Kinetic Studies of the Oxidation of Horse Heart Ferrocytochrome *c*, *Pseudomonas aeruginosa* Ferrocytochrome c_{551}, $Co(terpy)_2^{2+}$, and $Ru(NH_3)_5py^{2+}$ by Tris(1,10-phenanthroline)cobalt(III) Ions

James V. McArdle, Kathryn Yocom, and Harry B. Gray*

Contribution No. 5436 from the Arthur Amos Noyes Laboratory of Chemical Physics, California Institute of Technology, Pasadena, California 91125.
Received September 14, 1976

Abstract: Kinetic studies of the oxidation of horse heart ferrocytochrome *c*, *Pseudomonas aeruginosa* ferrocytochrome c_{551}, $Co(terpy)_2^{2+}$, and $Ru(NH_3)_5py^{2+}$ by tris complexes of 1,10-phenanthroline and its 5-chloro, 5,6-dimethyl, 4,7-dimethyl, and 4,7-diphenyl-4′-sulfonate derivatives with Co(III) have been performed. The rate constant for the oxidation of ferrocytochrome c_{551} by $Co(phen)_3^{3+}$ is 5.3×10^4 M^{-1} s^{-1} (25 °C, pH 7.0, μ = 0.1 M), which is about 30 times larger than that for the corresponding reaction of horse heart ferrocytochrome *c*. The activation parameters for the reaction between ferrocytochrome c_{551} and $Co(phen)_3^{3+}$ are $\Delta H^{\ddagger}$ = 12.3 kcal/mol and $\Delta S^{\ddagger}$ = 4 cal/(mol deg), compared to $\Delta H^{\ddagger}$ = 11.3 kcal/mol and $\Delta S^{\ddagger}$ = −6 cal/(mol deg) for the oxidation of the horse heart protein. Kinetic parameters for the oxidation of $Co(terpy)_2^{2+}$ and $Ru(NH_3)_5py^{2+}$ by $Co(phen)_3^{3+}$ are as follows: $Co(terpy)_2^{2+}$, $k = 4.2 \times 10^2$ M^{-1} s^{-1} (25 °C, pH 7.0, μ = 0.5 M), $\Delta H^{\ddagger}$ = 6.6 kcal/mol, and $\Delta S^{\ddagger}$ = −24 cal/(mol deg); $Ru(NH_3)_5py^{2+}$, $k = 1.9 \times 10^3$ M^{-1} s^{-1} (25 °C, pH 7.0, μ = 0.5 M), $\Delta H^{\ddagger}$ = 5 kcal/mol, and $\Delta S^{\ddagger}$ = −24 cal/(mol deg). The observed kinetic parameters for the cytochrome oxidations are interpreted to mean that considerable protein structural change is induced by $Co(phen)_3^{3+}$ in the transition state for electron transfer and that the oxidant has better access to the heme *c* in ferrocytochrome c_{551} than it does in horse heart ferrocytochrome *c*. Among the tripositive Co(III) reagents, the rate of oxidation of both ferrocytochromes decreases according to phen > 5,6-Me_2-phen > 5-Cl-phen > 4,7-Me_2-phen. The parameters for the $Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$ oxidation of ferrocytochrome c_{551} are $k = 3.17 \times 10^3$ M^{-1} s^{-1} (25 °C, pH 7.0, μ = 0.1 M), $\Delta H^{\ddagger}$ = 10.9 kcal/mol, and $\Delta S^{\ddagger}$ = −6 cal/(mol deg), whereas those for the corresponding horse heart ferrocytochrome *c* reaction are $k = 2.76 \times 10^1$ M^{-1} s^{-1} (25 °C, pH 7.0, μ = 0.1 M), $\Delta H^{\ddagger}$ = 14.6 kcal/mol, and $\Delta S^{\ddagger}$ = −3 cal/(mol deg). Linear compensation behavior in a plot of $\Delta H^{\ddagger}$ vs. $\Delta S^{\ddagger}$ is found for the oxidations of ferrocytochrome c_{551}, and it is suggested that the low reactivity of $Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$ is attributable to a longer phen edge-heme edge distance in the transition state. A similar plot of $\Delta H^{\ddagger}$ vs. $\Delta S^{\ddagger}$ for the horse heart protein reactions does not yield an acceptable isokinetic correlation; specifically, the $Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$ activation parameters are way out of line, indicating that the substrate-protein fit in the transition state in that case is particularly poor. Comparisons of kinetic parameters for the $Co[4,7\text{-}(Ph\text{-}SO_3)_2\text{-}phen]_3^{3-}$ oxidations show that electrostatic charge effects are not very large in the case of the two ferrocytochromes. Additional electron-transfer reactivity correlations have been made through the Marcus theory. Calculated cross-reaction rate constants for $Co(phen)_3^{3+}$ oxidations of ferrocytochrome c_{551} ($k_{12} = 2.1 \times 10^5$ M^{-1} s^{-1}) and azurin ($k_{12} = 6.2 \times 10^3$ M^{-1} s^{-1}) are in good agreement with measured values (azurin, $k_{12} = 3.2 \times 10^3$ M^{-1} s^{-1}).

