were stored at 4 °C overnight to precipitate KCl and filtered, the solvents were evaporated under vacuum, and the solutions were filtered again, washed with ether, and dried with anhydrous $MgSO_4$.

The properties of the optically active alcohols prepared from the enzymatically produced esters by using the aforedescribed procedures are listed in Table II.

Formation of Epoxides from 9 and 10. The procedure used was a modification of the method of Blau et al.[17] and was identical with that described in (iii) of the preceding section except that 6.3 g of **9** and 8.2 g of **10** were used instead of their butyric esters.

In the case of **9**, (*S*)-(−)-**9**[31] (see Table II) was converted to 2.4 g of (*S*)-(−)-propylene oxide (43% overall yield, 93% purity by GC) with $[\alpha]^{25}_D$ −4.9° (*c* 1, chloroform) which corresponds to ee 68%[34] (lit.[35] $[\alpha]^{25}_D$ −7.2° (*c* 1, chloroform)). The (*R*)-(+)-1-chloro-2-propyl butyrate[31] enzymatically produced (see Table II) was converted to 2.7 g of (*R*)-(+)-propylene oxide (50% overall yield, 94% purity by GC) with $[\alpha]^{25}_D$ +4.8° (*c* 1, chloroform), which corresponds to ee 67%[34] (lit.[35] $[\alpha]_D{}^{25}$ +7.2° (*c* 1, chloroform)).

In the case of **10**, (*S*)-(+)-**10**[31] (see Table II) was converted to 3.6 g of (*S*)-(+)-epichlorohydrin (51% overall yield, 95% purity by GC) with $[\alpha]^{25}_D$ +23.5° (*c* 1.2, MeOH)). The (*R*)-(−)-2,3-dichloro-1-propyl butyrates[31] enzymatically produced (see Table II) was converted to 3.0 g of (*R*)-(−)-epichlorohydrin (43% overall yield, 95% by GC) with $[\alpha]^{25}_D$ −23.0° (*c* 1.5, MeOH), which corresponds to ee 67%[34] (lit.[22] $[\alpha]^{25}_D$ −34.3° (*c* 1.5, MeOH)).

(31) The absolute configurations of optically active **9** and **10** (and their butyric esters) are not available as such. Therefore we determined the absolute configurations of the enzymatically produced **9**, **10**, and their esters on the basis of those of the epoxides derived from the alcohols, using the Cahn-Prelog-Ingold rule.

(32) Mori, K.; Sasaki, M.; Tamada, S.; Suguro, T.; Masuda, S. *Heterocycles* **1978**, *10*, 111-115.

(33) Mori, K.; Sasaki, M.; Tamada, S.; Suguro, T.; Masuda, S. *Tetrahedron* **1979**, *35*, 1601-1605.

(34) The most likely reason for a relatively low ee obtained is temperature-induced racemization of optically active **9** and **10** (and their butyric esters). In agreement with this hypothesis, when the enzymatically produced **9** was distilled at a lower vacuum (20 mmHg) and higher temperature (38 °C) only 10% ee was obtained for the resultant propylene oxide.

(35) See ref 30, p 997.

Kinetic Studies on the Oxidation of Calf Liver Cytochrome b_5 with Inorganic Complexes

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Abstract: The complexes $Co(edta)^-$, $Co(NH_3)_6^{3+}$, $Pt(NH_3)_6^{4+}$, and $(NH_3)_5CoNH_2Co(NH_3)_5^{5+}$ have been used as oxidants for cytochrome b_5(II) at 25 °C, pH 7.4, I = 0.10 M (NaCl). With $Co(edta)^-$ a simple rate law first order in both reactants (k = 14.1 M^{-1} s^{-1}) is observed. The effects of pH (5.4-7.9) and temperature ($\Delta H^{\ddagger}$ = 8.6 kcal mol^{-1}, $\Delta S^{\ddagger}$ = −24.4 cal K^{-1} mol^{-1}) were studied. With the other oxidants a less than first-order dependence on oxidant (≥10-fold excess) is observed, consistent with association (K) prior to electron transfer (k_{et}). Values obtained are for $Co(NH_3)_6^{3+}$ (600 M^{-1}, 0.075 s^{-1}), $Pt(NH_3)_6^{4+}$ (14 800 M^{-1}, 0.080 s^{-1}), and $(NH_3)_5CoNH_2Co(NH_3)_5^{5+}$ (16 600 M^{-1}, 3.8 s^{-1}). Competitive inhibition is observed with the positively charged oxidants on addition of redox-inactive $Cr(en)_3^{3+}$ (K_{Cr} = 309 M^{-1}). With $Co(edta)^-$, however, an increase in rate constants is observed on addition of $Cr(en)_3^{3+}$. These results can be accounted for in terms of reaction by all four oxidants at a specific functional site on the protein, which includes the exposed heme edge, and are strongly influenced by acidic residues in this region. The magnitude of K values suggests an effective charge of 3−/4− at this binding site.

