Reorganization Energy of Blue Copper: Effects of Temperature and Driving Force on the Rates of Electron Transfer in Ruthenium- and Osmium-Modified Azurins

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Azurin is a small blue copper protein that mediates electron transfer (ET)[2] in the denitrifying chains of certain bacteria. Its unique spectroscopic properties are the result of the electronic nature and geometric arrangement of the ligands in the Cu(II) site,[3] which are essentially unchanged in the Cu(I) state.[4] The site geometry is that of a trigonal bipyramid, with three strong ligands in a plane (Cys112, His46, His117) and two weaker axial interactions (Met121 and the carbonyl group of Gly45). Site-directed mutagenesis experiments have demonstrated that the cysteine ligand is an absolute requirement for a blue site,[5] whereas the other interactions are of less importance.[6,7] Changes in the weak interactions, particularly Met121, can, however, tune the reduction potential of the site.[8,9] As long as the protein retains a strong blue color, it is a facile ET agent.[6]

Here we report studies of intramolecular ET in wild-type and mutant forms of *Pseudomonas aeruginosa* azurin, in which His83 has been modified with Ru(II) complexes [and, in one case, with an Os(II) complex].[10] The electronic absorption spectrum of the ruthenated protein is the sum of the spectra of the ruthenium complex and of unmodified azurin (Figure 1), demonstrating that the electronic structure of the blue Cu(II) site is not perturbed by the ruthenium label on His83. This is confirmed by EPR spectra (data not shown), which display the same hyperfine structure as native azurin, and by the fact that the reduction potential of the Cu(II) site is the same in native and modified proteins (Table 1).[11] In addition, the crystal structure of ruthenated azurin[12] has shown that the geometry of the Cu(II) site is virtually identical with that of native azurin.

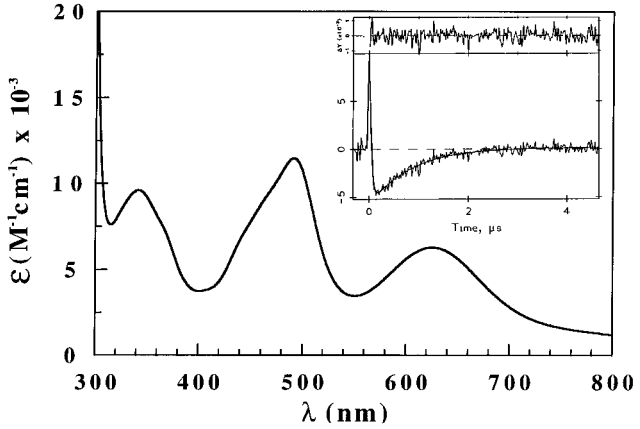

Figure 1. Electronic absorption spectrum of $Ru(bpy)_2(im)(His83)$-AzCu(II) Inset: transient absorption kinetics of a fully oxidized protein solution (~20 μM; KP_i, pH 7.0) following laser excitation (480 nm, ~20 ns pulse width, ~2 mJ/pulse). The solution was deoxygenated by multiple pump/fill cycles with Ar using a Schlenk line. Kinetics were monitored at 632.8 nm (10 mW HeNe laser). The fast event corresponds to excited-state decay; this was measured independently and held constant during the fitting procedure. Emission decay rates: T (K), $\tau \times 10^{-7}$ (s^{-1}); 275.5(1), 2.30; 279.4(1), 2.41; 283.3(1), 2.41; 286.6(1), 2.54; 293.4(1), 2.73; 298.9(1), 2.83; 309.3(1), 3.01. In each case, fits to a biexponential decay function (smooth line) gave k_{ET} [Cu(I) → Ru(III)] = 1.2(1) × 10^6 s^{-1}.

Values for the reduction potentials of both the copper and ruthenium centers of $Ru(bpy)_2(im)(His83)Az$ were determined by direct electrochemical methods[11] and are summarized in Table 1. We have determined the temperature dependence of the reduction potentials of both the Cu(II) and the Ru(III) [or Os(III)] sites, thereby obtaining the enthalpy and entropy changes associated with the redox reactions. As shown in Figure 2, there is only a slight increase in driving force for this reaction as the temperature is raised.

