Bis(dipicolinate) Complexes of Cobalt(III) and Iron(II) as New Probes of Metalloprotein Electron-Transfer Reactivity. Analysis of Reactions Involving Cytochrome *c* and Cytochrome c_{551}

A. Grant Mauk, Catherine L. Coyle, Emilio Bordignon, and Harry B. Gray*

Contribution No. 5948 from the Arthur Amos Noyes Laboratory of Chemical Physics, California Institute of Technology, Pasadena, California 91125.
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Abstract: The electron-transfer reactions of horse heart cytochrome *c* and *Pseudomonas aeruginosa* cytochrome c_{551} with bis(dipicolinato)cobaltate(III) ($Co(dipic)_2^-$) and bis(dipicolinato)ferrate(II) ($Fe(dipic)_2^{2-}$) have been studied and found to demonstrate second-order kinetic behavior. The rate constants (M^{-1} s^{-1}) [25 °C, μ = 0.2 M, pH 7.0 (phosphate)], $\Delta H^{\pm}$ (kcal/mol), and $\Delta S^{\pm}$ (eu) for these reactions are: 1.11 (4) × 10^4, 4.4 (8), and −25 (3) for ferrocytochrome *c* and $Co(dipic)_2^-$; 1.5 (3) × 10^4, 7.2 (3), and −15.4 (1.2) for ferricytochrome *c* and $Fe(dipic)_2^{2-}$; 5.8 (1) × 10^2, 3.6 (6), and −34 (2) for ferrocytochrome c_{551} and $Co(dipic)_2^-$; and 3.21 (4) × 10^3, 9.6 (6), and −10 (2) for ferricytochrome c_{551} and $Fe(dipic)_2^{2-}$. The calculated electrostatics-corrected protein self-exchange rate constants (k_{11}^{corr}) based on the cytochrome/$Fe(dipic)_2^{2-}$ kinetic data are over an order of magnitude greater than those previously obtained based on $Fe(EDTA)^{2-}$, consistent with the conclusion that the presence of the hydrophobic, π-conducting pyridine ligand in the complex promotes electron transfer by permitting reagent penetration toward the metal center in the protein. The k_{11}^{corr} values based on the oxidant $Co(dipic)_2^-$ are substantially larger than those based on $Fe(dipic)_2^{2-}$, suggesting that the electron-transfer reactivity of $Co(dipic)_2^-$ is enhanced by protein–reagent interactions.

Introduction

In recent years we have employed Marcus theory to elucidate the factors governing the rates of electron-transfer reactions between inorganic complexes and metalloproteins.[1-3] One limitation of this approach is that relatively few complexes are available with the electrochemical, solubility, and structural properties that are required to probe metalloprotein reactivity. Ideally, an assortment of probe reagents varying in charge, reduction potential, and ligand structure should be available to permit systematic characterization of metalloprotein active sites.

An important conclusion from previous work is that complexes containing hydrophobic, π-conducting ligands are much more reactive with the redox centers in metalloproteins than are redox agents with hydrophilic electron-transfer surfaces.[1-4] This conclusion is based on limited data, however, and in no case have reagents containing the same hydrophobic, π-conducting ligands been studied both as oxidants and reductants. Thus, we decided to investigate the kinetics of both oxidation and reduction of metalloproteins by metal complexes containing the ligand 2,6-dicarboxylpyridine (dipicolinate, or dipic), which was chosen because the pyridine groups were expected to enhance electron-transfer reactivity. The results of our kinetic study of the reactions of $Fe(dipic)_2^{2-}$ and $Co(dipic)_2^-$ with horse heart cytochrome *c* and with *Pseudomonas aeruginosa* cytochrome c_{551} are presented here.

Experimental Section

Horse heart cytochrome *c* (type VI) was purchased from Sigma and used directly. Cytochrome c_{551} from *Pseudomonas aeruginosa* was purified by the method of Ambler and Wynn.[5] Solutions of reduced cytochromes were prepared by reduction with $Fe(EDTA)^{2-}$, passage over Sephadex G-25, and storage under argon immediately before use. Protein solutions were bubbled slowly with vanadous-scrubbed argon prior to transfer into the drive syringe.