Cytochrome b_5 is a membrane-bound monoheme protein having a wide range of redox functions.[1] It can be detached from its membrane by detergent to give a protein of ca. 135 residues, which aggregates in solution. Much work has been carried out on the 93-residue (heme containing) soluble protein which is obtained by proteolysis.[2] This includes X-ray diffraction studies on the protein with Fe in the oxidized cytochrome b_5(III) (1.5-Å resolution) and reduced cytochrome b_5(II) (2.8 Å) states.[3] A preferred isolation procedure is that used in the present work involving trypsin digest, which gives an 84-residue protein. To obtain this the 50 C-terminal residues, which contain a high percentage of hydrophobic residues and attaches the protein to the membrane (it is also responsible for the aggregation), and 2 N-terminal residues are split off, leaving the 84-residue water-soluble heme peptide fragment, which is very resistant to further attack by trypsin. It has been demonstrated that trypsin digest of the 93-residue protein removes 7 C-terminal and 2 N-terminal residues to give the same 84-residue protein.[2] The heme group is situated in a hydrophobic pocket. There are no peptide to porphyrin linkages, but two histidines (39 and 63 in the 93-membered protein) are axial ligands to the Fe. There are no cysteines or methionines. The Fe is low spin in both the II (colored orange) and III (red) states. A redox potential of 20 mV has been reported for the protein in solution and when bound to microsomes.[4a] Recent studies indicate a value of 5 mV for the 84-residue protein at 25 °C, pH 7-8, I = 0.10 M (phosphate).[4b] From the amino acid composition[5] the charge on cytochrome b_5(II) and b_5(III) is estimated as 10− and 9−, respectively. A feature of the structure is the ring of acidic residues around the exposed heme edge.[1] The possible significance of two propionate groups attached to the porphyrin, both of which are exposed to solvent in hemoglobin but one of which is almost totally buried in cytochrome b_5, has been considered.[6]

A preliminary account of this work has appeared.[7] The reduction of cytochrome b_5(III) with $Fe^{II}(edta)^{2-}$ has been reported previously.[8] There have been no previous kinetic studies on the

(1) Mathews, F. S.; Czerwinski, E. W.; Argos, P. "The Porphyrins"; Dolphin, D., Ed.; 1979; Vol. VII, pp 107-147.

(2) Strittmatter, P.; Axols, J. *J. Biol. Chem.* **1966**, *241*, 4787.

(3) Argos, P.; Mathews, F. S. *J. Biol. Chem.* **1975**, *250*, 474 and references therein.

(4) (a) Weber, H.; Weis, W.; Staudinger, H. *Hoppe-Seyler's Z. Physiol. Chem.*, **1971**, *352*, 109. (b) Reid, L. S.; Taniguchi, V. T.; Gray, H. B.; Mauk, A. G. *J. Am. Chem. Soc.* **1982**, *104*, 7576.

(5) (a) Tsugita, A.; Kabayashi M.; Tani, S.; Kyo, S.; Rashid, M. A.; Yoshida, Y.; Kajchara, T.; Hagihara, B. *Proc. Natl. Acad. Sci. U.S.A.* **1970**, *67*, 442. (b) Ozols, J.; Strittmatter, P. *J. Biol. Chem.* **1969**, *244*, 6617.

(6) Reference 1, p 140.

(7) Chapman, S. K.; Davies, D. M.; Vuik, C. P. J.; Sykes, A. G.; *J. Chem. Soc., Chem. Commun.* **1983**, 868.

(8) Reid, L. S.; Mauk, A. G. *J. Am. Chem. Soc.* **1982**, *104*, 841.

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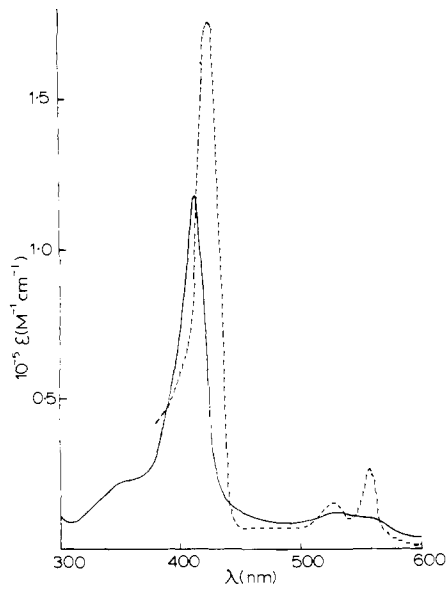

Figure 1. UV–visible spectra of calf liver cytochrome b_5 with reduced (---) and oxidized (—) forms.

oxidation of cytochrome b_5(II) with inorganic complexes.

Experimental Section

Protein. Cytochrome b_5 was isolated from the liver of yearling steer as the whole protein in its oxidized form (ca. 135 amino acid residues) by a literature procedure.[9] Minor modifications to the procedure were as follows. After precipitation of the microsomes with $CaCl_2$ the suspension was not centrifuged immediately but left to settle overnight at 4 °C. Some 80% of the clear red-brown supernatant was siphoned off and the rest centrifuged at 8500 rpm for 7–10 min to give a tight pellet of yellow-biege microsomes. The second DEAE-cellulose column fractions were not assayed for purity by gel electrophoresis. Instead their absorbance at 413 nm was measured and fractions having absorbance >25% that of the most concentrated fraction were retained. After the final Sephadex G-75 gel filtration column fractions with absorbance ratio $A_{413}/A_{280} \geq 1.8$ were retained.

