scaffold) and radical-radical recombination (major decay pathway of aqueous $W^\cdot$ and $Y^\cdot$). Equilibrium structural fluctuations in the protein matrix are likely to be involved in both the PCET of radical formation (connecting X_{32} to the primary proton acceptor) and decay (connecting two $X_{32}^\cdot$ residues). These observations set the stage for future investigations aimed at delineating the role of the protein ensemble on PCET and radical-stabilization reactions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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18. The apparent pK of reduced Y_{32} ($pK_{RED} = 11.3$)⁶ is above the isoelectric point of the protein ($pI \sim 8$) while the apparent pK_{RED} 's of three different F_nY_{32} residues (7.2 – 8.0)¹¹ are slightly below or at the pI . The net negative charge induced at the alkaline conditions used for the pH titration of α_3Y increases the pK_{RED} of Y_{32} ($pK_{RED} + 1.3$ relative to $Y(aq)$) as compared to the pK_{RED} 's of the F_nY_{32} residues ($pK_{RED} + 0.83 \pm 0.06$ relative to the corresponding $F_nYs(aq)$).
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Figure 1.

Panels (A) to (H) display background-corrected protein film voltammograms of α_3 W (in color) and α_3 Y (I_{net} black; reproduced from ref 11) collected using a SW frequency of 210, 240 and 270 Hz at pH 9.9 (top row), 8.4 (middle) and 6.9 (bottom). Each voltammogram was obtained by submerging the tip of the freshly polished working electrode in protein solution for 30 s, transferring the electrode to the buffer-containing electro-chemical cell, and initiating the measurement (see SI for experimental details). Panel (I) plots that the average E_{net} (210–270 Hz) for $\text{W}_{32}(\text{N}/\text{NH})$ (red) and $\text{Y}_{32}(\text{O}/\text{OH})$ ¹¹ (blue) vs pH. The α_3 scaffold is redox inert to at least 1.5 V vs NHE and does not produce a Faradaic current.^{8,11–13}

Figure 2.

Pourbaix plots of α_3 W and α_3 Y. Thermodynamic potentials were obtained by protein film SWV (α_3 W red circles, Figure 1; α_3 Y blue circles, reproduced from ref 11). pK_{OX} and pK_{RED} values were derived from solution pH titrations, as described in the main text.

Figure 3.

$W_{32}\cdot$ absorption spectra, formation kinetics, and decay kinetics at pH 5.5 (top row) and 8.5 (bottom row). (A and D) TA difference spectra of $W_{32}\cdot$ recorded after a LED excitation pulse of samples containing 500–510 μM α_3W , 35 μM $[Ru(bpy)_3]^{2+}$ and 4 mM $[Co(NH_3)_5Cl]^{2+}$. (B and E) TA difference kinetic traces recorded at 450 (green), 510 (light-orange) and 560 (red-orange) nm following a 7 ns laser pulse. The fits (black) were used to determine PCET rate constants (450, 510 and 560 nm) and $W_{32}\cdot$ yield (510 nm). (C and F) $W_{32}\cdot$ decay kinetics recorded after a LED pulse at 510 nm for different initial concentrations of $W_{32}\cdot$ (pH 5.5: 8, 11 and 26 μM ; 2 mm probe path) and (pH 8.5: 3, 12, 38 and 55 μM ; 4 mm path).

Figure 4.

Properties of the W₃₂ site in α_3 W. (A) The W₃₂ residue is wedged into the hydrophobic core of the protein with an average SASA²³ of < 4% (1–7% across the NMR structural ensemble).⁷ The indole N and H atoms have an average SASA of 4% and 34%, respectively. Two additional ring hydrogens exhibit a SASA of 8% and 4%, while all other W₃₂ atoms are completely buried. A residue depth analysis²⁴ of the α_3 W NMR structure predicts a depth of 5.9 ± 0.3 Å (W₃₂, all atoms), 5.6 ± 0.3 Å (side chain atoms) and 4.1 ± 0.4 Å (indole N). As a comparison, Y₃₂ has no effective SASA ($0.2 \pm 0.2\%$) and an average depth of 7.7 ± 0.3 Å (all atoms), 8.1 ± 0.4 Å (side chain atoms) and 6.3 ± 0.4 Å (phenol O).⁹ The SASA and residue depth analyses differ in that SASA reflect the average solvent accessibility of a specific residue or atom, while the depth describes the closest distance between a specific residue or atom to bulk solvent. Four surface residues, E₁₃, E₃₃, K₂₉ and K₃₆, contain atoms that are within 4 Å of the indole N. None of the carboxylic oxygen atoms are close enough to form a direct H-bond with the indole H. The shortest carboxyl O/indole H distance is 5.6 ± 1.1 Å. Figure (B) models the radical state of α_3 W. The aromatic ring is colored to illustrate the spin-density distribution of W $\cdot$ with high positive spin densities at the indole N (blue) and at the CG, CE and CH (orange) atoms.²⁵ Residues shown in green have atoms