Kinetic studies of the oxidation of horse heart ferrocytochrome *c* by $Co(phen)_3^{3+}$ support a mechanistic model in which electron transfer to the inorganic oxidant occurs in the vicinity of the partially exposed heme edge of the protein.[1] As contact between the heme and 1,10-phenanthroline edges is a key feature of this mechanism, it was felt that kinetic investigation of the oxidation of ferrocytochrome *c* by several Co(III) complexes containing modified 1,10-phenanthrolines[2] would yield useful information. Such a study was therefore undertaken for both horse heart ferrocytochrome *c* and *Pseudomonas aeruginosa* ferrocytochrome c_{551}. The latter cytochrome is of particular interest in reactivity comparisons, as it posseses the same reduction potential[3] and a structure[4] that is rather closely related (although the polypeptide chain length is shorter, 82 amino acid residues[5-7] vs. 104[8]) to the horse heart protein. In addition, cytochrome c_{551} exhibits a net negative charge in pH 7 aqueous solutions.[5]

The modified 1,10-phenanthrolines that were chosen as ligands for the Co(III) oxidants are 5-chloro-1,10-phenanthroline (5-Cl-phen); 5,6-dimethyl-1,10-phenanthroline (5,6-Me_2-phen); 4,7-dimethyl-1,10-phenanthroline (4,7-Me_2-phen); and 4,7-di(phenyl-4′-sulfonate)-1,10-phenanthroline [[4,7-$(Ph\text{-}SO_3)_2$-phen]$^{2-}$]. For comparison to the protein results, the kinetics of electron transfer involving each of the oxidants with the inorganic reductants $Co(terpy)_2^{2+}$ and $Ru(NH)_3)_5py^{2+}$ were also investigated.

Experimental Section

Reagent grade chemicals were used throughout. Deionized distilled water was used in the preparation of all solutions used for synthetic or kinetics experiments. Nitrogen gas was passed through two chromous scrubbing towers to remove oxidizing impurities. Horse heart cytochrome *c* (Type VI) obtained from Sigma Chemical Co. was used without further purification. Cytochrome c_{551} from *Pseudomonas aeruginosa* was purified according to the method of Ambler and Wynn.[7] The absorbance ratio A_{521}/A_{551} for cytochrome c_{551} at the conclusion of the purification procedure was 0.58, compared to the published range of 0.58–0.62 for the purified protein.[5] Polyacrylamide gel electrophoresis of the purified protein yielded a single band with a calculated molecular weight of 9500 (±10%), which agrees well with previously reported results.[5-7]

The $Fe(EDTA)^{2-}$ solutions were prepared and characterized exactly as previously reported.[1] Tris complexes of 1,10-phenanthroline and its derivatives with Co(III) were prepared by a method analogous to that of Pfeiffer and Werdelmann[9] and characterized by analyses and by spectroscopic[10] and reduction potential measurements (Table I). The $Co(terpy)_2Cl\cdot H_2O$ complex was prepared according to the method of Baker et al.[11] A method described in detail elsewhere[12] was used to synthesize $[Ru(NH_3)_5py](PF_6)_2$. Electronic absorption spectral measurements on $[Ru(NH_3)_5py]^{2+/3+}$ were performed by D. Cummins.

Solutions of reduced protein were prepared by adding a 20-fold excess of $Fe(EDTA)^{2-}$ to nitrogen-saturated, buffered solutions of the oxidized protein. Excess $Fe(EDTA)^{2-}$ and $Fe(EDTA)^{-}$ were removed from the reduced protein solutions using a hollow fiber Dow beaker dialyzer obtained from Bio-Rad Laboratories. Protein solutions of 75–100 mL were dialyzed under a constant flow of nitrogen against approximately 1.75 L of nitrogen-saturated buffer solution of ionic strength 0.1 M. Alternatively, reduced protein solutions were purified using Sephadex G-25 gel filtration beads obtained from Sigma

Table I. Analytical, Spectroscopic, and Reduction Potential Data for the Cobalt(III) Complexes

Complex	Calcd, % Co	C	N	H	Found, % Co	C	N	H	λ, nm[a]	$10^{-4}\epsilon$, M^{-1} cm^{-1} [a]	E, mV[b]
$[Co(5\text{-}Cl\text{-}phen)_3]Cl_3 \cdot 5H_2O$	6.55	48.08	9.35	3.48	6.11	48.60	9.50	3.86	270.5	8.87	430
									247.5	2.54	
$[Co(5,6\text{-}Me_2\text{-}phen)_3]Cl_3 \cdot 7H_2O$	6.43	55.01	9.18	5.50	6.46	55.12	9.21	5.36	288.5	6.50	420
									263.0	2.91	
$[Co(4,7\text{-}Me_2\text{-}phen)_3]Cl_3 \cdot 14H_2O$	5.65	48.40	8.06	6.20	5.05	48.08	8.50	5.98	282.0	8.33	340
									274.5	8.96	
									251.0	3.81	
$Na_6[Co[4,7\text{-}(Ph\text{-}SO_3)_2\text{-}phen]_3]Cl_3$ $8H_2O$	3.07	45.06	4.38	3.05	3.07	43.83	4.40	3.96	293.0	12.0	330
									257.5	3.84	

[a] Aqueous solutions at 25 °C. [b] Cyclic voltammetric measurements in 0.05 M NaCl aqueous solutions: A. R. Bowen, unpublished results.

