Comparison of the Electron-Transfer Reactivities of Tris(oxalato)cobaltate(III) ($Co(ox)_3^{3-}$) and Tris(1,10-phenanthroline)cobalt(III) ($Co(phen)_3^{3+}$) with Metalloproteins

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Abstract: A comparative kinetic study of the electron-transfer reactivities of the oxidants $Co(phen)_3^{3+}$ and $Co(ox)_3^{3-}$ with metalloproteins has been performed. At 25 °C, pH 7.0, μ = 0.5 M (phosphate), the rate constant ratio $k[Co(phen)_3^{3+}]/k[Co(ox)_3^{3-}]$ increases in the order: cuprous stellacyanin (2.5×10^2) < horse heart ferrocytochrome *c* (6.0×10^2) < cuprous plastocyanin (4.3×10^3) < ferrocytochrome *c*-551 (4.3×10^4) < cuprous azurin (1.5×10^5) < reduced high potential iron–sulfur protein (3.4×10^5). It is shown that the reactivity ratio $k[Co(phen)_3^{3+}]/k[Co(ox)_3^{3-}]$ provides a quantitative measure of the accessibility of the metalloprotein active center to outer-sphere contact with redox agents. Apparent metalloprotein self-exchange rate constants derived by applying relative Marcus theory to the $Co(ox)_3^{3-}$ rate data agree well with those obtained previously for another hydrophilic reagent, $Fe(EDTA)^{2-}$. The $Co(ox)_3^{3-}$ rate data therefore support the use of $k_{11}^{corr}(Fe(EDTA)^{2-})$ to define the lower limit of the "kinetic accessibility" scale for a metalloprotein.

Introduction

The Marcus formalism for outer-sphere electron transfer provides a convenient framework for systematically classifying and interpreting the electron-transfer reactivities of complex ion redox agents with metalloproteins.[1] Apparent self-exchange electron-transfer rate constants (k_{11}) calculated for a particular metalloprotein on the basis of cross-reaction rates and reduction potentials for various redox agents often span a wide range, providing a quantitative measure of the extent to which factors other than thermodynamic driving force and the self-exchange rate of the reagent influence its electron-transfer reactivity with the metalloprotein.[1] Large differences in k_{11} values for a given metalloprotein may persist even after compensating for differences in the electrostatic work required to bring the various redox agents into the precursor complex for electron transfer.[1,2] Thus, electrostatics-corrected apparent self-exchange rate constants (k_{11}^{corr}) for *Pseudomonas aeruginosa* azurin based on cross reactions with the reductant $Fe(EDTA)^{2-}$ and the oxidant $Co(phen)_3^{3+}$ are 1.2×10^{-2} and 1.6×10^4 M^{-1} s^{-1} (25 °C), respectively.[1]

The range in k_{11}^{corr} values for a particular metalloprotein provides a quantitative measure of the "kinetic accessibility" of its active site.[1,3] Thus, metalloproteins in which the metal site is substantially buried within the polypeptide structure exhibit wider k_{11}^{corr} ranges than those in which the active site is more exposed to attack by redox agents. Complexes with hydrophobic, π-conducting ligands (i.e., $Co(phen)_3^{3+}$) generally exhibit a much higher redox reactivity with metalloproteins than those, like $Fe(EDTA)^{2-}$, whose hydrophilicity hinders close approach between the reagent and the more hydrophobic metalloprotein active center. On this basis, k_{11}^{corr} values derived from cross reactions with $Fe(EDTA)^{2-}$ have been used to define the lower limit of the "kinetic accessibility" scale.[1,3]

As a test of this procedure it would be useful to determine whether k_{11}^{corr} values derived from cross reactions with other hydrophilic reagents, particularly oxidants, agree well with those for the reductant $Fe(EDTA)^{2-}$. If "kinetic accessibility" is indeed a meaningful concept, then the electron-transfer reactivities of all hydrophilic redox agents, as measured by k_{11}^{corr}, should be essentially similar, with variations in ligand structure or the direction of the electron-transfer process (metalloprotein oxidation or reduction) having little effect. This hypothesis rests upon two reasonable assumptions: namely, (1) the extent of nonelectrostatic interactions between hydrophilic reagents and metalloproteins is negligibly small and (2) transmission coefficients for electron transfer to or from hydrophilic redox agents uniformly constrained to attack the active site from the metalloprotein surface should be comparable, as the extent of orbital overlap between the redox partners in this case presumably is governed largely by the metal site-to-surface distance.

