transition. Although $D < 0$ for reduced desulforedoxin, the calculated value $\Delta = 0.6$ cm^{-1} [19] is too large for observation of an $S = 2$ EPR signal. We have verified that no such signal is observed from reduced desulforedoxin.[14] The possibility remains, however, that the similarities in spin Hamiltonian parameters of the major and minor species of $[Fe(SCH_2CH_2OH)_4]^{2-}$ to those of the two proteins reflect two energetic minima in the FeS_4 ligand field rather than coincidence. Such minima could occur as a result of Jahn-Teller effects.[17,18] The differing sets of spin Hamiltonian parameters for reduced rubredoxin and reduced desulforedoxin were originally interpreted as reflecting different orbital ground states: predominantly d_{z^2} for rubredoxin and predominantly $d_{x^2-y^2}$ for desulforedoxin.[19,20] These different ground states presumably reflect different FeS_4 stereochemistries. A more recent analysis[26] assumes D_2 symmetry for the FeS_4 site and uses a common set of ligand field energies for the two reduced proteins. The differing sets of spin Hamiltonian parameters, including the opposite signs of D, can be fit reasonably well by small variations in the degree of mixing the d_{z^2} and $d_{x^2-y^2}$ orbitals. Under D_2 symmetry the variation in the mixing parameter reflects essentially a variation in the S-Fe-S angles. Both interpretations are consistent with the idea that such angular variations are responsible for the two (at least) species observed in magnetic Mössbauer spectra of $[Fe(SCH_2CH_2OH)_4]^{2-}$. Thus, the observability of $S = 2$ EPR signals from $Fe(SR)_4$ sites appears to be highly sensitive to structural parameters whose variations have relatively low energy barriers. Indeed, the crystal structures of ferrous $Fe(SR)_4$ complexes show numerous S-Fe-S angles between 96 and 125°.[7,17,18] It is also noteworthy that the most angularly constraining ligand used in this study, namely, D,L-dihydrolipoate (which can form a six-membered S,S'-bidentate chelate ring vs seven-membered for dithiothreitol), forms the ferrous $Fe(SR)_4$ complex *not* exhibiting an $S = 2$ EPR signal. The fact that the ferrous $Fe(SR)_4$ complex of the tripeptide glutathione (γ-L-glutamyl-L-cysteinylglycine) shows an $S = 2$ EPR signal means that cysteine thiolate ligation permits observation of such a signal. The results presented here show that pseudotetrahedral high-spin ferrous complexes can give rise to $S = 2$ EPR signals. However, observability of such signals from biological $Fe(Cys\text{-}S)_4$ sites is likely to be strongly dependent on the protein matrix.[27]

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Registry No. $[Fe(SCH_2CH_2OH)_4]^{2-}$, 119109-48-7; $Ba[Fe(SCH_2CH_2OH)_4]$, 119109-49-8; $[Fe(SCH_2CH_2OH)_4]^{-}$, 119109-50-1.

Supplementary Material Available: For $Ba[Fe(SCH_2CH_2OH)_4]$ tables listing atomic coordinates, bond distances and angles, and anisotropic thermal parameters (1 page); a table of F_o and F_c values (3 pages). Ordering information is given on any current masthead page.

(26) Bertrand, P.; Gayda, J.-P. *Biochim. Biophys. Acta* **1988**, *954*, 347-350.

(27) EPR signals with line shapes and g values nearly identical with those of Figures 1 and 2 have recently been observed in partially iron-loaded samples of metallothionen, a cysteine-rich, metal-scavenging protein.[28]

(28) Werth, M. T.; Johnson, M. K. *Biochemistry*, in press.

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pH Dependence of Rate Constants for Reactions of Cytochrome *c* with Inorganic Redox Partners and Mechanistic Implications

