GGENTA has less tendency to lose its amide proton than the other ligands considering the distance between the amido groups, which indicates that the relative orientation of the two amides, similar to that in peptide linkages, seems to restrict this ionization. When two carbonyls are adjacent to each other, as in BAOTA, the proton ionization of the second amide group is considerably enhanced.

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Kinetic Studies on Electron-Transfer Reactions of *Brassica oleracea* Cytochrome *f* with Inorganic Oxidants of Varying Charge (5- to 3+)

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Electron-transfer reactions of *Brassica oleracea* cytochrome *f* with a range of inorganic oxidants (charges 5- to 3+) have been studied. Rate constants (25 °C, M^{-1} s^{-1}), I = 0.10 M (NaCl), which are invariant for any one oxidant over the pH range 6.5–8.0, and activation parameters $\Delta H^{\ddagger}$ (kcal mol^{-1}) and $\Delta S^{\ddagger}$ (cal K^{-1} mol^{-1}), are as follows: $[(CN)_5FeCNCo(CN)_5]^{5-}$, 4.9×10^5, -3.2, -42.9; $[Co(dipic)_2]^-$, 9.4×10^4, 2.3, -28.1; $[Co(bpy)_2(O_2CMe)_2]^+$, 16.8, 14.2, -5.4; $[Co(phen)_3]^{3+}$, 3.5×10^5, 10.9, 3.5. The rate constant for the $[Fe(CN)_6]^{3-}$ oxidation of *B. oleracea* cytochrome *f* is in good agreement with that previously reported for *Brassica komatsuna*: 1.7×10^5, -0.9, -38. First-order rate constants (25 °C) for the 5- oxidant (present in large excess) show a nonlinear dependence on oxidant concentration consistent with association K (4.1×10^3 M^{-1}) prior to electron transfer: k_{et} (122 s^{-1}) at pH 7.5. Although the protein (pI 5.5) is negatively charged at pH 7.5, the effect of temperature and ionic strength on K implicates a positively charged binding site on cytochrome *f*. The redox-inactive complexes $[Zr(C_2O_4)_4]^{4-}$, association constant K_B = 530 M^{-1}, and $[Mo(CN)_8]^{4-}$, K_B = 490 M^{-1}, exhibit competitive inhibition for the 5- oxidant at 25 °C, I = 0.10 M (NaCl). The effect of pH (4.0–9.5) on the $[Co(phen)_3]^{3+}$ reaction, pK_a's 5.1 and 9.7, mirrors effects observed for $[Fe(CN)_6]^{3-}$, suggesting that there is a common binding site, protonation of which influences rate constants according to the charge on the oxidant. Activation and thermodynamic parameters are compared to those previously obtained for reactions of other metalloproteins.

Introduction

Cytochrome *f* is a single heme *c*-type cytochrome (mol wt ~33 000) found in the chloroplasts of higher plants, where it is bound to the thylakoid membrane. It was first reported by Hill and Scarisbrick[1] and has been isolated from parsley,[2,3] spinach,[4-6] and tobacco leaves[7] in an oligomeric form. More recently, the protein has been isolated in a monomeric form, with no apparent tendency to aggregate from Japanese radish,[8] charlock,[9] and cabbage,[9,10] all of which are members of the family Cruciferae. Implications of recent sequenching information have been considered.[11] Cabbage leaves are the source of protein for studies describe in this paper. Cytochrome *f* has a reduction potential of ~360 mV and isoelectronic point (charlock) of 5.5.[9] Its function is to transfer electrons from the plastoquinone pool to plastocyanin in the photosynthetic electron-transport chain between photosystems II and I. No X-ray crystal structure information is yet available.

Although fairly extensive studies on the in vivo function of cytochrome *f* have appeared,[12] the properties of purified cytochrome *f* (and in particular its reactivity) have to date been comparatively neglected. Takabe et al.[13] have studied the electron-transfer reaction of *Brassica komatsuna* cytochrome *f* with $[Fe(CN)_6]^{3-}$. Here we report investigations on the reactions of *Brassica oleracea* cytochrome *f* with five inorganic oxidants of varying charge (5- to 3+) including $[Fe(CN)_6]^{3-}$, in order to assess effects of pH, ionic strength, and temperature. The pattern of behavior that emerges is of interest compared to that of other cytochromes and as a preliminary to studies on the reaction of cytochrome *f* with plastocyanin. The competitive inhibition, which has been identified with two redox-inactive complexes, is also of interest in this latter context.

Experimental Section

Preparations. Protein. Cytochrome *f* from Durham early-spring cabbage (*B. oleracea*) was purified by the literature method,[9,10] with modifications as indicated. Reagent grade chemicals and singly distilled water were used throughout. All chromatographic separations were carried out at ~4 °C. Batches of destalked frozen leaves (950 g) were homogenized with 0.2 M Na_2HPO_4/10 mM EDTA (150 cm^3) and butan-2-one (1500 cm^3) at -20 °C in a 1-gal Waring blender for 60 s at medium speed. The upper green phase was poured off after allowing the homogenate to settle in a beaker. The lower phase was squeezed through two layers of muslin and centrifuged for 10 min at 10 000 rpm (4 °C). The resulting lower orange aqueous phase was removed by aspiration.

Cytochrome *f* was precipitated from the aqueous extract by addition of 1.3 vol of acetone cooled to -20 °C. The precipitate was collected by centrifugation at 10 000 rpm for 10 min (4 °C); the reddish pellets were suspended in a minimum volume of 40 mM Na_2HPO_4/2 mM EDTA and centrifuged at 10 000 rpm for 10 min (4 °C). The clear reddish supernatant was desalted on a Sephadex G-25 (coarse) column (50 × 8.0 cm), equilibrated with 1 mM sodium phosphate buffer at pH 7.5. The eluate was applied to a 10 × 5 cm (diethylamino)ethylcellulose (DE52) column equilibrated with 1 mM sodium phosphate buffer pH 7.5. The cytochrome was eluted with 50 mM sodium phosphate buffer pH 7.5 (this column separates out a considerable amount of brown material). The