We report in this paper a comparative kinetic study of the electron-transfer reactivities of $Co(phen)_3^{3+}$ and the tris(oxalato)cobaltate(III), ion, $Co(ox)_3^{3-}$, with three blue copper proteins [cuprous stellacyanin (St(I)), plastocyanin (Pc(I)), and azurin (Az(I))], two cytochromes [horse heart ferrocytochrome *c* (cyt *c*(II)), ferrocytochrome *c*-551 (cyt *c*-551(II))], and an iron–sulfur protein [reduced high potential iron protein (HiPIP(r))]. The significance of the rate constant ratio $k[Co(phen)_3^{3+}]/k[Co(ox)_3^{3-}]$ is discussed, and k_{11}^{corr} values based on cross reactions with the hydrophilic oxidant $Co(ox)_3^{3-}$ are compared with those derived previously from the $Fe(EDTA)^{2-}$ reductions of the metalloproteins.

Experimental Section

Reagents. $K_3Co(ox)_3 \cdot 3.5H_2O$[4] and $[Co(phen)_3](ClO_4)_3 \cdot 2H_2O$[5] were prepared by literature methods. The former compound was stored in the dark to protect against photoinduced decomposition, but it was still necessary to recrystallize the salt from water–ethanol shortly before use to remove thermal decomposition products. UV–visible spectra of $Co(ox)_3^{3-}$ (ϵ_{605} = 175 M^{-1} cm^{-1}, ϵ_{425} = 230 M^{-1} cm^{-1})[6] and $Co(phen)_3^{3+}$ (ϵ_{350} = 3700 M^{-1} cm^{-1}, ϵ_{330} = 4680 M^{-1} cm^{-1})[7] solutions confirmed the purity of their salts, and were used routinely to determine the concentrations of these species. The Co content of $K_3Co(ox)_3 \cdot 3.5H_2O$, determined spectrophotometrically[8] following decomposition in boiling NaOH solution, was in excellent agreement with the theoretical value (% Co: calcd, 11.7; found, 11.7). The thermal stability of $Co(ox)_3^{3-}$ under our reaction conditions was found to be excellent; less than a 1% change in A_{605} was noted for solutions stored for 24 h in the dark. $Co(terpy)_2(ClO_4)_2$ was prepared according to the method of Baker et al.[9]

Sigma type VI horse heart cytochrome *c* was used as supplied. Stellacyanin was extracted from lacquer acetone powder (*Rhus ver-*

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nicifera) and purified by the method of Reinhammar.[10] Plastocyanin was solubilized from spinach leaves and partially purified (through the ammonium sulfate precipitation step) using the procedure of Bochert and Wessels.[11] The purification was completed by the method of Yocum et al.[12] HiPIP was obtained from *Chromatium vinosum* cells according to the procedure of Bartsch.[13] Azurin from *Pseudomonas aeruginosa* was purified by the method of Ambler and Brown[14] as modified by Rosenberg.[15] *Pseudomonas aeruginosa* cytochrome *c*-551 was isolated and purified according to the method of Ambler and Wynn.[16]

Preparation of Solutions. An ionic strength 0.5 M, pH 7.0 sodium phosphate buffer solution prepared with triply distilled water was used throughout. Oxidant solutions contained in serum-capped bottles were routinely shielded from light with aluminum foil and deoxygenated by purging with chromous-scrubbed N_2; to avoid contamination with iron, these solutions were purged or transferred (Hamilton gas-tight syringe) using Teflon needles only. To ensure constancy in ionic strength within a series of runs, the oxidant concentration was maintained at ≤5 mM. Accordingly, the ionic strength contribution from the oxidant never exceeded 6% of that from the phosphate buffer.

Anaerobic metalloprotein solutions were prepared by passing a stream of N_2 over the top of the stirred solution for at least 1 h. Metalloprotein solutions (except cytochrome *c* and plastocyanin) were adjusted to the correct pH and ionic strength by dialyzing against triply distilled water and diluting in a volumetric flask containing the amounts of $NaH_2PO_4 \cdot H_2O$ and Na_2HPO_4 required to give pH 7.0, μ = 0.5 M. Cytochrome *c* and $Co(terpy)_2(ClO_4)_2$ solutions were prepared by weighing the appropriate amount into pH 7.0, μ = 0.5 M buffer, while plastocyanin was dialyzed against several changes of this buffer before use. Solutions of ferrocytochrome *c*, ferrocytochrome *c*-551, cuprous stellacyanin, and cuprous azurin were obtained by adding the equivalent amount of sodium ascorbate to the deoxygenated, oxidized proteins. High potential iron protein was reduced with dithiothreitol, then dialyzed against the standard buffer solution to remove excess reductant. Cuprous plastocyanin was obtained directly from the purification procedure.[11,12]

Kinetic Measurements. The oxidation of cyt *c*(II), cyt *c*-551(II), HiPIP(r), St(I), Pc(I), and Az(I) by $Co(phen)_3^{3+}$ and $Co(ox)_3^{3-}$ was monitored at 550, 551, 480, 604, 597, and 625 nm, respectively. Pseudo-first-order conditions were employed, maintaining at least a tenfold excess of the oxidant over the metalloprotein concentration sufficient to give a 0.1 absorbance change at the indicated wavelength. Kinetic studies of the $Co(ox)_3^{3-}$–$Co(terpy)_2^{2+}$ reaction (510 nm) were also carried out with pseudo-first-order excesses of the oxidant, holding $[Co(terpy)_2^{2+}]_0$ constant at 36 μM.