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Rate constants for the oxidation of horse cytochrome *c*(II) with $[Co(phen)_3]^{3+}$ and $[Co(terpy)_2]^{3+}$ decrease (~30%) on decreasing the pH from 8 to 5, giving a pK_a value of 6.7 (average). No corresponding decrease is observed with tuna cytochrome *c*(II) (which has His26 but no His33) or with His33 diethyl pyrocarbonate (DEPC) modified horse cytochrome *c*(II). *Candida krusei* cytochrome *c*(II) gives a pK_a of 6.9, which is likewise assigned to His33. No dependence on pH is observed with the negatively charged oxidants $[Fe(CN)_6]^{3-}$ and $[Co(dipic)_2]^{-}$ or surprisingly with $[Co(terpy)_2]^{2+}$, $[Ru(NH_3)_5py]^{2+}$, and $[Co(sep)]^{2+}$ as reductants for horse cytochrome *c*(III). Previous work using CDNP-modified horse cytochrome *c* derivatives has indicated that the positively charged redox partners $[Co(phen)_3]^{3+}$ (oxidant) and $[Co(sep)]^{2+}$ (reductant) react preferentially at site II on the front face of the molecule to the right-hand side of the solvent-accessible heme edge (the latter vertical with axially coordinated Met to the left), whereas negatively charged $[Fe(CN)_6]^{3-}$ reacts at site III to the left-hand side and across the top section of the exposed heme edge. His33 is close to site II, consistent with the effect of protonation of this residue on reactivity with the 3+ oxidants. Reduction potentials for horse cytochrome *c* determined electrochemically in the presence of a mediator show no variation with pH over the range 7.5-5. Protonation of His33, the imidazole ring of which is 12 Å from Fe, has no effect on the reduction potential therefore, and its effect on reactivity is a local effect. It is not immediately clear why there is no dependence on pH for the reaction of cytochrome *c*(III) with the three positively charged reductants. Possible contributing factors include some sort of conformational change, with a resultant shift in the site for electron transfer in the case of for example $[Co(terpy)_2]^{2+}$ as compared to $[Co(terpy)_2]^{3+}$, and effects stemming from different degrees of solvation at or near His33. His33 of horse cytochrome *c* gives virtually identical pK_a's and rates of DEPC modification for both the oxidized and reduced forms, indicating similar degrees of accessibility.

Introduction

Studies aimed at understanding the reactivity of cytochrome *c* (~104 amino acids; MW 12 400) with small inorganic redox partners are a subject of continuing interest.[1,2] Horse-heart cytochrome *c* is the main subject of the present study, where comparisons with tuna and *Candida krusei* cytochrome *c*'s have proved invaluable in assigning the histidine involvement.[3-6] Amino acid sequences for all three cytochrome *c*'s have been determined (and compared),[2] and X-ray structures of tuna[7] and rice[8] cyto-

(1) Moore, G. R.; Pettigrew, G. W. *Cytochromes c*; Springer-Verlag: New York, 1987.

(2) Moore, G. R.; Eley, C. G. S.; Williams, G. *Adv. Inorg. Bioinorg. Mech.* **1984**, *3*, 1-96.

(3) Margoliash, E.; Smith, E. L.; Kreil, G.; Tuppy, H. *Nature (London)* **1961**, *192*, 1125.

(4) Kreil, G. *Hoppe-Seyler's Z. Physiol. Chem.* **1963**, 334, 154; **1965**, *340*, 86.

(5) Narita, K.; Titani, K. *J. Biochem. (Tokyo)* **1968**, *63*, 226.

(6) Lederer, F. *Eur. J. Biochem.* **1972**, *31*, 144.

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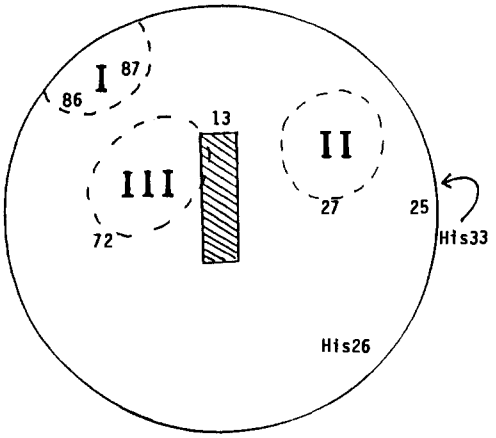


Figure 1. Approximate location of sites for reaction on front (hemispherical) face of cytochrome *c*, axial Met80 to left-hand side of exposed heme edge. Positions of relevant lysines (and histidines) are indicated.

chrome *c*'s as well as horse cytochrome *c* (the latter at lower resolution)[9] have been reported. The protein has a high positive overall charge (estimated at +8 and +9 for the Fe(II) and Fe(III) oxidation states, respectively) due to the large number of lysine residues. A large body of evidence points to the solvent-exposed heme edge of the protein as being relevant to electron transfer with a wide range of inorganic[10-15] and protein redox partners.[16-30] The lysines are distributed in a highly asymmetrical manner on the front face of the molecule around the exposed heme edge.[11,30,32]