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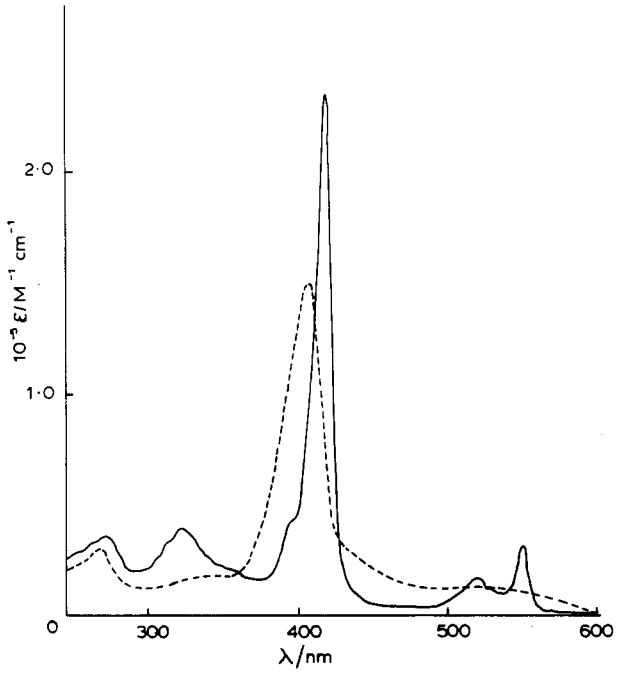

Figure 1. UV–visible spectra of cytochrome *f*(II) (—) and cytochrome *f*(III) (---), pH 7.5 (Tris/HCl), I = 0.10 M (NaCl).

eluates containing cytochrome *f* were combined, concentrated to about 200 cm^3 on an Amicon hollow-fiber dialyzer/concentrator, and applied to a second DE52 column (14 × 5 cm) equilibrated with 1 mM sodium phosphate buffer pH 7.5. The column was washed with 10 mM sodium phosphate buffer until there was marked separation between the orange-pink cytochrome *f* band and other colored bands. The protein was then eluted with 20 mM sodium phosphate buffer, pH 7.5. Fractions with absorbance ratios (A_{554}/A_{280}) 0.30–0.50 were combined, concentrated to 20 cm^3, and applied in 10-cm^3 batches to a Sephadex G-75 column equilibrated with 10 mM sodium phosphate buffer at pH 7.5. The protein was eluted with the same buffer at a flow rate of 30 cm^3/h. Fractions with ratios (A_{554}/A_{280}) from 0.61 to 0.85 on addition of DL-dithiothreitol were combined and applied to an hydroxyapatite column (17.6 × 2.6 cm) (Ultrogel, LKB 2204-110) equilibrated with 1 mM sodium phosphate buffer pH 7.5. The column was washed with 1 column vol each of 5, 10, and 20 mM sodium phosphate buffer, pH 7.5. The cytochrome was eluted with 50 mM sodium phosphate buffer at pH 7.5. Samples with absorbance ratio higher than 0.83 were used for kinetics. Cytochrome *f* can be reduced as described below. Spectra of the two oxidation states of cytochrome *f*, designated here as cyt *f*(II) (pink) and cyt *f*(III) (orange-pink) are shown in Figure 1. These are closely similar to those reported by Matsuzaki et al.[10] for cytochrome *f* obtained from *B. komatsuna*. The absorbance maxima at 554 nm (ϵ = 32 000 M^{-1} cm^{-1}) for cyt *f*(III) was used to determine concentrations in these studies.

Complexes. These were obtained and purified to known spectra (λ/nm, $\epsilon/M^{-1}\,cm^{-1}$) by procedures already described: barium (μ-cyano)(pentacyanoferrate(III))pentacyanocobaltate(III), $Ba_5[(CN)_5FeCNCo(CN)_5]_2\cdot 6H_2O$, 300 (1480);[14] ammonium bis(pyridine-2,6-dicarboxylato)cobaltate(III), i.e. ammonium bis(dipicolinato)cobaltate(III), $NH_4[Co(dipic)_2]$, 510 (610);[15] bis(acetato)bis(bipyridyl)cobalt(III)perchlorate, $[Co(bpy)_2(O_2CMe)_2]ClO_4$, 509 (123);[16] tris(1,10-phenanthroline)cobalt(III) perchlorate, $[Co(phen)_3](ClO_4)_3\cdot 2H_2O$ 332 (4550);[17] potassium tetrakis(oxalato)zirconate(IV), $K_4[Zr(C_2O_4)_4]\cdot 5H_2O$ (oxalate content confirmed by titration);[18] potassium octacyanomolybdate(IV), $K_4[Mo(CN)_8]\cdot 2H_2O$, 367 (170);[19] hexaammineplatinum(IV) chloride, $[Pt(NH_3)_6]Cl_4\cdot H_2O$, 260 (129);[20] ($\mu$-amido)bis[pentaamminecobalt(III)] bromide, $[(NH_3)_5CoNH_2Co(NH_3)_5]Br_5$, 360 (705), 505 (420);[21] tris(ethylenediamine)chromium(III) chloride, $[Cr(en)_3]Cl_3\cdot 3H_2O$, 351 (63), 457 (73).[22] Potassium hexacyanoferrate(III), $K_3[Fe(CN)_6]$ (BDH, Analar), peaks at 300 nm (1600) and 420 (1010), was used without further purification. Because the perchlorate salt of $[Co(phen)_3]^{3+}$ has limited solubility, the perchlorate was exchanged by chloride using a Dowex 1 × 4 (50–100 dry mesh) anion resin in the chloride form and crystallized to give $[Co(phen)_3]Cl_3\cdot 7H_2O$.[23]

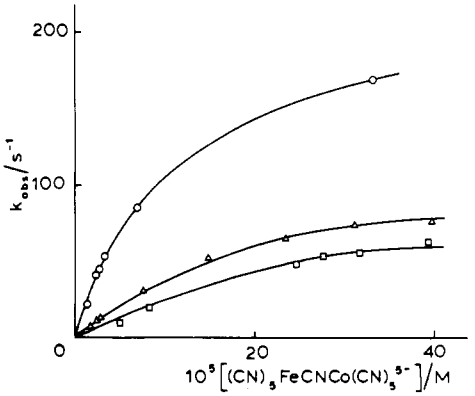

Figure 2. Variation of k_{obsd} (25 °C) with oxidant concentration for the $[(CN)_5FeCNCo(CN)_5]^{5-}$ oxidation of cytochrome *f*(II), at pH 5.0 (○), 7.5 (△), and 9.4 (□), I = 0.10 M (NaCl).

