Journal of Bioenergetics and Biomembranes, Vol. 27, No. 3, 1995

Electron Transfer in Ruthenium-Modified Proteins

Morten J. Bjerrum, Danilo R. Casimiro, I-Jy Chang, Angel J. Di Bilio, Harry B. Gray,[1] Michael G. Hill, Ralf Langen, Gary A. Mines, Lars K. Skov, Jay R. Winkler,[1] and Deborah S. Wuttke

Received February 27, 1995

Photochemical techniques have been used to measure the kinetics of intramolecular electron transfer in $Ru(bpy)_2(im)(His)^{2+}$-modified (bpy = 2,2′-bipyridine; im = imidazole) cytochrome *c* and azurin. A driving-force study with the His33 derivatives of cytochrome *c* indicates that the reorganization energy (λ) for $Fe^{2+} \rightarrow Ru^{3+}$ ET reactions is 0.8 eV. Reductions of the ferriheme by either an excited complex, $*Ru^{2+}$, or a reduced complex, Ru^{+}, are anomalously fast and may involve formation of an electronically excited ferroheme. The distance dependence of $Fe^{2+} \rightarrow Ru^{3+}$ and $Cu^{+} \rightarrow Ru^{3+}$ electron transfer in 12 different Ru-modified cytochromes and azurins has been analyzed using a tunneling-pathway model. The ET rates in 10 of the 12 systems exhibit an exponential dependence on metal–metal separation (decay constant of 1.06 $Å^{-1}$) that is consistent with predictions of the pathway model.

KEY WORDS: Electron transfer; cytochrome *c;* azurin; ruthenium; electronic coupling; driving-force dependence.

INTRODUCTION

Chemical modification of proteins has long been used in biochemical studies of structure and function. $Ru(NH_3)_5(OH_2)^{2+}$ was first employed as a His-selective protein-modification reagent with ribonuclease A (Matthews *et al.*, 1978). The goal of this study was the development of an environment-sensitive spectroscopic label for studies of protein folding. Environment-sensitive absorption spectra are not the sole attribute that Ru complexes bring to biochemistry: the $Ru^{3+/2+}$ redox couple is nearly ideal for investigations of electron-transfer (ET) reactions. Low-spin, pseudo-octahedral Ru complexes exhibit very small structural changes upon interconversion between the Ru^{2+} and Ru^{3+} formal oxidation states. Hence the inner-sphere reorganization barriers to their ET reactions tend to be small (Brown and Sutin, 1979). Furthermore, with appropriate choice of ligands, the $Ru^{3+/2+}$ reduction potential can range from <0 to >1.5 V vs. NHE. The combination of covalent protein modification and favorable ET properties paved the way for the first measurement of long-range electron transfer in $Ru(NH_3)_5$(His33)-cytochrome *c* (Winkler *et al.*, 1982). Many measurements of long-range ET in Ru-ammine-modified proteins were performed in the ensuing years, and these have been reviewed recently (Winkler and Gray, 1992).

Ru-bpy complexes (e.g., $Ru(bpy)_2(im)(His)^{2+}$; bpy = 2,2′-bipyridine; im = imidazole) exhibit the same attractive ET properties as Ru-ammine complexes, but have an additional feature not found with the ammines: long-lived, luminescent metal-to-ligand charge-transfer (MLCT) excited states. These excited states permit a wider range of electron-transfer measurements than is possible with nonluminescent complexes. In addition, the π-acidic bpy ligands raise the $Ru^{3+/2+}$ reduction potential to >1 V vs. NHE. Potent oxidants of this type allow measurements of intramolecular ET rates at driving forces near the activationless limit (*vide infra*), which leads to more reliable estimates of fundamental ET parameters. Results of ET

[1] Corresponding authors: Contribution from the Beckman Institute, California Institute of Technology, Pasadena, California 91125.

0145-479X/95/0600-0295$07.50/0 © 1995 Plenum Publishing Corporation

measurements in Ru-bpy- and Ru-phen-modified (phen = 1,10-phenanthroline) cytochrome *c* (cyt *c*) and azurin will be reviewed in this article.

The starting point for most discussions of ET reactions in modified proteins is the semiclassical expression describing the rate constant for electron transfer between two fixed redox sites [Eq. (1)] (Marcus and Sutin, 1985). This equation

$$k_{ET} = \sqrt{\frac{4\pi^3}{h^2\lambda k_B T}} H_{AB}^2 \exp\left(-\frac{(\Delta G^\circ + \lambda)^2}{4\lambda k_B T}\right) \quad (1)$$

predicts that rates should exhibit a Gaussian dependence on reaction free-energy change, and that at the optimum driving force ($-\Delta G^\circ = \lambda$), rates will be limited solely by the magnitude of the reactant-product electronic coupling at the transition state (H_{AB}). Inspection of Eq. (1) reveals that in the low-driving-force regime ($-\Delta G^\circ \ll \lambda$) the variation of ET rates with changes in driving force provides very little information about λ. Hence, studies of high-driving-force ET reactions involving strong oxidants or reductants (e.g., Ru-bpy complexes) are ideally suited for extracting reorganization and electronic-coupling parameters.

