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Photogeneration and Quenching of Tryptophan Radical in Azurin

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Abstract

Tryptophan and tyrosine can form radical intermediates that enable long-range, multistep electron transfer (ET) reactions in proteins. This report describes the mechanisms of formation and quenching of a neutral tryptophan radical in azurin, a blue-copper protein that contains native tyrosine (Y108 and Y72) and tryptophan (W48) residues. A long-lived neutral tryptophan radical W48• is formed upon UV-photoexcitation of a zinc(II)-substituted azurin mutant in the presence of an external electron acceptor. The quantum yield of W48• formation (Φ) depends upon the tyrosine residues in the protein. A tyrosine-deficient mutant, Zn^{II}Az48W, exhibited a value of $\Phi = 0.080$ with a Co(III) electron acceptor. A nearly identical quantum yield was observed when the electron acceptor was the analogous tyrosine-free, copper(II) mutant; this result for the Zn^{II}Az48W:Cu^{II}Az48W mixture suggests there is an interprotein ET path. A single tyrosine residue at one of the native positions reduced the quantum yield to 0.062 (Y108) or 0.067 (Y72). Wild-type azurin with two tyrosine residues exhibited a quantum yield of $\Phi = 0.045$. These data indicate that tyrosine is able to quench the tryptophan radical in azurin.

Graphical abstract

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Supporting Information: Absorption spectra, difference spectra, UVRR spectra, proposed intermolecular ET path, and pK_a titrations. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Introduction

Electron transfer (ET) reactions are essential to life. In proteins, long-range ET is made possible by a variety of redox-active molecules that are critical for overcoming the large kinetic barriers of charge separation over long distances.^{1–3} For example, the light reactions of photosynthesis involve multiple electron transport events facilitated by metal cofactors, including hemes and iron–sulfur clusters, as well as organic molecules, such as quinones and amino acids. One essential property of the organic ET cofactors is their ability to form radicals with relative ease. While the reactivity and stability of quinone and flavin radicals have been studied extensively, far less is known about amino acid radicals (e.g., cysteine, tryptophan, and tyrosine) that are essential for multistep ET reactions in enzymes such as photosystem II, ribonucleotide reductase, and DNA photolyase.^{4–10}

Aromatic amino acids form both charged and uncharged radical intermediates, and their oxidation reactions are described broadly as proton-coupled electron transfer (PCET) mechanisms in which ET and proton transfer (PT) occur in concerted or coupled stepwise manners. Cation radicals have been observed in biological systems, and in some cases, enzymes have evolved to stabilize these high-energy charged species. For example, the tryptophan cation radical has been reported for yeast cytochrome *c* peroxidase, DNA photolyase, and lignin peroxidase.^{11–16} In other cases, charged organic radical intermediates are avoided via rapid or concerted PT. The formation of the neutral tyrosine radical in PSII is one such example where only the neutral intermediate has been observed.⁸ PCET is more complicated than isolated ET for reasons that include the wide range of time scales that are involved, quantum mechanical aspects of both electron and proton transport, and the coupling among solvent, electron, and proton.^{17–21} The theoretical classification of a particular redox event as concerted or stepwise PCET is not straightforward,¹⁷ and experimental data are needed to help define the mechanism. The oxidation of tryptophan is generally considered a stepwise PCET event, and both neutral and cation radicals have been observed. In contrast, the tyrosine radical has only been observed in neutral form, and the evidence gathered so far is consistent with a concerted PCET mechanism.^{22–24}

The generation and characterization of amino acid radicals in proteins are challenging tasks because of the transient and unstable nature of these intermediates. We and others have focused on azurin from *Pseudomonas aeruginosa* as a model system for studying ET reactions that involve aromatic amino acids and the metal center or labels.^{25–28} This 14 kDa protein is a type I blue copper protein with a characteristic ligand-to-metal charge transfer (LMCT) band centered at 628 nm. Recently, our group reported photoinduced formation of a long-lived neutral tryptophan radical at position 48 that was accompanied by simultaneous reduction of Cu(II) to Cu(I) in a tyrosine-deficient azurin mutant.²⁸ The surprising stability of this neutral tryptophan radical in azurin allowed us and others to characterize its absorption, resonance Raman, and EPR spectra, and to compare these spectra to another stable tryptophan radical generated at position 108.^{27–30} In these prior reports, the mechanism of formation of W48• was hypothesized to be an intramolecular process. Here, we present evidence that both intra- and intermolecular charge transfer events take place.

We have also investigated the role of tyrosine residues in the formation and quenching of the tryptophan radical in azurin. The topic is of importance because a “wire” of aromatic residues within a protein would provide a viable path for long-range ET in the absence of metal cofactors. Tyrosine-to-tryptophan ET has been investigated for small model peptides or model compounds,^{22,31–33} but reports are rare for this kind of ET in larger protein systems. One reason for the lack of systematic study is that, despite the favorable thermodynamics of this reaction, only a few enzymes have been proposed to utilize a tyrosine-to-tryptophan path, including photolyase, cryptochrome, and ribonuclease reductase.^{4,34,35} In the present study of azurin, we probe the role of tyrosine residues in reducing a transient tryptophan cation radical. These results are discussed in the context of the local environment near the tyrosine residues. Our interest stems from the potential to utilize tyrosine and tryptophan as intermediates in engineered long-range ET reactions as a mechanism for stabilizing long-lived, charge-separated states.

Materials and Methods

Materials were purchased from Fisher Scientific and Sigma-Aldrich, and were used as received unless otherwise noted.

Sample Preparation

Expression, isolation, and purification of *Pseudomonas aeruginosa* azurin samples were carried out as previously described.^{25,26} Wild-type (WT) azurin, which contains W48+Y72+Y108, and four mutants were generated; all mutants studied here are stable and were handled at room temperature. The mutants include azurin with a single tryptophan residue at the native position 48 and no tyrosine residues [Y72F/Y108F], azurin with a single tryptophan at position 48 and a single tyrosine residue at position 72 [Y108F] or 108 [Y72F], and azurin without the native tryptophan or tyrosine residues [W48F/Y72F/Y108F]; these mutants are referred to as **XAz48W**, **XAz48W/72Y**, **XAz48W/108Y**, and **XAzFFF**, respectively, where X = Cu^{II}, Zn^{II}, or Cu^I metal center. Wild-type is denoted **XAzWT**.

The appropriate metal center was inserted into the wild-type and mutants following extraction of copper with cyanide, as described previously.³⁶ After initial purification via fast-protein liquid chromatography (FPLC), the sample contained a mixture of Cu^{II}- and Zn^{II}-azurin; removal of the metal from the protein was achieved by dialysis in a cyanide solution that contained 400 mM potassium cyanide and 100 mM potassium phosphate at pH 8.0 or 500 mM potassium cyanide and 200 mM carbonate buffer at pH 9.0. The sample was dialyzed for 4 h and then placed in a fresh cyanide solution for another 4 h. After this second dialysis, the sample was placed in 500 mL of 100 mM potassium phosphate pH 8.0 buffer three times for 4 h each to remove the cyanide from the protein solution. The metal-free, apoazurin samples were then exchanged into 10 mM Tris buffer, pH 7.4, for 4 h, and finally reconstituted with 7.5 mM CuSO₄ or ZnSO₄ in 50 mM Tris-HCl buffer, pH 7.5, two times for 4 h each to make high purity Cu(II)- or Zn(II)-azurin samples **Cu^{II}AzWT**, **Cu^{II}Az48W**, **Cu^{II}Az48W/72Y**, **Cu^{II}Az48W/108Y**, **Cu^{II}AzFFF**, **Zn^{II}AzWT**, **Zn^{II}Az48W**, **Zn^{II}Az48W/72Y**, and **Zn^{II}Az48W/108Y**. This method resulted in >99% purity in terms of metal content as assessed by inductively coupled plasma optical emission spectrometry (ICP-OES).

