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Chemistry

Metalloprotein electron transfer reactions: Analysis of reactivity of horse heart cytochrome *c* with inorganic complexes

(electrostatic interactions/kinetics)

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ABSTRACT **The reactions of horse heart cytochrome *c* with $Fe(ethylenediaminetetraacetate)^{2-}$, $Co(1,10\text{-phenanthroline})_3^{3+}$, $Ru(NH_3)_6^{2+}$, and $Fe(CN)_6^{3-}$ have been analyzed within the formalism of the Marcus theory of outer-sphere electron transfer, including compensation for electrostatic interactions. Calculated protein self-exchange rate constants based on crossreactions are found to vary over three orders of magnitude, decreasing according to $Fe(CN)_6^{3-} > Co(phen)_3^{3+} > Ru(NH_3)_6^{2+} > Fe(EDTA)^{2-}$. The reactivity order suggests that the mechanism of electron transfer involves attack by the small molecule reagents near the most nearly exposed region of the heme; this attack is affected by electrostatic interactions with the positively charged protein, by hydrophobic interactions that permit reagent penetration of the protein surface, and by the availability of π symmetry ligand (or extended metal) orbitals that can overlap with the π redox orbitals of the heme group.**

It has been suggested previously that electron transfer between horse heart cytochrome *c* and certain inorganic redox agents takes place by an outer-sphere mechanism at the heme edge that is partially exposed at the protein surface (1–3). In a particularly striking example, the calculated protein self-exchange rate constant (k_{11}) based on oxidation of ferrocytochrome *c* by $Co(phen)_3^{3+}$ (phen = 1,10-phenanthroline) has been shown to be in good agreement with the measured value, suggesting that the crossreaction involves a very similar mechanism (2). It should not necessarily be expected, however, that all reagents will have equal access to the heme edge in solution, as examination of structural models reveals that hydrophobic residues surround it. Indeed, the degree of access to the heme edge needs to be determined for a variety of substrates of various sizes, charges, and surface properties.

The purpose of this paper is to present a detailed analysis of the kinetics of the electron transfer reactions of cytochrome *c* with $Fe(CN)_6^{3-}$, $Ru(NH_3)_6^{2+}$, $Co(phen)_3^{3+}$, and $Fe(EDTA)^{2-}$ (EDTA = ethylenediaminetetraacetate). We shall employ the Marcus theory of outer-sphere electron transfer reactions to compensate for the variation in driving force and inherent reactivity of the reagents. Special attention will be directed to the evaluation of the electrostatic interactions between the protein and each reagent, thereby allowing an estimate to be made of the magnitudes of nonelectrostatic contributions to the activation free energies for the reactions.

THEORY

The Marcus theory correlates the crossreaction rate constant (k_{12}) with the electron exchange rate constants for the two reactants (k_{11} and k_{22}) and the equilibrium constant (K) through the expressions

$$k_{12} = (k_{11}k_{22}Kf)^{1/2} \qquad [1]$$

$$\log f = (\log K)^2/[4 \log (k_{11}k_{22}/Z^2)] \qquad [2]$$

where the factor f is quite near 1 for the reactions to be considered here because they have rather small equilibrium constants (Z is the collision frequency) (4). With the inclusion of adiabaticity factors p, Eq. 1 becomes $k_{12} = p_{12}(k_{11}k_{22}Kf/p_{11}p_{22})^{1/2}$ (4). For the purposes of this treatment, it will be assumed that the reactions are adiabatic ($p_{11} = p_{22} = p_{12} = 1$), or at least uniformly nonadiabatic ($p_{12}^2 = p_{11}p_{22}$). Eq. 1 may be applied as written, and the predicted k_{12} value may be calculated from known values of k_{11}, k_{22}, and K. In this approach, any deviation of the calculated from the observed values can be attributed to either the protein's or the reagent's undergoing a different activation process than in the self-exchange reaction, or to interaction energies between the reagent and protein which are not cancelled by the interactions in the exchange processes. Alternatively, it may be assumed that the activation process for reagent electron transfer, characterized by the exchange rate k_{22}, is approximately a constant. Under this assumption Eq. 1 can be solved for k_{11} (the subscript 1 refers to the protein in all formulas). In this treatment the calculated k_{11} reflects the activation process the protein must undergo as well as any contributions from protein–reagent interaction that are not cancelled by the interactions in the reagent self-exchange; if the protein employs the same mechanism of electron transfer in all reactions, the calculated k_{11} values should be invariant.