Buffers. The following buffers (~10^{-2} M) were used: pH 4.0–5.0, sodium acetate/acetic acid; pH 5.5–6.5, Mes/NaOH; pH 7.0–8.9, Tris/HCl; pH 9.0–9.8, sodium tetraborate (hereafter borate) borate/NaOH. The reagents Mes and Tris, abbreviations for 2-(*N*-morpholino)ethanesulfonic acid and tris(hydroxymethyl)aminomethane, respectively, were obtained from Sigma Chemicals. The pH of solutions was checked on a Radiometer (PHM 62) pH meter fitted with a combined electrode. Ionic strengths were adjusted with NaCl to 0.10 M except as stated. Previous studies on the reaction of *B. komatsuna* cytochrome *f* with $[Fe(CN)_6]^{3-}$ as oxidant at pH 7 (10^{-2} M potassium phosphate as buffer)[13] were first checked with cytochrome *f* from *B. oleracea*.

Stoichiometries. The inorganic complexes $[(CN)_5FeCNCo(CN)_5]^{5-}$, $[Fe(CN)_6]^{3-}$, $[Co(dipic)_2]^-$, $[Co(bpy)_2(O_2CMe)_2]^+$, and $[Co(phen)_3]^{3+}$ are all 1-equiv oxidants. Only the Fe(III) in the 5- binuclear complex is redox active in the present context. With the oxidants in large excess, spectrophotometric changes were consistent with 1:1 reactions as in (1), using a $\Delta\epsilon$ of 166 $mM^{-1}\,cm^{-1}$ for cyt *f*(II) and cyt *f*(III) at 422 nm (lit.[13] value 160 $mM^{-1}\,cm^{-1}$).

$$\text{cyt } f(\text{II}) \rightarrow \text{cyt } f(\text{III}) + e^- \quad (1)$$

Kinetic Studies. A Dionex D-110 stopped-flow spectrophotometer was used to monitor absorbance changes at the 422-nm cytochrome *f*(II) peak (Figure 1). Cytochrome *f* was reduced with DL-dithiothreitol prior to use and the reagent removed by anion-exchange chromatography using a short DE52 column. The reduced form could be readily stored at ~4 °C under semirigorous air-free conditions (i.e., N_2) for ~6 h. Concentrations of cytochrome *f*(II) were in the range (0.2–1.0) × 10^{-6} M. Inorganic oxidants were always in at least 10-fold excess of the protein. Plots of absorbance (A) changes, ln ($A_t - A_\infty$), against time were linear for 3–4 half-lives consistent with the rate law (2), which defines first-

$$-d[\text{cyt } f(\text{II})]/dt = k_{obsd}[\text{cyt } f(\text{II})] \quad (2)$$

order rate constants k_{obsd}. The stopped flow was equipped with a logarithmic amplifier, the output of which was stored digitally with a Datalab DL901 transient record. A Commodore Pet 2001-16K was interfaced to the recorder so that log ($A_t - A_\infty$) against time graphs could be displayed. Each rate constant listed is the mean of at least four determinations.

Treatment of Data. Nonlinear and linear least-squares fits of data (unit weighting) were used as appropriate.

Results

$[Fe(CN)_6]^{3-}$ as Oxidant. First-order rate constants at 25 °C (Table I)[24] with cytochrome *f* from *B. oleracea* exhibit a linear dependence on $[Fe(CN)_6^{3-}]$. Hence, a second-order rate constant k of (1.75 ± 0.15) × 10^5 M^{-1} s^{-1} was obtained for pH 7.5

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Table V. Summary of Association Constants (K) and Rate Constants (k_{et}) for the $[(CN)_5FeCNCo(CN)_5]^{5-}$ Oxidation of Cytochrome f(II) (Temperature 25 °C Except As Stated)

I, M	pH	$10^{-6}k$, M^{-1} s^{-1}	K, M^{-1}	k_{et}, s^{-1}
0.025	7.5	2.13	26200	65
0.10	5.0	2.03	8900	227
	7.5 (20 °C)	0.54	5200	103
	7.5 (25 °C)	0.49	4060	122
	7.5 (30 °C)	0.46	3200	145
	7.5 (40 °C)	0.41	2040	199
	9.4	0.36	3100	115
0.20	7.5	0.21	1170	180

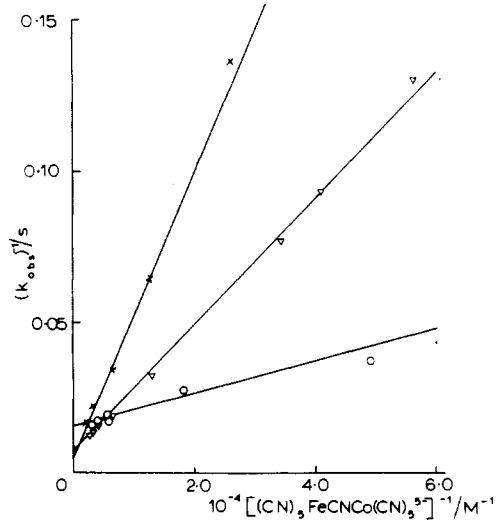

Figure 3. Dependence of k_{obsd} (25 °C) on oxidant concentration for the $[(CN)_5FeCNCo(CN)_5]^{5-}$ oxidation of cytochrome f(II) at pH 7.5 (Tris/HCl), ionic strength 0.25 M (○), 0.10 M (▽), and 0.025 M (×), adjusted with NaCl.

(Tris/HCl), I = 0.10 M (NaCl), in good agreement with the value 1.7×10^5 M^{-1} s^{-1} (20 °C, $\Delta H^{\ddagger}$ = −0.9 kcal mol^{-1}) for cytochrome f from *B. komatsuna*.[13] Replacement of Tris/HCl with phosphate buffer (which was used in ref 13) gave a rate constant (Table I)[24] of $(1.5 \pm 0.2) \times 10^5$ M^{-1} s^{-1}.