Cu^IAz48W and **Cu^IAzFFF** were prepared by placing a $\sim 1 \times 1 \text{ cm}^2$ platinum mesh into a solution of Cu^{II}Az48W or Cu^{II}AzFFF that was deoxygenated with five pump/N₂ purge cycles. This sample was stirred for 12–15 h under H₂(g), and thorough reduction of Cu^{II} to Cu^I was evident from the disappearance of the characteristic LMCT band of Cu^{II}-azurin by UV–vis spectroscopy. All azurin samples were stored as 0.5–2 mM stock Cu- or Zn-azurin in 50 mM sodium acetate, pH 4.5, buffer.

Photolysis experiments were performed on azurin samples prepared from stock solutions of pure Cu- or Zn-azurin. For experiments that utilized exogenous electron acceptors, an appropriate aliquot of 5 mM [Co^{III}(NH₃)₅Cl]Cl₂ or 2 mM [Ru^{III}(NH₃)₆]Cl₃ in 20 mM phosphate buffer, pH 7.2, was added to each sample of Cu- or Zn-azurin. The 1.2 mL protein + acceptor sample was placed in a round-bottom flask attached to atmosphere-controlled $2 \times 10 \text{ mm}^2$ or $4 \times 10 \text{ mm}^2$ cuvettes and deoxygenated with repeated pump/purge cycles using N₂ or N₂O gas to backfill the cuvette. Final protein concentrations were 50–140 μM in 20 mM potassium phosphate buffer, pH 7.2 (in a final volume of 0.6–1.0 mL), and the protein:quencher ratio ranged between 1:4 and 4:1.

Photolysis of Azurin Mutants

White light from a CeraLux 175 W xenon arc lamp (Luxtel LLC, Danvers, MA) was directed into a monochromator set at 292 nm and spectral bandpass of 10 nm; this center wavelength was selected to minimize photolysis of tyrosine residues. The output beam was approximately 4 mm (width) $\times$ 12 mm (height). Samples were placed as close as possible to the exit slit and photolyzed along the 2 or 4 mm path of the cuvette using a power of 500–900 μW . The total photolysis time varied from 15 to 45 min, and absorption spectra were typically collected after 3, 5, 10, 15, 20, 25, 30, 35, 40, and 45 min of photolysis. Samples were continuously stirred by a microstir bar, and the 2 mm cuvette was additionally gently shaken by hand approximately every 30 s. Manual shaking was not needed for the 4 mm cuvette.

Absorption Spectroscopy

Absorption spectra were obtained using a UV–vis–NIR spectrophotometer (Shimadzu UV-3600) with a 1 nm spectral bandwidth. The corrected difference absorption spectrum of each sample was determined by subtracting a pre-photolysis scan from the post-photolysis spectrum. Pre-photolysis concentrations of azurin that contained Cu^{II} were determined on the basis of a 628 nm LMCT molar absorption coefficient of $5900 \text{ M}^{-1} \text{ cm}^{-1}$. The molar absorption coefficient at 280 nm was utilized to determine concentrations of all samples that did not exhibit this strong LMCT band, as well as to calculate the fraction of light absorbed by the sample. Relevant values for ϵ_{280} (in units of $\text{M}^{-1} \text{ cm}^{-1}$) are as follows: 550 for [Co^{III}(NH₃)₅Cl]²⁺, 300 for [Ru^{III}(NH₃)₆]³⁺, 6690 for Cu^{II}Az48W and Zn^{II}Az48W, 10520 for Cu^{II}WT and Zn^{II}AzWT, 9450 for Cu^{II}Az48W/72Y and Zn^{II}Az48W/72Y, 7940 for Cu^{II}Az48W/108Y and Zn^{II}Az48W/108Y, and 9110 for Cu^IAz48W. The concentrations of Cu^{II}AzFFF and Cu^IAzFFF were determined using ϵ_{265} values of 2470 and 4760 $\text{M}^{-1} \text{ cm}^{-1}$, respectively. These 280 and 265 nm absorption coefficients of the azurin mutants were based on the value of $5900 \text{ M}^{-1} \text{ cm}^{-1}$ for the Cu^{II} peak as well as published spectra of Cu^I and Cu^{II} azurin.^{37–39} The absorption coefficients of [Co^{III}(NH₃)₅Cl]²⁺ and [Ru^{III}(NH₃)₆]³⁺ were

determined using the Beer–Lambert law. Absorption spectra of Cu^{II}AzWT, Cu^{II}Az48W, Cu^{II}Az48W/72Y, Cu^{II}Az48W/108Y, and Cu^{II}AzFFF as well as difference spectra that reflect the isolated absorption profiles of Y72, Y108, and W48 are shown in the Supporting Information. Reduction of [Co^{III}(NH₃)₅Cl]²⁺ creates a [Co^{II}(H₂O)₆]²⁺ photoproduct that has negligible absorption in the spectral regions of interest; therefore, small changes of this acceptor concentration were neglected. Spectra of the Co^{III} and Co^{II} species as well as a model tryptophan compound are shown in the Supporting Information; the Co^{II} species was generated by dissolving Co(Cl)₂ in water.

Ultraviolet Resonance Raman Spectroscopy

Instrumentation for ultraviolet resonance Raman spectroscopy has been described in detail elsewhere.⁴⁰ Briefly, a 1 kHz Ti:sapphire laser provided >1 W average power at 912 nm; the fundamental was directed through a lithium triborate (LBO) crystal, and the resulting 456 nm beam was then passed through a β -barium borate (BBO) crystal to produce >8 mW of 228 nm UV excitation. This wavelength was found to be optimal for enhancement of tryptophan and tyrosine residues. The power at the sample was ~3 mW. Scattered light was collected in a ~135° backscattering geometry and focused onto the entrance slit of a prism-based prefilter. Raman scattered light was dispersed in a 0.5 m spectrograph equipped with a 3600 gr/mm grating and imaged onto a CCD detector. The spectral response was determined by a deuterium lamp. The bandpass of the spectrograph was less than 15 cm⁻¹, and the accuracy of peak assignments was ~2 cm⁻¹, as determined from ethanol calibration spectra. Previous UVRR power dependence studies of azurin indicated that the current experiments were performed under conditions of linear response (data not shown).

Samples were pumped through a vertically oriented, 100 μ m i.d. quartz microcapillary at a linear flow rate of 340 μ m/ms to ensure a fresh sample for each laser pulse, and discarded after a single pass through the laser beam to eliminate artifacts from photolyzed sample. Fifteen-minute UVRR spectra were collected for Cu^{II}Az48W, Cu^{II}Az48W/108Y, Cu^{II}Az48W/72Y, Cu^{II}AzWT, and L-tyrosine. The azurin concentration was 50 μ M, and samples were contained in 20 mM phosphate (H₂O) at pH 7.2 or 20 mM phosphate (D₂O) at pD 7.6. Spectra of buffer-only solutions were also acquired and subtracted from corresponding azurin spectra. Center frequencies for the overlapping Y8a/Y8b modes in the difference spectra of azurin mutants were determined by Gaussian decomposition of difference spectra.