ET between Cu(I) and Ru(III) [or Os(III)] in the labeled proteins was triggered both by direct photoinduction and by a flash/quench method.[13] The same value for the rate constant (k_{ET}) was found by the two methods, when measurements were made at wavelengths typical for Cu(II) as well as for Ru(II)/Ru(III) [or Os(II)/Os(III)]; an example of the kinetic traces is

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(2) Abbreviations: ET, electron transfer; Az, azurin; im, imidazole; bpy, 2,2′-bipyridine; phen, 1,10-phenanthroline; trpy, 2,2′:6′,2″-terpyridine; P_i, phosphate; Tris, tris(hydroxymethyl)aminomethane.

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(10) $Ru(N-N)_2(X)(His83)Az$ (N−N = bpy, phen; X = H_2O, im) was prepared as described earlier (Regan, J. J.; Di Bilio, A. J.; Langen, R.; Skov, L. V.; Winkler, J. R.; Gray, H. B.; Onuchic, J. N. *Chem. Biol.* **1995**, *2*, 489−496). The cyanide derivatives were obtained by reacting $Ru(N-N)(H_2O)(His83)Az$ with 5−10 mM sodium cyanide at pH 9.0 in 50 mM Tris buffer (along with 50 mM NaCl and 1 mM $CuSO_4$) for several days at 4 °C. (A low concentration of cyanide was required to avoid denaturing azurin.) $Ru(trpy)(N-N)(His83)Az$ was synthesized by reacting azurin with an excess (5−10 mM, pH 7−8) of $[Ru(trpy)(N-N)(H_2O)]^{2+}$ for nearly 48 h at 30 °C. A similar procedure was used to prepare $Os(trpy)(bpy)(His83)$-Az. $[Ru(trpy)(N-N)(H_2O)]^{2+}$ and $[Os(trpy)(bpy)(H_2O)]^{2+}$ were synthesized by literature methods: Ware, D. C.; Lay, P. A.; Taube, H. *Inorg. Synth.* **1986**, *24*, 299−306. Takeuchi, K. J.; Thompson, M. S.; Pipes, D. W.; Meyer, T. J. *Inorg. Chem.* **1984**, *23*, 1845−1851. All modified proteins were purified extensively by ion-exchange chromatography (FPLC) and characterized by absorption spectroscopy. The MLCT absorption bands in the modified azurins were at the same wavelengths as in the respective model complexes. λ_{max} (nm): $[Ru(bpy)_2(CN)(im)]^+$, 322 (sh), 416 (sh), 452; $[Ru(phen)_2(CN)$-$(im)]^+$,[17] 310 (sh), 420 (sh), 442; $[Ru(trpy)(bpy)(im)]^{2+}$, 360 (sh), 430 (sh), 476, 550 (sh), 600 (sh); $[Ru(trpy)(phen)(im)]^{2+}$, 420 (sh), 466, 550 (sh), 600 (sh); $[Os(trpy)(bpy)(im)]^{2+}$, 360 (sh), 480 (sh), 502, 590 (sh), 742.

(11) All electrochemical experiments were performed with a Princeton Applied Research Model 173 potentiostat driven by a PAR Model 175 Universal Programmer. Cyclic voltammetry (CV) was performed with a modified three-electrode configuration consisting of an edge-plane graphite (EPG) working electrode, platinum wire auxiliary electrode, and an SCE reference electrode. All three compartments were filled with 0.1 M sodium phosphate buffer at pH 7.0. Variable-temperature CV was carried out using a nonisothermal cell, in which the electrode was thermostated at 25 °C in an insulated beaker containing 3 M KCl and connected to the reference compartment of the electrochemical cell via an agar salt bridge (3 M KCl); the reference compartment was further separated from the working compartment by a Luggin capillary. Prior to use, the EPG electrode was polished with 0.3 mm alumina, sonicated for 15 min, and rinsed with distilled water. All ΔH and ΔS values for copper reduction and ruthenium oxidation are referenced to NHE, assuming $\Delta S_{NHE} = -130$ J/(K-mol).