All reactions involving $Co(phen)_3^{3+}$ and those of $Co(ox)_3^{3-}$ with $Co(terpy)_2^{2+}$, St(I), and cyt *c*(II) were followed on a Durrum D-110 stopped-flow spectrophotometer. Kinetic data were collected as photographs of absorbance-time traces on a Tektronix Model 564B storage oscilloscope or as traces from a Hewlett-Packard Model 7004 B X-Y recorder. The reactions of $Co(ox)_3^{3-}$ with cyt *c*-551(II), HiPIP(r), Pc(I), and Az(I) were monitored on a Cary 17 spectrophotometer, mixing the reactants in a deoxygenated, serum-capped 1-cm cell and recording the absorbance-time trace using the 0.2 slidewire. Some data for the $Co(ox)_3^{3-}$–Pc(I) reaction were acquired with an Aminco DW-2a spectrophotometer. In each case, the reactants were thermostated at 25.0 ± 0.2 °C before mixing and throughout kinetics determinations.

Data Analysis. In most cases, observed first-order rate constants (k_{obsd}) were obtained from the least-squares slopes of ln $|A_t - A_\infty|$ vs. time plots. These first-order analytical plots were found to be linear for at least 3 half-lives in all reactions except that of cyt *c*-551(II) with $Co(ox)_3^{3-}$ (vide infra). The reactions of Az(I) and HiPIP(r) with low concentrations of $Co(ox)_3^{3-}$ were so slow as to make determination of the final absorbance (A_∞) considerably uncertain. Rate constants for these slow runs were extracted from Guggenheim plots[17] based on data obtained during the first 2 half-lives of the reactions. Most reported k_{obsd} values are the average of at least two determinations.

Results and Discussion

Rate data obtained for the reactions of $Co(ox)_3^{3-}$ and $Co(phen)_3^{3+}$ with metalloproteins are summarized in Table I, along with data for the $Co(ox)_3^{3-}$–$Co(terpy)_2^{2+}$ reaction.

Table I. Rate Constants for the Oxidation of Metalloproteins and $Co(terpy)_2^{2+}$ by $Co(ox)_3^{3-}$ and $Co(phen)_3^{3+}$ [a,b]

reductant	oxidant	10^3[oxidant], M	k_{obsd}, s^{-1}
St(I)	$Co(ox)_3^{3-}$	0.125	0.080 (0.004)
		0.250	0.166 (0.004)
		0.500	0.341 (0.008)
		0.750	0.529 (0.005)
		1.00	0.709 (0.021)
		2.50	1.72 (0.05)
		5.00	3.69
Pc(I)	$Co(phen)_3^{3+}$	0.0684	0.087 (0.001)
		0.201	0.237 (0.003)
		0.326	0.374 (0.018)
		0.652	0.728 (0.007)
		1.30	1.41 (0.01)
		2.61	2.67 (0.19)
Pc(I)	$Co(ox)_3^{3-}$	0.463	1.25 (0.01) × 10^{-4}
		1.00	2.41 (0.09) × 10^{-4}
		2.50	6.19 (0.08) × 10^{-4}
		5.00	1.21 (0.04) × 10^{-3}
Az(I)	$Co(phen)_3^{3+}$	1.03	4.50 (0.15)
		3.10	13.5 (0.1)
Az(I)	$Co(ox)_3^{3-}$	0.90	3.03 (0.02) × 10^{-5}
		2.00	5.15 (0.35) × 10^{-5}
		3.80	9.93 (0.40) × 10^{-5}
		4.95	1.49 (0.10) × 10^{-4}
cyt *c*(II)	$Co(phen)_3^{3+}$	0.283	0.883 (0.008)
		1.13	3.79 (0.03)
		2.26	7.95 (0.18)
cyt *c*(II)	$Co(ox)_3^{3-}$	0.489	1.84 (0.01) × 10^{-3}
		0.994	4.88 (0.05) × 10^{-3}
		2.37	1.29 (0.03) × 10^{-2}
		4.73	2.54 (0.01) × 10^{-2}
cyt *c*-551(II)	$Co(phen)_3^{3+}$	1.03	60.6 (2.2)
		3.10	199
cyt *c*-551(II)	$Co(ox)_3^{3-}$	0.457	7.39 (0.55) × 10^{-4}
		0.914	1.32 (0.12) × 10^{-3}
		2.29[c]	3.10 (0.11) × 10^{-3}
		4.47[c]	6.23 (0.11) × 10^{-3}
HiPIP(r)	$Co(phen)_3^{3+}$	0.157	0.41 (0.02)
		0.555	1.41 (0.02)
		1.13	2.97 (0.03)
		2.26	6.14 (0.28)
HiPIP(r)	$Co(ox)_3^{3-}$	0.934	7.15 × 10^{-6}
		2.31	1.61 (0.04) × 10^{-5}
		4.58	3.60 (0.20) × 10^{-5}
$Co(terpy)_2^{2+}$	$Co(ox)_3^{3-}$	0.486	0.0807 (0.0006)
		0.943	0.163 (0.002)
		2.40	0.406 (0.003)
		4.79	0.771 (0.012)