Potassium Ferrioxalate Actinometry

In order to determine the number of photons impinging on a sample, a potassium ferrioxalate actinometric system was used.^{41,42} A 500 μ L solution of 0.006 M potassium ferrioxalate in 0.05 M H₂SO₄ was irradiated in a 2 $\times$ 10 mm² cuvette along the 2 mm path with 850 μ W of UV light centered at 292 nm (spectral bandpass of 10 nm). Irradiation times ranged from 1 to 2 min, and the solution was shaken by hand every 30 s while being mixed with a stir bar. A 200 μ L aliquot of this irradiated solution was added to 800 μ L of 0.1% phenanthroline in 0.5 M H₂SO₄, and an absorption spectrum was measured. The absorbance at 510 nm was measured and a calculated quantum yield was compared to the published quantum yield of 1.24 for 297/302 and 313 nm excitation.^{41–43} These actinometry

experiments revealed a correction factor of 1.04 for the power measured at the exit slit by a calibrated Ophir power meter and photodiode head (PD-300UV).

Calculation of W48• Quantum Yields

Effective quantum yields were derived from knowledge of the rate of formation of neutral tryptophan radicals, $k(\text{W48}\bullet)$, and the rate of excitation of tryptophan molecules, $k(\text{W48}^*)$, in the first 15 min of photolysis where radical production was linear:

$$\Phi = \frac{k(\text{W48}\bullet)}{k(\text{W48}^*)} \quad (1)$$

The half-life of the radical is ~ 7 h, and therefore, we neglected population decay during this 15 min photolysis period (Supporting Information). Given the challenges associated with determining absolute reaction quantum yields, the quantum yields reported here are better described as *effective* quantum yields that are subject to the assumptions described below. We anticipate that the numerical values of the yields will depend on some experimental conditions, such as spectral bandpass, but expect the relative yields and trends among mutants to be unaffected by the specific experimental conditions.

The rate of radical formation $k(\text{W48}\bullet)$ in eq 1 was assessed by absorption spectroscopy. The molar absorption coefficient of the radical at the 515 nm maximum in the visible region (ϵ_{515}) was determined with the assumption of lossless (i.e., 1:1) conversion between closed-shell tryptophan and its radical counterpart. We relied on long-time (40 min) experiments which created a significant population of radical, and at least 60% of the closed-shell tryptophan was depleted. This procedure set a lower limit for the value of ϵ_{515} for W48• because the estimated radical concentration is the maximum possible concentration. The fact that we utilize a lower limit for the value of ϵ_{515} has the consequence that the numerator in the quantum yield expression is an upper limit.

The denominator $k(\text{W48}^*)$ (rate of formation of excited tryptophan molecules) was determined by calculating the number of photons absorbed per second by tryptophan molecules in the sample solution; this solution may contain other UV-absorbing moieties, such as acceptor and tyrosine residues, and therefore, a correction for the inner-filter effect was included in the calculation. The fraction of the total absorbed light that could be attributed to W48 follows from the Beer–Lambert law for a multicomponent system

$$k(\text{W48}^*) = I_{\text{abs}}^{\text{tot}} \times \frac{\epsilon_{292}^{\text{W48}} \times [\text{W48}]}{\sum_i \epsilon_{292}^i \times [i]} \quad (2)$$

where $I_{\text{abs}}^{\text{tot}}$ is the average total number of photons absorbed by the sample per second, $\epsilon_{292}^{\text{W48}}$ is the molar absorption coefficient of W48 at 292 nm, $[\text{W48}]$ is the concentration of W48, ϵ_{292}^i is the molar absorption coefficient of each species i in the sample solution, including

W48, tyrosines, Co^{III} , etc., and $[i]$ is the concentration of species i . For example, in a solution containing $\text{Zn}^{\text{II}}\text{Az48W}$ and Co^{III} species, the rate of photons absorbed by W48 is $I_{\text{abs}}^{\text{tot}} \times ((\epsilon_{292}^{\text{W48}} \times [\text{Zn}^{\text{II}}\text{Az48W}]) / ((\epsilon_{292}^{\text{Zn}^{\text{II}}\text{Az48W}} \times [\text{Zn}^{\text{II}}\text{Az48W}]) + (\epsilon_{292}^{\text{Co}^{\text{III}}} \times [\text{Co}^{\text{III}}]))))$. The molar absorption coefficients at 292 nm were determined from the absorption spectra and knowledge of ϵ_{280} (from above). It is important to note that, in the numerator, $\epsilon_{292}^{\text{W48}}$ is the absorption coefficient for the single tryptophan residue and does not have contributions from phenylalanines or tyrosines in the protein. Here, we use a value of $\epsilon_{292}^{\text{W48}} = 5260 \text{ cm}^{-1} \text{ M}^{-1}$, obtained via $\epsilon_{292}^{\text{Zn}^{\text{II}}\text{Az48W}} - \epsilon_{292}^{\text{Zn}^{\text{II}}\text{AzFFF}}$ (or $5820\text{--}560 \text{ cm}^{-1} \text{ M}^{-1}$). The average total number of photons absorbed by the sample, $I_{\text{abs}}^{\text{tot}}$ is given as $I_0(1 - 10^{-\text{abs}}) \times \text{CF}$, where I_0 is the incident power converted to photons per second (after correction from actinometry) and abs is the absorbance of the sample in a 2 or 4 mm pathlength cuvette from UV-vis absorption spectroscopy. CF is a correction factor that converts the fraction of absorbed light at a single wavelength (i.e., 292 nm) to an average fraction of absorbed light over the 10 nm spectral bandpass of 287–297 nm; the absorption profile of the azurin mutants varied over this 10 nm window, so CF values ranged from 0.79 to 0.88. Decrease of the absorbance value at 292 nm over the 15 min photolysis interval was typically less than 25% but not taken into account in this calculation. This decrease in absorbance would have the effect of decreasing $I_{\text{abs}}^{\text{tot}}$, which is equivalent to decreasing the denominator in the expression for quantum yield.

The expected corrections to the molar absorption coefficient for the radical, ϵ_{515} , and evolution of the spectral profile during photolysis will have opposing effects on the quantum yield; the former correction will systematically decrease the yields from the values reported here, whereas the latter will increase them. Hence, under the conditions and assumptions described herein, we report effective quantum yields. These yields are reported as averages of several trials, and errors are reported as the standard deviation of the mean when the number of trials, N , is greater than or equal to 6. When $N = 2$, the reported error encompasses the range in values of the two trials.

Results

Photolysis of $\text{Zn}^{\text{II}}\text{Az48W}$, $\text{Cu}^{\text{II}}\text{Az48W}$, and $\text{Zn}^{\text{II}}\text{Az48W} + \text{Cu}^{\text{II}}\text{Az48W}$ Mixtures

Samples that contained only pure $\text{Zn}^{\text{II}}\text{Az48W}$ or pure $\text{Cu}^{\text{II}}\text{Az48W}$ in the absence of any external electron acceptor produced no detectable radical when photolyzed (Figure 1). The minor (<5%) losses of absorption seen for some samples are likely caused by degradation of the protein. In contrast to pure Zn- or Cu-azurin solutions, the $\text{Zn}^{\text{II}}\text{Az48W} + \text{Cu}^{\text{II}}\text{Az48W}$ mixture resulted in the formation of the neutral tryptophan radical. Photolysis of 0.6:1, 1:1, 2:1, and 3:1 mixtures of $\text{Zn}^{\text{II}}\text{Az48W}:\text{Cu}^{\text{II}}\text{Az48W}$, with $[\text{Cu}^{\text{II}}\text{Az48W}]$ held constant at approximately $50 \mu\text{M}$, resulted in an increase of absorption at 485 and 515 nm accompanied by a decrease in Cu^{II} absorbance; an isosbestic point also appears at 528 nm. Representative results for the 3:1 mixture are shown in Figure 2. An important result based upon the investigations of $\text{Zn}^{\text{II}}\text{Az48W} + \text{Cu}^{\text{II}}\text{Az48W}$ mixtures is that nominally “pure” $\text{Cu}^{\text{II}}\text{Az}$ samples, if untreated by cyanide, contain a significant amount of $\text{Zn}^{\text{II}}\text{Az}$. Also, the isosbestic point indicates that there are only two distinct absorbing species in the visible region, namely, the copper center and tryptophan neutral radical.