The cytochrome b_5 whole protein was converted to the 84-residue peptide by tryptic digest at 4 °C. Fractions of protein as specified above were dialyzed three times against 10× the volume of 0.020 M phosphate buffer at pH 7.6. Calcium chloride solution (8 mL of 2×10^{-5} M per 100 mL of protein solution) was added, followed by trypsin (Sigma Type X1, DPCC treated, 3.5 mg per 100 mL of dialyzed protein solution) in buffer solution (20 mL), and the mixture was stirred for 44 h. The solution was then loaded onto a DE-52 column (2 cm by 35 cm long) preequilibrated with 0.020 M phosphate buffer at pH 7.6. After the solution was washed with 400 mL of the same buffer, the cytochrome b_5 was eluted with 1200 mL of buffered KCl, the concentration being increased from 0 to 0.35 M. The 84-residue cytochrome b_5 was collected in 3–5-mL fractions, and those fractions with $A_{413}/A_{280} \geq 5.7$ were retained. Solutions of protein (spectrum in Figure 1) were standardized assuming $\epsilon = 1.17 \times 10^5$ M^{-1} cm^{-1} at 413 nm.

Complexes. These were prepared by literature procedures and characterized by their UV–visible absorbance spectra in aqueous solution, λ_{max}/nm (ϵ/(M^{-1} cm^{-1})): sodium ethylenediaminetetracetatocobaltate(III), $Na[Co(edta)]\cdot 4H_2O$, 381 (216), 535 (320);[10] [hexaamminecobalt(III)] chloride, $[Co(NH_3)_6]Cl_3$, 339 (46.4), 473 (57.1);[11] [hexaammineplatinum(IV)] chloride, $[Pt(NH_3)_6]Cl_4\cdot H_2O$, 260 (129);[12] ($\mu$-amido)bis[pentaamminecobalt(III)] bromide, $[(NH_3)_5CoNH_2Co(NH_3)_5]Br_5$, 360 (705), 505 (420);[13] tris[(ethylenediamine)chromium(III)] chloride, $[Cr(en)_3]Cl_3\cdot 3H_2O$, 351 (63), 457 (73).[14]

Buffers. Most studies used 2.0×10^{-2} M phosphate buffer, $H_2PO_4^-/HPO_4^{2-}$. For the reactions with $[Pt(NH_3)_6]^{4+}$ phosphate gave a precipitate and extensive association with $[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$ was assumed. Instead a 0.010 M solution of HCl adjusted to the required pH with tris(hydroxymethyl)aminomethane (Sigma, Trizma), here referred to as Tris, was used. Check runs with $[Co(edta)]^-$ as oxidant indicated satisfactory agreement of rate constants with both buffers. The pH of solutions was checked by means of a Radiometer (PHM 62) instrument fitted with a combined electrode.

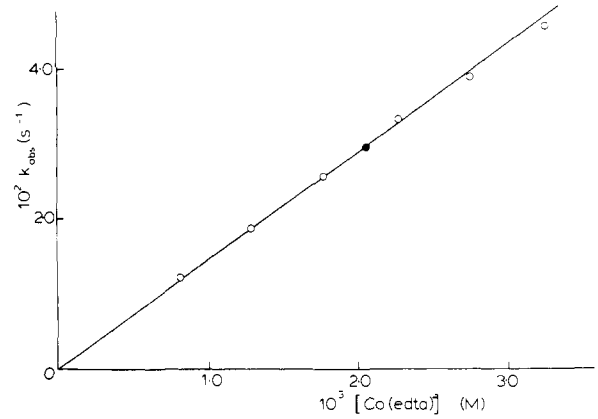

Figure 2. Linear dependence of rate constants, k_{obsd} (25 °C), for the $[Co(edta)]^-$ oxidation of cytochrome b_5(II) with oxidant concentration at pH 7.9 with Tris/HCl (○) and phosphate (●) buffers, I = 0.10 M (NaCl).

Kinetics. Solutions were made up to ionic strength I = 0.10 M with sodium chloride. The inorganic complex was in at least 10-fold excess. Reactions were monitored at the cytochrome b_5(II) absorbance peak of 555 nm (Figure 1) on Durrum-Gibson and Dionex D-110 stopped-flow spectrophotometers. Extra precautions to exclude traces of O_2 included a flow of N_2 from behind the pistons of the drive syringes. One of the reservoir syringes was replaced by a Teflon fitting with 20-cm length of 0.7-mm internal diameter Teflon tubing to enable direct loading of the drive syringe with solutions of inorganic complex. All solutions were degassed for at least 15 min (per 25 mL of solution) prior to transfer to the stopped flow. In the case of cytochrome b_5(III) solutions gentle bubbling (for 15 min) was followed by a period in which N_2 was passed over solutions. The drive syringes were rinsed three times with degassed buffer prior to use. Cytochrome b_5(III) was reduced with sodium dithionite after loading the reservoir syringe with ca. 8 mL of 6–10 mM solution. Buffer in the drive syringe was flushed out and replaced by ca. 2 mL of cytochrome b_5(III) from the reservoir syringe. The latter was then disconnected and inverted, and a small crystal of dithionite dropped through the syringe nozzle into the remainder of the cytochrome b_5(III). After reconnecting, the protein was mixed rapidly between the drive and reservoir syringes 20–30 times to bring about reduction. Excess dithionite was readily detected in the $[Co(edta)]^-$ and $[Co(NH_3)_6]^{3+}$ studies because it gave rise to nonexponential absorbance changes in subsequent kinetic runs. An excess of dithionite caused the most problems in the $[Co(edta)]^-$ study because the latter absorbs appreciably at 555 nm and is reduced by dithionite on the same time scale as cytochrome b_5(III). Excess dithionite could be removed by addition of more cytochrome b_5(III). The absorbance changes were either photographed from the oscilloscope or stored digitally by using a Datalab DL901 transient recorder which was interfaced to a Commodore PET 2001-16K desk-top computer. A simple program permitted a display of ln ($A_t - A_\infty$) against time plots and first-order rate constant k_{obsd}. Providing all the above precautions were observed and excess dithionite and traces of oxygen were avoided, first-order plots were linear to 3–4 half-lives. All absorbance changes with monomeric Co(III) oxidants were consistent with a 1:1 stoichiometry as in (1). The primary product $Co(NH_3)_6^{3+}$ formed