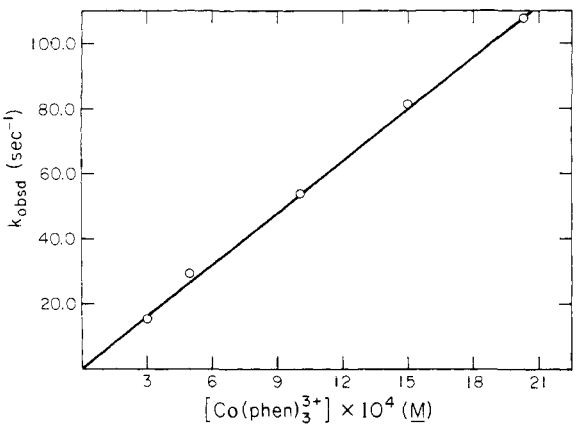

Figure 1. Plot of k_{obsd} vs. $[Co(phen)_3^{3+}]$ for the oxidation of *P. aeruginosa* ferrocytochrome c_{551} [25 °C, pH 7.0 (phosphate), μ = 0.1 M (NaCl)].

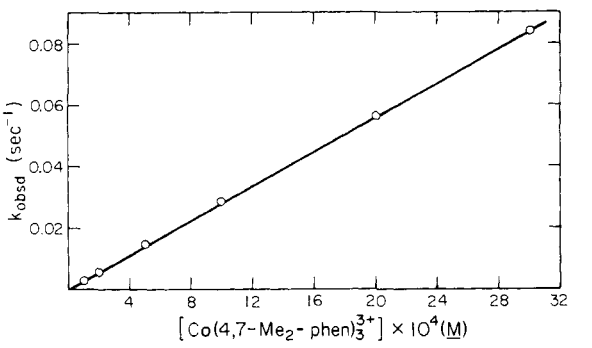

Figure 2. Plot of k_{obsd} vs. $[Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}]$ for the oxidation of horse heart ferrocytochrome *c* [25 °C, pH 7.0 (phosphate), μ = 0.1 M (NaCl)].

Chemical Co. The reduced protein was loaded onto the column, eluted with the appropriate buffer, and diluted to the desired volume.

Preparation of Solutions. Buffered solutions in the range pH 6–9 were used for the kinetics measurements. Type VI cytochrome *c* solutions were stored in nitrogen-purged, serum-capped bottles. Nitrogen was passed only slowly through the cytochrome *c* solutions or above them to prevent protein denaturation. Buffers were prepared to contribute 0.05 M to the ionic strength of the solutions. Both reactant solutions contained identical buffering systems, except any reaction that involved $Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$. It was found that, after a period of time, buffered solutions of $Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$ became cloudy. It was therefore necessary to store $Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$ in deionized distilled water and to perform buffer jump experiments when $Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$ was involved. In such cases, the buffer of the reductant was prepared to give the proper pH and ionic strength *after* mixing of the reactant solutions.

Reactant solutions were stored in serum-capped, round-bottom flasks fitted with a nitrogen inlet tube and a glass luer-lock fitting, thereby allowing introduction of the solutions into the stopped-flow apparatus through an all glass and Kel-F Teflon system. Both reactant solutions were nitrogen-saturated for at least 15 min prior to introduction into the stopped-flow machine.

Kinetics Measurements and Data Analysis. A Durrum Model 110 stopped-flow spectrophotometer was used in all kinetics experiments. Data collection was performed as described earlier.[1] The rates of oxidation were monitored at the following wavelengths: ferrocytochrome *c*, 550 nm ($\Delta\epsilon$ 18.5 × 10^3 M^{-1} cm^{-1});[13] ferrocytochrome c_{551}, 551 nm; $Co(terpy)_2^{2+}$, 510 nm ($\Delta\epsilon$ 1.4 × 10^3 M^{-1} cm^{-1});[14] $Ru(NH_3)_5py^{2+}$, 428 nm ($\Delta\epsilon$ 5.87 × 10^3 M^{-1} cm^{-1}). The concentration of the oxidant was varied between 9.0 × 10^{-5} and 4.0 × 10^{-3} M and was always kept in large excess over the reductant. At least four kinetic runs were performed with each oxidant solution. Because of the problem of clouding mentioned above, reliable absorbance values for the completed reactions of $Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$ could not be obtained. In such cases, the Guggenheim method[15] was used to calculate k_{obsd}.