The quantum yields for formation of the neutral radical, W48•, in Zn^{II}Az48W and Cu^{II}Az48W mixtures were determined using the lower limit value of ϵ_{515} of 2200 cm⁻¹ M⁻¹ for the radical as described above (see the Supporting Information). Radical yields increased linearly with photolysis time up to ~20 min. Additionally, the increasing concentration of W48• correlated with the decreasing concentration of Cu^{II} across a 10-fold variation in the ratio of Zn^{II}Az48W:Cu^{II}Az48W (Supporting Information). The quantum yield for a 1:1 mixture was found to be 0.083 ± 0.003 ($N = 8$). The quantum yields are summarized in Table 1.

Photolysis of Cu^IAz48W and CuAzFFF

Additional experiments with different azurin mutants and mixtures were pursued to determine whether the tryptophan radical could be generated in Cu^IAz48W, and to investigate the general photophysical response of protein in the absence of W48. In one experiment, a mixture of 1:1 Cu^IAz48W:Cu^{II}Az48W was photolyzed. After 25 min of photolysis, there was no observable production of W48• ($\Phi = 0$), but the Cu^{II} absorbance band at 628 nm increased by approximately 10% of the initial absorbance. In another experiment, a mixture of Zn^{II}AzW48+Cu^{II}AzFFF was photolyzed, and this mixture produced W48• in a manner similar to Zn^{II}W48+Cu^{II}Az48W mixtures. Absorption spectra are included in the Supporting Information. Photolysis experiments on the following pure samples yielded no radical, but other minor spectral changes were noted: Cu^IAz48W (increase in Cu^{II} band), Cu^IAzFFF (increase in Cu^{II} band), and Cu^{II}AzFFF (decrease in Cu^{II} band).

Photolysis of Zn^{II}Az48W and CuAz48W with Ru^{III}(NH₃)₆ Electron Acceptor

The finding that W48• could only be generated in the presence of a Cu^{II}Az electron acceptor motivated us to study other known electron scavengers to analyze the ET event associated with formation of the tryptophan neutral radical. An electron acceptor, Ru^{III}(NH₃)₆Cl₃, was added to Zn^{II}Az48W, Cu^{II}Az48W, or Cu^IAz48W. One possible complication with this acceptor is that it undergoes reversible redox chemistry (the [Ru-(NH₃)₆]^{3+/2+} reduction potential is 0.10 V vs NHE⁴⁴), and therefore could potentially re-reduce a transient tryptophan cation radical. A 1:1 mixture of Zn^{II}Az48W:[Ru^{III}(NH₃)₆]³⁺ exhibited a quantum yield of 0.067 ± 0.006 ($N = 2$). In a mixture of Cu^{II}Az48W and [Ru^{III}(NH₃)₆]³⁺, there was no detectable generation of radical, and the decay of the LMCT band was minimal; this loss in LMCT signal was comparable to that which was observed in pure Cu^{II}Az48W (no acceptor) and attributed to photodecomposition. Finally, the mixture of Cu^IAz48W and [Ru^{III}(NH₃)₆]³⁺ behaved qualitatively similar to the Cu^IAz48W + Cu^{II}Az48W combination in that there was a slight increase in the intensity of the LMCT band.

Photolysis of Zn^{II}Az48W and Cu^{II}Az48W with [Co^{III}(NH₃)₅Cl]²⁺ Electron Acceptor

Another molecule commonly used as an exogenous electron scavenger is [Co^{III}(NH₃)₅Cl]²⁺. The advantage of this acceptor relative to Cu^{II}Az48W and Ru-based acceptors is that reduction of [Co^{III}(NH₃)₅Cl]²⁺ is irreversible because the Co^{II} product transforms to [Co^{II}(H₂O)₆]²⁺ and does not undergo further redox chemistry after the initial electron

scavenging event.^{26,45,46} An additional benefit of this acceptor is that the absorption in the UV and visible regions is minimal.

Photolysis of a 1:1 mixture of Cu^{II}Az48W and [Co^{III}(NH₃)₅Cl]²⁺ did not result in formation of tryptophan radical (see the Supporting Information). Instead, there was a minor decrease in the 628 nm band and an increase in absorbance in the UV region. These changes were subtle, and less than 5% of the initial absorbance.

In contrast, tryptophan radical was generated in photolyzed mixtures of Zn^{II}Az48W and [Co^{III}(NH₃)₅Cl]²⁺. The buildup of W48• in these solutions was apparent from the growth of absorption bands at 325, 360, 485, and 515 nm; the reduction of Co^{III} to Co^{II} and loss of closed-shell tryptophan were evident from the loss of absorbance at most wavelengths in the spectral range 230–300 nm. Representative absorption spectra and difference spectra of a 1:1 mixture are shown in Figure 3.

The yield of radical formation in the Zn^{II}Az48W + [Co^{III}(NH₃)₅Cl]²⁺ mixtures was similar to that of the Zn^{II}Az48W + Cu^{II}Az48W mixture. A 1:1 mixture exhibited $\Phi = 0.080 \pm 0.002$ ($N = 31$). For a given Zn^{II}Az48W concentration of 40–70 μM with varying amounts of acceptor, the yield increased linearly up to a 1:1 ratio of Zn^{II}Az48W + [Co^{III}(NH₃)₅Cl]²⁺; further raising the ratio beyond 1:1 did not affect the yield, which remained 0.080 ± 0.002 ($N = 6$) at 1:2 to 1:4 ratios. The higher quantum yield obtained with Co(III) relative to Ru(III) is consistent with the fact Co(III) undergoes irreversible reduction, whereas Ru(III) redox chemistry is reversible.

Photolysis of Zn^{II}Az48W and Cu^{II}Az48W with N₂O

N₂O is a well-known scavenger of solvated electron^{47,48} and was employed as a possible acceptor in a final series of experiments. The motivation for these experiments was to investigate the possibility of intermolecular electron ejection from W48, followed by PT, to generate W48•. The binary mixtures of Zn^{II}Az48W + N₂O and Cu^{II}Az48W + N₂O did not generate a radical; however, there was an increase in the UV region of Zn^{II}Az48W and an approximate 10% decrease in the LMCT band in Cu^{II}Az48W. Photolysis of the ternary mixture of Zn^{II}Az48W + Cu^{II}Az48W + N₂O caused the generation of W48• and loss of Cu(II) LMCT. The ternary mixture of Zn^{II}Az48W + [Co^{III}(NH₃)₅Cl]²⁺ + N₂O also generated W48•, as was seen in the absence of N₂O (see the Supporting Information). The ternary Cu^{II}Az48W + [Co^{III}(NH₃)₅Cl]²⁺ + N₂O did not show signal of the radical, as seen without N₂O. In summary, the addition of N₂O did not affect the production of W48•; photolysis results in the absence and presence of N₂O were qualitatively similar.

pH Effects on W48• Generation in Zn^{II}Az48W

Photolysis experiments of 1:1 Zn^{II}Az48W + [Co^{III}(NH₃)₅Cl]²⁺ with $\sim 50 \mu\text{M}$ [Zn^{II}Az48W] were performed at pH 4.5 and 9.0 to complement the data collected at pH 7.2. The quantum yields at pH 4.5 and 9.0 are $\Phi = 0.095 \pm 0.004$ ($N = 2$) and 0.071 ± 0.009 ($N = 2$), respectively. In the absence of Co^{III}, photolysis of Zn^{II}Az48W at pH 4.5 did not generate a radical.