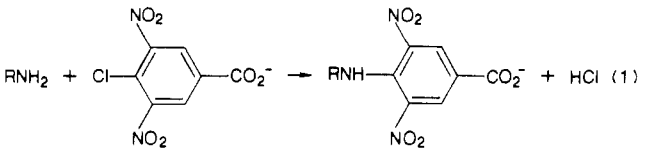
Table I. Reduction Potentials Relevant to This Work

couple	$E°$/mV[a]
cytochrome *c*(III/II)	273[b]
$[Co(phen)_3]^{3+/2+}$	370
$[Co(terpy)_2]^{3+/2+}$	270
$[Fe(CN)_6]^{3-/4-}$	410
$[Co(dipic)_2]^{-/2-}$	747
$[Co(sep)]^{3+/2+}$	−260 (−300)

[a] Source of reduction potentials ref 14 and listing in: Sykes, A. G. *Chem. Soc. Rev.* **1985**, *14*, 283. [b] This work and ref 45.

Studies on lysine single-modified derivatives, as well as extensive NMR investigations, have proved informative in defining more precisely specific regions adjacent to the exposed heme edge as redox partner binding sites.[1,2]

Three sites have been defined that are referred to in the simplified representation of the molecule (Figure 1) with the heme edge vertical and the axial methionine to the left-hand side. Residues in the horse cytochrome *c* amino acid sequence are referred to. Site I is positioned at the top left of the front face of the molecule and includes residues 65 and 89. Lysines 5, 86, and 87 may well be involved as binding groups. This site is preferred by the physiological protein reaction partners.[11] Site II lies to the right of the heme and includes amino acids Val11, Ala15, and Thr19. Lysine residues 7, 25, and 27 also help define this region, as indicated in studies with lysine-modified derivatives. Site III lies to the left of the heme edge, and resonances of Ile81, Phe82, and Ala83 are affected by binding at this site. Lysine residues 13, 72, and 86 further define this locality. From NMR studies it is known that sites I and III have a high affinity for anionic reactants such as $[Fe(CN)_6]^{3-}$[33] and that kinetically site III appears to be most relevant.[10,12] With $[Co(phen)_3]^{3+}$ as oxidant however, there is evidence implicating site II.[10,12] This may be associated with the presence of the acidic residue Glu21 with, in the case of the horse CDNP-modified Lys27 (or 25) derivatives, a second negatively charged group in close proximity (eq 1). There are fewer positively charged lysines in this region.



To date, the effect of pH in the range 5–8 on the reactivity of small inorganic reactants with cytochrome *c* has been almost completely ignored.[34] Our initial experiments with $[Co(phen)_3]^{3+}$ as oxidant for cytochrome *c*(II) indicated a well-defined pH effect, corresponding to an acid dissociation pK_a of ~6.7. The latter can be assigned to His33, a residue adjacent to site II. In view of recent work on the Ru modification of His33,[35] and its possible relevance in electron-transfer processes, it was decided to further explore this effect.

Experimental Section

Protein. Horse-heart, tuna, and *Candida krusei* cytochrome *c*'s were obtained from Sigma Chemicals and purified by the procedure described.[36] Fractions with UV/visible absorbance (*A*) ratios A_{550}(re-