that are within 4 Å of the spin centers and they are all aliphatic. Structural depictions were prepared using PyMOL Molecular Graphics System, Version 1.7 (Schrödinger, LLC).

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$W_{32} \cdot$ and $W \cdot$ (aq) formation and decay kinetics and ratios of k_{PCET} and k_{DECAY} for W_{32} , Y_{32} , W (aq) and Y (aq)

Table 1

System	pL	$k_{\text{PCET}} (\text{M}^{-1} \text{s}^{-1}) (a)$	$k_{\text{sub}}, k_{\text{dim}} (\text{M}^{-1} \text{s}^{-1})$	yield $W_{32} (d)$	KIE
$W_{32}/[\text{Ru}(\text{bpy})_3]^{3+}$	pH 5.5	$(3.5 \pm 0.5) \times 10^5$	$(b)(0.2 \pm 0.05) \times 10^4, (c)(1.6 \pm 0.2) \times 10^4$	0.8 ± 0.1	2.2 ± 0.4
	pD 5.5	$(1.7 \pm 0.4) \times 10^5$	–	1.1 ± 0.1	
	pH 8.5	$(4.6 \pm 0.9) \times 10^5$	$(b)(1.6 \pm 0.3) \times 10^2, (c)(42 \pm 4) \times 10^2$	0.9 ± 0.2	3.1 ± 0.5
	pD 8.5	$(1.8 \pm 0.2) \times 10^5$	–	0.8 ± 0.1	
$W_{32}/[\text{Ru}(\text{dmb})_3]^{3+}$	pH 5.5	$(5.3 \pm 1.1) \times 10^2$	–	–	1.4 ± 0.2
	pD 5.5	$(3.8 \pm 0.6) \times 10^2$	–	–	
$W/[\text{Ru}(\text{bpy})_3]^{3+}$	pH 5.5	$(1.2 \pm 0.2) \times 10^9$	$(c)(1.2 \pm 0.2) \times 10^9$	1.2 ± 0.2	–
	pH 8.5	$(1.4 \pm 0.2) \times 10^9$	$(c)(8.5 \pm 1.0) \times 10^8$	1.2 ± 0.2	–
Ratio (k_{PCET})	pH 5.5	pH 8.5	Ratio (k_{DECAY})	pH 5.5	pH 8.5
$k_{\text{PCET}}(W/W_{32})$	3×10^3	3×10^3	$k_{\text{DECAY}}(W/W_{32})$	7×10^4	20×10^4
$k_{\text{PCET}}(Y/Y_{32})$	0.2×10^3	1×10^3	$k_{\text{DECAY}}(Y/Y_{32})$	1×10^4	6×10^4
$k_{\text{PCET}}(W_{32}/Y_{32})$	30	3	$k_{\text{DECAY}}(W/Y)$	1	0.4
$k_{\text{PCET}}(W/Y)$	500	7	$k_{\text{dim}}(W_{32})/k_{\text{dim}}(Y_{32})$	1	0.4

(a) From Figures S5, S7B and S8B.

(b) k_{sub} , bimolecular reaction between $W_{32} \cdot$ and a reduced $\alpha_3 W$.

(c) k_{dim} , $W_{32} \cdot$ – $W_{32} \cdot$ dimerization.

(d) Relative to the $[\text{Ru}(\text{bpy})_3]^{2+}$ yield (6–10 μM /flash) and using an $\epsilon 510(W_{32})$ of $2300 \text{ M}^{-1} \text{ cm}^{-1}$ and an $\epsilon 510([\text{Ru}(\text{bpy})_3]^{2+})$ of $-2600 \text{ M}^{-1} \text{ cm}^{-1}$.