Results

First-order plots of the absorbance-time data observed for the oxidations of ferrocytochrome *c* and ferrocytochrome c_{551} were found to be linear for greater than 90% of the reactions in all cases. The plots of observed first-order rate constants vs. the concentration of the cobalt(III) complexes all yield straight lines with zero intercepts. Examples are shown in Figures 1 and 2. The rate law for the oxidation of either reduced protein by any one of the Co(III) oxidants is therefore

$$\frac{-d[cyt(II)]}{dt} = k[Co(III)][cyt(II)]$$

The second-order rate constant for the oxidation of ferrocytochrome c_{551} by $Co(phen)_3^{3+}$ in 0.05 M phosphate buffer is 5.30 ± 0.05 × 10^4 M^{-1} s^{-1} (25 °C, pH 7.0, μ = 0.1 M (NaCl)). The second-order rate constants obtained for the oxidation of ferrocytochrome c_{551} by $Co(phen)_3^{3+}$ at various pH values in phosphate and Tris buffers do not vary substantially (Table II). The rate of oxidation does decrease somewhat with increasing ionic strength at pH 7.0 and 25 °C as follows: k = 1 × 10^5 M^{-1} s^{-1}, μ = 0.02 M (NaCl); k = 7.5 × 10^4 M^{-1} s^{-1}, μ = 0.06 M; k = 5.3 × 10^4 M^{-1} s^{-1}, μ = 0.1 M; k = 5.2

Table II. Rate Constants (k) for the Oxidation of *P. aeruginosa* Ferrocytochrome c_{551} by Tris(1,10-phenanthroline)cobalt(III) at 25 °C (μ = 0.1 M)

pH	Buffer	$10^{-4}k$, M^{-1} s^{-1}
6.0	41 mM phosphate (K^+)	3.67
7.0	24 mM phosphate (K^+)	5.30
7.0	54 mM Tris (HCl)	5.36
7.8	18 mM phosphate (K^+)	9.44
9.0	414 mM Tris (HCl)	8.52

Table III. Kinetic Parameters for the Oxidation of Ferrocytochrome *c* and Ferrocytochrome c_{551} [25 °C, pH 7.0 (Phosphate), μ = 0.1 M (NaCl)]

Protein	Oxidant	$10^{-3}k$, M^{-1} s^{-1}	$\Delta H^{\ddagger}$, kcal/mol	$\Delta S^{\ddagger}$, cal/(mol deg)
Ferrocytochrome *c*	$Co(phen)_3^{3+}$	1.50 (5)	11.3 (4)	−6 (1)
	$Co(5\text{-Cl-phen})_3^{3+}$	0.126 (5)	9.6 (4)	−16 (1)
	$Co(5,6\text{-Me}_2\text{-phen})_3^{3+}$	0.266 (5)	12.4 (4)	−6 (1)
	$Co(4,7\text{-Me}_2\text{-phen})_3^{3+}$	0.0276 (5)	14.6 (4)	−3 (1)
	$Co[4,7\text{-(Ph-SO}_3)_2\text{-phen}]_3^{3-}$	2.87 (5)	12.8 (4)	0 (1)
Ferrocytochrome c_{551}	$Co(phen)_3^{3+}$	53.0 (5)	12.3 (4)	4 (1)
	$Co(5\text{-Cl-phen})_3^{3+}$	4.42 (5)	9.4 (4)	−10 (1)
	$Co(5,6\text{-Me}_2\text{-phen})_3^{3+}$	27.0 (5)	13.6 (4)	7 (1)
	$Co(4,7\text{-Me}_2\text{-phen})_3^{3+}$	3.17 (5)	10.9 (4)	−6 (1)
	$Co[4,7\text{-(Ph-SO}_3)_2\text{-phen}]_3^{3-}$	27.5 (5)	13.8 (4)	8 (1)

Table IV. Kinetic Parameters for the Oxidation of $Co(terpy)_2^{2+}$ and $Ru(NH_3)_5py^{2+}$ [25 °C, pH 7.0 (Phosphate), μ = 0.5 M (NaCl)]

Reductant	Oxidant	$10^{-2}k$, M^{-1} s^{-1}	k_2, s^{-1}	$\Delta H^{\ddagger}$, kcal/mol	$\Delta S^{\ddagger}$, cal/(mol deg)
$Co(terpy)_2^{2+}$	$Co(phen)_3^{3+}$	4.16 (8)		6.6 (5)	−24 (1)
	$Co(5\text{-Cl-phen})_3^{3+}$	0.77 (8)		10.9 (5)	−13 (1)
	$Co(5,6\text{-Me}_2\text{-phen})_3^{3+}$	1.22 (8)		6.7 (5)	−26 (1)
	$Co(4,7\text{-Me}_2\text{-phen})_3^{3+}$	1.04 (8)		11.6 (5)	−11 (1)
	$Co[4,7\text{-(Ph-SO}_3)_2\text{-phen}]_3^{3-}$	265		2.6 (5)	−29 (1)
$Ru(NH_3)_5py^{2+}$	$Co(phen)_3^{3+}$	19.1 (7)[a]	0.04 (5)	5 (1)	−24 (2)
	$Co(5\text{-Cl-phen})_3^{3+}$	9.0 (7)[a]	0.15 (5)	3 (1)	−35 (2)
	$Co(5,6\text{-Me}_2\text{-phen})_3^{3+}$	3.3 (7)[a]	0.05 (5)	7 (1)	−24 (2)
	$Co(4,7\text{-Me}_2\text{-phen})_3^{3+}$	3.0 (7)[a]	0.02 (5)	8 (1)	−17 (2)
	$Co[4,7\text{-(Ph-SO}_3)_2\text{-phen}]_3^{3-}$	200[a]		2 (1)	−32 (2)

[a] k_1 values.