[a] 25 °C, pH 7.0, μ = 0.5 M (phosphate). [b] Average deviations from the mean shown in parentheses. [c] Based on the last 50% of ln ($A_t - A_\infty$) vs. time plots (see text).

First-order analytical plots for the $Co(ox)_3^{3-}$–cyt *c*-551(II) reaction were found to deviate from linearity when $[Co(ox)_3^{3-}]$ was 2.29 or 4.47 mM. Thus, slight curvature was noted in the ln ($A_t - A_\infty$) plots throughout the first 50% of the 551-nm absorbance change, while the last half of the plots yielded lines with reproducible slopes. At present the reasons for this deviation from first-order behavior are not well understood. We have tentatively calculated k_{obsd} for these runs from the slopes of the line determined by the last 50% of the absorbance-time data. This analysis may in fact not be appropriate if the initial curvature represents an authentic induction period in the $Co(ox)_3^{3-}$–cyt *c*-551(II) reaction.

The data set out in Table I demonstrate that all of the reactions under consideration exhibit first-order dependences on both the reducing agent and the cobalt(III) oxidant. Second-order rate constants derived from the least-squares anal-

Table II. Comparison of Rate Parameters for Oxidations by $Co(phen)_3^{3+}$ and $Co(ox)_3^{3-}$ [a,b]

reductant	k, M^{-1} s^{-1}		$\frac{k(Co(phen)_3^{3+})}{k(Co(ox)_3^{3-})}$
	$Co(phen)_3^{3+}$	$Co(ox)_3^{3-}$	
stellacyanin(I) (*Rhus vernicifera*)	1.8×10^5 [c]	$7.3\ (0.3) \times 10^2$	2.5×10^2
plastocyanin(I) (spinach)	$1.02\ (0.03) \times 10^3$	$2.4\ (0.1) \times 10^{-1}$	4.3×10^3
azurin(I) (*Pseudomonas aeruginosa*)	$4.4\ (0.2) \times 10^3$	$2.9\ (0.3) \times 10^{-2}$	1.5×10^5
cyt *c*(II) (horse heart)	$3.3\ (0.2) \times 10^3$	$5.5\ (0.2) \times 10^0$	6.0×10^2
cyt *c*-551(II) (*Pseudomonas aeruginosa*)	$6.0\ (0.3) \times 10^4$	$1.4\ (0.1) \times 10^0$	4.3×10^4
HiPIP(r) (*Chromatium*)	$2.73\ (0.04) \times 10^3$	$8\ (1) \times 10^{-3}$	3.4×10^5
$Co(terpy)_2^{2+}$	4.16×10^2 [d]	$1.60\ (0.04) \times 10^2$	2.6×10^0

[a] 25 °C, pH 7.0, μ = 0.5 M (phosphate) unless otherwise specified. [b] Uncertainties given in parentheses. [c] Reference 18. [d] μ = 0.5 M (NaCl); ref 20.

ysis of linear k_{obsd} vs. [Co(III)] plots are contained in Table II. Since first-order $Co(phen)_3^{3+}$ dependences have already been established for the reactions of this oxidant with Az(I),[18] cyt *c*(II),[19] and cyt *c*-551(II),[20] k_{obsd} was obtained for only two or three $Co(phen)_3^{3+}$ concentrations in each case. The second-order rate constant k was then calculated as the average value of $k_{obsd}/[Co(phen)_3^{3+}]$.