METHODS

Although other techniques are available, most measurements of intramolecular ET kinetics rely upon some form of pulsed-laser-initiated electron-transfer event. The methodology that we have employed with Ru-bpy-modified proteins is illustrated in Fig. 1. In a typical reaction sequence, a laser-excited Ru-bpy complex ($^*Ru^{2+}$) directly donates (accepts) an electron to (from) a redox partner (*ET), generating the oxidized (reduced) Ru-bpy complex and the reduced (oxidized) partner (Fig. 1: **1→2→3** (**6→4→5**)). The intermediate formed in this photoinduced ET reaction decays in a subsequent charge-recombination reaction to regenerate the original donor–acceptor complex (**3→1** (**5→6**)). This scheme is viable only when the *ET rate competes effectively with excited-state deactivation. The excited-state lifetime of a model of a Ru-bpy-modified protein, $Ru(bpy)_2(im)_2^{2+}$, is relatively short (τ = 70 ns in H_2O (Chang *et al.*, 1991)) and only *ET rates $>2 \times 10^6$ s^{-1} will produce measurable changes in the observed excited-state decay kinetics. If this were the only technique available, the short excited-state lifetime of the Ru-bpy complex would limit measurements of ET rates to systems in which the Ru-complex and protein active site were relatively well coupled.

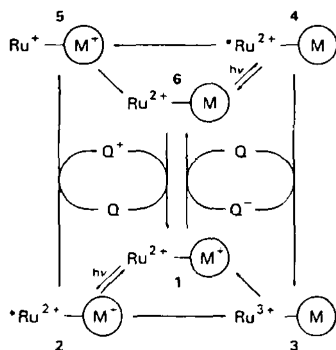

Fig. 1. Reaction scheme for measuring intramolecular ET rates in Ru-bpy-modified metalloproteins.

A second approach, however, circumvents this problem. In the so-called flash-quench procedure (Fig. 1: oxidative, **6→4→3→1**; reductive, **1→2→5→6**), a quencher (Q) is added to the solution to react with $^*Ru^{2+}$ in a bimolecular electron-transfer reaction. This bimolecular quenching reaction generates the same intermediates as intramolecular quenching reactions (**3** or **5**) but with greater efficiency. Once generated, the intermediate will react via an intramolecular ET reaction. Then, on a much longer timescale, the reduced (oxidized) quencher will react with the oxidized (reduced) protein to regenerate the original complex (**1↔6**). Even longer time windows can be examined if irreversible quenchers are employed. The combination of these two strategies for studying intramolecular ET rates permits measurements of ET rates on timescales ranging from picoseconds to seconds.

Cytochrome *c*

Driving-Force Variations

We have continued to employ His33-labeled cytochrome *c* as a prototype system for studies of electron transfer in Ru-modified proteins. The Ru-bpy derivative, $Ru(bpy)_2(im)$(His33)-cyt *c*, is prepared by incubating horse heart cytochrome *c* with $Ru(bpy)_2(CO_3)$ (Durham *et al.*, 1990; Millett and Durham, 1991; Chang *et al.*, 1991). The rate constant for $Fe^{2+} \to Ru^{3+}$ ET in this protein was first measured by an oxidative flash-quench procedure (Fig. 1: **6→4→3→1**): $k_{ET} = 2.6 \times 10^6$ s^{-1} ($-\Delta G^\circ = 0.81$ eV) (Chang *et al.*, 1991; Mines *et al.*, 1995). ET rates at

five additional driving forces have now been measured using proteins labeled at His33 with substituted Ru-bpy complexes (Table I). The driving-force dependence of the $Fe^{2+} \rightarrow Ru^{3+}$ ET can be described by Eq. (1) using $\lambda = 0.8$ eV and $H_{AB} = 0.09$ cm^{-1} (Fig. 2). The magnitude of λ is about one-third smaller than that found in studies of Ru-ammine modified cytochrome *c* (Meade *et al.*, 1989), and this is in accord with the smaller reorganization energy expected for Ru-bpy complexes (Brown and Sutin, 1979). Interestingly, the value of λ extracted from the driving-force study is quite close to that predicted using the Marcus cross relation (Winkler and Gray, 1992). A similar value ($\lambda = 0.93$ eV) was reported for ET in $Ru(bpy)_3^{2+}$-modified cytochrome b_5 (Scott *et al.*, 1993, 1994).

A reductive flash-quench approach (Fig. 1: **1→2→5→6**) has been employed in measurements of $Ru^{+} \rightarrow Fe^{3+}$ ET rates in two Ru(His33)-modified cytochromes *c* (Mines *et al.*, 1993, 1995). We have also extracted the rate of $*Ru^{2+} \rightarrow Fe^{3+}$ ET in two His33 derivatives by measuring the yield of $Ru^{3+}-Fe^{2+}$-cyt *c* formed following $*Ru^{2+}$ decay. These reactions proceed at very high driving forces (≥ 1.1 eV) with rates $\geq 2 \times 10^5$ s^{-1} (Table I). The rates for the two highest-driving-force reactions do not fit on the same driving-force curve as the $Fe^{2+} \rightarrow Ru^{3+}$ rates (Fig. 2); the ferriheme reduction rates are much faster than expected for the high driving forces involved.