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Table 1. [Cu(I) → M(III)] (M = Ru, Os) ET Data (298 K) for Modified Azurins

protein	k_{ET} (s^{-1})	$-\Delta G°$ (eV)[a]	pH	τ[b]
Ru(bpy)$_2$(im)(His83)Az	1.2(1) × 10^6	0.76(1)	7.0	(100, 36)
Ru(bpy)$_2$(CN)(His83)Az	1.2(1) × 10^6	0.72(2)	7.0	*c*
Ru(phen)$_2$(CN)(His83)Az	3.5(1) × 10^6	0.71(2)	7.0	(408, 98)
Ru(trpy)(bpy)(His83)Az	2.0(1) × 10^6	0.76(2)	7.0	(89, 63)
Ru(trpy)(phen)(His83)Az	2.7(1) × 10^6	0.78(2)	7.0	(103, 65)
Ru(bpy)$_2$(im)(His83)-(Met121Leu)Az	5.6(1) × 10^5	0.67(2)	7.0	*c*
Ru(trpy)(bpy)(His83)-(Met121Asp)Az	1.3(1) × 10^6	0.79(2)	7.5	(*c*, 85)
Ru(trpy)(bpy)(His83)-(Met121Asp)Az	1.7(1) × 10^6	0.76(2)	5.0	(*c*, 70)
Os(trpy)(bpy)(His83)Az	1.7(2) × 10^5	0.39(2)	7.0	*c*

[a] $E°$'{[Ru(bpy)$_2$(im)(His83)AzCu$^{2+/+}$] = 0.326 V ($\Delta H°$ = −63.7 kJ/mol; $\Delta S°$ = −108.4 J/(K-mol)); [Ru$^{3+/2+}$(bpy)$_2$(im)(His83)Az] = 1.082 V ($\Delta H°$ = −128.9 kJ/mol; $\Delta S°$ = −81.2 J/(K-mol)); [Ru$^{3+/2+}$(bpy)$_2$-(im)(CN)] = 1.05 V; [Ru$^{3+/2+}$(phen)$_2$(im)(CN)] = 1.04 V (ref 17); [Ru$^{3+/2+}$(trpy)(bpy)(im)] = 1.09 V; [Ru$^{3+/2+}$(trpy)(phen)(im)] = 1.11 V; [(Met121Leu)AzCu$^{2+/+}$] = 0.438, 0.412 V (pH 5.0, 7.0, ref 8); [(Met121Asp)AzCu$^{2+/+}$] = 0.333, 0.319 V (pH 6.0, 7.0, ref 8); [Os$^{3+/2+}$(trpy)(bpy)(im)] = 0.717 V ($\Delta H°$ = −88.9 kJ/mol; $\Delta S°$ = −66.2 J/(K-mol))} vs NHE. [b] Luminescence decay lifetimes for reduced and oxidized derivatives. [c] Not measured.

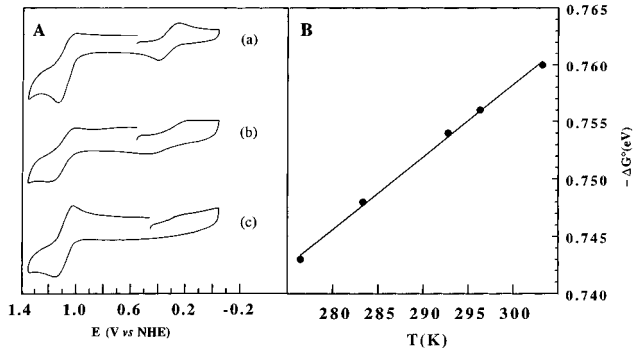

Figure 2. (A) Cyclic voltammograms of a ∼1 mM solution of Ru(bpy)$_2$(im)(His83)AzCu(II) in NaP$_i$ (μ = 0.1 M, pH 7.0).[11] Due to its high reduction potential, the Ru$^{3+/2+}$ couple is reversible only at low temperature and/or fast scan rates: (a) n = 0.1 V/s, T = 303 K; (b) n = 0.5 V/s, T = 303 K; (c) n = 0.1 V/s, T = 288 K. (B) Driving force as a function of temperature for the Cu(I) → Ru(III) reaction.