Photolysis of Zn^{II}WT, Zn^{II}Az48W/72Y, and Zn^{II}Az48W/108Y

Zinc-substituted wild-type azurin and mutants containing tyrosine residues were photolyzed in the presence of [Co^{III}(NH₃)₅Cl]²⁺ in 1:1 ratios to examine the role of the native tyrosine residues (Y72 and Y108) on the quantum yield of radical formation. At pH 4.5, 7.2, and 9.0, the quantum yields for Zn^{II}WT are 0.074 ± 0.003 ($N=2$), 0.045 ± 0.004 ($N=8$), and 0.053 ± 0.003 ($N=2$), respectively. At pH 7.2, Zn^{II}Az48W/108Y and Zn^{II}Az48W/72Y exhibited $\Phi = 0.062 \pm 0.007$ ($N=6$) and 0.067 ± 0.004 ($N=6$), respectively. Representative difference spectra that have been normalized to total incident power are shown in Figure 4, and quantum yields are included in Table 1.

Ultraviolet Resonance Raman Spectra on Azurin Mutants

UVRR spectra provide valuable insights into the local environment and H-bonding structures of amino acids. UVRR spectra of the single-tyrosine mutants are shown as Supporting Information. Tyrosine normal modes are indicated with the standard nomenclature scheme. Spectra of Cu^{II}Az48W/108Y at various time points following dilution into D₂O buffer were also measured to determine if there is H/D exchange in the partially solvent-exposed Y108. It was found that, immediately (~1 min) after addition of D₂O, the vibrational bands of Y108 were consistent with that of fully exchanged phenol, unlike what is observed for the solvent-excluded W48. Subtractions of Cu^{II}Az48W signal from Cu^{II}AzWT, Cu^{II}Az48W/72Y, and Cu^{II}Az48W/108Y spectra were performed to isolate the tyrosine bands from those of the tryptophan modes (see the Supporting Information).

We focus on vibrational modes and relative intensities that have been shown to be hydrogen-bond indicators in tyrosine UVRR spectra. The Y8b mode consists of ring-breathing coupled to –OH bending, and its frequency decreases with increasing strength of hydrogen-bond donation.⁴⁹ In the case of fully deprotonated tyrosinate, this mode is found at $\sim 1560\text{ cm}^{-1}$. For Tyr72, Tyr108, and L-tyrosine, it appears at 1605, 1602, and 1602 cm^{-1} , respectively, and these results are consistent with tyrosine residues that are not strong hydrogen bond donors. However, the Y8b peak is a low-intensity peak with 228 nm excitation, and overlaps with tryptophan and phenylalanine modes. The Y7a' and Y7a mode frequencies also reflect hydrogen bonding of the phenolic hydrogen; these are intense bands in a less congested region of the spectrum. The Y7a' mode appears at $1265\text{--}1275\text{ cm}^{-1}$ for hydrogen-bond donating species and $1230\text{--}1240\text{ cm}^{-1}$ for hydrogen-bond accepting species, while the Y7a mode frequency shows the opposite trend in the range $1205\text{--}1215\text{ cm}^{-1}$.⁵⁰ In the Y72 mutant, the Y7a' and Y7a modes appear at 1259 and 1210 cm^{-1} , respectively; the Y7a' and Y7a modes are observed at 1257 and 1215 cm^{-1} for the Y108 mutant. These results indicate that both tyrosine residues may participate in weak hydrogen bonds. An additional hydrogen-bonding marker in the tyrosine UVRR spectrum may be found in the Fermi doublet between the Y1 and 2xY16a modes.⁵¹ For off-resonance Raman spectra, it has been shown that the intensity ratio $I_{850}:I_{830}$ is less than 1 for hydrogen bond donation and greater than 1 for hydrogen bond acceptance, and this trend persists in the resonance Raman spectra of tyrosine.⁴⁹ Both azurin mutants show an intensity ratio of ~ 1 in the UVRR spectra, which indicates weak hydrogen bond donation and acceptance.

Discussion

The observation of a relatively stable, photogenerated tryptophan radical within a protein is unusual. There are numerous factors that are important for the generation and stability of an amino acid radical in a protein, such as the ease of de- and reprotonation, local environment, and mechanisms of electron transfer. In the case of Zn-substituted azurin, the neutral W48• species at native position 48 is generated with 8.0% yield upon absorption of 292 nm light in the presence of electron acceptors. Surprisingly, the radical survives for several hours despite the high reduction potential of 1.015 V vs NHE (pH 7.0) for tryptophan model compounds.²⁴ It is worth noting that the single tryptophan residue in azurin has been studied previously for its unique photophysical properties, including the low fluorescence Stokes shift and long phosphorescence lifetime, that stem from solvent exclusion of W48 in the hydrophobic protein core. The sheltering of W48 is also likely a reason for the stability of its radical. Kinetic barriers to reprotonation may slow the loss of the neutral radical, and this process will be further investigated in a future study. In the present report, we focus on the mechanisms by which this radical is generated and quenched.

Intermolecular Mechanism for Formation of W48•

A key question that motivates the current experiments is the following: Is an electron ejected from W48 via an *intra*- or *intermolecular* pathway? In a previous report, we hypothesized that the neutral tryptophan radical is generated by direct, intramolecular ET from W48 to Cu^{II} in a single azurin unit, and that the resulting transient cation radical, W48•⁺, can deprotonate to form the neutral radical, W48•.²⁸ Alternatively, back-ET from Cu^I reforms the closed-shell species of W48 (see Figure 5, path A). The relative rates of back-ET and deprotonation would determine the yield of formation of the neutral radical. In addition to this intramolecular mechanism, an intermolecular mechanism can form tryptophan radicals because indole is known to eject an electron upon UV photolysis.^{52–54}

Subsequent to our earlier report, we found that the Cu^{II}Az48W samples contained significant amounts of Zn^{II}Az48W impurity (40–60%). Furthermore, control experiments with pure Zn^{II}Az48W did not produce a radical. The presence of Zn^{II}Az despite purification of the sample and addition of excess Cu(II) is a well-documented problem. Favorable binding constants, greater stability of Zn^{II}Az over Cu^{II}Az, and a kinetic barrier to Zn displacement all result in a large concentration of Zn^{II}Az.^{55,56} Difficulties associated with obtaining pure Cu^{II}Az sample have led us and others to an additional purification step in which cyanide is used to remove all bound metals from azurin, and the intended metal is subsequently reintroduced to the apo-Az sample.^{36–38} Addition of this cyanide step increases sample purity substantially: a mixture of Cu^{II}Az (40%) and Zn^{II}Az (60%) is purified to >99% Cu^{II}Az (or >99% Zn^{II}Az) as assessed by ICP OES.

As reported above, photolysis of pure Zn^{II}Az48W or pure Cu^{II}Az48W yielded no observable radical (Figure 1). On the other hand, a mixture of Zn^{II}Az48W+Cu^{II}Az48W yielded a significant amount of tryptophan neutral radical (Figure 2). The formation of a radical was accompanied by near quantitative bleach of Cu^{II} to Cu^I (Supporting Information). Together, these observations indicate that the ET reaction must be intermolecular, with Zn^{II}Az48W as the electron donor and Cu^{II}Az48W as the acceptor (see Figure 5, path B). The observation

that W48• was also generated in Zn^{II}Az48W + Cu^{II}AzFFF mixtures supports the conclusion that the tryptophan radical resides in Zn^{II}Az48W, not in Cu^{II}Az48W. This intermolecular electron transfer mechanism is supported by additional experiments with other exogenous electron acceptors in the presence of Zn^{II}Az48W, including Ru^{III} and Co^{III} compounds (Figure 5, path B). These findings correct our earlier hypothesis that intramolecular ET in Cu(II)Az is predominant in the radical formation. The intramolecular ET pathway likely proceeds in Cu(II)Az; however, the back ET is too fast to allow buildup of the neutral radical.