$$\text{cyt } b_5\text{(II)} + \text{Co(III)} \rightarrow \text{cyt } b_5\text{(III)} + \text{Co(II)} \quad (1)$$

in the reaction with $[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$ reacts relatively slowly and does not participate further in the reaction (2). With the 2-equiv

$$\text{cyt } b_5\text{(II)} + \text{Co(III)}_2 \rightarrow \text{cyt } b_5\text{(III)} + \text{Co(II)} + \text{Co(III)} \quad (2)$$

$[Pt(NH_3)_6]^{4+}$ oxidant, the Pt(III) intermediate formed in (3) is assumed

$$\text{cyt } b_5\text{(II)} + \text{Pt(IV)} \rightarrow \text{cyt } b_5\text{(III)} + \text{Pt(III)} \quad (3)$$

to be a transient with (4) rapid and not rate determining. Measured

$$\text{cyt } b_5\text{(II)} + \text{Pt(III)} \xrightarrow{\text{fast}} \text{cyt } b_5\text{(III)} + \text{Pt(II)} \quad (4)$$

first-order rate constants were halved to allow for (4), giving the values listed.

(9) Strittmatter, P.; Fleming, P.; Connors, M.; Corcoran, D.; *Methods Enzymol.* **1978**, *52*, 97.
(10) Schwarzenbach, G. *Helv. Chim. Acta* **1949**, *32*, 839.
(11) Bjerrum, J., Reynolds, J. P. *Inorg. Synth.*, **1946**, *2*, 216.
(12) Essen, L. N. *Inorg. Synth.* **1973**, *15*, 93.
(13) Davies, R.; Mori, M.; Sykes, A. G.; Weil, J. A. *Inorg. Synth.* **1970**, *12*, 212.
(14) Gillard, R. D.; Mitchell, P. R. *Inorg. Synth.* **1972**, *14*, 184.

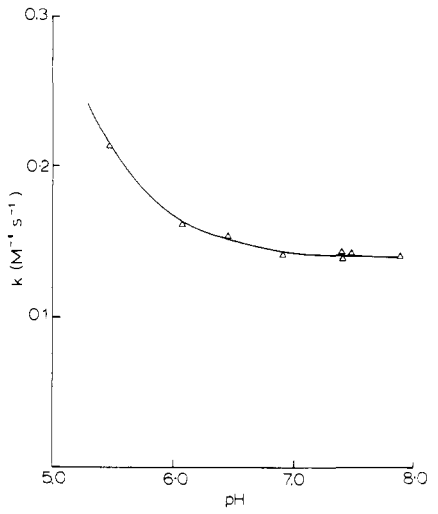

Figure 3. pH dependence (phosphate buffer) of second-order rate constants k (25 °C) for the $[Co(edta)]^-$ oxidation of cytochrome b_5(II), I = 0.10 M (NaCl).

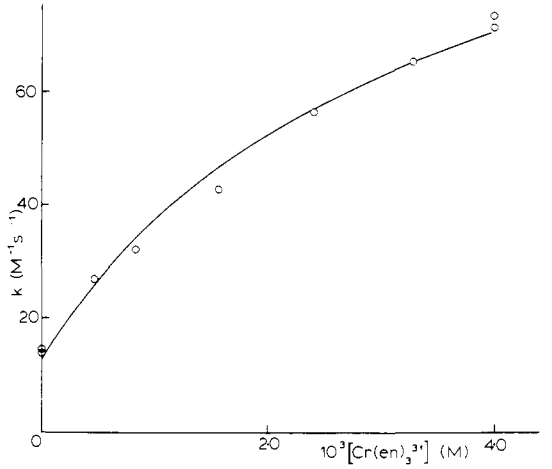

Figure 4. Effect of redox inactive $[Cr(en)_3]^{3+}$ on the second-order rate constants k (25 °C) for the $[Co(edta)]^-$ oxidation of cytochrome b_5(II) at pH 7.4 (phosphate), I = 0.10 M (NaCl).

Treatment of Data. Experimental data were fitted to eq 6, 10, 13, and 14 by using an unweighted nonlinear least-squares program.