Bis(dipicolinato)ferrate(II) was prepared anaerobically in solution in a manner similar to that used for preparation of $Fe(EDTA)^{2-}$.[6] An argon-purged aqueous solution of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ was added to degassed phosphate buffer containing a 40% excess of dipicolinic acid (Aldrich Chemical Co.). Portions of the stock reductant solution were diluted with degassed buffer. All solution transfers and dilutions were performed using Hamilton gas-tight syringes. Ionic strength and pH were controlled entirely with sodium phosphate. No adjustment of pH was required. The Fe(II) complex of dipicolinate has a reddish-orange color with λ_{max} 480 nm ($\epsilon \sim 1600$ M^{-1} cm^{-1}) in phosphate buffer (pH 7, μ = 0.2 M).

Bis(dipicolinato)cobaltate(III) was prepared by the following method: Dipicolinic acid (10 g) was suspended in 10 mL of water and 200 mL of concentrated NH_4OH was added. The mixture was heated to 60–70 °C and taken to dryness. Water (50 mL) was added and the mixture taken to dryness; this step was repeated once. The product was dissolved in 500 mL of water with heating. After cooling, a solution of $Co(NO_3)_2 \cdot 6H_2O$ (8.7 g in 10 mL of water) was added slowly with stirring. After 1 h, 50 mL of 30% H_2O_2 was added and the solution was stirred for 3–4 h at room temperature. The precipitate was filtered off and the filtrate heated to 60–70 °C with stirring until the solution became slightly turbid. The solution was cooled and allowed to stand at room temperature for several hours. The resulting precipitate (~6 g) was filtered and washed with ethanol and ether. Additional product may be isolated from the mother liquor. The bis(dipicolinato)cobaltate(III) complex is scarlet-red with λ_{max} 510 nm (ϵ 630 M^{-1} cm^{-1}); these properties are in agreement with those reported for the complex by Hartkamp.[7] Anal. Calcd for $C_{14}H_6N_2O_8Co$: C, 41.29; H, 2.48; N, 10.32; mol wt 406.93. Found: C, 41.08; H, 2.55; N, 10.28.

All kinetic measurements were made on a Durrum Model D-110 stopped-flow spectrophotometer interfaced to a PDP-10 computer for data acquisition and analysis. Transfer of reactant solutions into the drive syringes was done under vanadous-scrubbed argon. All reactions were run under pseudo-first-order conditions with the reagent in 25- to 250-fold concentration excess over protein. The changes in the oxidation states of cytochromes *c* and c_{551} were followed at 550 and 551 nm, respectively.

Results and Discussion

All first-order plots were linear for at least 90% of the reaction. The dependences of observed first-order rate constants on reagent concentration for the four reactions are shown in Figure 1. No rate saturation was observed over the concentration range examined. Second-order rate constants obtained from weighted least-squares fits of the data in Figure 1 are as follows (k_{12} values at 25 °C, μ = 0.2 M, pH 7.0 (phosphate)): ferricytochrome *c*/$Fe(dipic)_2^{2-}$, 1.5 (3) × 10^4 M^{-1} s^{-1}; ferrocytochrome *c*/$Co(dipic)_2^-$, 1.11 (4) × 10^4 M^{-1} s^{-1}; ferri-

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Table I. Calculated Protein Self-Exchange Rate Constants (25 °C, μ = 0.1 M, pH 7.0)

protein	reagent	Z_1	w_{12}[a]	w_{11}	w_{21}	w_{22}	ΔG_{11}^{*corr}, kcal/mol	k_{11}^{corr}, M^{-1} s^{-1}
cyt *c*	$Fe(dipic)_2^{2-}$	7.5/6.5	−0.269	0.406	−0.116	0.308	13.8[b]	2.2×10^2
cyt *c*	$Fe(EDTA)^{2-}$	7.5/6.5	−0.567	0.406	−0.246	0.493	16.4[c]	6.2
cyt *c*	$Co(dipic)_2^-$	6.5/7.5	−0.116	0.406	−0.269	0.308	9.7[d]	2.4×10^5
cyt *c*	$Co(phen)_3^{3+}$	6.5/7.5	0.490	0.406	0.377	0.507	13.6[e]	7.1×10^2
cyt c_{551}	$Fe(dipic)_2^{2-}$	−2/−3	0.094	0.079	0.070	0.308	15.1	4.5×10^1
cyt c_{551}	$Fe(EDTA)^{2-}$	−2/−3	0.194	0.079	0.146	0.493	17.0[e]	2.0
cytc_{551}	$Co(dipic)_2^-$	−3/−2	0.070	0.079	0.094	0.308	12.7	2.9×10^3
cyt c_{551}	$Co(phen)_3^{3+}$	−3/−2	−0.287	0.079	−0.128	0.507	10.3[e]	1.8×10^5