Our kinetic results for the Az(I)-$Co(phen)_3^{3+}$ reaction agree quite well with those of Sykes and co-workers,[21] but we do not detect the rate saturation reported by these workers in the oxidation of Pc(I) by $Co(phen)_3^{3+}$ (k_{et} = 17.9 s^{-1}, $K \approx 167$ M^{-1}; pH 7.5, μ = 0.1 M (NaCl)).[22] Thus, k_{obsd} varies linearly with $[Co(phen)_3^{3+}]$ over a 38-fold concentration range in the oxidation of spinach plastocyanin at ionic strength 0.5 M. Nevertheless, the agreement between our second-order rate constant for the Pc(I)-$Co(phen)_3^{3+}$ reaction (1.02×10^3 M^{-1} s^{-1}) and that obtained by Segal and Sykes (3.0×10^3 M^{-1} s^{-1}, parsley plastocyanin)[22] seems reasonable on consideration of the different reaction media and protein sources employed. The absence of rate saturation in our studies is not surprising, as a high ionic strength reaction medium was employed specifically to discourage precursor complex formation arising from electrostatic interactions between the oxidant and the metalloprotein.

Cuprous stellacyanin forms an exceptionally strong precursor complex with the anionic oxidant $Co(EDTA)^-$, leading to rate saturation at high $[Co(EDTA)^-]$.[23] By contrast, the reaction of $Co(ox)_3^{3-}$ with St(I) under the same conditions (25 °C, pH 7.0, μ = 0.5 M) is characterized by a first-order oxidant dependence over a 40-fold concentration range. Rate saturation is in fact not observed in the $Co(ox)_3^{3-}$-St(I) reaction even when the ionic strength is decreased to 0.1 M, presumably enhancing the electrostatic attraction between the strongly basic metalloprotein (pI = 9.86)[10] and the anionic oxidant. Thus, a k_{obsd} vs. $[Co(ox)_3^{3-}]$ plot based on data[24] obtained at 25.1 °C, pH 7.2 (0.001 M phosphate), μ = 0.1 M (NaCl), is linear within the range 0.114 mM $\leq [Co(ox)_3^{3-}] \leq$ 4.78 mM, yielding a second-order rate constant ($6.5 \pm 0.1 \times 10^2$ M^{-1} s^{-1}) essentially identical with that found at μ = 0.5 M. Nonelectrostatic protein-oxidant interactions therefore must be largely responsible for the unusually high stability of the $Co(EDTA)^-$-St(I) precursor complex.[25]

Rate constants for the $Co(ox)_3^{3-}$ oxidation of St(I), Pc(I), Az(I), cyt *c*(II), cyt *c*-551(II), and HiPIP(r) span nearly a 10^5-fold range (8×10^{-3} to 7.3×10^2 M^{-1} s^{-1}). By contrast, a 500-fold smaller interval is covered by rate constants for the $Co(phen)_3^{3+}$ oxidation of these same six metalloproteins (1.02×10^3 to 1.8×10^5 M^{-1} s^{-1}). More importantly, the reactivity ratio $R = k[Co(phen)_3^{3+}]/k[Co(ox)_3^{3-}]$ is not constant throughout the series, as would be expected if the equation of relative Marcus theory[26] for outer-sphere electron transfer pertained rigorously. Indeed, this ratio varies by a factor of 1.4 $\times 10^3$ throughout the sequence HiPIP(r) > Az(I) > cyt *c*-551(II) > Pc(I) > cyt *c*(II) > St(I). The relationship between electron-transfer rate and thermodynamic driving force given by relative Marcus theory is (for reactions with small driving force at 25 °C):

$$k_{12} = [k_{11}k_{22}\exp(38.94(\Delta E))]^{1/2} \quad (1)$$

where k_{11} and k_{22} are the self-exchange electron-transfer rate constants for the metalloprotein and its redox partner, respectively. The cross-reaction rate constant is designated by k_{12}, and ΔE is the difference between the reduction potentials of the protein and the oxidant in volts. On this basis the ratio R may be expressed:

$$R = \left[\frac{k_{22}[Co(phen)_3^{3+/2+}]}{k_{22}[Co(ox)_3^{3-/4-}]}\exp(38.94(\Delta E'))\right]^{1/2} \quad (2)$$

where $\Delta E'$ now is the difference between the reduction potentials of $Co(phen)_3^{3+}$ and $Co(ox)_3^{3-}$. Since none of the parameters in this equation depend on the nature of the reductant, R should be a constant for all electron donors that meet the weak overlap criterion on which the Marcus equation is based. Electrostatic interactions between the reductant and oxidant are not accounted for in eq 2, but corrections for this effect are very small at ionic strength 0.5 M (vide infra).