Results

$[Co(edta)]^-$ as Oxidant. First-order rate constants k_{obsd} (Table I)[15] at pH 7.9 give a linear first-order dependence on $[Co(edta)]^-$ (Figure 2). The simple rate law (5) applies, therefore, and it

$$\text{rate} = k[\text{cyt } b_5(\text{II})][\text{Co(III)}] \quad (5)$$

can be concluded that K for association of the two reactants prior to electron transfer (see below) is <70 M^{-1}. At this pH good agreement is observed for runs in phosphate and Tris buffers. The dependence of rate constants k on pH (see Figure 3) could be fitted to (6), where k_H and k_o are rate constants for the protonated and

$$k = \frac{k_H[H^+] + k_oK_a}{K_a + [H^+]} \quad (6)$$

unprotonated protein, respectively, and K_a is the acid dissociation constant of the protonated form, to give k_H = 33.2 ± 4.0 M^{-1} s^{-1}, k_o = 14.0 ± 0.1 M^{-1} s^{-1}, and pK_a = 5.23 ± 0.14. From the temperature dependence of k at pH 7.4 (Table II),[15] the activation parameters $\Delta H^{\ddagger}$ = 8.6 ± 0.3 kcal mol^{-1} and $\Delta S^{\ddagger}$ = −24.4 ± 0.9 cal K^{-1} mol^{-1} were obtained. The reaction at pH 7.4 is accelerated by the presence of redox inactive $[Cr(en)_3]^{3+}$ (Table III).[15] Figure 4 illustrates the variation of second-order rate constants. The reaction sequence 7–9 gives (10). From a nonlinear least-squares

$$\text{cyt } b_5(\text{II}) + [Cr(en)_3]^{3+} \rightleftharpoons \text{cyt } b_5(\text{II}), [Cr(en)_3]^{3+} \quad (7)$$

$$\text{cyt } b_5(\text{II}) + [Co(edta)]^- \xrightarrow{k_o} \text{products} \quad (8)$$

$$\text{cyt } b_5(\text{II}), [Cr(en)_3]^{3+} + [Co(edta)]^- \xrightarrow{k_{Cr}} \text{products} \quad (9)$$

$$k = \frac{k_o + k_{Cr}K_{Cr}[Cr(en)_3]^{3+}}{1 + K_{Cr}[Cr(en)_3]^{3+}} \quad (10)$$

fit k_o = 14.2 ± 1.1 M^{-1} s^{-1}, k_{Cr} = 153 ± 13 M^{-1} s^{-1}, and K_{Cr} = 180 ± 25 M^{-1}. A satisfactory fit is also obtained with K_{Cr} = 309 M^{-1}, which is the value of K_{Cr} from the data with $[Co(NH_3)_6]^{3+}$ as oxidant, when k_o = 13.0 ± 1.2 M^{-1} s^{-1} and k_{Cr} = 118 ± 2 M^{-1} s^{-1}. Because the data with $[Co(NH_3)_6]^{3+}$ are believed to be the more accurate, the latter set is preferred.

$[Co(NH_3)_6]^{3+}$ as Oxidant. First-order rate constants k_{obsd} (Table IV)[15] gives a nonlinear dependence on $[Co(NH_3)_6]^{3+}$ (Figure 5). The behavior observed is consistent with the sequence 11–12, which

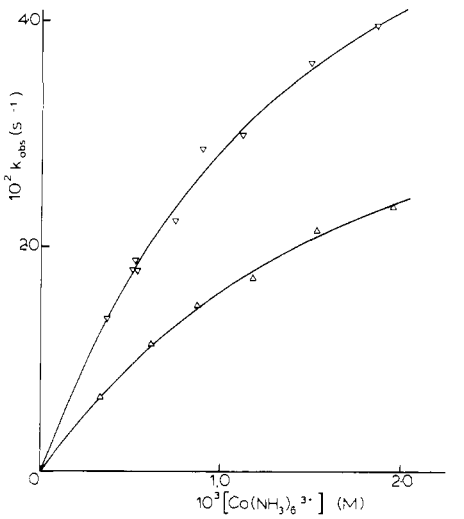

Figure 5. Variation of rate constants, k_{obsd} (25 °C), for the $[Co(NH_3)_6]^{3+}$ oxidation of cytochrome b_5(II) with oxidant concentration at pH 7.4 (▽) and 5.42 (△) (phosphate buffer), I = 0.10 M (NaCl).

$$\text{cyt } b_5(\text{II}) + [Co(NH_3)_6]^{3+} \rightleftharpoons \text{cyt } b_5(\text{II}), [Co(NH_3)_6]^{3+} \quad (11)$$

$$\text{cyt } b_5(\text{II}), [Co(NH_3)_6]^{3+} \xrightarrow{k_{et}} \text{products} \quad (12)$$

gives expression 13. From a fit of data at 25 °C, pH 7.4

$$k_{obsd} = \frac{Kk_{et}[\text{oxidant}]}{1 + K[\text{oxidant}]} \quad (13)$$

(phosphate), K = 600 ± 60 M^{-1}, and k_{et} = 0.075 ± 0.005 s^{-1}. At pH 5.42, K = 455 ± 70 M^{-1} and k_{et} = 0.051 ± 0.005 s^{-1}, and both K and k_{et} decrease as protonation of the protein takes place.