Our data suggest that the electron acceptor forms a complex with the Zn^{II}AzW48 donor. Non-specific binding of the Co^{III} or Ru^{III} compounds to azurin is reasonable given the presence of localized patches of anionic residues on the protein surface. The strongest evidence for a bound complex is the finding that the yield for radical formation reaches a maximum at a ratio of protein:acceptor of 1:1. This observation suggests that, on average, there is a single effective electron acceptor per azurin unit. Additional supporting evidence for a protein–acceptor complex as opposed to bimolecular quenching of a free, solvated electron comes from N₂O experiments. The presence of ~25 mM of this electron scavenger (based on Henry's constant of $k_H = 2.5 \times 10^{-2} \text{ M atm}^{-1}$ and 1 atm of N₂O gas⁵⁷) does not promote oxidation of tryptophan. This observation suggests that a solvated electron is not generated in this system. Instead, the neutral radical is formed when Co^{III} is added to the Zn^{II}Az48W+N₂O solution (see the Supporting Information). The quantum yield for formation of W48• in the ternary Zn^{II}Az48W+N₂O+Co^{III} solution is 0.059, which is lower than the yield in the absence of N₂O ($\Phi = 0.080$). The reason for this variation in quantum yield is unknown but may reflect interactions among N₂O, Co^{III}, and protein that affect the binding of Co^{III} to azurin. A final piece of evidence against a solvated electron mechanism is the observation that photolysis at low pH, in the absence of electron acceptor, does not result in the formation of a radical. Acidic solution is also known to quench solvated electron,⁵⁸ and the quantum yield for formation of solvated electron by aqueous indole changes less than 10% across the pH range 3.5–11.0.^{52,59} Therefore, if a solvated electron mechanism were occurring for Zn^{II}Az48W, one would expect an increase in concentration of tryptophan radical at low pH. However, no radical was observed in pH 4.5. These four results support electron transfer within a protein– acceptor complex, rather than via a solvated electron.

Intramolecular Electron Transfer

Surprisingly, photolysis of Cu^IAz48W or Cu^{II}Az48W in the presence of Co^{III} did not result in the formation of a radical; qualitatively similar results were obtained in the absence of Co^{III}. Instead, there were small changes in the 628 nm Cu^{II} band. The magnitudes of these changes can be compared to those of control samples that did not contain tryptophan, Cu^IAzFFF and Cu^{II}AzFFF, and are shown in the Supporting Information.

The observed redox chemistry of the Cu center can be attributed to several mechanisms that may be occurring in solution, and shown in Figure 5: path A, ET from Cu^I to photogenerated W48•⁺; path C, direct electron ejection⁶⁰ from Cu^I to generate Cu^{II}; and path D, ET from Cu^I to photogenerated W48*.⁵⁵ Protein degradation is also possible. In the case of Cu^IAzFFF, the increase in Cu^{II} signal is attributed to path C in which an electron is ejected

directly from the Cu^I center. For Cu^IAz48W, however, we propose that the two different electron ejection events of paths A and C lead to the increase in Cu^{II} signal. This hypothesis is based on the observation that the magnitude of Cu^{II} increase is greater for Cu^IAz48W than for Cu^IAzFFF, which indicates that the tryptophan residue plays a role in copper oxidation. Specifically, electron ejection from W48 to external acceptor creates a transient cation radical, W48^{•+}, which can be rapidly reduced by ET from Cu^I because of the large driving force. Two possible ET pathways between W48 and the Cu center have been discussed.²⁸ The rate of Cu^I-to-W48^{•+} ET must be faster than deprotonation of W48^{•+}, since no neutral radical, W48[•], is observed. Path D may be possible for Cu^IAz48W, but this reductive quenching mechanism is likely thermodynamically unfavorable, and no net loss or gain of Cu^I is expected. In the case of Cu^{II}Az48W, path A is expected to occur, though, in this case, the initial photoinduced event is rapid ET from photoexcited W48 to Cu^{II}, followed by rapid back-ET, i.e., recombination, from Cu^I to W48^{•+}. In this hypothesis, one expects no net change in Cu^{II} signal if the forward- and backET events occur with unity yield. The slight decrease in Cu^{II} signal in photolyzed Cu^{II}Az48W may reflect UV-induced protein degradation, because this decrease in Cu^{II} signal is also observed in Cu^{II}AzFFF.

Intramolecular ET has been proposed as a mechanism to quench tryptophan fluorescence in a number of proteins, including azurin.^{61–63} The inclusion of certain metal centers affects the photophysical properties of azurin: Zinc- and apoazurin exhibit significantly longer fluorescence lifetimes and fluorescence quantum yields than Cu^{II} and Cu^I azurin.^{37,61} This trend is interpreted in terms of charge transfer reactions that are possible for Cu^{II} and Cu^I but not Zn^{II} or apoazurin. Oxidative quenching of W48 shown as path A in Figure 5 is proposed to take place in Cu^{II}Az, with favorable forward- and back-ET rate constants of $\sim 10^9$ s⁻¹.^{28,61,64} Our data, particularly the comparison of radical yields of Zn^{II}Az48W and Cu^{II}Az48W, support this oxidative quenching mechanism in Cu^{II}Az. In the case of Cu^IAz, reductive quenching also in the form of fast, reversible charge separation has been proposed (Figure 5, path D).⁶¹ Our findings do not provide evidence for the transient appearance of an anion radical for Cu^IAz48W, but we nonetheless do not exclude this charge-separation event.

Quenching of W48[•] by Tyrosine Residues

Tyrosine has been shown to quench tryptophan radicals in synthetic dipeptides,^{22,31} but this is not a prevalent mechanism in proteins. From a thermodynamic perspective, tyrosine-to-tryptophan ET is favorable, and requires deprotonation of tyrosine because tyrosine undergoes concerted PCET reactions.^{24,33} Kinetically, however, this process can be hindered because of the PT requirement. We observed that the quantum yield for formation of W48[•] decreases in the presence of tyrosine residues. The yield is greatest for Zn^{II}Az48W (no tyrosine), decreased for Zn^{II}Az48W/72Y and Zn^{II}Az48W/108Y (one tyrosine), and lowest for Zn^{II}AzWT (two tyrosines); see Figure 4. This trend was maintained at three different pH values of 4.5, 7.2, and 9.0. These findings suggest that tyrosine residues reduce the tryptophan cation radical. We propose that the native Y72 and Y108 residues transfer an electron to the photogenerated tryptophan cation radical, W48^{•+}; for this ET to occur, the tyrosine-to-tryptophan ET rate must compete with the deprotonation rate of W48^{•+} and thus impede the formation of W48[•] (Figure 5, path E). Reduction of W48^{•+} generates closed shell W48 as well as a transient tyrosine neutral radical (Y72[•] or Y108[•]) that is undetectable

on the time scale of our present UV–vis absorption experiments. We are currently pursuing EPR and more advanced absorption studies to directly detect these tyrosine radicals.

Electron Transfer Paths

We are interested in understanding the intra- and intermolecular ET paths for generation and quenching of tryptophan radical. Four different donor–acceptor pairs, and hence ET paths, are the focus of the present work. The ET paths are described in Figure 5: Intermolecular ET between tryptophan-48 and an external electron acceptor (path B); intramolecular ET between tryptophan-48 and the copper center (paths A and D); intramolecular ET between tyrosine-108 and tryptophan-48 (path E); and intramolecular ET between tyrosine-72 and tryptophan-48 (path E).