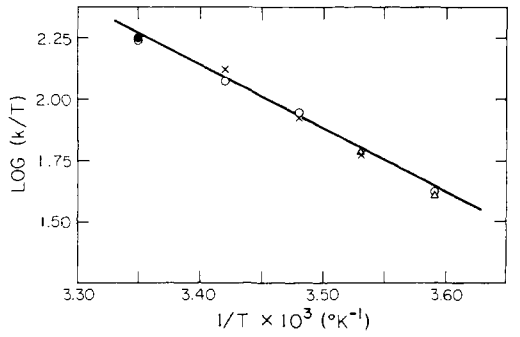

Figure 3. Eyring plot of the rate data for the oxidation of *P. aeruginosa* ferrocytochrome c_{551} by $Co(phen)_3^{3+}$ [pH 7.0 (phosphate), μ = 0.1 M (NaCl)]: (●) $[Co(phen)_3^{3+}]$, 2.0×10^{-3} M; (○) $[Co(phen)_3^{3+}]$, 1.0×10^{-3} M; (×) $[Co(phen)_3^{3+}]$, 3×10^{-4} M; (△) $[Co(phen)_3^{3+}]$, 5×10^{-4} M.

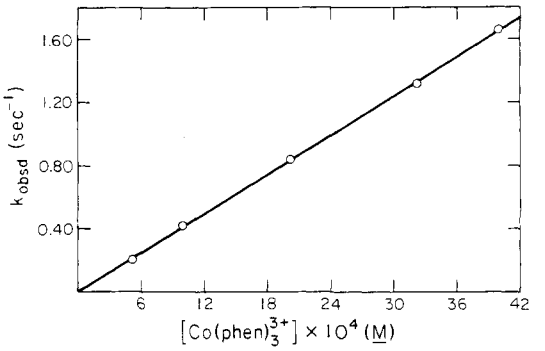

Figure 4. Plot of k_{obsd} vs. $[Co(phen)_3^{3+}]$ for the oxidation of $Co(terpy)_2^{2+}$ [25 °C, pH 7.0 (phosphate), μ = 0.5 M (NaCl)].

$\times 10^4$ M^{-1} s^{-1}, μ = 0.15 M; $k = 4.5 \times 10^4$ M^{-1} s^{-1}, μ = 0.20 M. The activation parameters at pH 7.0 in phosphate buffer obtained from a plot of log (k/T) vs. $1/T$ are $\Delta H^{\ddagger} = 12.3 \pm 0.5$ kcal/mol and $\Delta S^{\ddagger} = 4 \pm 1$ cal/(mol deg) (Figure 3). Kinetic data for the reactions between the cytochromes and the various oxidants are collected in Table III.

A plot of observed first-order rate constants vs. $[Co(phen)_3^{3+}]$ for the oxidation of $Co(terpy)_2^{2+}$ was found to be linear with a zero intercept (Figure 4). Similar behavior was found for the other oxidants, the rate law being

$$\frac{-d[Co(terpy)_2^{2+}]}{dt} = k[Co(terpy)_2^{2+}][Co(III)]$$

A plot of observed first-order rate constants vs. $[Co(phen)_3^{3+}]$ for the oxidation of $Ru(NH_3)_5py^{2+}$ was also observed to be linear. However, this and similar plots for the various Co(III) oxidants were found to have nonzero intercepts, indicating a rate law of the form

$$-d[Ru(NH_3)_5py^{2+}]/dt = k_1[Ru(NH_3)_5py^{2+}][Co(III)] + k_2[Ru(NH_3)_5py^{2+}]$$

Photoinduced oxidation is thought to be responsible for the nonzero intercepts, as strong inhibition resulted upon storing all solutions of $Ru(NH_3)_5py^{2+}$ in the dark prior to the stopped-flow experiments. The kinetic parameters for oxidation of both $Co(terpy)_2^{2+}$ and $Ru(NH_3)_5py^{2+}$ are summarized in Table IV.