Redox-inactive $[Cr(en)_3]^{3+}$ produces a retardation of rate constant k_{obsd} (Figure 6). From a fit of data at pH 7.4 (phosphate) (Table V)[15] to (14), which is obtained by consideration

$$k_{obsd} = \frac{Kk_{et}[Co(NH_3)_6]^{3+}}{1 + K[Co(NH_3)_6]^{3+} + K_{Cr}[Cr(en)_3]^{3+}} \quad (14)$$

of (7), (11), and (12), and by use of values of K and k_{et} as above, K_{Cr} = 309 ± 14 M^{-1} at 25 °C.

$[Pt(NH_3)_6]^{4+}$ as Oxidant. From the variation of first-order rate constants k_{obsd} (Table VI)[15] with $[Pt(NH_3)_6]^{4+}$ and by carrying out a fit to (13) (Figure 7), K = 14 800 ± 1100 M^{-1} and k_{et} = 0.080 ± 0.003 s^{-1} at 25 °C, pH 7.4 (Tris/HCl). The effect of redox-inactive $[Cr(en)_3]^{3+}$ 3.2 × 10^{-3} M, the rate constant k_{obsd} decreased from 0.074 s^{-1} to 0.046 s^{-1}, consistent with competitive inhibition.

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

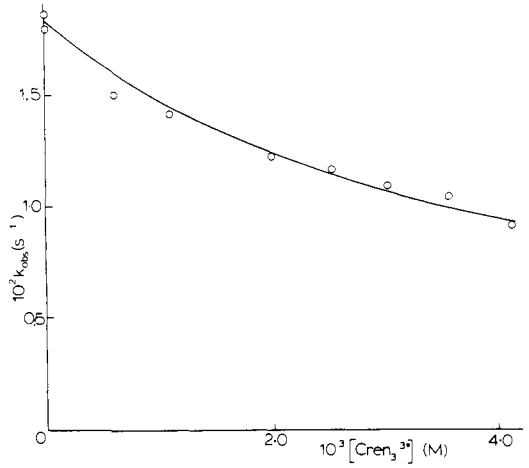

Figure 6. Effect of redox inactive $[Cr(en)_3]^{3+}$ on the rate constants, k_{obsd} (25 °C), for the $[Co(NH_3)_6]^{3+}$ (0.54×10^{-3} M) oxidation of cytochrome b_5 at pH 7.4 (phosphate), I = 0.10 M (NaCl).

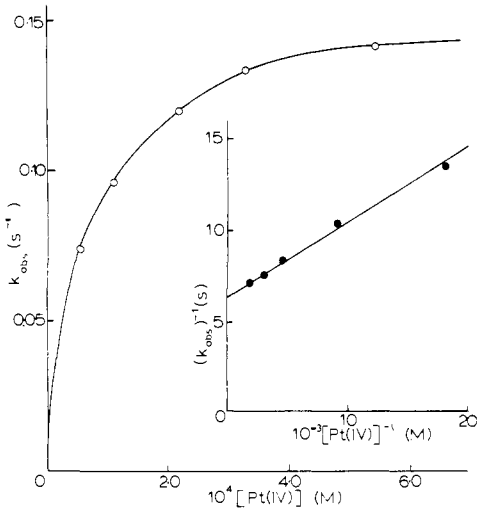

Figure 7. Variation of rate constants, k_{obsd} (25 °C), for the $[Pt(NH_3)_6]^{4+}$ oxidation of cytochrome b_5(II) with oxidant concentration and (inset) the corresponding reciprocal plot for eq 13 at pH 7.4 (phosphate), I = 0.10 M (NaCl).

$[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$ as Oxidant. A less than first-order dependence of k_{obsd} (Table VII)[15] on oxidant concentration was again observed. From the fit to an equation of the same form as (13) (Figure 8) K = 16 600 ± 1600 M^{-1}, k_{et} = 3.8 ± 0.1 s^{-1} at 25 °C, pH 7.4 (Tris/HCl). Competitive inhibition was observed for a run with $[Co^{III}_2]$ = 4.0×10^{-5} M and $[Cr(en)_3]^{3+}$ = 3.2×10^{-3} M, and the rate constant k_{obsd} decreased from 1.46 to 0.61 s^{-1}.

Discussion

Kinetic studies on the oxidation of cytochrome b_5(II) with $[Co(NH_3)_6]^{3+}$, $[Pt(NH_3)_6]^{4+}$, and $[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$ are here interpreted in terms of association (K) prior to electron transfer (k_{et}). From the magnitude of the K values (Table VIII) and by use of a simple electrostatic treatment, it can be concluded that a negative binding site of charge 3−/4− is effective.[16] We note that K for $[Pt(NH_3)_6]^{4+}$ is much larger than that for $[Co(NH_3)_6]^{3+}$ at pH 7.4 even though the 3+ conjugate-base form predominates,[17] suggesting that the effective charge density is as for the 4+ complex. Charged residues on cytochrome b_5 are known to be distributed unevenly over the protein surface.[18] Eleven of the acidic residues in the 84-residue fragment are located in the top half of the molecule where the heme is situated, whereas the basic groups tend to be located in the lower portion. A number of acidic residues are positioned around the exposed heme edge. Acid residues in the peptide sequences 37–48 and 56–60 which could give rise to a 3−/4− negative patch are Glu 37, 38, 43, 44, 48 and Glu 56, 59 and Asp 60. Of these Glu 43 and 59 are involved in salt bridge formation and therefore may not be relevant.[18] (The numbers used here are those applying to the 93-residue protein[2] the crystal structure of which has been reported; subtract 2 from all numbers to convert to the 84-residue protein.)