There are three possible explanations for the anomalously fast $*Ru^{2+} \rightarrow Fe^{3+}$ and $Ru^{+} \rightarrow Fe^{3+}$ reactions. The Gaussian free-energy dependence described by Eq. (1) is based on a model that treats nuclear motions classically (Marcus and Sutin, 1985); quantum-mechanical models lead to qualitatively similar results, but the magnitude of the inverted effect is substantially attenuated (Brunschwig and Sutin, 1987). An unrealistically large amount of high-frequency reorganization, however, is required to account for the $*Ru^{2+} \rightarrow Fe^{3+}$ and $Ru^{+} \rightarrow Fe^{3+}$ ET rates (Mines *et al.*, 1995).

Alternatively, the electronic coupling could be larger for the $*Ru^{2+} \rightarrow Fe^{3+}$ and $Ru^{+} \rightarrow Fe^{3+}$ ET reactions than for $Fe^{2+} \rightarrow Ru^{3+}$ ET. Simple descriptions of $*Ru^{2+}$ and Ru^{+} complexes localize the transferring electron in a π^* orbital of a bpy ligand, whereas the hole in the Ru^{3+} complex is metal-localized (Sutin and Creutz, 1978). The Ru^{3+} center is directly bound to the protein through the His ligand, but the bpy ligands meet the protein only through van der Waals contacts. We have found in a study of $Ru(bpy)_2$(His-39)(His58)$^{2+}$-modified cyt *c* that the $*Ru^{2+} \rightarrow Fe^{3+}$ ET is slower ($k_{ET} = 2.4 \times 10^5$ s^{-1}) than in either of the monodentate analogs (i.e., $Ru(bpy)_2(im)(HisX)^{2+}$, $Ru(bpy)_2(im)(HisY)^{2+}$: His39, $k_{ET} = 1.1 \times 10^6$ s^{-1}; His58, $k_{ET} = 3.2 \times 10^5$ s^{-1}) (Casimiro *et al.*, 1993a). This observation suggests that in the chelated complex the bpy ligands are directed away from the protein, weakening the electronic coupling. We expect the bpy-protein contacts to produce generally poorer couplings for $*Ru^{2+} \rightarrow Fe^{3+}$ and $Ru^{+} \rightarrow Fe^{3+}$ ET than the Ru^{3+}-His contacts yield in $Fe^{2+} \rightarrow Ru^{3+}$ reactions. The high ferriheme reduction rates are, therefore, unlikely to be the result of unusually strong electronic-coupling interactions.

The third possibility is that the $*Ru^{2+}$ and Ru^{+} reactions are so highly exergonic that they lead to the formation of electronically excited products. This situation is simply another manifestation of the inverted effect in which a lower-driving-force reaction is faster than a high-driving-force process (Marcus and Sutin, 1985). When an ET reaction forms excited products, the actual reaction driving force is lower (by an amount equal to the energy of the excited state formed in the reaction) than that determined on the basis of the redox potentials of the two sites

Table I. Rates and Driving Forces for Intraprotein ET in His33-Labeled Cytochrome *c*

Complex	Reaction	k_{ET} s^{-1}	$-\Delta G^\circ$ eV[a]
$Ru(4,4',5,5'-(CH_3)_4$-bpy$)_2$(im)(His)$^{2+}$	$Fe^{2+} \rightarrow Ru^{3+}$	1.6×10^6	0.59
$Ru(4,4'-(CH_3)_2$-bpy$)_2$(im)(His)$^{2+}$	$Fe^{2+} \rightarrow Ru^{3+}$	2.0×10^6	0.70
$Ru(bpy)_2(im)(His)^{2+}$	$Fe^{2+} \rightarrow Ru^{3+}$	2.6×10^6	0.81
	$*Ru^{2+} \rightarrow Fe^{3+}$	2.0×10^5	1.25
$Ru(4,4'-(CONH(C_2H_5))_2$-bpy$)_2$(im)(His)$^{2+}$	$Fe^{2+} \rightarrow Ru^{3+}$	1.1×10^6	1.05
	$Ru^{+} \rightarrow Fe^{3+}$	2.3×10^5	1.1[b]
$Ru(phen)_2(im)(His)^{2+}$	$Fe^{2+} \rightarrow Ru^{3+}$	3.5×10^6	0.75
$Ru(phen)_2(CN)(His)^{+}$	$Fe^{2+} \rightarrow Ru^{3+}$	1.0×10^7	0.78
	$Ru^{+} \rightarrow Fe^{3+}$	4.5×10^5	1.6[c]
	$*Ru^{2+} \rightarrow Fe^{3+}$	2.0×10^5	1.4[c]

[a] Driving forces were calculated using 0.26 V vs. NHE for the $Fe^{3+/2+}$–cyt *c* potential. $Ru^{3+/2+}$ potentials were determined directly with the modified proteins, or were taken from measurements on model complexes (Mines *et al.*, 1995).

[b] Estimate based on measurements of the metal-to-ligand charge-transfer absorption maximum and the $Ru^{3+/2+}$ potential (Dodsworth and Lever, 1986; Kalyanasundarum, 1982).

[c] Estimate based on the $Ru(phen)_3^{2+/+}$ potential (Kalyanasundarum, 1982).