In addition to the inherent energy of activation for the protein, the k_{11} value also includes contributions from the electrostatic interactions between the protein and the reagent. An important manifestation of electrostatic interactions is the ionic strength dependence of reactions between ions. The transition

Table 1. Properties of the reagents (25°, μ 0.1 M, pH 7)

Reagent	E^0 (mV)	k_{11} (M^{-1} s^{-1})	R(A)
$Fe(EDTA)^{2-}$	120[a]	3 (10^4)[b]	4[c]
$Co(phen)_3^{3+}$	370[d]	4.5 (10^1)[e]	7[f]
$Ru(NH_3)_6^{2+}$	51[g]	8.0 (10^2)[h]	3[i]
$Fe(CN)_6^{3-}$	433[j]	2.0 (10^4)[k]	4.5[l]

[a] Ref. 14.
[b] From the $Fe(EDTA)^{2-}/Fe(CyDTA)^-$ (CyDTA = cyclohexanediaminetetraacetate) crossreaction; ref. 15.
[c] Ref. 16.
[d] D. Cummins and H. B. Gray, to be published.
[e] Calculated from data obtained by Neumann, as quoted in ref. 17.
[f] Ref. 18.
[g] Ref. 19.
[h] Ref. 20 (for μ 0.013 M).
[i] Ref. 21.
[j] Ref. 22 (interpolated for μ 0.18 M).
[k] Ref. 23 (for μ 0.2 M).
[l] Ref. 24.

Abbreviations: EDTA, ethylenediaminetetraacetate; phen, 1,10-phenanthroline.

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Table 2. Ionic strength dependence fits of reaction data

	Eq. 5			Eq. 4				Eq. 17		
Reagent	Z_1	k_0 (M^{-1} s^{-1})	SEM	R_1 (A)	Z_1	k_1 (M^{-1} s^{-1})	SEM	Z_1	k_0 (M^{-1} s^{-1})	SEM
$Fe(EDTA)^{2-}$	1.7	$2.7(10^5)$	$8.1(10^2)$	16.6	5.5	$8.0(10^5)$	$1.2(10^3)$	8.1	$8.6(10^3)$	$1.9(10^3)$
				8	3.4	$4.7(10^5)$	$1.0(10^3)$	4.4	$4.0(10^3)$	$1.4(10^3)$
				2	2.3	$3.2(10^5)$	$8.2(10^2)$	2.8	530	$1.1(10^3)$
$Co(phen)_3^{3+}$	0.42	$6.0(10^2)$	12	16.6	5.8	2.9	18	4.7	$2.9(10^3)$	21
				8	1.7	$2.2(10^2)$	13	2.2	$3.5(10^3)$	16
				2	0.2	$9.5(10^2)$	13	1.2	$5.5(10^3)$	14
$Fe(CN)_6^{3-}$	0.64	$6.9(10^7)$	$1.2(10^6)$	16.6	2.0	$2.2(10^8)$	$5.1(10^5)$	6.6	$3.8(10^6)$	$5.8(10^5)$
				8	1.7	$1.9(10^8)$	$5.4(10^5)$	3.0	$2.6(10^6)$	$4.0(10^5)$
				2	1.6	$1.8(10^8)$	$5.5(10^5)$	1.6	$8.9(10^5)$	$6.0(10^5)$

SEM is standard error of the mean.