The intermolecular ET paths that result in creation of W48• are not known. If the Co^{III} compound is bound to azurin, there are several possible locations where the cationic Co^{III} compound may bind to a solvent-exposed anionic residue. The observation that the quantum yield for W48• formation reaches a maximum at a ratio of 1:1 $\text{Zn}^{\text{II}}\text{Az48W}$:acceptor suggests there is a dominant ET pathway at a single binding site. When the electron acceptor is $\text{Cu}^{\text{II}}\text{Az}$, the situation is different because an azurin dimer has been proposed to form via the large hydrophobic patch on the protein surface.^{65,66} Experiments that illustrated relatively fast electron self-exchange rates for the dimer focused on a high concentration of azurin, on the order of 1–2 mM.⁶⁷ Recently, a noncovalent dimer of rhenium-labeled azurin was reported to undergo tryptophan-mediated electron hopping through the protein interface; the dimer was found to exist at low concentrations near 70 μM .⁶⁸ The present observation that reduction of $\text{Cu}^{\text{II}}\text{Az}$ proceeds quantitatively with formation of W48• strongly supports the presence of a dimer that enables interprotein ET under the present experimental concentrations of $\sim 50 \mu\text{M}$. An ET path through a dimer protein interface is proposed in the Supporting Information. In this dimer model, the ET path along the backbone and side chain is as follows: W48 in $\text{Zn}^{\text{II}}\text{Az48W} \rightarrow \text{T84} \rightarrow \text{H83} \rightarrow$ across the protein–protein interface to N47 in $\text{Cu}^{\text{II}}\text{Az48W} \rightarrow \text{Cys112} \rightarrow \text{Cu}^{\text{II}}$. The path from W48 to H83 includes a jump across a short hydrogen bond, and the path from N47 to the copper center is consistent with previous work based on electrodes.⁶⁹ Kinetic studies to determine the rates of interprotein ET are ongoing.

The three intramolecular ET events can be discussed in the context of the high-resolution crystal structures of azurin. In our previous report, we discussed two possible paths for Trp48-to-copper ET, an 8-bond pathway from indole to copper that requires tunneling through a hydrogen bond between Asn47 and Cys112 or an 11-bond pathway to copper via Val49 that requires tunneling through a hydrogen bond between Val49 and Phe111.²⁸ In both cases, the nonbonded distance from donor to acceptor is only 10 Å, and the ET rate is expected to be high.^{1,3}

Intramolecular ET from tyrosine to tryptophan can also be explained with reasonable paths, shown in Figure 6. Tyr108-to-Trp48 ET may be achieved through an 11-bond pathway from Tyr108 to indole that requires tunneling through a π – π interaction between Phe110 and Trp48 (3.87 Å from indole nitrogen to the centroid of Phe110). The Tyr72-to-Trp48 path is longer, with 14 bonds that include a hydrogen bond jump from Asp71 to Asn47. The

nonbonded distance between the centroids of Tyr108 (Tyr72) and Trp 48 is 11.32 Å (17.54 Å). Despite the longer path between Tyr72 and Trp48, ET may still be efficient between these aromatic residues because part of the Tyr72–Trp48 path overlaps with the strongly coupled bonds that allow efficient Trp48-to-copper²⁸ ET. These tyrosine-to-tryptophan ET paths were predicted using the HARLEM program, which predicted that the HDA coupling constant for the Tyr108–Trp48 path is larger than that for the Tyr72–Trp48 path.⁷⁰

Tyrosine Environment

Both tyrosine residues exhibit anomalously high pK_a values of 11.4 and 12.5 based on ¹³C NMR spectroscopy.⁷¹ Results from our lab on Cu^{II}Az48W/72Y ($pK_a = 11.6$) and Cu^{II}Az48W/108Y ($pK_a = 13.3$) are higher than these earlier findings but support the trend of enhanced pK_a values (Supporting Information). The high pK_a values could arise from hydrogen bonding, electrostatics, and/or hydrophobicity of the tyrosine environment.^{72,73} These types of effects on tyrosine pK_a are complicated but have been observed in previous studies. For example, an unusually high pK_a value for a nitrotyrosine in ribonucleotide reductase (NO₂-Tyr122) was attributed to the presence of an aspartate residue that disfavored the charged tyrosinate species.⁷⁴ A native tyrosine in Rh. *sphaeroides* was also shown to exhibit a variable pK_a value that reflected the local protein environment; replacement of a nearby histidine with glutamate subsequently increased the tyrosine pK_a by 2 units. The negatively charged carboxylate group on glutamate likely acts as a hydrogen bond acceptor and prevents ionization through electrostatic repulsion.⁷⁵

These reported effects of a nearby anionic residue can explain the high pK_a of Tyr108, where Glu106 is near the hydroxyl group of Tyr108 according to the crystal structure (O–O distance of 2.73 Å). However, in solution, it is not clear that Glu106 and Tyr108 form a hydrogen bond. UVR results do not support the presence of a strong H-bond, and instead, it is possible that Glu106 forms a salt-bridge with a nearby Lys103. The environment around Tyr108 is also enriched in polar residues; two lysine residues, two glutamate residues, and one glutamine residue are in the immediate vicinity. These residues may create a well-ordered environment where the phenolic oxygen can participate as both an H-bond acceptor and donor. This amphoteric nature of tyrosine would likely result in the observed spectroscopic signatures.⁵¹ There are no charged residues in the vicinity of Tyr72, and thus, the high pK_a value may be a direct result of unfavorable electrostatics.

In addition to the influence of a nearby anionic residue, another contribution to the high pK_a values is the hydrophobic pockets that surround Tyr108 and Tyr72. Both of these residues are in hydrophobic environments, as assessed by the crystal structure and supported by the red-shifted absorption maxima of 278 and 280 nm relative to model compound L-tyrosine in water (275 nm).^{76,77} A hydrophobic environment would disfavor the loss of a proton to solvent because of limited solvent accessibility as well as destabilization of the resulting anion.

An inverse correlation between pK_a and reduction potential has been reported for the case of tyrosine and its analogues, suggesting a decrease in the reduction potential of Tyr108 and Tyr72 relative to free tyrosine $E^\circ(\text{Tyr}^\bullet, \text{H}^+/\text{TyrOH}) = 0.93 \text{ V vs NHE, pH } 7$.^{24,73,78} On the other hand, the hydrophobic environment of W48 likely increases the reduction potential of

W48^{•+} relative to model compounds ($E^\circ(\text{TrpH}^{\bullet+}/\text{TrpH}) = 1.15 \text{ V vs NHE, pH } 2$),²⁴ further increasing the driving force for tyrosine ET to tryptophan. Ultimately, the high pK_a values and possible hydrogen bonding within a hydrophobic environment may affect the driving force for PCET between either Tyr72 or Tyr108 and W48^{•+}; however, electrostatic considerations of local environments add uncertainty to this hypothesis.

The effects of pH on ET and PCET reactions for biological reactions are complex. In the current experiments, changes in pH affect the reduction potential of the acceptor in a well-known manner described by Pourbaix.⁷⁹ Our results support the expected increase in the reduction potential of Co^{III} compound at low pH; the quantum yield for radical formation in Zn^{II}Az48W is higher at acidic pH than at basic pH, consistent with a larger driving force for ET. The pH trend for Zn^{II}AzWT is more complex. On the one hand, the quantum yields of Zn^{II}AzWT are lower than those of Zn^{II}Az48W at all investigated pH values, supporting the hypothesis that tyrosine reduces the tryptophan cation radical. However, the pH trend for Zn^{II}AzWT is somewhat surprising: At basic pH, we would expect that the combination of decreased reduction potential⁸⁰ and enhanced population of tyrosinate might facilitate more efficient ET from tyrosine to the tryptophan cation radical, therefore resulting in a reduced quantum yield for tryptophan radical formation relative to pH 7.2. In azurin, the unusually high pK_a values for the tyrosine residues result in a tyrosinate population of less than 1% for both residues at pH 9.0, and the observed quantum yield *increased* slightly at pH 9.0 relative to 7.2. This finding indicates that the effects of pH are complicated, and protein structural changes may significantly impact ET efficiencies.