given in Figure 1 (inset). Furthermore, the observed rate is independent of the protein concentration, demonstrating that it represents an intramolecular reaction.

We have analyzed our results in terms of semiclassical ET theory (eq 1).[14] Since we know $\Delta G°$ as a function temperature

$$k_{ET} = (4\pi^3/h^2\lambda RT)^{1/2}(H_{AB})^2 \exp[-(\Delta G° + \lambda)^2/4\lambda RT] \quad (1)$$

for Ru(bpy)$_2$(im)(His83)Az, values for the reorganization energy (λ) and the tunneling matrix element (H_{AB}) can be obtained from the temperature dependence of the observed rate constant. The modest variation of k_{ET} with temperature places λ in the range 0.6−0.8 eV, and a value near 0.8 eV is consistent with the dependence of the rate constant on $-\Delta G°$ [λ = 0.80(5) eV and H_{AB} = 0.067(5) cm^{-1} for Ru(bpy)$_2$(X)(His83)Az (X = im, CN) and M(trpy)(bpy)(His83)Az (M = Ru, Os)].[15] With λ(RuAz) = 0.80 eV, and taking λ(Ru) = 0.78 eV,[16−18] we find λ(Az) = 0.82 eV. The minor variations in k_{ET} for the different Ru-modified proteins (Table 1) may reflect small differences in coupling between the polypeptide bridge and the Ru complexes.[17,19]

As the total reorganization energy is only 0.82 eV, and much of that is undoubtedly associated with outer-sphere (solvent/protein) reorientation,[14] the inner-sphere reorganization energy of the blue site must be very small (≤0.2 eV).[20] A relatively small λ for the copper is required for facile long-range ET with redox couples such as S_2^-/S_2. Analysis of the temperature dependence of intramolecular ET from a Cys3-Cys26 disulfide radical anion to the azurin blue copper gives a reorganization energy of ∼1 eV.[21] Taking λ(Az) = 0.82 eV, we obtain ∼1.2 eV for $\lambda(S_2^-/S_2)$. This relatively high reorganization energy is not unreasonable in view of the large decrease in the S−S bond distance that should accompany depopulation of the $\sigma^*(S_2)$ orbital. For comparison, $\lambda(O_2^-/O_2)$ in aqueous solution has been estimated to be ≥2 eV.[22]

Analysis of both driving force and temperature dependences of intramolecular ET rates shows that the reorganization energies of azurin and cytochrome *c* are in the 0.7−0.8 eV range,[16−18] as required for function. In the Cu_A subunit of cytochrome oxidase, the reorganization energy is further decreased because of electron delocalization over the two copper atoms in the redox center.[23−29] Again, this is consistent with its function, which is to mediate ET between cytochrome *c* and the proton-pumping machinery of the oxidase. An electron can enter and leave Cu_A by different routes,[24] a necessity because the membrane-bound protein cannot rotate. In azurin and other blue copper proteins, on the other hand, the electron can be transferred to and from the copper via the same path.

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(16) Calculated from λ(RuAz) = [λ(Az) + λ(Ru)]/2;[14] the value of λ-(Ru) is assumed to be the same as that extracted from the dependence of ET rates on driving force in Ru(bpy)$_2$(im)(His33)cytochrome *c* (λ(Rucyt *c*) = 0.74 eV;[17] λ(cyt *c*) = 0.70 eV[18]).

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