state formalism treats the ionic strength dependence as the result of the changing activity coefficients of the reactants (5). Assuming the Debye–Hückel treatment for these activity coefficients, the resulting equation is

$$\ln k = \ln k_0 - \frac{Z_1^2\alpha\sqrt{\mu}}{1+\kappa R_1} - \frac{Z_2^2\alpha\sqrt{\mu}}{1+\kappa R_2} + \frac{(Z_1+Z_2)^2\alpha\sqrt{\mu}}{1+\kappa R_\pm} \quad [3]$$

where k is the rate at ionic strength μ, the Zs are the charges on the reactants, the Rs are the radii of the reactants (1 and 2) and the transition state, and α is a constant with the value 1.17 and κ is 0.329 $\sqrt{\mu}$ Å^{-1} (water, 25°). If it is assumed that the radii of the protein and the activated complex are the same ($R_1 = R_\pm$), then Eq. 3 reduces to

$$\ln k = \ln k_0 + \frac{(2Z_1Z_2 + Z_2^2)\alpha\sqrt{\mu}}{1+\kappa R_1} - \frac{Z_2^2\alpha\sqrt{\mu}}{1+\kappa R_2}. \quad [4]$$

The often-employed relationship (Eq. 5) results

$$\ln k = \ln k_0 + 2Z_1Z_2\alpha\sqrt{\mu} \quad [5]$$

only if all radii are assumed to be equal and the ionic strength is low enough such that $1 \gg \kappa R$.

Another approach to treating the ionic strength dependence of electron transfer reactions is to use the equations of Marcus theory and an appropriate function for the coulombic interaction (6). The free energy change for the crossreaction, ΔG_{12}^0, can be separated into an electrostatic contribution, w^0, and a term independent of such interaction, ΔG_r^0. The w^0 term in turn may be expressed as the difference between the electrostatic work to bring the reactants together, w_{12}, and that to bring the products together, w_{21},

$$\Delta G_{12}^0 = \Delta G_r^0 + w_{12} - w_{21}. \quad [6]$$

Similarly, the three activation free energies, ΔG_{12}^* for the crossreaction and ΔG_{11}^* and ΔG_{22}^* for the two exchange reactions, may be expressed as the sum of a work term and an electrostatics-independent term

$$\Delta G_{11}^* = \Delta G_{11}^{**} + w_{11} \quad [7]$$

$$\Delta G_{22}^* = \Delta G_{22}^{**} + w_{22} \quad [8]$$

$$\Delta G_{12}^* = \Delta G_{12}^{**} + w_{12}. \quad [9]$$

For the crossreaction, the part of the activation energy that is independent of electrostatic effects is expressed†

$$\Delta G_{12}^{**} = (\Delta G_{11}^{**} + \Delta G_{22}^{**} + \Delta G_r^0)/2$$

$$= (\Delta G_{11}^* + \Delta G_{22}^* + \Delta G_{12}^0 - w_{12} + w_{21} - w_{11} - w_{22})/2. \quad [10]$$

† Inclusion of the f term of Eq. 1 results in replacement of ΔG_r^0 by $\Delta G_r^0(1+\alpha)$, where $\alpha = \Delta G_r^0/4(\Delta G_{11}^{**} + \Delta G_{22}^{**})$; see ref. 4. Correction for α was made in all work term calculations.

The predicted crossreaction activation free energy is then

$$\Delta G_{12}^* = (\Delta G_{11}^* + \Delta G_{22}^* + \Delta G_{12}^0 + w_{12} + w_{21} - w_{11} - w_{22})/2. \quad [11]$$

Solving for the predicted protein self-exchange activation energy results in the equation

$$\Delta G_{11}^* = 2\Delta G_{12}^* - \Delta G_{22}^* - \Delta G_{12}^0 - w_{21} - w_{12} + w_{11} + w_{22}. \quad [12]$$

Each of the work terms represents the energy required to bring two species from infinite separation to the interaction distance in the activated complex. As the calculation of such terms will prove difficult enough to handle, further refinements, such as protein conformation and charge distribution changes on forming the activated complex, will not be considered. The possible importance of changes in charge distribution and dipolar interactions is documented and should be considered in more detailed calculations (7, 8).