(7) Takano, T.; Dickerson, R. E. *J. Mol. Biol.* **1981**, *153*, 79.
(8) Ochi, H.; Hata, Y.; Tanaka, N.; Kakudo, M.; Sakurai, T.; Aihara, S.; Morita, Y. *J. Mol. Biol.* **1983**, *166*, 407.
(9) Dickerson, R. E.; Timkovich, R. In *The Enzymes*, 3rd ed.; Boyer, P., Ed.; Academic Press: New York, 1975; Vol. II, pp 397–547.
(10) Butler, J.; Chapman, S. K.; Davies, D. M.; Sykes, A. G.; Speck, S. H.; Osheroff, N; Margoliash, E. *J. Biol. Chem.* **1983**, *258*, 6400.
(11) Koppenol, W. H.; Margoliash, E. *J. Biol. Chem.* **1982**, *257*, 4426.
(12) Butler, J.; Davies, D. M.; Sykes, A. G.; Koppenol, W. H.; Osheroff, N.; Margoliash, E. *J. Am. Chem. Soc.* **1981**, *103*, 469.
(13) Ahmed, A. J.; Millett, F. *J. Biol. Chem.* **1981**, *256*, 1611.
(14) Armstrong, G. D.; Chambers, J. A.; Sykes, A. G. *J. Chem. Soc., Dalton Trans.* **1986**, 755.
(15) Butler, J.; Koppenol, W. H.; Margoliash, E. *J. Biol. Chem.* **1982**, *257*, 10747.
(16) Staudenmayer, N.; Smith, M. B.; Spies, F. K.; Millett, F. *Biochemistry* **1976**, *15*, 3198.
(17) Staudenmayer, N.; Ng, S.; Smith, M. B.; Millett, F. *Biochemistry* **1977**, *16*, 600.
(18) Smith, H. T.; Staudenmayer, N.; Millett, F. *Biochemistry* **1977**, *16*, 4975.
(19) Ng, S.; Smith, M. B.; Smith, H. T.; Millett, F. *Biochemistry* **1977**, *16*, 4975.
(20) Ferguson-Miller, S.; Brautigan, D. L.; Margoliash, E. *J. Biol. Chem.* **1978**, *253*, 149.
(21) Kang, C. H.; Brautigan, D. L.; Osheroff, N.; Margoliash, E. *J. Biol. Chem.* **1978**, *253*, 6502.
(22) Ahmed, A. J.; Smith, H. T.; Smith, M. B.; Millett, F. S. *Biochemistry* **1978**, *17*, 2479.
(23) Speck, S. H.; Ferguson-Miller, S.; Osheroff, N.; Margoliash, E. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 155.
(24) Stonehuerner, J.; Williams, J. B.; Millett, F. *Biochemistry* **1979**, *18*, 5422.
(25) Webb, M.; Stonehuerner, J.; Millett, F. *Biochim. Biophys. Acta* **1980**, *593*, 290.
(26) Smith, M. B.; Stonehuerner, J.; Ahmed, A. J.; Staudenmayer, N.; Millett, F. *Biochim. Biophys. Acta* **1980**, *592*, 303.
(27) Konig, B. W.; Osheroff, N.; Wilms, J.; Muijsers, A. O.; Dekker, H. L.; Margoliash, E. *FEBS Lett.* **1980**, *111*, 395.
(28) Speck, S. H.; Koppenol, W. H.; Dethmers, J. K.; Osheroff, N.; Margoliash, E.; Rajagopalan, R. V. *J. Biol. Chem.* **1981**, *256*, 7394.
(29) Augustin, M. A.; Chapman, S. K.; Davies, D. M.; Sykes, A. G.; Speck, S. H.; Margoliash, E. *J. Biol. Chem.* **1983**, *258*, 6405.
(30) Armstrong, G. D.; Chapman, S. K.; Sisley, M. J.; Sykes, A. G.; Aitken, A.; Osheroff, N. O.; Margoliash, E. *Biochemistry* **1986**, *25*, 6947–6951.
(31) Dickerson, R. E.; Takano, T.; Eisenberg, D.; Kallai, O. B.; Samson, L.; Cooper, A.; Margoliash, E. *J. Biol. Chem.* **1971**, *246*, 1511.
(32) Salemme, F. R.; Kraut, J.; Kamen, M. D. *J. Biol. Chem.* **1973**, *248*, 7701.
(33) Eley, C. G. S.; Moore, G. R.; Williams, G.; Williams, R. J. P. *Eur. J. Biochem.* **1982**, *124*, 295.
(34) (a) McArdle, J. V.; Gray, H. B.; Creutz, C.; Sutin, N. *J. Am. Chem. Soc.* **1974**, *96*, 5737. (b) Cummins, D.; Gray, H. B. *J. Am. Chem. Soc.* **1977**, *99*, 5158.
(35) (a) Winkler, J. R.; Nocera, D. G.; Yocum, K. M.; Bordignon, E.; Gray, H. B. *J. Am. Chem. Soc.* **1982**, *104*, 5798. (b) Yocum, K. M.; Shelton, J. B.; Shelton, J. R.; Schroeder, W. A.; Worosila, G.; Isied, S. S.; Bordignon, E.; Gray, H. B. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 7052. (c) Nocera, D. G.; Winkler, J. R.; Yocum, K. M.; Bordignon, E.; Gray, H. B. *J. Am. Chem. Soc.* **1984**, *106*, 5145. (d) Isied, S. S.; Kuehn, C.; Worosila, G. *J. Am. Chem. Soc.* **1984**, *106*, 1722. (e) Bechtold, R.; Gardineer, M. B.; Kazmi, A.; van Hemelryck, B.; Isied, S. S. *J. Phys. Chem.* **1986**, *90*, 3800.
(36) Brautigan, D. L.; Ferguson-Miller, S.; Margoliash, E. *Methods Enzymol.* **1978**, *53*, 128.