Discussion

Ferrocytochrome c_{551} is oxidized by $Co(phen)_3^{3+}$ more than 30 times faster than the horse heart protein. In both cases the $\Delta H^{\ddagger}$ values are relatively large, suggesting that the cytochromes undergo substantial conformational changes under the influence of $Co(phen)_3^{3+}$. It is likely that there are favorable nonelectrostatic substrate-protein interactions in the transition state for electron transfer. Such interactions between the phen rings and hydrophobic protein groups have been proposed to account for the finding that the electrostatics-corrected self-exchange rate constant for horse heart cyto-

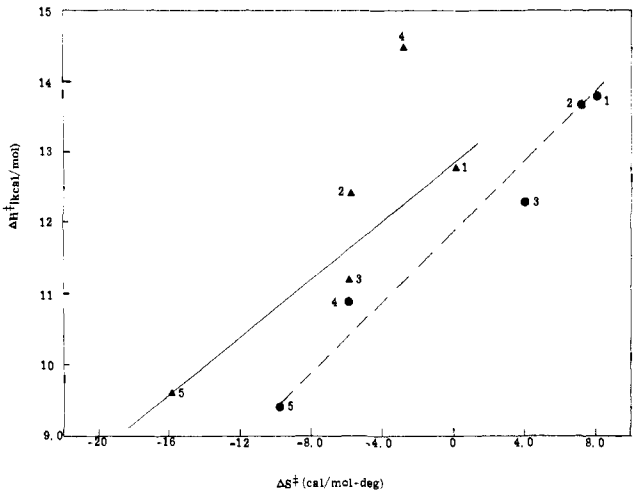

Figure 5. Plot of $\Delta H^{\ddagger}$ vs. $\Delta S^{\ddagger}$: (—, ▲) ferrocytochrome *c*; (- - -, ●) ferrocytochrome c_{551}; (1) $Co[4,7\text{-}(Ph\text{-}SO_3)_2\text{-}phen]_3^{3-}$; (2) $Co(5,6\text{-}Me_2\text{-}phen)_3^{3+}$; (3) $Co(phen)_3^{3+}$; (4) $Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$; (5) $Co(5\text{-}Cl\text{-}phen)_3^{3+}$.

chrome *c* based on $Co(phen)_3^{3+}$ is significantly larger than that based on $Fe(EDTA)^{2-}$ ($7 \times 10^2 > 6\ M^{-1}\ s^{-1}$).[16]

The $\Delta H^{\ddagger}$ of the ferrocytochrome c_{551}-$Co(phen)_3^{3+}$ reaction is higher by 1 kcal/mol, and the $\Delta S^{\ddagger}$ is more positive by 10 cal/(mol deg), than corresponding quantities for the oxidation of the horse heart protein. The higher enthalpy of activation suggests that in the transition state a larger conformational change is induced in ferrocytochrome c_{551} by the oxidant. A greater amount of protein-oxidant interaction in the transition state leads to a more positive entropy of activation, owing to increased loss of ordered water bound at the surface of the protein and $Co(phen)_3^{3+}$. Wood has previously proposed loss of ordered water molecules to explain the observed positive entropies of activation for certain protein-protein electron-transfer reactions.[17]

Introduction of substituents at various edge positions of the phen ring in the tripositive Co(III) reagents leads to a decrease in the rate of oxidation of both ferrocytochrome *c* and ferrocytochrome c_{551}, according to phen > 5,6-Me_2-phen > 5-Cl-phen > 4,7-Me_2-phen. The modest rate reductions for the 5,6-Me_2-phen and 5-Cl-phen derivatives are probably attributable to a slight steric hindrance to phen edge-heme edge overlap in the transition state for both cytochromes.

More dramatic effects on the rates are observed for the oxidations of the proteins by $Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$. In contrast to the reference small-molecule reductants, $Co(terpy)_2^{2+}$ and $Ru(NH_3)_5py^{2+}$, where little differences in rate are found between $Co(5,6\text{-}Me_2\text{-}phen)_3^{3+}$ and $Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$, both cytochromes are oxidized relatively slowly by the 4,7-Me_2-phen derivative. The sluggishness of the oxidation of ferrocytochrome *c* by $Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$ is particularly pronounced, as in that case the rate falls to 1/60th the value observed for $Co(phen)_3^{3+}$. Examination of molecular models shows that 4,7-Me_2 substitution quite effectively blocks the outer phen-ring edge (more so than in 5,6-Me_2-phen), which suggests that achieving good phen edge-heme edge overlap could be particularly difficult for $Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$. In such a situation electron transfer from the protein redox center to Co(III) would reasonably occur over a somewhat longer distance than in the corresponding $Co(phen)_3^{3+}$ reaction. Longer distance electron transfer might not be an unreasonable alternative, as it should be characterized by a lower $\Delta H^{\ddagger}$ associated with protein structural change than that required by close substrate-heme edge contact.

In order to examine the above mechanistic questions further, we have plotted $\Delta H^{\ddagger}$ against $\Delta S^{\ddagger}$ for the oxidation of each of the cytochromes by the series of Co(III) reagents (Figure 5). A good fit of the horse heart ferrocytochrome *c* data results only when the $Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$ point is omitted. (If all five data points are included, a correlation coefficient of 0.825 is obtained, whereas the correlation coefficient is 0.949 without the $Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$ data point; a correlation coefficient of at least 0.95 has been considered demonstrative of a true isokinetic correlation.[18]) The four points on the ferrocytochrome *c* plot yield a β value (slope) of 204 K, whereas the five-point ferrocytochrome c_{551} plot gives a β value of 226 K (correlation coefficient 0.985).