Studies with $Co(edta)^-$ as oxidant for cytochrome b_5 do not give limiting kinetics, and it has been concluded that $K < 70$ M^{-1}. The pH dependence (Figure 3), like that previously reported for the $Fe(edta)^{2-}$ reduction of cytochrome b_5(III), shows no tendency to level out at the lowest pH studied. From a nonlinear least-squares fit of data to eq 6 a protein pK_a of 5.20 is obtained. We have no information as to whether this effect stems from protonation at or near to the binding site (in which case it might correspond to the first of a number of possible protonations) or to a single protonation effect with modification of E^0 for the active site. The value obtained for the $Fe(EDTA)^{2-}$ reduction of cytochrome b_5(III) is 5.85.[8] Activation parameters for the cytochrome b_5(II) + $Co(edta)^-$ reaction ($\Delta H^{\ddagger}$ = 8.6 kcal mol^{-1}; $\Delta S^{\ddagger}$

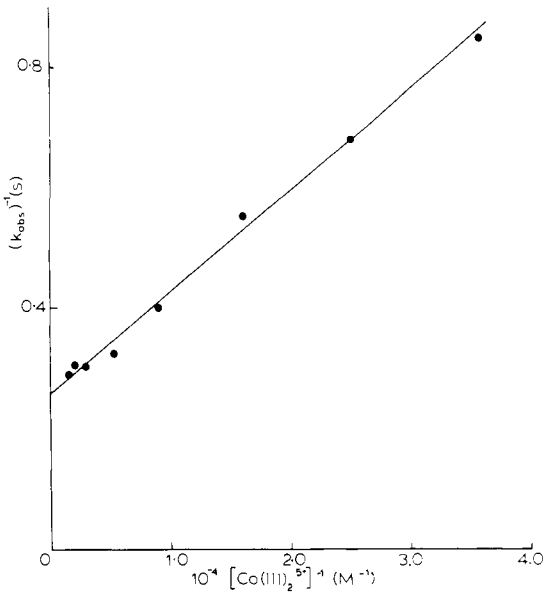

Figure 8. Reciprocal plot of rate constants (25 °C) for the $[(NH_3)_5CoNH_2CoNH_2Co(NH_3)_5]^{5+}$ oxidation of cytochrome b_5(II) illustrating the dependence on oxidant and the applicability of eq 13, I = 0.10 M (NaCl).

Table VIII. Summary of Data from Kinetic Studies on the Oxidation of Cytochrome b_5(II) with Inorganic Complexes at 25 °C, I = 0.10 M (NaCl)

complex	pH	k, M^{-1} s^{-1}	K, M^{-1}	k_{et}, s^{-1}
$[Co(edta)]^-$	7.4[a]	14.1[b]		
$[Cr(en)_3]^{3+}$ [c]	7.4[a]		309	
$[Co(NH_3)_6]^{3+}$	7.4[a]	45.0	600	0.075
	5.4[a]	23.5	460	0.051
$[Pt(NH_3)_6]^{4+}$	7.4[d]	1.18×10^3	14 800	0.080
$[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$	7.4[d]	6.2×10^4	16 600	3.8

[a] Phosphate buffer (2×10^{-2} M). [b] $\Delta H^{\ddagger}$ = 8.6 kcal mol^{-1}; $\Delta S^{\ddagger}$ = −24.4 cal K^{-1} mol^{-1}. [c] Redox inactive complex. [d] Tris/HCl buffer (10^{-2} M).

(16) Chapman, S. K.; Sinclair-Day, J. D.; Sykes, A. G.; Tam, S. C.; Williams, R. J. P. *J. Chem. Soc., Chem. Commun.* **1983**, 1152.

(17) The pK_a of $[Pt(NH_3)_6]^{4+}$ has been determined: S. K.; Chapman, Watson, A. D.; Sykes, A. G. *J. Chem. Soc., Dalton Trans.*, **1983**, 2543 (pK_a = 7.1); Johnson, R. C.; Basolo, F.; Pearson, R. G. *J. Inorg. Nucl. Chem.* **1962**, *24*, 59 (pK_a = 7.2).

(18) (a) Reference 1, p 125–126. (b) Reference 1, p 113.

(19) (a) Armstrong, F. A.; Sykes, A. G. *J. Am. Chem. Soc.* **1978**, *100*, 7710. (b) Armstrong, F. A.; Henderson, R. A.; Sykes, A. G. *Ibid.* **1979**, *101*, 6912. (c) Armstrong, F. A.; Henderson, R. A.; Ong, H. W. K.; Sykes, A. G. *Biochim. Biophys. Acta* **1982**, *681*, 161. (d) Armstrong, F. A. *Adv. Inorg. Bioinorg. Mech.* **1982**, *1*, 65–120.