$[(CN)_5FeCNCo(CN)_5]^{5-}$ as Oxidant. First-order rate constants k_{obsd} at different pHs (Table II)[24] exhibit a nonlinear dependence on oxidant concentration (Figure 2). This is consistent with a two-stage process involving association of the reactants (eq 3),

$$\text{cyt } f\text{(II)} + \text{oxidant} \overset{K}{\rightleftharpoons} \text{cyt } f\text{(II)},\text{oxidant} \quad (3)$$

$$\text{cyt } f\text{(II)},\text{oxidant} \xrightarrow{k_{et}} \text{products} \quad (4)$$

prior to electron transfer (eq 4). The dependence (5) can be

$$k_{obsd} = Kk_{et}[\text{oxidant}]/(1 + K[\text{oxidant}]) \quad (5)$$

derived from (3) and (4), and plots of $1/k_{obsd}$ against $[\text{oxidant}]^{-1}$ are linear, with slope and intercept corresponding to $1/Kk_{et}$ and $1/k_{et}$, respectively. Variations of K and k_{et} with pH were investigated (see listing in Table V). Changes in k ($=Kk_{et}$) on going to higher (9.4) and lower (5.0) pHs are consistent with findings with $[Fe(CN)_6]^{3-}$ as oxidant, see Figure 5. Similarly, experiments in which the temperature (Table III)[24] and ionic strength (Table IV,[24] reciprocal plot illustrated in Figure 3) were varied gave further information regarding K and k_{et} (Table V). From the temperature dependence for K, ΔH° = −8.5 ± 1.0 kcal mol^{-1} and ΔS° = −12.0 ± 3.3 cal K^{-1} mol^{-1}, and for k_{et}, $\Delta H_{et}^{\ddagger}$ = 5.3 ± 0.7 kcal mol^{-1} and $\Delta S_{et}^{\ddagger}$ = −30.9 ± 2.3 cal K^{-1} mol^{-1}.

The blocking, i.e. competitive inhibition, exhibited by redox-inactive $[Zr(C_2O_4)_4]^{4-}$ on rate constants (Table VI)[24] for the

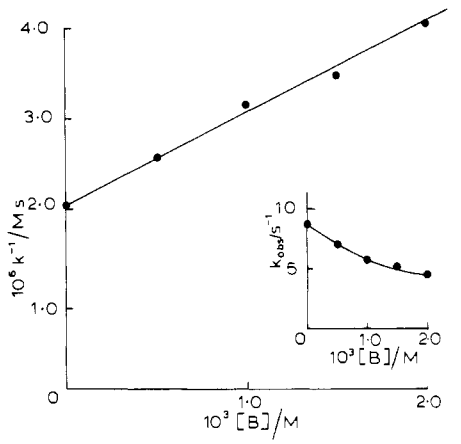

Figure 4. Blocking, i.e. competitive inhibition, of redox-inactive $[Zr(C_2O_4)_4]^{4-}$ on rate constants (25 °C) for the $[(CN)_5FeCNCo(CN)_5]^{5-}$ oxidation of cytochrome f(II) at pH 7.5 (Tris/HCl), I = 0.10 M (NaCl).

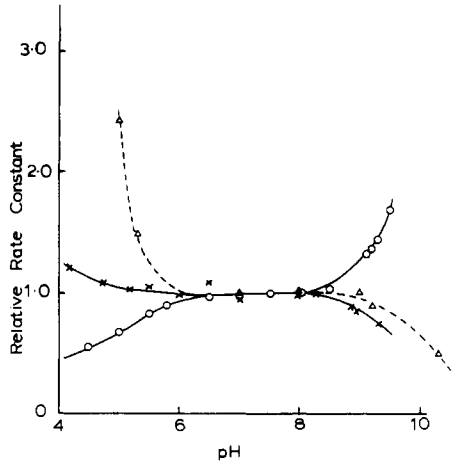

Figure 5. Effect of pH on rate constants (25 °C), I = 0.10 M (NaCl), for the oxidation of cytochrome f(II) with $[Co(dipic)_2]^-$ (×), $[Co(phen)_3]^{3+}$ (○), and $Fe(CN)_6^{3-}$ (△, at 20 °C from ref 13). So that rate constants can be compared, relative values (1.0 at pH 7–8) are indicated.

$[(CN)_5FeCNCo(CN)_5]^{5-}$ reaction is illustrated in Figure 4. The effect can be accounted for by inclusion of the association step (6), where B represents the redox-inactive complex. As in previous

$$\text{cyt } f\text{(II)} + \text{B} \overset{K_B}{\rightleftharpoons} \text{cyt } f\text{(II)},\text{B} \quad (6)$$

studies[25] a graph of $[\text{oxidant}]/k_{obsd}$, i.e. k^{-1}, against [B] is linear for a given oxidant (Figure 4). Assuming that the adduct in (6) is redox inactive, then eq 3, 4, and 6 give (7). With the inclusion

$$k_{obsd}/[\text{oxidant}] = Kk_{et}/(1 + K_B[\text{B}] + K[\text{oxidant}]) \quad (7)$$

of K[oxidant], K_B = 530 ± 60 M^{-1}. Redox-inactive $[Mo(CN)_8]^{4-}$ gives a similar effect (Table VI)[24] with K_B = 490 ± 40 M^{-1}.

$[Co(dipic)_2]^-$ as Oxidant. For the range of oxidant concentrations investigated, a linear dependence of k_{obsd} on $[Co(dipic)_2]^-$ was observed, from which it could be concluded that K < 120 M^{-1} at 25 °C. Second-order rate constants, k, give a mild dependence on pH (Table VII),[24] which is illustrated alongside that for $[Fe(CN)_6]^{3-}$ in Figure 5. From the temperature dependence of k_{obsd} at pH 7.0 (Table VIII),[24] activation parameters for k are $\Delta H^{\ddagger}$ = 2.3 ± 0.2 kcal mol^{-1} and $\Delta S^{\ddagger}$ = −28.1 ± 0.6 cal K^{-1} mol^{-1}.

$[Co(bpy)_2(O_2CMe)_2]^+$ as Oxidant. First-order rate constants k_{obsd} (Table IX)[24] give a linear dependence on oxidant concentrations (K < 50 M^{-1} at 25 °C). Activation parameters for k at pH 7.5 are $\Delta H^{\ddagger}$ = 14.2 ± 0.8 kcal mol^{-1} and $\Delta S^{\ddagger}$ = −5.4 ± 2.8 cal K^{-1} mol^{-1}.