Table II. Second-Order Rate Constants (k(25 °C)) for the $[Co(phen)_3]^{3+}$ ((0.9–2.2) × 10^{-4} M) Oxidation of Horse, Tuna, and *C. krusei* and DEPC-Modified Horse and *C. krusei* cytochrome *c*(II)'s (~1.5 × 10^{-6} M), I = 0.10 M (NaCl)

(a) Horse Cytochrome *c*(II)														
pH	3.71	4.37	5.10	5.10	5.75	6.44	6.64	7.00	7.36	7.66	7.96	8.31	8.31	8.31
$10^{-3}d/M^{-1}$ s^{-1}	1.60	1.21	1.12	1.18	1.15	1.34	1.32	1.50	1.54	1.72	1.75	1.68	1.77	1.77
(b) Tuna Cytochrome *c*(II)														
pH	3.95	4.25	4.25	4.57	5.10	5.91	6.80	7.51	8.31	8.31	8.94			
$10^{-3}k/M^{-1}$ s^{-1}	2.07	1.75	1.90	1.60	1.63	1.57	1.52	1.57	1.56	1.64	1.55			
(c) *C. krusei* Cytochrome *c*(II)														
pH	3.79	4.76	5.21	5.21	5.44	5.91	5.91	6.40	6.76	7.15	7.35	7.62	7.90	8.45
$10^{-3}k/M^{-1}$ s^{-1}	3.19	2.44	2.43	2.34	2.37	2.38	2.46	2.49	2.58	2.68	2.70	2.73	2.80	2.85
(d) DEPC-Modified Horse Cytochrome *c*(II)														
pH	4.66	5.51	5.51	6.27	7.04	7.50								
$10^{-3}k/M^{-1}$ s^{-1}	1.72	1.79	1.65	1.76	1.85	1.78								
(e) DEPC-Modified *C. krusei* Cytochrome *c*(II)														
pH	5.26	5.11	6.40	7.05	8.15									
$10^{-3}k/M^{-1}$ s^{-1}	2.71	2.81	2.88	2.86	2.85									

duced) to A_{280}(oxidized) >1.15 in the case of horse and *C. krusei* cytochrome *c*'s and >1.00 for tuna cytochrome *c* were used in kinetic experiments. Concentrations were determined from the absorbance at 416 nm (1.29 × 10^5 M^{-1} cm^{-1}) for reduced protein.

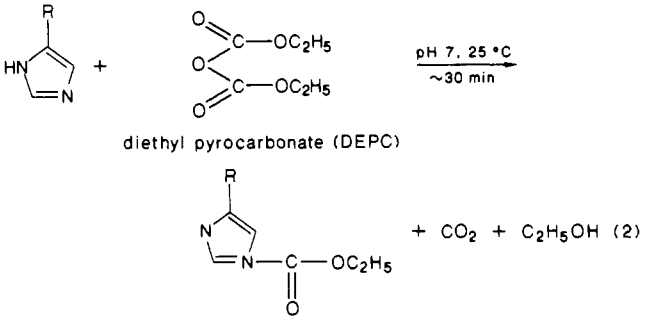
Complexes. Complexes were prepared and recrystallized to the following known spectral λ/nm (ε/M^{-1} cm^{-1}): tris(1,10-phenanthroline)-cobalt(III) chloride, $[Co(phen)_3]Cl_3 \cdot 7H_2O$, 330 (4660), 350 (3620);[37] bis(2,2′:6′,2″-terpyridine)cobalt(III) perchlorate, $[Co(terpy)_2](ClO_4)_3 \cdot H_2O$, 278 (4.6 × 10^4), 338 (2.1 × 10^4);[38] ammonium bis(dipicolinato)-cobaltate(III), $(NH_4)[Co(dipic)_2]$, 510 (680);[39] bis(2,2′:6′,2″-terpyridine)cobalt(II) perchlorate, $[Co(terpy)_2](ClO_4)_2 \cdot H_2O$, 445 (1578), 505 (1387);[40] pentaammine(pyridine)ruthenium(II) perchlorate, $[Ru(NH_3)_5py](ClO_4)_2$, 407 (7.8 × 10^3);[41] [Co(sep)]$Cl_3 \cdot H_2O$, where sep denotes the sepulchrate cage ligand 1,3,6,8,10,13,16,19-octaazabicyclo-[6.6.6]eicosane, 340 (116), 472 (109).[42]