The parameters required for the calculation of R are: $Co(phen)_3^{3+/2+}$, k_{22} = 4.5 × 10^1 M^{-1} s^{-1} (μ = 0.1 M (KNO_3)),[9] E^0 = 0.370 V (μ = 0.1 M);[3] $Co(ox)_3^{3-/4-}$, k_{22} = 2.8×10^{-7} M^{-1} s^{-1} (μ = 0.1 M),[27] E^0 = 0.570 V (μ = 1.0 M (KCl)).[28] From these parameters, a "normal" R value of 2.6×10^2 may be estimated, in close agreement with the observed ratio for stellacyanin. Consistent with previous findings,[1,3] stellacyanin is the only one of the six metalloproteins for which Marcus theory successfully correlates cross-reaction and self-exchange electron-transfer rates. The ordering of metalloproteins according to the ratio $k[Co(phen)_3^{3+}]/k[Co(ox)_3^{3-}]$ correlates well with that based on the "kinetic accessibility" scale formulated by Wherland and Gray:[1] Az~HiPIP < cyt *c*-551 < cyt *c* < Pc ≪ St. Only the relative positions of plastocyanin and cytochrome *c* are interchanged between the two series. This small discrepancy probably is not significant, as a different source of plastocyanin (bean) was used in the studies from which the "kinetic accessibility" of plastocyanin was derived.

The reactivity ratio R thus provides an alternative measure of "kinetic accessibility" to that derived from the range in k_{11}^{corr} values calculated for the protein on the basis of rate data for its reactions with a diverse set of redox agents. Indeed, the same factors responsible for the lack of constancy in k_{11}^{corr} (nonadiabicity, nonelectrostatic protein-reagent interactions) also would give rise to larger than "normal" R values. Nonelectrostatic interactions between the protein and the hydrophobic 1,10-phenanthroline ligands of $Co(phen)_3^{3+}$ will become increasingly important as the distance of penetration required to achieve satisfactory overlap between the donor orbital of the reductant and the phenanthroline π system increases. Nonelectrostatic interactions between metalloproteins and the strongly hydrophilic $Co(ox)_3^{3-}$ ion are not expected to be substantial. Indeed, $Co(ox)_3^{3-}$ should face a prohibitive

Table III. Marcus Calculations for the Reactions of $Co(ox)_3^{3-}$ with Selected Metalloproteins[a]

protein	k_{12}, $M^{-1}\ s^{-1}$	ΔE^0, V	Z_1/Z_1'	Z_2/Z_2'	R, Å	w_{12}[b]	w_{21}	w_{11}	k_{11}^{corr}, $M^{-1}\ s^{-1}$	k_{11}^{corr},[c] $M^{-1}\ s^{-1}$	r[d]
stellacyanin	$7.3(10^2)$	0.386	0/0[e]	−3/−4	19.5	0	0	0	$2.5(10^4)$	$2.3(10^5)$	9.2
plastocyanin	$2.4(10^{-1})$	0.22	−10/−9	−3/−4	15.8	0.274	0.328	0.882	$5.4(10^{-1})$	3.4(10)[f]	63
azurin	$2.9(10^{-2})$	0.266	−2/−1	−3/−4	17.2	0.046	0.031	0.015	$2.6(10^{-3})$	$1.2(10^{-2})$	4.6
cytochrome *c*	$5.5(10^0)$	0.31	6.5/7.5	−3/−4	16.6	−0.161	−0.248	0.405	4.6	6.2	1.3
cytochrome *c*-551	$1.4(10^0)$	0.31	−3/−2	−3/−4	14.4	0.099	0.088	0.079	1.4	2.0	1.4
HiPIP	$8.0(10^{-3})$	0.22	−3.5/−2.5	−3/−4	15.5	0.100	0.095	0.091	$1.1(10^{-3})$	$1.3(10^{-2})$	11.8

[a] 25 °C, pH 7.0 (phosphate). Second-order rate constants measured at 0.5 M ionic strength and k_{11}^{corr} values corrected to 0.10 M. w_{22} = 2.408 kcal/mol. E^0 for $Co(ox)_3^{4-/3-}$ assumed to be 570 mV (Hin-Fat, L.; Higginson, W. C. E. *J. Chem. Soc. A* **1967**, 298). The self-exchange rate of $Co(ox)_3^{4-/3-}$ was estimated from the cross reaction with $Co(terpy)_2^{2+/3+}$ to be $2.83(10^{-7})$ $M^{-1}\ s^{-1}$ at 25 °C and 0.1 M ionic strength. [b] The radius of $Co(ox)_3^{4-/3-}$ was assumed to be 4.5 Å. All work terms are given as kcal/mol. [c] Calculation based on the cross reaction of the protein with $Fe(EDTA)^{2-}$. [d] The ratio of the protein self-exchange rate calculated from the $Fe(EDTA)^{2-}$ cross reaction divided by that obtained from the $Co(ox)_3^{3-}$ cross reaction. [e] The protein charge calculated from the amino acid sequence and making certain assumptions about the carbohydrate contribution to the charge is 6/7. As previous studies have found no rate dependence on ionic strength, the present calculations assume zero electrostatic charge for this protein. [f] Derived from k_{12} for the reduction of bean plastocyanin.