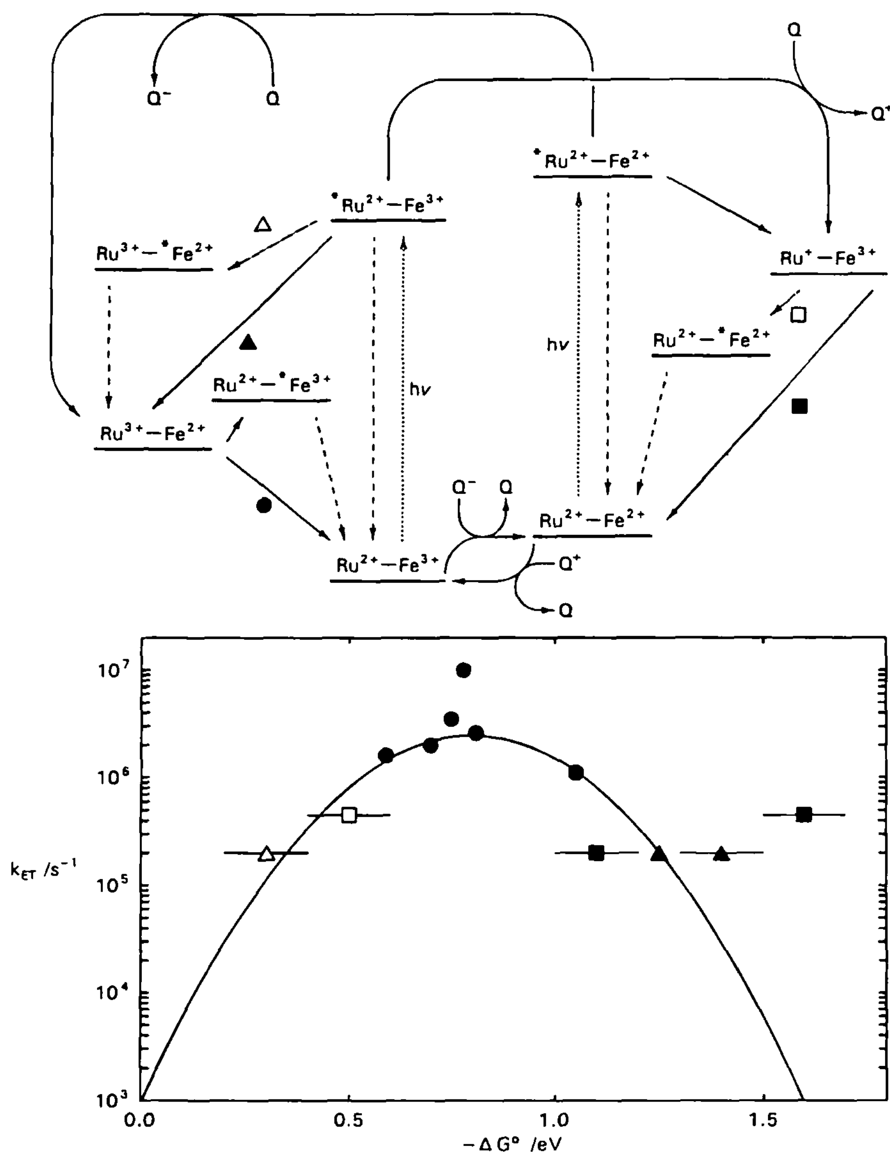

Fig. 2. (Upper) Possible reaction sequences following Ru-bpy excitation in Ru-modified cytochromes: ···› excitation; - - - → excited-state deactivation (nonET); ——→ ET reactions. (Lower) Driving-force dependence of intramolecular ET rates in His33-modified cytochromes *c*: $Fe^{2+} \rightarrow Ru^{3+}$ (●); $Ru^{+} \rightarrow Fe^{3+}$ (■,□); $*Ru^{2+} \rightarrow Fe^{3+}$ (▲,△). Open symbols refer to a reaction in which a 1.1-eV excited ferroheme is formed.

(McCleskey *et al.*, 1992). In the case of $*Ru^{2+} \rightarrow Fe^{3+}$ and $Ru^{+} \rightarrow Fe^{3+}$ ET, excited states of the Ru^{2+} complex and the ferroheme must be considered (Heacock, II, *et al.*, 1994). The lowest-lying excited states of the Ru^{2+} complex are ~ 2 eV above the ground state and cannot be populated in ET reactions between Ru^{+} and the ferriheme. The origin of the lowest-lying absorption band in ferrocytochrome *c*, however, is found near 1.3 eV, and spin-forbidden states will lie at even lower energies (Mines *et al.*, 1995). Hence, if a 1.1-eV excited state of ferrocytochrome *c* is formed upon reduction of ferricytochrome *c* by $*Ru^{2+}$ or Ru^{+}, then the actual reaction driving forces will be in the 0.3–0.5-eV range and the observed rate constants are more consistent with results obtained for $Fe^{2+} \rightarrow Ru^{3+}$ ET (Fig. 2).