For $[Co(sep)]^{3+}$ it was necessary to convert the chloride to the sulphate salt prior to electrochemical reduction. The procedure used was to dissolve [Co(sep)]$Cl_3 \cdot H_2O$ (1 g) in 2.5 mL of concentrated H_2SO_4. After 1–2 h the dark orange solution was poured into methanol (50 mL). The resultant pale orange precipitate was filtered off and recrystallized from 0.1 M H_2SO_4 (10–15 mL; 75 °C). The product was analyzed for $[Co(sep)]^{3+}$ by using the spectrum as above and by titration with NaOH. It generally corresponded to the formula $[Co(sep)](SO_4)(HSO_4) \cdot 2H_2O$. Solutions of $[Co(sep)]^{2+}$ (10 mM) in 20 mM Tris buffer were prepared with sufficient Na_2SO_4 to give I = 0.10 M on completion of the reduction. Run solutions were prepared by appropriate dilution with air-free buffer and standardized by titration against $[Fe(CN)_6]^{3-}$ ($\Delta\epsilon$ = 1010 M^{-1} cm^{-1} at 420 nm) or by precise dilution of a spectrophotometrically standardized stock solution. Amounts of free sulfate introduced were (2–8) × 10^{-4} M. Potassium ferricyanide, $K_3[Fe(CN)_6]$ (BDH, AnalaR), was used without further purification, 420 nm (1010 M^{-1} cm^{-1}). Relevant reduction potentials are given in Table I. All the complexes gave satisfactory stability over the range pH 5–8.

Buffers. The following buffers (from Sigma Chemicals) were used: tris(hydroxymethyl)aminoethane (Tris), pK_a = 8.08 (25 °C), pH range 7.1–9.1 adjusted by addition of 0.1 M HCl; *N*-(2-hydroxyethyl)-piperazine-*N*′-ethanesulfonic acid (HEPES), pK_a = 7.5, pH range 6.8–8.2 adjusted with NaOH; *N*-morpholineethanesulfonic acid (MES), pK_a = 6.2, pH range 5.2–6.8 adjusted with NaOH; acetic acid/acetate as required (BDH, AnalaR), pK_a = 4.76, pH range 4.0–5.6. Reaction solutions were ~20 mM in buffer and were made up to ionic strength I = 0.10 M (NaCl).

Kinetics. Reactions were studied using a Dionex D-110 stopped-flow spectrophotometer. Changes in the cytochrome *c* absorbance at 416 nm ($\Delta\epsilon$ = 4.0 × 10^4 M^{-1} cm^{-1}; reduced form more strongly absorbing) or in the case of $[Fe(CN)_6]^{3-}$ and $[Ru(NH_3)_5py]^{2+}$ at 550 nm ($\Delta\epsilon$ = 1.9 × 10^4) were monitored. The pH-jump method was used, in which the protein solution at pH 7.2 maintained by minimal buffer (2 mM Tris-HCl) was mixed with a solution of complex in 40 mM buffer. Both solutions were at I = 0.10 M (NaCl). The pH of the solutions after mixing was checked on a Radiometer PHM62 pH meter equipped with a Russell CWR 320 combination electrode. Variations in this procedure had no effect. All reactions were studied with the inorganic complex in large >10-fold excess.

DEPC Modification. The reaction is shown in (2). Solutions of 0.2 M diethyl pyrocarbonate in absolute ethanol were prepared from the solid



and stored under N_2 at 4 °C. Solutions were standardized against imidazole in phosphate buffer, pH 6.5–70, $\Delta\epsilon$ = 3200 M^{-1} cm^{-1} at 240 nm.[43]

The modification procedure was as follows. An equal volume of 3 × 10^{-5} M cytochrome *c* (typically 1.5 mL) in 0.1 M phosphate buffer at pH 7.0 was placed in each of two optical cells. To one cell was added sufficient DEPC to give a 15–30-fold excess of DEPC. To the other was added an equal volume of ethanol. Changes of UV/vis spectra at 230–300 nm were recorded at 2 min intervals, until there was no further increase in absorbance (30 min). From the increase in absorbance at 238 nm ($\Delta\epsilon$ = 2750 M^{-1} cm^{-1}),[44] the number of histidines undergoing modification was determined. If the modified protein was to be used for kinetics, excess reagent was removed and the buffer exchanged by diafiltration to give 2 mM Tris-HCl buffer, I = 0.10M (NaCl).