(24) See paragraph at end of paper regarding supplementary material.

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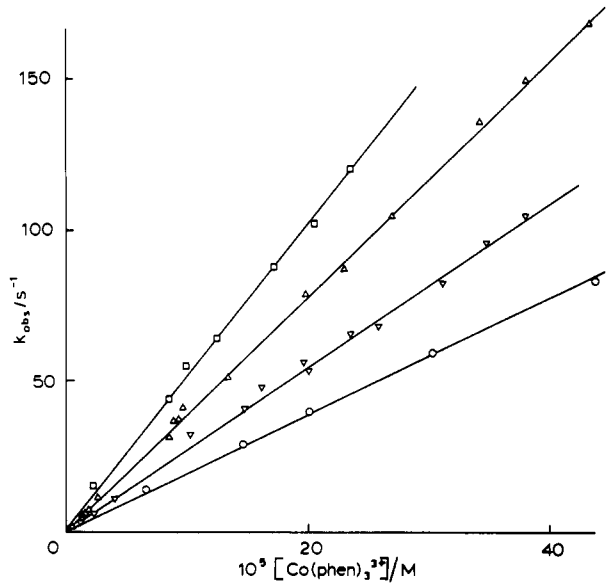

Figure 6. Dependence of k_{obsd} on oxidant concentration for the $[Co(phen)_3]^{3+}$ oxidation of cytochrome *f*(II) at pH 7.5 (Tris/HCl), $I = 0.10$ M (NaCl), at temperatures 15 °C (○), 20 °C (▽), 25 °C (△), and 30 °C (□).

$[Co(phen)_3]^{3+}$ as Oxidant. First-order rate constants k_{obsd} (Table X)[24] give a linear dependence on $[Co(phen)_3^{3+}]$ (Figure 6) from which it is concluded $K < 200$ M^{-1} at 25 °C. The rate constants determined are at the limit of the stopped-flow range, and it was not possible to further increase the concentration of oxidant. Activation parameters for k at pH 7.5 are $\Delta H^{\ddagger} = 10.9 \pm 0.2$ kcal mol^{-1} and $\Delta S^{\ddagger} = 3.5 \pm 0.7$ cal K^{-1} mol^{-1}. The variation of k with pH (Table XI)[24] mirrors that observed for $[Fe(CN)_6]^{3-}$ (Figure 5). From a fit of rate constants at pH 4.5–7.0 using a procedure previously indicated,[27] a cyt *f*(II) acid dissociation pK_a of 5.12 ± 0.12 is obtained. Likewise, for rate constants at pH ≥ 8 a pK_a of 9.7 ± 0.4 is indicated.

No competitive inhibition was observed at pH 7.5 with the redox-inactive complexes $[Pt(NH_3)_6]^{4+}$ (2.0×10^{-4} M), $[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$ (9.0×10^{-4} M), and $[Cr(en)_3]^{3+}$ (3.0×10^{-3} M).

Discussion

Cytochrome *f* used in these studies was isolated from cabbage leaves, *B. oleracea*. A brief investigation of the reaction with $[Fe(CN)_6]^{3-}$ as oxidant has shown that rate constants are very similar to those obtained for cytochrome *f* from *B. komatsuna*.[13] Since phosphate can associate with proteins, e.g. cytochrome *c*,[26] Tris/HCl buffer was preferred for the pH range 7–8.9. It was demonstrated that with phosphate as buffer (10^{-2} M) the rate constant is within 15% of the value in Tris/HCl.

A range of oxidants of varying charge (E° values as shown) $[(CN)_5FeCNCo(CN)_5]^{5-}$ (0.45 V),[27] $[Fe(CN)_6]^{3-}$ (0.41 V), $[Co(dipic_2)]^-$ (0.75 V), $[Co(bpy)_2(O_2CMe)_2]^+$ (E^0 not known), and $[Co(phen)_3]^{3+}$ (0.37 V) were used in this study,[28] all of which are suitable oxidants for cytochrome *f*(II) (0.37 V)[10] providing the inorganic oxidant is in large (≥10-fold) excess. Rate constants with $[Co(edta)]^-$ (0.38 V) as oxidant (1.0×10^{-4} M) were not sufficiently rapid for stopped-flow studies (little or no reaction within 200 s at 25 °C). Activation parameters obtained for the different complexes, and applying to the pH 6.5–8.0 region (within which rate constants are invariant), are listed in Table XII. A steady progression in $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ values with charge is noted.

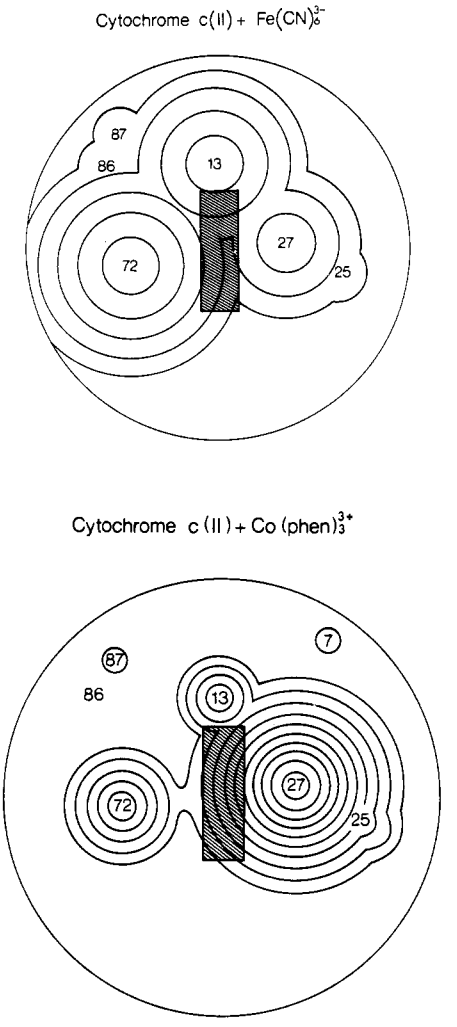

Figure 7. Reactivity contour maps for the oxidation of cytochrome *c*(II) with (top) $[Fe(CN)_6]^{3-}$ and (bottom) $[Co(phen)_3]^{3+}$, planar projections on front hemisphere of protein. The rectangular area represents the exposed heme edge, and the numbers refer to positions of α-carbon atoms of lysines that were in turn modified.[30]