Electronic-Coupling Strengths

Understanding how a heterogeneous polypeptide mediates the electronic coupling between redox sites in a protein is a subject of considerable experimental and theoretical interest. Dutton and coworkers, in a survey of a variety of biological ET systems, found a strong empirical correlation between electronic-coupling strength and edge-to-edge donor–acceptor distances (R_E) (Moser *et al.*, 1992; Farid *et al.*, 1993). Specifically, they found that k_{max} decayed exponentially with increasing R_E; the empirical decay constant (β) was 1.4 $Å^{-1}$. Although this empirical correlation provides a useful starting point for discussions of the distance dependence of ET rates, it implies that the composition of the intervening protein does not affect the coupling between the redox sites that it separates. Indeed, the exponential distance dependence is in accord with a model that treats the protein as a homogeneous square tunneling barrier (Hopfield, 1974).

Studies of ET in Ru-modified cytochrome *c* are at variance with this simple interpretation of electronic coupling in proteins. ET rates have been measured in eight different $Ru(bpy)_2(im)(HisX)^{2+}$ derivatives of wild-type and mutant cytochromes *c* (Table II) (Chang *et al.*, 1991; Wuttke *et al.*, 1992; Casimiro *et al.*, 1993b; Karpishin *et al.*, 1994). Maximum ET rates do not correlate well with a 1.4 $Å^{-1}$ distance decay and it seems clear that a model that takes into account the structure of the bridging medium is required. Beratan and Onuchic have developed a tunneling-pathway model to describe electronic couplings between redox sites in proteins (Onuchic *et al.*, 1992; Beratan *et al.*, 1991; Onuchic *et al.*, 1991; Onuchic and Beratan, 1990). In this model, the many complex interactions between the atoms that comprise the protein are reduced to just three: covalent bonds, hydrogen bonds, and through-space contacts. A structurally characterized protein can be reconstructed in terms of this limited set of interactions and a pathway searching algorithm can be employed to identify optimum coupling paths between electron donor and acceptor. We have found in Ru-modified cytochrome *c* that a small number of well-defined pathways (often just one) dominates the coupling between the Ru-center and the heme (Casimiro *et al.*, 1993b,c). The relative coupling strengths determined from the pathway analysis can be recast in terms of an effective tunneling pathlength ($\sigma\ell$). The tunneling-pathway model predicts that maximum ET rates will decay exponentially with $\sigma\ell$. The decay constant (β') is determined by the coupling decay across a single covalent bond (ϵ_c), a quantity that is a function of the energy of the tunneling electron. A value of $\epsilon_c = 0.6$ implies a decay constant $\beta' = 0.73$ $Å^{-1}$ (Casimiro *et al.*, 1993b). Plots of log k_{max} vs. $\sigma\ell$ for Ru-bpy-modified cytochromes exhibit excellent linear correlations (Fig. 3). More involved treatments of electronic coupling in Ru-modified cytochrome *c* have also provided remarkably good agreement between calculated and experimental coupling strengths (Siddarth and Marcus, 1993; Gruschus and

Table II. Dependence of $Fe^{2+} \rightarrow Ru^{3+}$ ET Rates and Electronic Couplings on Binding Site in $Ru(bpy)_2(im)(HisX)$ Cytochromes *c*

X^a	k_{ET}, s^{-1}	H_{AB}, cm^{-1b}	R_M, Å	$\sigma\ell$, Å
39 (Y, n)	3.3×10^6	0.11	20.3	28.0
33 (H, n)	2.6×10^6	0.097	17.9	27.9
66 (Y, m)	1.0×10^6	0.06	18.9	25.2
72 (H, m)	9.0×10^5	0.057	13.8	30.2
58 (Y, m)	5.2×10^4	0.014	20.2	29.8
54′ (Y, m)^c	5.7×10^4	0.014	21.5	32.7
54 (Y, m)	3.1×10^4	0.011	22.5	33.7
62 (Y, m)	1.0×10^4	0.006	20.2	37.2

[a] Letters in parentheses indicate the source of the cytochrome *c* (Y = yeast iso-1-cytochrome *c*; H = horse heart cytochrome *c*) and whether the modified His residue is a native (n) or mutant (m) group.
[b] H_{AB} calculated using $\lambda = 0.8$ eV.
[c] Asn52Ile, Lys54His double mutant of yeast cytochrome *c* (Karpishin *et al.*, 1994).

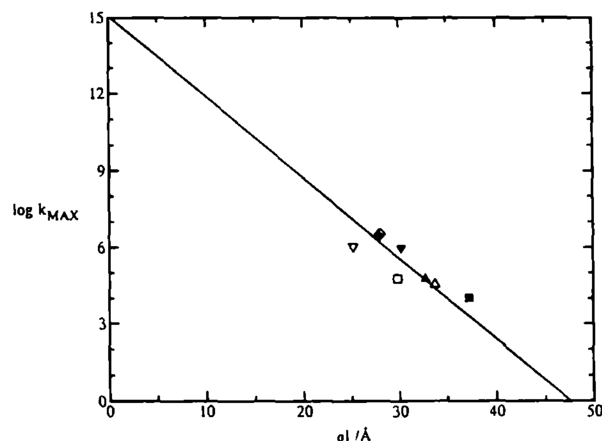

Fig. 3. Tunneling-pathlength dependence of maximum ET rates in Ru-bpy-modified cytochromes *c*: His39 (◇); His33 (◆); His66 (▽); His72 (▼); His58 (□); His54′ (▲); His54 (△); His62 (■).