For simplicity, we shall assume the protein to be a sphere with a totally symmetric charge distribution. The dielectric within the sphere must be lower than that of the medium, but its value is not required (9). The equation for the potential of the reactants in the activated complex relative to infinite separation is

$$V = \frac{1}{2}\left[\frac{e^{\kappa R_1}}{1+\kappa R_1} + \frac{e^{\kappa R_2}}{1+\kappa R_2}\right]\left[\frac{Z_1Z_2e^2}{\epsilon}\right]\left[\frac{e^{-\kappa r}}{r}\right] \quad [13]$$

where ϵ is the dielectric constant of water (78.3 at 25°), r is the distance between the centers of the reagent and protein in the activated complex, e is the charge on an electron and the rest of the quantities are as previously defined (10). Eq. 13 reduces to

$$V = 2.1175\left[\frac{e^{-\kappa R_1}}{1+\kappa R_2} + \frac{e^{-\kappa R_2}}{1+\kappa R_1}\right]\left[\frac{Z_1Z_2}{R_1+R_2}\right] \quad [14]$$

when the values of the constants are substituted and r is approximated as the sum of R_1 and R_2.

In order to evaluate Eq. 14, the parameters R and Z must be selected. For the small molecule reagents these values are easily determined from the molecular formula and from x-ray structural data. For a protein of molecular weight M_r, the radius may be estimated from Eq. 15 (11):

$$R = 0.717\ M_r^{1/3} \quad [15]$$

The charge on the protein may be estimated from the amino acid composition, assuming that all of the glutamates and aspartates are ionized, that all of the lysines are protonated, and that arginine is in its monopositive form; it may be further assumed that half of the histidines are protonated. Specific amino

Table 3. Calculated protein electron self-exchange rate constants

Reagent	R_1 (A)	Z_1[a]	k_{12} (M^{-1} s^{-1})	w_{12}	w_{21}	w_{11}	w_{22}
$Fe(EDTA)^{2-}$	16.6	7.5/6.5	2.6(10^4)[e]	−0.567	−0.246	0.406	0.493
	8	2/1	2.6(10^4)	−0.471	−0.118	0.126	0.493
	2	1	2.6(10^4)	−0.790	−0.395	0.712	0.493
$Co(phen)_3^{3+}$	16.6	6.5/7.5	1.5(10^3)[f]	0.490	0.377	0.406	0.507
	8	1/2	1.5(10^3)	0.218	0.291	0.126	0.507
	2	1	1.5(10^3)	0.614	0.409	0.712	0.507
$Ru(NH_3)_6^{2+}$	16.6	7.5/6.5	3.8(10^4)[g]	0.655	0.851	0.406	3.402[h]
	8	2/1	3.8(10^4)	0.563	0.422	0.126	3.402
	2	1	3.8(10^4)	1.037	1.556	0.712	3.402
$Fe(CN)_6^{3-}$	16.6	6.5/7.5	6.7(10^6)[i]	−0.433	−0.667	0.406	1.752
	8	1/2	6.7(10^6)	−0.230	−0.614	0.126	1.752
	2	1	6.7(10^6)	−0.862	−1.149	0.712	1.752

[a] Where two charges are given they are for the reactant and the product.
[b] All energies are in kcal/mol. 1 kcal = 4.184 J.
[c] Calculated for μ 0.1 M.
[d] For k_{11} 3.5(10^2) M^{-1} s^{-1}; ref. 26.
[e] Ref. 1.

acids that have their pK values shifted, as well as the contribution to the charge from the metal ion site, require individual attention for each protein considered.

The Marcus theory treatment of electrostatic interactions discussed above leads directly to an expression for the ionic strength dependence of a crossreaction. The free energy of activation for a crossreaction may be written

$$\Delta G_{12}^{*} = \frac{1}{2}(\Delta G_{11}^{**} + \Delta G_{22}^{**} + \Delta G_r^0) + w_{12} \quad [16]$$

where the ionic strength dependence term is w_{12}. Substituting from Eq. 13 for the work term and converting to rate constant form, we have

$$\ln k = \ln k_0 - 3.576\left[\frac{e^{-\kappa R_1}}{1+\kappa R_2} + \frac{e^{-\kappa R_2}}{1+\kappa R_1}\right]\left[\frac{Z_1 Z_2}{R_1 + R_2}\right] \quad [17]$$

where k_0 is the rate constant at infinite ionic strength.