enthalpic barrier to penetration of the protein surface, preferring instead to accept nonadiabicity in the electron-transfer process due to the poor redox orbital overlap achieved when the oxidant attacks the active site from the surface. The 500-fold wider range in $k[Co(ox)_3^{3-}]$ relative to that in $k[Co(phen)_3^{3+}]$ confirms this hypothesis, as the reactivity of the former reagent clearly is more sensitive to differences in the metal site-to-surface distance. For example, rate constants for the oxidation of cyt *c*(II) and HiPIP(r) by $Co(phen)_3^{3+}$ are essentially identical even though the FeS_4S_4* cluster of HiPIP is considerably more buried (by ca. 2 Å)[1] than is the heme edge of cytochrome *c*. The difference in site accessibilities is clearly manifested in the $Co(ox)_3^{3-}$ results, however, as cyt *c*(II) now enjoys nearly a 700-fold reactivity advantage over HiPIP(r).

The reactivities of the hydrophilic reagents $Fe(EDTA)^{2-}$ and $Co(ox)_3^{3-}$ with metalloproteins are best compared using electrostatics-corrected apparent metalloprotein self-exchange rate constants, calculated through the Marcus theory analysis of Wherland and Gray.[1] Table III presents this comparison of k_{11}^{corr} values based on $Fe(EDTA)^{2-}$ reductions and $Co(ox)_3^{3-}$ oxidations for each of the six proteins considered. Protein charges were estimated on the basis of the amino acid composition in each case. Considering the uncertainties in reagent radii, charges, and electrostatic work terms inherent in the calculation, k_{11}^{corr} values based on cross reactions with $Fe(EDTA)^{2-}$ and $Co(ox)_3^{3-}$ agree remarkably well. It should be noted that $k_{11}^{corr}(Co(ox)_3^{3-})$ is somewhat smaller than $k_{11}^{corr}(Fe(EDTA)^{2-})$ for each of the metalloproteins considered, but the difference amounts to no more than one order of magnitude except in the case of plastocyanin. The 63-fold difference in k_{11}^{corr} values for plastocyanin may reflect, in part, the differing redox reactivities of the bean $(Fe(EDTA)^{2-})$ and spinach $(Co(ox)_3^{3-})$ proteins. The factor of 9.2 discrepancy between k_{11}^{corr} estimates for stellacyanin is surprising, as values based on cross reactions with $[Ru(NH_3)_5py]^{3+}$ (1.7×10^5 $M^{-1}\ s^{-1}$), $Fe(EDTA)^{2-}$ (2.3×10^5 $M^{-1}\ s^{-1}$), and $Co(phen)_3^{3+}$ (3.0×10^5 $M^{-1}\ s^{-1}$) do not differ appreciably.[3] The higher redox reactivity of $Fe(EDTA)^{2-}$ with stellacyanin relative to that of $Co(ox)_3^{3-}$ reasonably could be attributed to stabilization of the precursor complex in the $Fe(EDTA)^{2-}$-St(II) system, considering Yoneda and Holwerda's report of strong association between this metalloprotein and several EDTA-containing species $(Co(EDTA)^-$, $Co(EDTA)^{2-}$, $H_2(EDTA)^{2-})$.[23]

The close agreement between $k_{11}^{corr}(Fe(EDTA)^{2-})$ and $k_{11}^{corr}(Co(ox)_3^{3-})$ estimates confirms the use of the former to define the lower limit of the "kinetic accessibility" scale. Thus, the insensitivity of k_{11}^{corr} to substantial differences in the structures and reaction types of the two hydrophilic redox agents suggests that all such reagents will exhibit reactivities similar to that of $Fe(EDTA)^{2-}$. In this limit k_{11}^{corr} does indeed appear to be primarily a function of protein characteristics, with the metal site-to-surface distance probably being the most important of these. Recently completed calculations[29] indicate that a quantitative relationship does in fact exist between k_{11}^{corr} and the distance of electron transfer employed by the metalloprotein with a particular hydrophilic reagent.

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Supplementary Material Available: A listing of observed rate constants for the oxidation of cuprous stellacyanin by $Co(ox)_3^{3-}$ at ionic strength 0.1 M (Supplementary Table I) (1 page). Ordering information is given on any current masthead page.