The linear compensation behavior found for the oxidation of ferrocytochrome c_{551} by the Co(III) reagents suggests that the aromatic-ring substituents simply perturb a common electron-transfer mechanism. The decrease in $\Delta H^{\ddagger}$ (accompanied by a more negative $\Delta S^{\ddagger}$) in the ferrocytochrome c_{551}-$Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$ reaction may be interpreted in terms of lessened protein-substrate interaction in the transition state, resulting in electron transfer at a larger phen edge-heme edge distance.

The compensation plot reveals that the mechanistic differences among the ferrocytochrome *c* oxidations are probably too large to be treated as perturbations on some reference transition state interaction. Clearly this must be so for the reaction involving $Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$ as oxidant, as the $\Delta S^{\ddagger}$ value is completely out of line (it is too negative) with the large $\Delta H^{\ddagger}$ observed. The mechanistic implication of this result is that the horse heart protein is able to accommodate $Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$ only by undergoing unnecessarily large structural alterations in the vicinity of the heme edge. Said in another way, the substrate-protcin fit in the case of ferrocytochrome *c* and $Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$ is particularly poor.

We next turn to a discussion of the kinetic data for the oxidations involving the negatively charged reagent, $Co[4,7\text{-}(Ph\text{-}SO_3)_2\text{-}phen]_3^{3-}$. As expected, large rate enhancements attributable to favorable electrostatic interactions are observed for the $Co[4,7\text{-}(Ph\text{-}SO_3)_2\text{-}phen]_3^{3-}$ oxidations of $Co(terpy)_2^{2+}$ and $Ru(NH_3)_5py^{2+}$, relative to the corresponding reactions of $Co(phen)_3^{3+}$ (Table IV). These rate enhancements may be traced in each case to substantially reduced $\Delta H^{\ddagger}$ values, again as would be predicted from simple electrostatic charge considerations. In contrast, electrostatic factors appear to be relatively unimportant in determining the reactivities of the two cytochromes with $Co[4,7\text{-}(Ph\text{-}SO_3)_2\text{-}phen]_3^{3-}$. Taking $Co(phen)_3^{3+}$ as a reference point, the very small variation in rate constant for each protein is determined by an increased $\Delta H^{\ddagger}$ and a more positive $\Delta S^{\ddagger}$ (Table III). The fact that protein oxidations by $Co[4,7\text{-}(Ph\text{-}SO_3)_2\text{-}phen]_3^{3-}$ are substantially faster than those by $Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$ deserves special comment. As both 4,7-substituted derivatives would be expected to have difficulty in effecting direct phen edge-heme edge overlap, it is likely that electron transfer to the Co(III) in $Co[4,7\text{-}(Ph\text{-}SO_3)_2\text{-}phen]_3^{3-}$ is facilitated by heme contact with the phenylsulfonate groups.

Additional electron-transfer rate correlations may be made through the use of the Marcus theory.[19] The relevant theoretical expression, which contains the self-exchange (k_{11} and k_{22}) and cross (k_{12}) electron-transfer rate constants, and the driving force ($\Delta E°$) for the reaction, is as follows:

$$\log k_{12} = 0.5[\log k_{11} + \log k_{22} + 16.9\Delta E°]$$

The self-exchange rate constant for cytochrome c_{551} may be calculated from the rate of oxidation of ferrocytochrome c_{551} by ferricytochrome *c* from horse heart (pH 7, 25 °C). Taking a k_{12} of $7.1 \times 10^4\ M^{-1}\ s^{-1}$,[3] a $\Delta E°$ of 0, and a k_{22} for horse heart cytochrome *c* of $3.5 \times 10^2\ M^{-1}\ s^{-1}$,[16] k_{11} for cytochrome c_{551} is calculated to be $1.4 \times 10^7\ M^{-1}\ s^{-1}$. The predicted value may be compared with a self-exchange rate constant of 1.2 ×

10^7 M^{-1} s^{-1}, which has been obtained[20] recently by NMR measurements on cytochrome c_{551} at 42 °C. Assuming a k_{11} of 1.4×10^7 M^{-1} s^{-1}, the k_{12} ferrocytochrome c_{551}-Co(phen)$_3^{3+}$ is calculated to be 2.1×10^5 M^{-1} s^{-1} ($k_{22} = 4.5 \times 10^1$ M^{-1} s^{-1};[16] $E_2 = 370$ mV;[16] $E_1 = 260$ mV[21]). The calculated rate constant is in reasonable agreement with the measured value of 5.3×10^4 M^{-1} s^{-1}.