[a] The radius of $M(dipic)_2^{n-}$ was estimated to be 5.2 Å; all *w* values are in kcal/mol. [b] Calculation based on $E°$ ($Fe(dipic)_2^{-/2-}$) = 278 mV[8] and $k_{22}^{corr} = 3.2 \times 10^5$ M^{-1} s^{-1}.[9] [c] From ref 2b. [d] Calculation based on $E°$ ($Co(dipic)_2^{-/2-}$) = 400 mV and $k_{22}^{corr} = 4.0 \times 10^{-1}$ M^{-1} s^{-1}. The k_{22}^{corr} was estimated from k_{12} ($Co(dipic)_2^-/Fe(EDTA)^{2-}$) = 2.3 (1) $\times 10^4$ M^{-1} (25 °C, μ = 0.2 M, pH 7.0 (phosphate)).[10] The calculated k_{11}^{corr} is not very sensitive to the exact value assumed for the reduction potential of $Co(dipic)_2^-$; thus, k_{11}^{corr} for cytochrome *c* based on $E°$ = 300 mV is 2.9×10^5 M^{-1} s^{-1}, and is 1.8×10^5 M^{-1} s^{-1} based on $E°$ = 600 mV. [e] From ref 11.

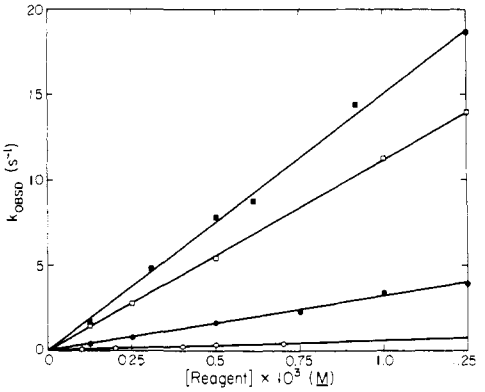

Figure 1. The dependences of the observed rate constants on the concentration of reagent at 25 °C, μ = 0.2 M, pH 7.0 (phosphate): (■) ferricytochrome *c*/$Fe(dipic)_2^{2-}$; (□) ferrocytochrome *c*/$Co(dipic)_2^-$; (●) ferricytochrome c_{551}/$Fe(dipic)_2^{2-}$; (○) ferrocytochrome c_{551}/$Co(dipic)_2^-$.

cytochrome c_{551}/$Fe(dipic)_2^{2-}$, 3.21 (4) $\times 10^3$ M^{-1} s^{-1}; ferrocytochrome c_{551}/$Co(dipic)_2^-$, 5.8 (1) $\times 10^2$ M^{-1} s^{-1}. Eyring plots for these reactions are shown in Figure 2, and the activation parameters obtained from these weighted fits are: ferricytochrome *c*/$Fe(dipic)_2^{2-}$, $\Delta H^{\ddagger}$ = 7.2 (3) kcal/mol, $\Delta S^{\ddagger}$ = −15.4 (1.2) eu; ferrocytochrome *c*/$Co(dipic)_2^-$, $\Delta H^{\ddagger}$ = 4.4 (8) kcal/mol, $\Delta S^{\ddagger}$ = −25 (3) eu; ferricytochrome c_{551}/$Fe(dipic)_2^{2-}$, $\Delta H^{\ddagger}$ = 9.6 kcal/mol, $\Delta S^{\ddagger}$ = −10 (2) eu; ferrocytochrome c_{551}/$Co(dipic)_2^-$, $\Delta H^{\ddagger}$ = 3.6 (6) kcal/mol, $\Delta S^{\ddagger}$ = −34 (2) eu.

The electrostatics-corrected self-exchange rate constants (k_{11}^{corr}) for the cytochromes were calculated as previously described.[2] The input[8-10] and output parameters are given in Table I along with analogous results obtained previously for $Fe(EDTA)^{2-}$ and $Co(phen)_3^{3+}$ reactions with the proteins.[1,2,11] The effect of ligand structure on protein electron-transfer reactivity is clearly seen by comparing the k_{11}^{corr} value based on the cytochrome *c*/$Fe(dipic)_2^{2-}$ reaction with that based on $Fe(EDTA)^{2-}$. The substantial increase in cytochrome electron-transfer reactivity found in the former case may be attributed to the presence of the pyridine ligand. This group presumably allows the Fe(II) complex to penetrate the hydrophobic region around the heme, which permits closer approach of the redox centers and accelerates electron transfer. Significantly, the k_{11}^{corr} calculated for cytochrome *c* based on its reaction with $Fe(dipic)_2^{2-}$ is not very different from the measured protein k_{11}[12] nor from the k_{11}^{corr} values based on $Co(phen)_3^{3+}$ oxidation[13] and $Ru(NH_3)_5BzIm^{2+}$ reduction,[14] indicating that all of these reaction pathways are similar.