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Liposomal Membranes. 2. Synthesis of a Novel Pyrene-Labeled Lecithin and Structural Studies on Liposomal Bilayers

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Abstract: A novel pyrene-labeled phosphatidylcholine, 1,2-bis[ω-(1-pyreno)decanoyl]-*sn*-glycero-3-phosphorylcholine (DPDL), was synthesized for use in structural studies on the liposomal bilayers. From the fluorescence measurements on DPDL incorporated into the phospholipid bilayers as well as in organic solvents and aqueous buffered solutions, the following interesting information was obtained. (1) The probe DPDL is intercalated in a well-ordered fashion in the very hydrophobic domain of egg and dipalmitoylphosphatidylcholine bilayers. (2) Without cholesterol and/or below the phase-transition temperature, DPDL is arranged with the lipid-separated conformation in the shrunk liposomal bilayers (Figure 9d). (3) Increasing the mobility of lipid molecules by the addition of cholesterol and/or by elevating temperatures causes a conformational change of DPDL in the bilayers from the lipid-separated conformation to the intimate one (Figure 9c).

In connection with the role of biological membranes as the barrier against permeation of materials or the suitable domain for the membrane-bound enzymes, an understanding of the static and dynamic structures of lipid model membranes is important. To date a considerable amount of data has been accumulated about the lipid bilayer structure by the use of various techniques such as NMR,[1-3] DSC,[4] ESR,[5,6] or X-ray diffraction.[4,7] In order to obtain more detailed information about the structure and microenvironment of the bilayers, it is necessary to utilize such a probe that has more resemblance in the structure to the natural phospholipids and can sit in good order in the lipid bilayers. For this reason the probe which is covalently bound to the suitable positions of the lecithin molecule seems more preferable, as has been shown in the studies with the spin-labeled phospholipids[1,8] or the fluorescent phosphatidylcholine.[9]

Fluorescent probe techniques have been extensively applied to problems involving the fluidity and phase transition,[9,10] mobility and lateral diffusion,[11,12] polarity,[13,14] or surface potential of phospholipid bilayers.[15] However, the previously used fluorescent probes such as 8-anilino-1-naphthalenesulfonate (ANS),[16,17] anthroyl fatty acid derivatives,[18] pyrenes,[14,19] 1,6-diphenyl-1,3,5-hexatriene (DPH),[9,11] or polyene fatty acids[10] had serious drawbacks together with their advantages. For example, some of these probes such as pyrene or DPH, which have less resemblance to the natural phospholipids, may disturb the intact structure of lipid bilayers. In addition, since they are not linked covalently to the lecithin they have considerable freedom to move in the bilayers. Hence, they may easily change their location and orientation when the conditions such as composition of the membrane and temperature are varied.[9,11] Waggoner and Stryer employed a covalently fluorescent probe-labeled lipid, dansylphosphatidylethanolamine, but it only gave information about the region close to the membrane surface since the dansyl group was bound on the polar head group of the lecithin.[13] A possible reason that the use of covalently probe-labeled lecithins has been rather limited is the relative difficulty of the chemical synthesis of such lipids, especially when the probe is introduced to the fatty acid chains.[10,20]

We have designed a novel, covalently pyrene-labeled lecithin, 1,2-bis[ω-(1-pyreno)decanoyl]-*sn*-glycero-3-phosphorylcholine (DPDL), to get information about the hydrophobic interior of liposomal bilayers. In order for the probe to fit best in the dipalmitoylphosphatidylcholine bilayers, the length of the polymethylene chains of the fatty acid residues is critical, and the appropriate length was chosen with the aid of a Corey-Pauling-Koltun (CPK) model (Figure 1).

Levine et al. have demonstrated by ^{13}C NMR spin-lattice relaxation measurements with covalently spin-labeled lipids that the lipid molecules are most tightly packed at the glycerol group.[1] The same conclusion has also been reached by studies on the lateral diffusion of the phospholipid molecules in bilayers.[21] Judging from these previous results, DPDL will be intercalated in a well-ordered fashion within the phospholipid bilayers, since the head group of the lecithin was not modified chemically at all. Moreover, the pyrene moiety will exhibit a relatively long lifetime and high quantum yield of fluorescence emission,[19,22] and, hence, should be suitable for fluorescence studies. High quantum efficiency of the probe allows the use of smaller amounts of the probe, which should reduce the probe's perturbation of the host lipid environment.[18] DPDL has another unique feature worth noting as a probe diagnosing the nature and state of lipid bilayers. DPDL carries a pyrene moiety at the end of each fatty acyl chain of the lipid molecule and will easily adopt a sandwich configuration of the pyrene moieties in the ground state. Hence, it can easily form an intramolecular exciplex from the ground-state dimer[23,25] and will provide useful information concerning the inter- and/or intramolecular interactions between the fatty acid chains located in a very hydrophobic domain. A small probe containing a single fluorescent group, ethyl ω-(1-pyreno)decanoate (EP), was also used for comparison. Using these probes, we have investigated the structure of phospholipid bilayers as well as the location and orientation of the probe molecules in the bilayers.

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