The rate of oxidation of ferrocytochrome c_{551} by its physiological partner, azurin, has been studied both by Wilson et al.[22] and by Rosen and Pecht.[23] Taking a k_{12} of 6×10^6 M^{-1} s^{-1},[22] and a reduction potential of 330 mV[21] for azurin, the self-exchange rate constant for the blue protein is calculated to be 2×10^5 M^{-1} s^{-1}. Using the Marcus equation again, the rate constant for the oxidation of azurin by Co(phen)$_3^{3+}$ is calculated to be 6×10^3 M^{-1} s^{-1}, which is in excellent agreement with the experimental value ($k_{12} = 3.2 \times 10^3$ M^{-1} s^{-1}).[24]

The agreement between experiment and theory found for the oxidations by Co(phen)$_3^{3+}$ is extraordinary. The success of the Marcus calculations implies that interactions of Co(phen)$_3^{3+}$ with cytochromes *c* or with azurin resemble very closely those of cytochromes *c* with themselves or with azurin. As cytochromes *c* and Co(phen)$_3^{3+}$ structurally resemble each other only in having hydrophobic, π-conjugated ligands, the rate correlations provide strong support for a mechanistic model in which electron transfer takes place through these ligands. In the case of a cytochrome *c*, of course, the logical point for electron transfer is the partially exposed heme edge of the protein.

The close resemblance of electron-transfer pathways employed by ferrocytochrome c_{551} and Co(phen)$_3^{3+}$ is further evidenced by their similar activation parameters for cross reactions with azurin (fairly large values of $\Delta H^{\ddagger}$, positive values of $\Delta S^{\ddagger}$).[23,24] Apparently, penetration to the redox center of azurin by a hydrophobic π-conjugated ligand system is required for efficient electron transfer. It is ironic that the redox center in azurin is so constructed that it apparently cannot meet this requirement in the case of self-penetration, as it has been observed that the rate of electron self-exchange for the blue protein is too slow to measure on the NMR time scale.[25]

Acknowledgments. We thank Drs. Diane Cummins and Barry Rothman for experimental assistance and Drs. Robert Holwerda and Scot Wherland for helpful discussions. This research was supported by the National Science Foundation.

References and Notes

(1) J. V. McArdle, H. B. Gray, C. Creutz, and N. Sutin, *J. Am. Chem. Soc.*, **96,** 5737 (1974).
(2) A. A. Schilt, "Analytical Applications of 1,10-Phenanthroline and Related Complexes", Pergamon Press, Oxford, 1969.
(3) R. A. Morton, J. Overnell, and H. A. Harbury, *J. Biol. Chem.*, **245,** 4653 (1970).
(4) R. E. Dickerson, R. Timkovich, and R. J. Almassy, *J. Mol. Biol.*, **100,** 473 (1976).
(5) R. P. Ambler, *Biochem. J.*, **89,** 341 (1963).
(6) R. P. Ambler, *Biochem. J.*, **89,** 349 (1963).
(7) R. P. Ambler and M. Wynn, *Biochem. J.*, **131,** 485 (1973).
(8) R. E. Dickerson and R. Timkovich in "The Enzymes", Vol. 11, 3rd ed, P. Boyer, Ed., Academic Press, New York, N.Y., 1975.
(9) P. Pfeiffer and B. Werdelmann, *Z. Anorg. Allg. Chem.*, **263,** 31 (1950).
(10) G. Favini and E. Paglia, *J. Inorg. Nucl. Chem.*, **8,** 155 (1958).
(11) B. R. Baker, F. Basolo, and H. M. Neumann, *J. Phys. Chem.*, **63,** 371 (1959).
(12) D. Cummins and H. B. Gray, *J. Am. Chem. Soc.*, in press (1977).
(13) E. Margoliash and N. Frohwirt, *Biochem. J.*, **71,** 570 (1969).
(14) R. Farina and R. G. Wilkins, *Inorg. Chem.*, **7,** 514 (1968).
(15) A. A. Frost and R. G. Pearson, "Kinetics and Mechanisms", 2nd ed, Wiley, New York, N.Y., 1961.
(16) S. Wherland and H. B. Gray, *Proc. Natl. Acad. Sci. U.S.A.*, **73,** 2950 (1976).
(17) P. M. Wood, *Biochim. Biophys. Acta*, **357,** 370 (1974).
(18) J. E. Leffler and E. Grunwald, "Rates and Equilibria of Organic Reactions", Wiley, New York, N.Y., 1963.
(19) R. A. Marcus, *J. Phys. Chem.*, **67,** 853 (1963).
(20) R. M. Keller, K. Wüthrich, and I. Pecht, *FEBS Lett.*, **70,** 180 (1976).
(21) R. A. Holwerda, S. Wherland, and H. B. Gray, *Annu. Rev. Biophys. Bioeng.*, **5,** 363 (1976).
(22) M. T. Wilson, C. Greenwood, M. Brunori, and E. Antonini, *Biochem. J.*, **145,** 449 (1975).
(23) P. Rosen and I. Pecht, *Biochemistry*, **15,** 775 (1976).
(24) J. V. McArdle, C. Coyle, H. B. Gray, G. S. Yoneda, and R. A. Holwerda, *J. Am. Chem. Soc.*, **99,** 2483 (1977).
(25) H. A. O. Hill, private communication.