RESULTS AND DISCUSSION

Application of Eq. 15 to cytochrome *c* from horse heart gives a radius of 16.6 Å (M_r = 12,500); this value compares favorably with the dimensions of the protein from the x-ray structural determination (25 × 30 × 35 Å) (12). The charges on cytochrome *c* are estimated to be +7.5 and +6.5 in the oxidized and reduced forms, respectively, from the sequence data (12), which show that there are three aspartates, nine glutamates, nineteen lysines, two arginines, and one histidine (the other two have low pK values); there is a contribution of −2 to the charge from the two propionate groups on the heme, and −1 from the terminal carboxylate group (the amino terminus is acetylated), and the metal site contributes +1 (oxidized) or 0 (reduced). The redox potential of cytochrome *c* is independent of ionic strength around 0.1 M at neutral pH, with a value of 260 mV (13). The properties of the four reagents (14–24) to be discussed in detail are given in Table 1.

The results of least squares fits of the ionic strength dependence data for the reactions of $Fe(EDTA)^{2-}$, $Fe(CN)_6^{3-}$, and $Co(phen)_3^{3+}$ with horse heart cytochrome *c* to Eqs. 4, 5, and 17 are set out in Table 2. For the latter two equations, the radius is fixed in the calculation. The fits assuming smaller radii are included to represent models in which the site of attack is near the point where the heme edge comes nearest to the surface of the protein. In one case, an active site radius of 2 Å is assumed (which includes the lysine-79 amino group, estimated charge +1); a second model takes a somewhat larger site (R = 8 Å), which includes lysine-79, a heme propionate, and the iron center (estimated net charges are +2 and +1, respectively, in the oxidized and reduced forms). The pattern that emerges from these and more extensive calculations (S. Wherland and H. B. Gray, unpublished results) is that fits with similar standard errors are obtained regardless of the radius chosen (i.e., as the radius is decreased, the best fit charge also decreases). Eqs. 4 and 17 give similar fits, although the latter usually yields a value of the charge somewhat closer to that predicted from the sequence. The values of the charge calculated from reactions involving protein oxidation are uniformly lower than those for the reduction of cytochrome *c*, pointing to the reasonableness of the inclusion of the charge on the metal center.

The electrostatics-corrected cytochrome *c* self-exchange rate constants (k_{11}^{corr}) calculated from crossreaction data (1–3, 25) are presented in Table 3. For comparison, calculated k_{12} values assuming a protein self-exchange rate of 3.5(10^2) M^{-1} s^{-1} (26) are included. It should be noted that the two site models give k_{11}^{corr} values similar to those obtained from the full charge/radius treatment, thereby providing justification for using the latter approach in making electrostatic corrections in general. The k_{11}^{corr} values for the four reagents fall into three categories, with $Fe(EDTA)^{2-}$ and $Ru(NH_3)_6^{2+}$ being lowest, $Co(phen)_3^{3+}$ intermediate, and $Fe(CN)_6^{3-}$ highest. The crossreaction with $Co(phen)_3^{3+}$ leads to a k_{11}^{corr} that most nearly agrees with the experimental value [or equivalently, the $Co(phen)_3^{3+}$ k_{12}^{calcd} accords closely with experiment]. Although the electrostatic correction is negligible for $Co(phen)_3^{3+}$, it is substantial in the other three cases.

Variations in electrostatics-corrected activation free energy (ΔG_{11}^{*corr}) may be attributed in part to nonelectrostatic interactions between the protein and the reagent. Such interactions are expected to facilitate electron transfer by permitting penetration of the hydrophobic residues that block approach to the heme, as well as possibly including interaction with the

Table 3. (*continued*)

Electrostatics				
Uncorrected		Corrected		
$\Delta G_{11}{}^{*b}$	k_{11} (M^{-1} s^{-1})	$\Delta G_{11}{}^{*corr c}$	$k_{11}{}^{corr}$ (M^{-1} s^{-1})	$k_{12}{}^{calcd d}$ (M^{-1} s^{-1})
14.63	$1.2(10^2)$	16.36	6.2	$2.0(10^5)$
14.63	$1.2(10^2)$	15.86	$1.4(10^1)$	
14.63	$1.2(10^2)$	17.05	1.9	
13.51	$7.6(10^2)$	13.55	$7.1(10^2)$	$1.1(10^3)$
13.51	$7.6(10^2)$	13.64	$6.2(10^2)$	
13.51	$7.6(10^2$	13.70	$5.6(10^2)$	
13.51	$7.6(10^2)$	15.82	$1.6(10^1)$	$1.8(10^5)$
13.51	$7.6(10^2)$	16.03	$1.1(10^1)$	
13.51	$7.6(10^2)$	15.06	$5.6(10^1)$	
8.634	$2.9(10^6)$	11.88	$1.2(10^4)$	$1.2(10^6)$
8.634	$2.9(10^6)$	11.33	$3.1(10^4)$	
8.634	$2.9(10^6)$	13.10	$1.5(10^3)$	