Table XII. Summary of Second-Order Rate Constants (25 °C) and Activation Parameters for the Oxidation of Cytochrome *f*(II) (pH 7.5, $I = 0.10$ M (NaCl))

oxidant	k, M^{-1} s^{-1}	$\Delta H^{\ddagger}$, kcal mol^{-1}	$\Delta S^{\ddagger}$, cal K^{-1} mol^{-1}
$[(CN)_5FeCNCo(CN)_5]^{5-}$	4.5×10^5	−3.2[a]	−42.9[b]
$[Fe(CN)_6]^{3-}$	1.7×10^5 [c,d]	−0.9[c]	−38[c]
$[Co(dipic)_2]^-$	9.4×10^4	2.3	−28.1
$[Co(bpy)_2(O_2CMe)_2]^+$	16.8	14.2	−5.4
$[Co(phen)_3]^{3+}$	3.5×10^5	10.9	3.5

[a] $\Delta H^0 = -8.5$ kcal mol^{-1}; $\Delta H_{et}^{\ddagger} = 5.3$ kcal mol^{-1}. [b] $\Delta S^0 = -12.0$ cal K^{-1} mol^{-1}; $\Delta S_{et}^{\ddagger} = -30.9$ cal K^{-1} mol^{-1}. [c] Cytochrome *f* from *B. komatsuna*;[13] rate constant at 20 °C. [d] Rate constant 1.75×10^{-5} M^{-1} s^{-1} at 25 °C with cytochrome *f* from *B. oleracea*.

In the case of the 5– oxidant, parameters for the association step (K) prior to electron transfer (k_{et}) have been evaluated. For this interpretation, $k = Kk_{et}$, and the temperature dependences of K and k_{et} clearly indicate that the negative overall $\Delta H^{\ddagger}$ (−3.3 kcal mol^{-1}) is made of a negative thermodynamic ΔH^0 for K (−8.5) and positive activation energy for k_{et} (5.3). For the first time, the effect of pH and I on K and k_{et} has been investigated. An increase in ionic strength from 0.025 to 0.20 M has the effect of increasing k_{et} for electron transfer within the adduct (eq 3) from 62 to 180 s^{-1}. This is a small effect most likely resulting from changes in ionic solvation that in an extreme case might result

(27) Chapman, S. K.; Sanemasa, I.; Watson, A. D.; Sykes, A. G. *J. Chem. Soc., Dalton Trans.* **1983**, 1949.
(28) See: Chapman, S. K.; Sanemasa, I.; Sykes, A. G. *J. Chem. Soc., Dalton Trans.* **1983**, 2549 and reference therein. The value 0.75 V for the $[Co(dipic)_2]^{-,2-}$ couple has recently been reported by: Williams, N. H.; Yandell, J. K. *Aust. J. Chem.* **1983**, *36*, 2377.

in some shift in the binding site of the inorganic redox partner. The effect of ionic strength on K is much bigger, with a 30-fold decrease for an increase in ionic strength from 0.025 to 0.20 M, consistent with a process in which charge is influential. The trend observed suggests that the binding site on cytochrome *f* is positively charged. This is at first surprising since the protein has a p*I* of 5.5 and at pH 7.5 has an overall negative charge. However, from amino acid sequence information[11] the highly charged and extensive main body of cytochrome *f* containing the heme and some 250 residues has only a small overall negative charge estimated to be ~1−. There are a substantial number of positively charged residues (~30),[9,10] some of which are in pairs that may come together in the tertiary structure to give a positive patch. Dickerson and Timkovich[29] have pointed out that a feature of known three-dimensional structures of cytochrome *c* is a ring of positive charges around an exposed heme edge. It is proposed that the binding site for the 5− oxidant on cytochrome *f* has these features. It is now clear[11] that cytochrome *f* is no longer to be regarded as an extensively hydrophobic membrane protein.

The question as to whether other oxidants use the same binding site as the 5− complex is now considered. It has been possible, with lysine-modified derivatives of cytochrome *c*, to demonstrate that $[Fe(CN)_6]^{3-}$ and $[Co(phen)_3]^{3+}$ both react at the solvent-accessible edge of the heme prosthetic group.[30] With $[Fe(CN)_6]^{3-}$ the reaction is more strongly influenced by modification at Lys72 a residue to the left of the exposed heme edge, whereas $[Co(phen)_3]^{3+}$ is more influenced by modification at Lys27 to the right of the heme. In the present study, the effects of pH provide strong support for binding sites in the same locality. With $[Co(phen)_3]^{3+}$ as oxidant (Figure 5), rate constants k (M^{-1} s^{-1}) vary with pH in such a way as to mirror closely effects observed with $[Fe(CN)_6]^{3-}$.[13] Effects with both oxidants as well as for $[Co(dipic)_2]^-$ (Figure 5) and the 5− complex at pH 5.0, 7.5, and 9.4 (Table V) are also in the direction expected if the charge on H^+ is a prime controlling influence. Protonation at or near to the binding site is implicated, the pK_a's of 5.1 and 9.7 suggesting acid dissociation processes at two different types of residue. A simple straightforward dependence on charge is not always observed when there is protonation, a particularly good example being with plastocyanin, PCu^I, when a proton-induced geometry charge at the active site results in a decrease in reactivity of the protein with both positively and negatively charged inorganic oxidants.[27]

The demonstration that the 5− complex associates strongly with cytochrome *f* suggested the use of redox-inactive complexes of high negative charge in competitive inhibition, i.e. blocking, experiments. The complexes $[Zr(C_2O_4)_4]^{4-}$ and $[Mo(CN)_8]^{4-}$ give blocking effects, K_B = 530 and 490 M^{-1}, respectively, at 25 °C, pH 7.5, I = 0.10 M (NaCl). At an ionic strength 0.20 M, which it has been necessary to use in related studies on the reaction of cyt *f*(II) with PCu^{II} (to slow down rates so that stopped-flow studies are possible), both effects are smaller and we had difficulty in obtaining precise data for K_B.