The results of the cytochrome *c* oxidation studies are particularly intriguing. The most unusual finding is that the k_{11}^{corr} based on the $Co(dipic)_2^-$ reaction is over two orders of magnitude greater than that obtained from the $Fe(dipic)_2^{2-}$ reaction. One possible reason for this enhanced electron-transfer reactivity of the ferrocytochrome *c*/$Co(dipic)_2^-$ pair is a breakdown in our assumption that k_{22} is an appropriate measure of the activation free energy attributable to the reagent. In a low-spin Co(III) complex, this energy barrier is relatively large, and it is not unreasonable to suppose that the

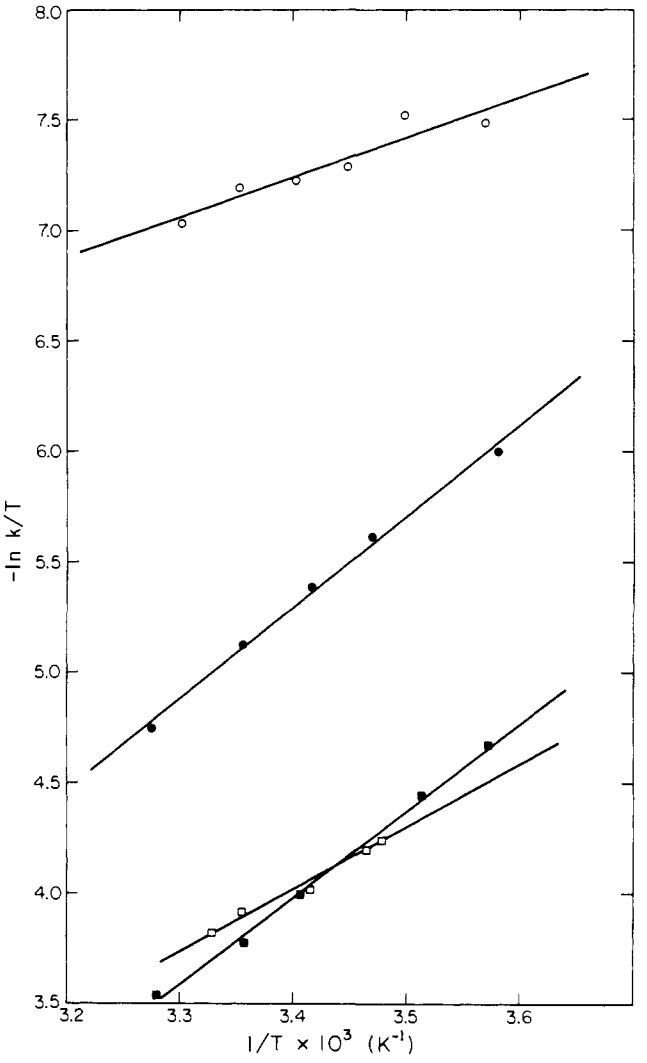

Figure 2. Eyring plots of the rate constant data for the reactions of $Fe(dipic)_2^{2-}$ and $Co(dipic)_2^-$ with cytochrome *c* and cytochrome c_{551} at μ = 0.2 M, pH 7.0 (phosphate). In each case [reagent] = 5×10^{-4} M, except for ferrocytochrome c_{551}/$Co(dipic)_2^-$ where [reagent] = 4×10^{-4} M.

barrier could be lowered by protein-reagent interaction in the activated complex. For example, penetration of the pyridine group of $Co(dipic)_2^-$ into the protein interior could easily produce a slight distortion of the Co(III) geometry, thereby reducing the large inner sphere reorganization barrier to electron transfer. Similar protein-reagent interaction would not be expected to have a comparable influence on the reactivity of the ferricytochrome $c/Fe(dipic)_2^{2-}$ pair.[15]