Kuki, 1993). As is the case with the tunneling-pathway model, these treatments find that the coupling strength is extremely sensitive to the detailed composition of the medium bridging the redox sites.

Azurin

Recent work has focused on ET in Ru-bpy-modified derivatives of wild-type and mutant azurins (Langen *et al.*, 1995; Di Bilio *et al.*, 1995). The active site in azurin is a type I blue copper center coordinated by three strong ligands in an equatorial plane (His46, His117, Cys112), and two weakly interacting axial groups (Met121, carbonyl O of Gly45). The protein has a β-barrel structure with individual β-strands that extend directly from the Cu ligands (Adman and Jensen, 1981). This unique structure provides an opportunity to probe electronic coupling mediated by a particular type of protein secondary structure.

A native surface His residue of *Pseudomonas aeruginosa* azurin (His83) has been modified by coordination to a $Ru(bpy)_2(im)^{2+}$ complex. The $Cu^+ \rightarrow Ru^{3+}$ ET rate measured in this modified protein is 1.1×10^6 s^{-1} (Table III) (Di Bilio *et al.*, 1995; Regan *et al.*, 1995). It is clear from Eq. (1) that the reorganization energy for an ET reaction can be determined from the temperature dependence of the observed rate constant. In the 3–35°C temperature range, the variation in the $Cu^+ \rightarrow Ru^{3+}$ ET rate is smaller than the experimental error in the measured values (<10%). It is tempting to assume that the negligible temperature variation of the ET rate indicates that this reaction occurs at a driving force where $-\Delta G° = \lambda$ [Eq. (1)]. Care must be taken, however, to account for the temperature dependence of $\Delta G°$. We have measured the redox potentials of both the Ru and Cu sites in $Ru(bpy)_2(im)(His83)^{2+}$ -azurin at temperatures in the 3–35°C range. We find that the $Cu^+ \rightarrow Ru^{3+}$ reaction driving force varies by only 13 mV over this temperature range. Fitting the data to Eq. (1) yields $\lambda = 0.87$ eV and $H_{AB} = 0.07$ cm^{-1} (Di Bilio *et al.*, 1995). The reorganization energy for Ru-bpy-modified azurin is quite close to that determined for Ru-bpy-modified cyt *c*, indicating that comparable degrees of nuclear reorientation accompany the reactions of these two electron-transfer proteins.

Using site-directed mutagenesis, surface His residues have been introduced at three locations (His122, His124, His126) on the β-strand extending from the Met121 ligand. The $Cu^+ \rightarrow Ru^{3+}$ ET rates measured for these three modified azurins span five orders of magnitude (Table III) (Langen *et al.*, 1995). In this instance, the ET rates correlate equally well with direct metal-to-metal distance (R_M) and $\sigma\ell$ (Fig. 4), implying that the two distance measures are linearly related. This is to be expected, since the β-strand structure is quite close to that of an extended hydrocarbon in which the direct distance between sites depends nearly linearly upon the number of carbon atoms separating them. An important distinction between the Ru-modified azurin data and Dutton's empirical distance correlation is the slope. A value of 1.1 $Å^{-1}$ is obtained for the coupling decay along the β-strand extending from Met121 in azurin; Dutton's distance decay parameter is 1.4 $Å^{-1}$ (Moser *et al.*, 1992; Farid *et al.*, 1993). Apparently, a β-strand is a more efficient mediator of electronic coupling than are the structures in the systems surveyed by Dutton.

Table III. Dependence of $Cu^+ \rightarrow Ru^{3+}$ ET Rates and Electronic Couplings on Binding Site in $Ru(bpy)_2(im)(HisX)$ Azurins

X	k_{ET}, s^{-1}	H_{AB}, cm^{-1a}	R_M, Å	$\sigma\ell$, Å
83	1.1×10^6	0.068	16.9	31.0
122	7.1×10^6	0.16	15.9	21.0
124	2.2×10^4	0.0088	20.6	29.4
126	1.3×10^2	0.0007	26.0	37.8

[a] H_{AB} calculated using $\lambda = 0.8$ eV.

DISCUSSION

Careful analysis of the variation of ET rates with Ru-binding site in Ru-modified cyt *c* and azurin demonstrates that the structure and composition of the intervening medium are critical in determining distant electronic couplings (Wuttke *et al.*, 1992; Casimiro *et al.*, 1993b; Karpishin *et al.*, 1994; Langen *et al.*, 1995). It is difficult to ignore, however, Dutton's empirical rate vs. distance correlation [12 orders of magnitude in rate spanning 20 Å in distance (Moser *et al.*, 1992; Farid *et al.*, 1993)], as well as the fact that in select subsets of our Ru-modified-protein database ET rates correlate equally well with R_M and $\sigma\ell$ (*vide supra*) (Langen *et al.*, 1995). Indeed, it is interesting to examine the correlation between $\sigma\ell$ and R_M for the twelve Ru-modified cytochromes and azurins (Fig. 4). Remarkably, for all but three of these proteins (Ru(His72)-cyt *c*, Ru(His62)-cyt *c*, and Ru(His83)-