Conclusion

Azurin is a valuable model protein that allows for interrogation of an unusually stable tryptophan radical at native position 48. The neutral radical, W48[•], is observed upon UV photolysis of a tyrosine deficient, Zn^{II}-substituted azurin mutant. The mechanism for formation of this neutral radical is intermolecular, and requires an external electron acceptor such as Co^{III}. In the presence of tyrosine residues, the quantum yield for formation of W48[•] is decreased, suggesting that the tyrosine residues are able to quench the tryptophan radical via an intramolecular ET mechanism. The observation of tyrosine-to-tryptophan ET in azurin is promising and enables continued study of the role of protein environment on PCET reactions along amino acid wires.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Absorption spectra of $\text{Zn}^{\text{II}}\text{Az48W}$ and $\text{Cu}^{\text{II}}\text{Az48W}$ in pH 7.2 buffer during 15 min photolysis at indicated time points. Spectra prior to photolysis are indicated as “prephoto”. Spectra for $\text{Zn}^{\text{II}}\text{Az48W}$ are offset 0.01, 0.02, 0.03, and 0.04 absorbance units for 3, 5, 10, and 15 min time points, respectively. Spectra for $\text{Cu}^{\text{II}}\text{Az48W}$ are offset 0.15, 0.16, 0.17, 0.18, and 0.19 absorbance units for prephoto, 3, 5, 10, and 15 min time points, respectively.

Figure 2. Absorption spectra of a 3:1 mixture of Zn^{II}Az48W:Cu^{II}Az48W in pH 7.2 buffer before photolysis (“pre-photo”) and after 3, 5, 10, and 15 min of photolysis.

Figure 3.

Top panel: Absorption spectra of 1:1 Zn^{II} Az48W: $[\text{Co}^{\text{III}}(\text{NH}_3)_5\text{Cl}]^{2+}$ in pH 7.2 buffer before and during a 40 min photolysis experiment. Bottom panel: Difference absorption spectra (post-photolysis) – (pre-photolysis) of Zn^{II} Az48W derived from spectra of the top panel. Arrows indicate spectral changes associated with increasing photolysis time. Inset: Change in absorbance at 515 nm as a function of photolysis time.

Figure 4.

Difference absorption spectra (post-photolysis) – (pre-photolysis) per mole of photons (Einstein) absorbed for $\text{Zn}^{\text{II}}\text{Az48W}$ (“W48”), $\text{Zn}^{\text{II}}\text{Az48W/72Y}$ (“W48+Y72”), $\text{Zn}^{\text{II}}\text{Az48W/108Y}$ (“W48+Y108”), and $\text{Zn}^{\text{II}}\text{AzWT}$ (“WT”) in the presence of $[\text{Co}^{\text{III}}(\text{NH}_3)_5\text{Cl}]^{2+}$, pH 7.2, after 15 min of photolysis. The ratio of azurin:acceptor is 1:1. Spectra have been scaled to 50 μM protein concentration (actual protein concentrations ranged from 38 to 62 μM).

A. Intramolecular charge transfer (oxidative): No W48• **B. Intermolecular ET with external acceptor. A_{ox} is Cu(II), Ru(III), or Co(III); A_{red} is reduced form: [W48•] high** **C. Direct electron ejection from Cu(I): No W48•** **D. Intramolecular charge transfer (reductive): No W48•** **E. Intramolecular Y-W48 ET. Y is Y108 or Y72: [W48•] low** **Figure 5.**

Relevant intra- and intermolecular ET events. Residues and redox-active metal center contained within a single azurin unit are denoted in the same color (red) and connected by a dash (-), e.g., “W48-Cu(II)”. Residues and redox-active metal centers on different azurin units are denoted by two different colors (red and blue) and separated by a plus sign (+), e.g., “W48+A_{ox}”. The redox-inactive metal center Zn(II) is omitted; e.g., “W48” is shorthand for Zn Az48W, and “Y108-W48” is shorthand for Zn^{II}Az48W/108Y. Intramolecular rate constants k'_{ET} and k''_{ET} describe rates for oxidative and reductive quenching of W48*, respectively; corresponding back-ET events are denoted as k'_{backET} and k''_{backET} . Intermolecular ET from W48 to external electron acceptor and deprotonation of resulting tryptophan cation radical, W48^{•+}, are described by k_{ET} and k_{deprot} , respectively. Intramolecular PCET from tyrosine to tryptophan is described by k_{ET2} . See text for a detailed description of paths A–E.

Figure 6.

Proposed charge transfer paths for Zn^{II}AzW48/72Y (top) and Zn^{II}AzW48/108Y (bottom) based on the crystal structure of azurin (PDB: 4AZU). Absorption of a UV photon results in electron ejection from W48 to an electron scavenger [Co^{III}(NH₃)₅Cl]²⁺, shown as external blue sphere with ligands, via an undetermined path. The resulting hole on W48 is transferred to tyrosine along indicated paths. Deprotonation of phenol accompanies oxidation of tyrosine residue. Zinc metal is shown as a yellow sphere in place of native copper in the crystal structure. See text for additional details.

Table 1
Quantum Yields of Formation of W48• after 15 min of Photolysis with 292 nm Light^a

sample	acceptor	ratio	pH	Φ
Zn ^{II} Az48W	none	--	7.2	0
Cu ^{II} Az48W	none	--	7.2	0
Zn ^{II} Az48W	Cu ^{II} Az48W	1:1	7.2	0.083 ± 0.003^b
Cu ^I Az48W	Cu ^{II} Az48W	1:1	7.2	0
Zn ^{II} Az48W	Ru ^{III}	1:1	7.2	0.067 ± 0.006^e
Cu ^I Az48W	Co ^{III}	1:1	7.2	0
Zn ^{II} Az48W	Co ^{III}	1:1	7.2	0.080 ± 0.002^c
Zn ^{II} Az48W	Co ^{III}	>1:2	7.2	0.080 ± 0.002^d
Zn ^{II} Az48W	Co ^{III}	1:1	4.5	0.095 ± 0.004^e
Zn ^{II} Az48W	Co ^{III}	1:1	9.0	0.071 ± 0.009^e
Zn ^{II} AzWT	Co ^{III}	1:1	7.2	0.045 ± 0.004^b
Zn ^{II} AzWT	Co ^{III}	1:1	4.5	0.074 ± 0.003^e
Zn ^{II} AzWT	Co ^{III}	1:1	9.0	0.053 ± 0.003^e
Zn ^{II} Az48W/108Y	Co ^{III}	1:1	7.2	0.062 ± 0.007^d
Zn ^{II} Az48W/72Y	Co ^{III}	1:1	7.2	0.067 ± 0.004^d

^aThe ratio is defined as protein:acceptor. Abbreviations for acceptors: Ru^{III} = [Ru^{III}(NH₃)₆]³⁺ and Co^{III} = [Co^{III}(NH₃)₅Cl]²⁺.

^bThe errors are reported as standard deviation of the mean where $N = 8$,

^cThe errors are reported as standard deviation of the mean where $N = 31$,

^dThe errors are reported as standard deviation of the mean where $N = 6$.

^eThese experiments were performed twice, and reported errors encompass the values of the measurements.