Cytochrome *c* Reduction Potential. The procedure as described in ref 45, with gold and platinum electrodes and (2-(3-pyridylmethylene)-hydrazino)carbothioamide (alternative name pyridine-3-carboxaldehyde thiosemicarbazone, from Lancaster Chemicals) as mediator, was used. Values of 274 mV (pH 5.1) and 273 mV (pH 7.1) were in agreement with the literature value of 274 mV (pH 7.5).[45] At pH 8.3 the reduction potential is 267 mV (see also ref 2, p 12), the decrease being attributed to the incidence of axial methionine dissociation.

Treatment of Data. The stopped-flow spectrophotometer was equipped with a logarithmic amplifier and the output stored digitally on a Datalab DL901 transient recorder. Reaction traces were displayed on an oscilloscope. A Commodore PET 2001-16K computer was interfaced to the transient recorder, and it enabled plots of absorbance (A) changes ln ($A_\infty - A_t$) to be displayed. Some of the later work was on an IBM PC/AT-X computer with data handling and fitting programs supplied by On-Line

(37) (a) Pryzystas, T. J.; Sutin, N. *J. Am. Chem. Soc.* **1973**, *95*, 5545. (b) Segal, M. G.; Sykes, A. G. *J. Am. Chem. Soc.* **1978**, *100*, 4585.
(38) Baker, B. R.; Basolo, F.; Neumann, H. M. *J. Phys. Chem.* **1959**, *63*, 371.
(39) Mauk, A. G.; Coyle, C. L.; Bordignon, E.; Gray, H. B. *J. Am. Chem. Soc.* **1979**, *101*, 5054.
(40) (a) Stanbury, D. M.; Ledicky, L. A. *J. Am. Chem. Soc.* **1984**, *106*, 2847. (b) Hogg, R.; Wilkins, R. G. *J. Chem. Soc.* **1962**, 341.
(41) Ford, P.; Rudd, De F. F.; Gaunder, R.; Taube, H. *J. Am. Chem. Soc.* **1968**, *90*, 1187.
(42) Creaser, I. I.; Geue, R. J.; Harrowfield, J. MacB.; Herlt, A. J.; Sargeson, A. M.; Snow, M. R.; Springborg, J. *J. Am. Chem. Soc.* **1982**, *104*, 6016.
(43) Miles, E. W. *Methods Enzymol.* **1977**, *47*, 431.
(44) Jackman, M. P.; Lim, M.-C.; Osvath, P.; de Silva, D. G. A. H.; Sykes, A. G. *Inorg. Chim. Acta* **1988**, *153*, 205.
(45) Hill, H. A. O.; Page, D. A.; Walton, N. J.; Whitford, D. *J. Electroanal. Chem. Interfacial Electrochem.* **1985**, *187*, 315.

Table III. Second-Order Rate Constants (k(25 °C)) for the $[Co(terpy)_2]^{3+}$ ((1.4–6.4) × 10^{-4}M) Oxidation of Native and DEPC-Modified Horse Cytochrome c(II)'s (~1.5 × 10^{-4} M), I = 0.10 M (NaCl)

(a) Native Horse Cytochrome c(II)												
pH	4.36	4.95	5.65	5.65	5.96	5.96	6.54	6.94	6.94	7.40	7.40	8.30	8.81
$10^{-2}k/M^{-1}$ s^{-1}	6.1	6.1	6.4	6.8	6.8	7.1	7.3	8.6	8.6	8.6	9.0	9.2	9.1
(b) DEPC-Modified Horse Cytochrome c(III)												
pH	5.04	5.04	5.52	6.10	6.50	7.01	7.75						
$10^{-2}k/M^{-1}$ s^{-1}	9.1	8.6	8.9	8.7	9.2	9.2	9.5						

Table IV. Second-Order Rate Constants (k(25 °C)) for the $[Fe(CN)_6]^{3-}$ (~3.2 × 10^{-5} M) and $[Co(dipic)_2]^-$ ((1.09–6.1) × 10^{-5} M) Oxidations of Horse Cytochrome c(II) (~1.5 × 10^{-6} M), I = 0.10 M (NaCl)