= −24.4 cal K^{-1} mol^{-1}) compare with those for the $Fe(edta)^{2-}$ + cytochrome b_5(III) reaction ($\Delta H^{\ddagger}$ = 5.4 kcal mol^{-1}; $\Delta S^{\ddagger}$ = −29.2 cal K^{-1} mol^{-1}), suggesting similar influences.

Further information has been obtained from the redox-inactive complex $[Cr(en)_3]^{3+}$. Thus, with the 3+, 4+, and 5+ oxidants competitive inhibition is observed (K_{Cr} = 309 M^{-1}), and it can be concluded that either a single specific site or kinetically indistinguishable sites are involved. The accelerated effect of $[Cr(en)_3]^{3+}$ on the $[Co(edta)]^-$ reduction and fit of data suggest that $Co(edta)^-$ is able to use this same site when redox-inactive $[Cr(en)_3]^{3+}$ is present and may also use this site when $[Cr(en)_3]^{3+}$ is not present, although this is not absolutely essential. The situation is very similar to that observed previously for the oxidation of [2Fe-2S] and 2[4Fe-4S] ferredoxins by inorganic oxidants.[19]

Some concern is often expressed as to whether studies with inorganic complexes relate to natural processes involving protein-protein reactions. As a part of this work[7] it has been demonstrated that $[Cr(en)_3]^{3+}$ not only inhibits the reaction of cytochrome b_5 with the 3+, 4+, and 5+ complexes but also blocks association with cytochrome c(III), which is believed to be a natural partner for cytochrome b_5. An overlap of the site used by cytochrome c with that used by the inorganic complexes is indicated. We have no reason to believe that the reactivity here outlined will change appreciably when cytochrome b_5 is present as the 134-residue protein or when it is membrane bound, since $E°$ values remain practically unchanged for these different situations.[4]

Much current information suggests that the exposed heme edge of a cytochrome is relevant to electron transfer. Crystal structure information is now available for cytochromes b_5,[1,3] c,[20] and c_{551},[21] a particular feature being that all three differ in the immediate environment of the heme edge. That of cytochrome b_5 has already been considered and the importance of negative residues surrounding the heme edge indicated. In cytochrome c the heme is surrounded by basic residues. Although $[Fe(CN)_6]^{3-}$ appears to react preferentially at a site close to Lys 72 (which in the normal view is to the left of the heme edge) of cytochrome c(III),[22] there is no compact positive patch such as would be needed for strong association with inorganic complexes. Thus, for reduction of cytochrome c(III) by $[Fe(CN)_6]^{4-}$ it has been demonstrated that $K < 200$ M^{-1}.[23] However NMR line-broadening studies indicate an association with $[Fe(CN)_6]^{3-}$ having K = 450 M^{-1} at I = 0.18 M.[24] This is believed to refer to a 3+/4+ charged patch in the 86-91 region which is unlikely to contribute extensively to electron transfer. The positive complex $[Co(phen)_3]^{3+}$ has been shown to react at a site close to Lys 27 on the right-hand side of the heme edge; overlap with the exposed heme edge occurs from this position as well as the site used by $[Fe(CN)_6]^{3-}$. The heme edge in cytochrome c_{551} is surrounded by hydrophobic residues, and further data illustrating the effect of these residues on reactions with inorganic complexes would be of interest. Recent studies in which "fixed-site" electron transfer from Ru(II) attached to the His 33 of cytochrome c through to the Fe(III) active site has been determined[25,26] highlight the possibility of small contributions resulting from long-distance (15 Å) electron transfer without use of the exposed heme edge.

An alternative explanation of (13) is in terms of the "dead-end" mechanism.[19] As in other studies the stance we have adopted is that discussion should proceed in terms of e.g. (11) and (12) until positive evidence for this alternative is obtained. The same numerical K values apply in both mechanisms.

The strategy described with $[Cr(en)_3]^{3+}$ as an inhibitor has possible applications with enzymes such as sulfite oxidase. The latter has cytochrome b_5 and Mo active sites,[27] and such an approach could have relevance in diagnosing which of these is used as electron "in" and "out" sites in electron-transfer processes.

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Registry No. Na[Co(edta)], 89196-49-6; $[Co(NH_3)_6]Cl_3$, 10534-89-1; $[Pt(NH_3)_6]Cl_4$, 16893-12-2; $[(NH_3)_5CoNH_2Co(NH_3)_5]Br_5$, 72273-61-1; $[Cr(en)_3]Cl_3$, 21510-38-3; cytochrome b_5, 9035-39-6.

Supplementary Material Available: A listing of rate constant Tables I-VII (7 pages). Ordering information is given on any current masthead page.

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Communications to the Editor

Preparation of 2-Methylenebenzocyclobutenone by the Flash Vacuum Pyrolysis of 3-((Benzoyloxy)methyl)benzofuran[1]

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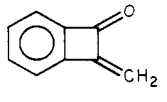
Although a few substituted methylenebenzocyclobutenones have been prepared,[2] the parent compound (**1**), which is one of the simple derivatives of cyclobutadiene, has not been reported. The flash vacuum pyrolysis (FVP) of furfuryl benzoate (**2**) is a convenient source of methylenecyclobutenone (**3**).[3] Several sub-



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