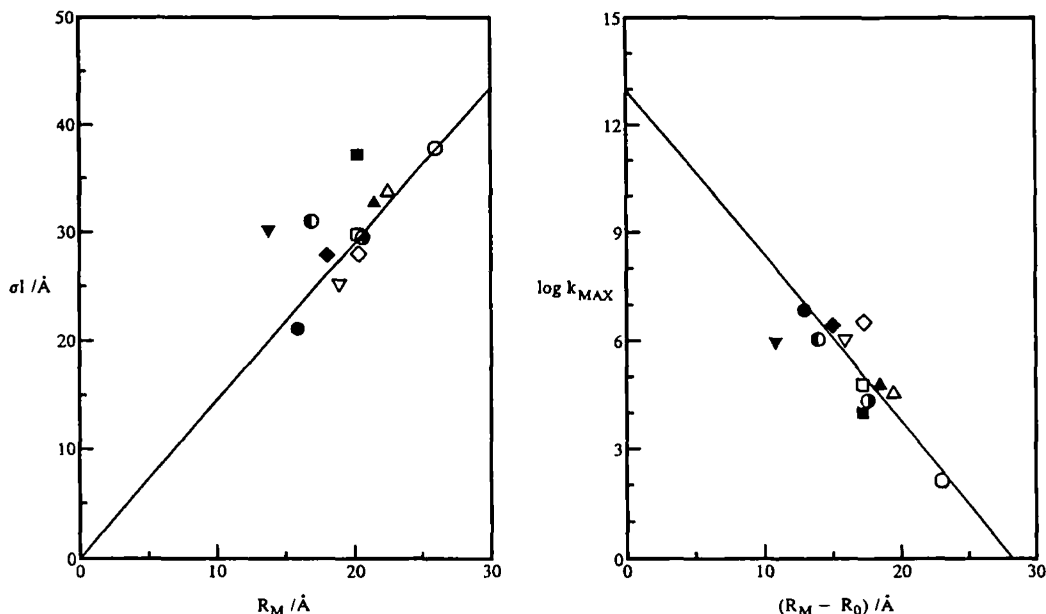

Fig. 4. (Left) Correlation of tunneling pathlengths with metal–metal separation distances in Ru-modified cytochromes *c* and azurins. (Right) Variation of maximum ET rates with R_M ($R_0 \equiv 3$ Å). Cytochrome *C:* His39 (◇); His33 (◆); His66 (▽); His72 (▼); His58 (□); His54' (▲); His54 (△); His62 (■). Azurin: His122 (●); His124 (◑); His126 (○); His83 (◐).

azurin), the $\sigma\ell$ vs. R_M relationship can be described by a single straight line with a slope of 1.45 $\sigma\ell$-Å/R_M-Å. The linear $\sigma\ell/R_M$ relationship predicts that, with just those three exceptions, maximum ET rates should vary exponentially with R_M with a decay constant of $\beta = 1.06$ Å^{-1}. The plot log k_{max} vs. R_M in Fig. 4 largely supports this analysis. This $\sigma\ell/R_M$ plot for Ru-modified cyt *c* and azurin represents relatively efficient mediation of electronic coupling; the corresponding predicted slope for an extended hydrocarbon polymer is 1.30 $\sigma\ell$-Å/R_M-Å with $\beta = 0.87$ Å^{-1}. We can conclude, then, that the surface sites on cytochrome *c* and azurin that we have examined so far are well coupled for ET to the metalloprotein active sites.

The maximum ET rate for $Ru(bpy)_2$(-im)(His83)$^{2+}$-azurin agrees better with the log k_{max} vs. R_M correlation than would be expected on the basis of the $\sigma\ell/R_M$ plot. The best tunneling pathway found for this system involves two hydrogen-bond-mediated β-strand crossings to the Cys112 strand (Regan *et al.*, 1995). The anomalously strong coupling found in the His83 derivative indicates that these β-sheet H-bonds are as effective as covalent bonds in mediating distant donor–acceptor interactions.

We have found Ru-bpy-modified proteins to be extremely useful for studying the fundamental aspects of long-range intraprotein ET reactions. Other investigators have employed them successfully in work on ET in protein–protein complexes (Geren *et al.*, 1991; Pan *et al.*, 1993; Scott *et al.*, 1993, 1994). The capacity for photochemical generation of potent oxidants and reductants bound directly to protein surfaces provides several promising avenues for further exploration.

ACKNOWLEDGMENTS

Our studies of electron transfer in Ru-modified proteins have been supported by the National Science Foundation, the National Institutes of Health, and the Arnold and Mabel Beckman Foundation.

REFERENCES

Adman, E. T., and Jensen, L. H. (1981). *Isr. J. Chem.* **21**, 8–12.
Beratan, D. N., Betts, J. N., and Onuchic, J. N. (1991). *Science* **252**, 1285–1288.
Brown, G. M., and Sutin, N. (1979). *J. Am. Chem. Soc.* **101**, 883–892.
Brunschwig, B. S., and Sutin, N. (1987). *Comments Inorg. Chem.* **6**, 209–235.
Casimiro, D. R., Muheim, A., Richards, J. R., Arnold, F. H., Winkler, J. R., and Gray, H. B. (1993). *J. Inorg. Biochem.* **51**, 232.
Casimiro, D. R., Richards, J. H., Winkler, J. R., and Gray, H. B. (1993). *J. Phys. Chem.* **97**, 13073–13077.