It has been demonstrated previously that charge effects are important in the interaction of inorganic complexes with metalloproteins.[31] The two correlations in Figures 8 and 9 lend support to this belief. The first involves activation parameters for k (Figure 8). Results obtained by this group only are illustrated,[32-37] an advantage being that conditions of ionic strength

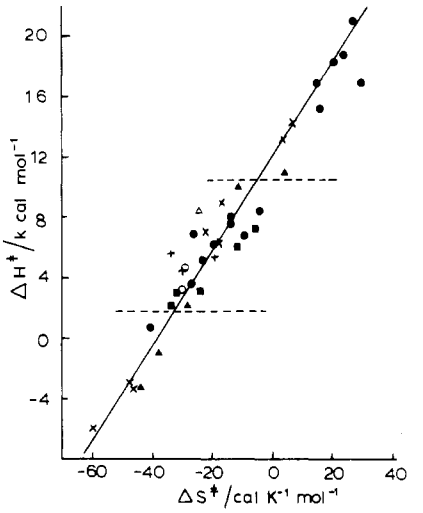

Figure 8. Correlation of activation parameters for the second-order rate constant k for electron-transfer reactions of metalloproteins with inorganic complexes. Reactions: Fe/S proteins, ●, ref 32; plastocyanin and azurin, ×, ref 33; stellacyanin, the only positively charged protein, +, ref 34; cytochrome *f*, ▲, this work; cytochrome b_5, △, ref 35. Two inner-sphere reactions of the [2 Fe–2 S] protein are included (○, ref 36). Points above the upper broken line are all with complexes of charge 3+ or 5+, and points below the lower broken line are all with complexes of charge 3− or 5−. Four points (■, ref 37) are for metalloprotein–metalloprotein reactions. All studies are at I = 0.10 M (NaCl) and pH at or close to pH 7.5.

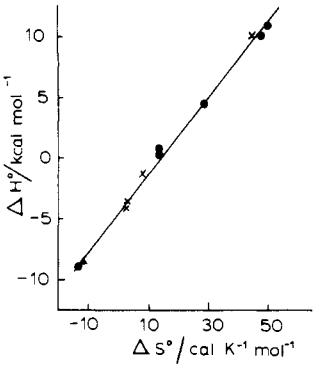

Figure 9. Correlation of thermodynamic parameters for association of inorganic complexes with metalloproteins; symbols and references as for Figure 7. Information indicated has been obtained from studies that display limiting kinetic behavior.

(0.10 M) and buffers are standardized. Activation parameters for reactions of cytochrome *c* (a second positively charged protein) do not appear in Figure 8, although values listed by Sutin[38] do in fact correlate well. Features to note in Figure 8 are that the correlation is not according to protein charge as such, E^0 values, size of rate constants ((13.9–5.6) × 10^6 M^{-1} s^{-1}), or whether the mechanism is outer or inner sphere. Nor does it appear to depend on whether the protein is the oxidant or reductant (small thermodynamic driving forces apply in many of the reactions). The charge of the inorganic reactant does appear to be important however, since points above the upper broken line are all for 3+/5+ complexes and points below the lower broken line are all for 3−/5− complexes. Moreover, the point for the reaction of the [2 Fe–2 S] protein with $[Co(acac)_3]$ (the only point for a neutral

(29) Dickerson, R. E.; Timkovich, R. In "The Enzymes"; 3rd ed.; Boyer, P., Ed.; Academic Press: New York, 1975; Vol. XIA, p 397.
(30) Butler, J.; Chapman, S. K.; Davies, D. M.; Sykes, A. G.; Speck, S. H.; Osheroff, N.; Margoliash, E. *J. Biol. Chem.* **1983**, *258*, 6400.
(31) Chapman, S. K.; Sinclair-Day, J. D.; Sykes, A. G.; Tam, S.-C.; Williams, R. J. P. *J. Chem. Soc., Chem. Commun.* **1983**, 1152.
(32) (a) Armstrong, F. A.; Henderson, R. A.; Sykes, A. G. *J. Am. Chem. Soc.* **1979**, *101*, 6912; **1980**, *102*, 6545. (b) Adzamli, I. K.; Davies, D. M.; Stanley, C. S.; Sykes, A. G. *J. Am. Chem. Soc.* **1981**, *103*, 5543. (c) Adzamli, I. K.; Petrou, A.; Sykes, A. G.; Rao, K. K.; Hall, D. O. *Biochem. J.* **1983**, *211*, 219.
(33) (a) Segal, M. G.; Sykes, A. G. *J. Am. Chem. Soc.* **1978**, *100*, 4385. *Am. Chem. Soc.* **1978**, *100*, 4385. For $[Fe(CN)_6]^{3-,4-}$ and $[Ru(NH_3)_5py]^{2+}$, see more recent studies.[27,28] (b) Lappin, A. G.; Segal, M. G.; Weatherburn, D. C.; Henderson, R. A.; Sykes, A. G. *J. Am. Chem. Soc.* **1979**, *101*, 2302.
(34) Sisley, M. J.; Segal, M. G.; Stanley, C. S.; Adzamli, I. K.; Sykes, A. G. *J. Am. Chem. Soc.* **1983**, *105*, 225.
(35) Chapman, S. K.; Davies, D. M.; Vuik, C. P. J.; Sykes, A. G. *J. Am. Chem. Soc.* **1984**, *106*, 2692.
(36) Adzamli, I. K.; Henderson, R. A.; Sinclair-Day, J. D.; Sykes, A. G. *Inorg. Chem.* **1984**, *23*, 3069.
(37) Augustin, M. A.; Chapman, S. K.; Davies, D. M.; Watson, A. D.; Sykes, A. G. *J. Inorg. Biochem.* **1984**, *20*, 281.
(38) Sutin, N. *Adv. Chem. Ser.* **1977**, *No. 162*, 156.

complex)[32] lies central at $\Delta H^{\ddagger} = 6.3$ kcal mol^{-1} and $\Delta S^{\ddagger} = -19.6$ cal K^{-1} mol^{-1}. Four points are included for metalloprotein–metalloprotein reactions. Sutin, from a similar inspection of a more limited set of data for cytochrome *c* reactions, has suggested that metalloprotein–metalloprotein reactions are associated with more positive $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ values.[38] This point does not appear to be confirmed from the more extensive comparison now possible. Implications of the correlation (and the line drawn) are that $\Delta G^{\ddagger} \sim 12$ kcal mol^{-1}, and the isokinetic temperature is 40 °C.