Examination of the activation parameters for the cytochrome reactions reinforces the conclusion that the mechanism of electron transfer to $Co(dipic)_2^-$ possesses unusual characteristics. Whereas the ferricytochrome $c/Fe(dipic)_2^{2-}$ activation parameters are quite normal for a bimolecular electron-transfer reaction, the $\Delta H^{\ddagger}$ of 4.4 kcal/mol for ferrocytochrome $c/Co(dipic)_2^-$ is unexpectedly small (for comparison, ferrocytochrome $c/Co(phen)_3^{3+}$ has a $\Delta H^{\ddagger}$ of 11 kcal/mol).[13] There is a possibility that the site of interaction of $Co(dipic)_2^-$ with cytochrome c is a greater distance from the heme than the site employed by $Fe(dipic)_2^{2-}$, but at that site more favorable protein-reagent interaction can occur. If this interaction leads to a lower activation barrier for Co(III), then the alternative pathway could enhance the heme c electron-transfer rate, as observed. Our postulate of a more remote, reagent-compatible interaction site for ferrocytochrome $c/Co(dipic)_2^-$ is fully consistent with the large negative value of $\Delta S^{\ddagger}$ (−25 eu) associated with the reaction.

The reactivity patterns of cytochrome c_{551} are similar. The k_{11}^{corr} based on $Fe(dipic)_2^{2-}$ is substantially larger than that based on $Fe(EDTA)^{2-}$, attesting to the favorable effect of π-conducting groups present in the former reagent. The k_{11}^{corr} (cytochrome c_{551}) obtained from the $Co(dipic)_2^-$ reaction is greater than that based on $Fe(dipic)_2^{2-}$, and again this may be attributed to a lower Co(III) activation barrier arising from reagent-protein interaction. In this case, however, the more exposed heme edge region might not be expected to induce as great a distortion of the cobalt geometry resulting in a smaller k_{11}^{corr} than that found for cytochrome c. Alternatively, the site of interaction of $Co(dipic)_2^-$ with ferrocytochrome c_{551} could be farther from the heme c than in the case of ferrocytochrome c, resulting in reduced electron-transfer catalysis. The latter interpretation is more consistent with the observed activation parameters for the ferrocytochrome $c_{551}/Co(dipic)_2^-$ reaction (small $\Delta H^{\ddagger}$, 3.6 kcal/mol; large negative $\Delta S^{\ddagger}$, −34 eu).

Our analysis of the reactions of $Fe(dipic)_2^{2-}$ and $Co(dipic)_2^-$ with cytochromes is important in the sense that it raises the possibility that different protein sites could be involved in reduction and oxidation steps, even with reagents possessing identical ligands. We intend to explore this matter further by analyzing the reactions of the bis(dipicolinate) complexes of Fe(II) and Co(III) with simple blue copper proteins. The copper protein investigation will be the subject of our next report.

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Supplementary Material Available: Listing of observed pseudo-first-order rate constants (1 page). Ordering information is given on any current masthead page.

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(14) From published data[4] on the ferricytochrome $c/Ru(NH_3)_5BzIm$ system ($k_{12} = 5.8 \times 10^4\ M^{-1}\ s^{-1}$, $k_{22} = 5 \times 10^4\ M^{-1}\ s^{-1}$, $\Delta E^{\circ} = 0.11$ V (pH ~7, 25 °C, $\mu = 0.1$ M)), we estimate k_{11}^{corr} to be $5 \times 10^2\ M^{-1}\ s^{-1}$.
(15) Electron transfer from $Fe(dipic)_2^{2-}$ involves a $d\pi$-type donor orbital ($Fe(dipic)_2^{2-}$ (high-spin d^6) → $Fe(dipic)_2^-$ (high-spin d^5)). Because the energies of the relatively nonbonding $d\pi$ orbitals would not vary significantly for small distortions of $Fe(dipic)_2^{2-}$, we would not expect the inner sphere reorganization barrier attributable to $Fe(dipic)_2^{2-}$ to be modified greatly by protein-reagent interaction. In this connection, we should note that our recent calculations on the electron-transfer reactions of myoglobin with the $Fe(EDTA)^{2-/-}$ and $Fe(CDTA)^{2-/-}$ couples have shown that, for each redox pair, the oxidant and reductant generate the same k_{11}^{corr} values (Mauk, A. G.; Gray, H. B. *Biochem. Biophys. Res. Commun.* **1979,** *86*, 206).