[f] Ref. 2.
[g] Ref. 3.
[h] For μ 0.013 M; ref. 20.
[i] For μ 0.18 M; ref. 25.

heme itself. Any breakdown of the assumptions employed in making the $k_{11}{}^{corr}$ calculations will also appear as variations in $\Delta G_{11}{}^{*corr}$. One obvious discrepancy is that cytochrome *c* does not have a totally symmetric charge distribution. That this particular factor should not make much difference is attested to by the small variation in the $k_{11}{}^{corr}$ values among the three assumed site models. Deviations from this source, however, are likely to be larger in protein–protein reactions. A more important factor that was oversimplified in the original model is the assumption that the reactions are adiabatic (or uniformly nonadiabatic). Considering adiabaticity to parallel the extent of orbital overlap (for the low-spin heme *c* center), it is to be expected that reagents with π symmetry ligand orbitals will promote reaction when they can be brought into position to overlap with the porphyrin π system. The compromise that must be reached in the activation process can be considered as attainment of the optimal amount of overlap between the heme *c* and reagent redox orbitals at the minimum enthalpic cost for protein penetration by the reagent.

We turn now to a brief discussion of the expected sources of deviation from strict Marcus theory behavior for the reactions of cytochrome *c* with the four reagents. The $Fe(EDTA)^{2-}$ ion is the least symmetric of the four, with a hydrophilic side comprised of carboxylates and a coordinated water, and a more hydrophobic region of methylene hydrogens. Penetration of the protein surface would probably occur along the hydrophobic section of the reagent, but the best chance for π overlap involves the carbonyl oxygens in the hydrophilic region; thus it is not surprising that the predicted $\Delta G_{11}{}^{*corr}$ is highest for this reagent. The second least reactive reagent is $Ru(NH_3)_6{}^{2+}$, which is uniformly hydrophilic; however, it possesses relatively expanded $d\pi$ orbitals, which probably allow efficient electron transfer at longer metal–heme edge distances than for $Fe(EDTA)^{2-}$. For $Co(phen)_3{}^{3+}$ the hydrophobic nature of the ligands may allow penetration of the heme-edge surface as well as favorable interactions with the porphyrin group itself. Effective π overlap will only be realized if the porphyrin and a phenanthroline ligand are precisely aligned. As the chelating ligands are held rigidly with respect to each other, the interaction of all three phenanthrolines will contribute to the preferred orientation of the ligand involved in the orbital overlap in the precursor complex; there is no reason to expect that the geometry that provides the most favorable hydrophobic interaction will also provide the best overlap, so some compromise must be reached. In contrast, the problem of the nonbridging ligands dictating the bridging alignment is not as critical in the case of $Fe(CN)_6{}^{3-}$, as cyanide is monodentate and has a cylindrically symmetrical π orbital set. Judging from the observation that a very favorable pathway for protein–reagent electron transfer exists, it is quite likely that at least one of the ligands of $Fe(CN)_6{}^{3-}$ penetrates the protein surface, thereby allowing direct FeCN-heme π overlap.

To sum up, the reactivity order of substrates, $Fe(EDTA)^{2-} < Ru(NH_3)_6{}^{2+} < Co(phen)_3{}^{3+} < Fe(CN)_6{}^{3-}$, is fully consistent with a mechanistic model in which cytochrome *c* electron transfer involves attack on the positively charged protein at the heme edge. The order may be understood in terms of the activation associated with penetration of the protecting hydrophobic residues at or near the protein surface, and the expected facility of overlap of heme *c*–reagent redox orbitals.

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