Since the rate constant k may be regarded as composite ($=Kk_{et}$) whether or not limiting kinetics are observed, it can be presumed that $\Delta H°$ and $\Delta S°$ are a part of the correlation in Figure 8. A graph of all known $\Delta H°$ and $\Delta S°$ values (Figure 9) gives an impressive correlation that is according to charge and consistent with $\Delta H°$ and $\Delta S°$ making major contribution to the separation of points in Figure 8. All the protein reactants in Figure 9 are negatively charged, with complexes 3+ (upper seven points) and 3– (lower points) and the triangle indicating parameters for the association of the 5– complex with cytochrome *f*. We also note that $\Delta H^{\ddagger}$ values for the reactions of plastocyanin, PCu^{I}, and azurin, ACu^{I}, with $[Fe(CN)_6]^{3-}$ and the 5– complex are negative and that the $\Delta H°$ component is likely to be negative therefore for these reactions.[27] It is of further interest that three of the points in Figure 9 are for the $[Co(4,7\text{-DPSphen})_3]^{3-}$ complex (4,7-DPSphen = 4,7-bis(phenylsulfonato)phenanthroline), which has a large size and unusual charge distribution. A correlation for $\Delta H_{et}^{\ddagger}$ and $\Delta S_{et}^{\ddagger}$, although giving a similar trend, shows much more scatter.

The correlation in Figure 9 implies a common $\Delta G°$ of –4.6 kcal mol^{-1} at a temperature close to 50 °C. On a cautionary note, we should add that although no $\Delta H°$ and $\Delta S°$ parameters are at present available for 4+ and 5+ complexes, it is unlikely that these will correlate in a similar fashion. Thus, K values for 4+ and 5+ complexes[31] of ~20 000 M^{-1} at 25 °C are unlikely to decrease to around 1000 M^{-1} at 50 °C to conform to the correlation.

Registry No. $[(CN)_5FeCNCo(CN)_5]^{5-}$, 53535-93-6; $[Fe(CN)_6]^{3-}$, 13408-62-3; $[Co(dipic)_2]^{-}$, 71605-21-5; $[Co(bpy)_2(O_2CMe)_2]^{+}$, 16986-92-8; $[Co(phen)_3]^{3+}$, 16788-34-4; $[Zr(C_2O_4)_4]^{4-}$, 21392-82-5; $[Mo(CN)_8]^{4-}$, 17923-49-8; cytochrome *f*, 9035-46-5.

Supplementary Material Available: Listings of rate constants, Tables I–IV and VI–XI (12 pages). Ordering information is given on any current masthead page.

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Selective Transport of Gaseous CO through Liquid Membranes Using an Iron(II) Macrocyclic Complex

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The equilibrium constant and rate constants for the reversible 1:1 complexation reaction

$$Fe^{II}(TIM)(C_6H_5CN)_2^{2+} + CO \rightleftharpoons^{K} Fe^{II}(TIM)(C_6H_5CN)(CO)^{2+} + C_6H_5CN$$

have been measured in benzonitrile. At 25 °C, $K_{eq} = K/[C_6H_5CN] = k_1'/k_{-3} = 420$ M^{-1}, $k_1' = 0.14$ M^{-1} s^{-1}, and $k_{-3} = 3.3 \times 10^{-4}$ s^{-1}. In CO-saturated solutions, the Fe(II) complex can be oxidized electrochemically by a $C_rE_rE_rC_i$ mechanism, which allows the diffusion coefficients of the complex and CO adduct to be determined. The reversible complexation reaction of this Fe(II) complex with carbon monoxide affords facilitated transport of CO across benzonitrile liquid membranes. For a membrane with a thickness of 0.072 cm, the transport rate for CO is increased by 14% over the purely diffusional rate. Since the Fe(II) complex does not bind N_2, O_2, CO_2, or H_2, the facilitated transport will be selective for CO in a variety of gaseous matrices. Selectivity is demonstrated for CO/O_2 gas mixtures. The rate constants for CO complexation and the diffusion coefficients for the Fe(II) complexes can be used as input parameters for a mathematical model that predicts the magnitude of the facilitated transport. Furthermore, the model and experimental work indicate that low solubility of the Fe(II) complex limits the magnitude of the facilitated transport in this case, as opposed to the thermodynamics or kinetics of the complexation reaction. The experimental and mathematical procedures described herein can be applied to any 1:1 complexation reaction between a soluble, nonvolatile carrier and a dissolved gas molecule to predict the magnitude of the facilitated transport across liquid membranes.

Introduction

Carbon monoxide is a key raw material in the synthetic routes for a variety of major chemical products including methanol, formaldehyde, acetic acid, isocyanates, aldehydes, formic acid, pesticides, and herbicides. An abundant and relatively inexpensive source of CO would greatly enhance further growth of CO as a raw material for chemical industry. Such growth could result in the CO-based chemical industry approaching the importance of the ethylene-based chemical industry.[1] It is estimated that 1.3×10^{11} kg of CO are released annually from industrial processes in the U.S. as gaseous effluents.[2] These gases can be used as fuel if the concentration of CO is high enough. Much of the dilute CO, however, is flared or discharged with minimum treatment. This unused CO is estimated to exceed 1.8×10^{10} kg (1.5×10^{17} J) per year. Separation and recovery of CO, even a fraction of the total available waste CO, would result in significant improvements via natural-gas displacement.

There are also small-scale requirements for separation and/or detection of CO that are important. Purification of life support system gases in self-contained environments is obviously needed to eliminate the toxic effects of CO on humans. Furthermore, underground mining and workplaces with combustion devices require CO detection and removal.

Rising energy and operating costs have underscored the need for energy-efficient and selective separation processes for gaseous mixtures. A variety of promising separation methods involve reversible chemical complexation, wherein a chemical carrier binds reversibly with the molecule to be separated (permeate).[3-5] One

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‡ National Bureau of Standards Center for Chemical Engineering.

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