Casimiro, D. R., Wong, L.-L., Colón, J. L., Zewert, T. E., Richards, J. H., Chang, I.-J., Winkler, J. R., and Gray, H. B. (1993). *J. Am. Chem. Soc.* **115**, 1485–1489.

Chang, I.-J., Gray, H. B., and Winkler, J. R. (1991). *J. Am. Chem. Soc.* **113**, 7056–7057.

Di Bilio, A., Skov, L. K., Hill, M. G., and Gray, H. B. (1995). To be submitted for publication.

Dodsworth, E. S., and Lever, A. B. P. (1986). *Chem. Phys. Lett.* **124**, 152–158.

Durham, B. D., Pan, L. P., Hahn, S., Long, J., and Millett, F. (1990). In *ACS Advances in Chemistry Series* (Johnson, M. K., King, R. B., Kurtz, D. M., Kutal, C., Norton, M. L., and Scott, R. A., eds.), American Chemical Society, Washington DC, pp. 180–193.

Farid, R. S., Moser, C. C., and Dutton, P. L. (1993). *Curr. Opin. Struct. Biol.* **3**, 225–233.

Geren, L., Hahm, S., Durham, B., and Millett, F. (1991). *Biochemistry* **30**, 9450–9457.

Gruschus, J. M., and Kuki, A. (1993). *J. Phys. Chem.* **97**, 5581–5593.

Heacock D. H., II, Harris, M. R., Durham, B., and Millett, F. (1994). *Inorg. Chim. Acta* **226**, 129–135.

Hopfield, J. J. (1974). *Proc. Natl. Acad. Sci. USA* **71**, 3640–3644.

Kalyanasundaram, K. (1982). *Coord. Chem. Rev.* **46**, 159–244.

Karpishin, T. B., Grinstaff, M. W., Jomar-Panicucci, S., McLendon, G., and Gray, H. B. (1994). *Structure* **2**, 415–422.

Langen, R., Chang, I.-J., Germanas, J. P., Richards, J. H., Winkler, J. R., and Gray, H. B. (1995). *Science* **268**, 1733–1735.

Marcus, R. A., and Sutin, N. (1985). *Biochim. Biophys. Acta* **811**, 265–322.

Matthews, C. R., Erickson, P. M., Van Vliet, D. L., and Petersheim, M. (1978). *J. Am. Chem. Soc.* **100**, 2260–2262.

McCleskey, T. M., Winkler, J. R., and Gray, H. B. (1992). *J. Am. Chem. Soc.* **114**, 6935–6937.

Meade, T. J., Gray, H. B., and Winkler, J. R. (1989). *J. Am. Chem. Soc.* **111**, 4353–4356.

Millett, F., and Durham, B. (1991). In *Metals in Biological Systems,* (ed.), Marcel Dekker, Inc., New York, pp. 223–264.

Mines, G. A., Winkler, J. R., Gray, H. B., and Chan, S. I. (1993). *J. Inorg. Biochem.* **51**, 236.

Mines, G. A., Bjerrum, M. J., Hill, M. G., Casimiro, D. R., Chang, I-J., Winkler, J. R., and Gray, H. B. (1995). *J. Am. Chem. Soc.*, submitted for publication.

Moser, C. C., Keske, J. M., Warncke, K., Farid, R. S., and Dutton, P. L. (1992). *Nature* **355**, 796–802.

Onuchic, J. N., and Beratan, D. N. (1990). *J. Chem. Phys.* **92**, 722–733.

Onuchic, J. N., Andrade, P. C. P., and Beratan, D. N. (1991). *J. Chem. Phys.* **95**, 1131–1138.

Onuchic, J. N., Beratan, D. N., Winkler, J. R., and Gray, H. B. (1992). *Annu. Rev. Biophys. Biomol. Struct.* **21**, 349–377.

Pan, L. P., Hibdon, S., Liu, R.-Q., Durham, B., and Millett, F. (1993). *Biochemistry* **32**, 8492–8498.

Regan, J. J., Di Bilio, A. J., Langen, R., Skov, L. K., Winkler, J. R., Gray, H. B., and Onuchic, J. N. (1995). *Chem. Biol.*, in press.

Scott, J. R., Willie, A., Mark, M., Stayton, P. S., Sligar, S. G., Durham, B., and Millett, F. (1993). *J. Am. Chem. Soc.* **115**, 6820–6824.

Scott, J. R., McLean, M., Sligar, S. G., Durham, B., and Millett, F. (1994). *J. Am. Chem. Soc.* **116**, 7356–7362.

Siddarth, P., and Marcus, R. A. (1993). *J. Phys. Chem.* **97**, 13078–13082.

Sutin, N., and Creutz, C. (1978). In *Inorganic and Organometallic Photochemistry.* (ed.), American Chemical Society, Washington, DC, pp. 1–27.

Winkler, J. R., and Gray, H. B. (1992). *Chem. Rev.* **92**, 369–379.

Winkler, J. R., Nocera, D. G., Yocom, K. M., Bordignon, E., and Gray, H. B. (1982). *J. Am. Chem. Soc.* **104**, 5798–5800.

Wuttke, D. S., Bjerrum, M. J., Winkler, J. R., and Gray, H. B. (1992). *Science* **256**, 1007–1009.