(a) Oxidation with $[Fe(CN)_6]^{3-}$													
pH	3.25	3.61	3.61	4.50	5.35	6.00	6.61	7.44	7.44	8.20	8.20	8.98	
$10^{-6}k/M^{-1}$ s^{-1}	23.4	15.6	19.6	10.0	9.5	8.8	9.2	9.2	8.8	9.0	7.5	8.4	
(b) Oxidation with $[Co(dipic)_2]^-$													
pH	3.46	3.50	3.65	3.65	4.03	4.03	4.50	5.51	5.51	6.10	6.63	7.33	8.04	8.54
$10^{-4}k/M^{-1}$ s^{-1}	6.46	2.84	2.65	2.85	1.46	1.65	1.33	0.96	1.04	1.02	1.06	1.04	1.00	0.98

Table V. Second-Order Rate Constants (k(25 °C)) for the $[Co(terpy)_2]^{2+}$, $[Ru(NH_3)_5py]^{2+}$, and $[Co(sep)]^{2+}$ Reductions of Horse Cytochrome c(III) (~1.5 × 10^{-6} M), I = 0.10 M (NaCl)

(a) Reduction with $[Co(terpy)_2]^{2+}$ ((1.0–2.0) × 10^{-4} M)													
pH	3.54	3.72	4.00	4.00	4.15	4.50	5.04	5.65	6.48	6.99	7.36	7.98	8.35
$10^{-3}k/M^{-1}$ s^{-1}	3.87	2.53	1.35	1.52	1.15	1.04	0.91	1.08	0.99	0.96	1.02	0.98	1.12
(b) Reduction with $[Ru(NH_3)_5py]^{2+}$ ((1.2–3.2) × 10^{-4} M)													
pH	5.61	6.24	6.24	6.50	7.03	7.45	8.08						
$10^{-3}k/M^{-1}$ s^{-1}	3.9	3.9	4.0	4.1	4.1	4.1	4.3						
(c) Reduction with $[Co(sep)]^{2+}$ ((1.1–4.0) × 10^{-4} M)													
pH	5.50	5.56	5.56	5.73	6.00	6.38	6.50	7.05	7.50	7.75	7.75		
$10^{-5}k/M^{-1}$ s^{-1}	3.52	3.32	3.68	3.36	3.33	3.37	3.62	3.57	3.18	3.10	3.53		

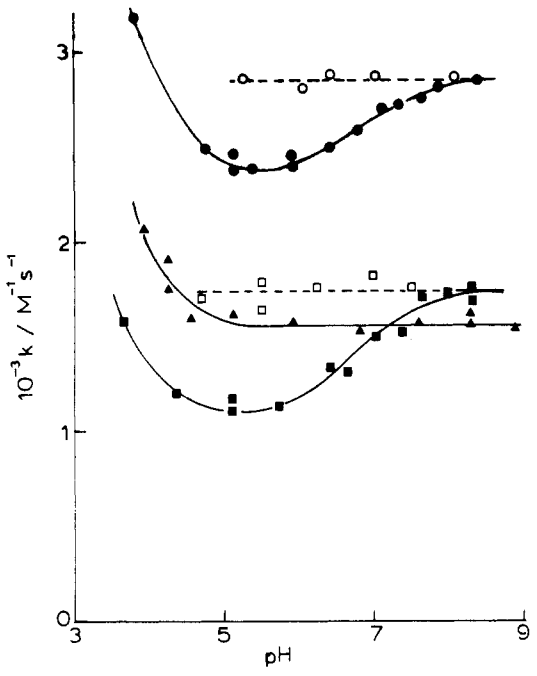


Figure 2. Variation of second-order rate constants, k(25 °C), for the $[Co(phen)_3]^{3+}$ oxidation of native (●) and DEPC-modified (○) horse cytochrome c's, native tuna cytochrome c (▲), and native (■) and DEPC-modified (□) *C. krusei* cytochrome c(II)'s (I = 0.10 M (NaCl)).

Instrument Systems (Jefferson, GA).

Results

For the 1:1 electron-transfer reactions studied, second-order rate constants, k(25 °C), are defined by the rate law (3). Results

$$\text{rate} = k[\text{cytochrome}][\text{complex}] \quad (3)$$

are listed in Tables I–V. For the conditions investigated no trend in k values was observed on varying the concentration of complex (reactant in excess). Rate constant dependences on pH were observed for the reaction of $[Co(phen)_3]^{3+}$ with horse and *C. krusei* cytochrome c(II), but not with tuna (Figure 2). The behavior observed corresponds to the reaction sequence (4)–(6), from which

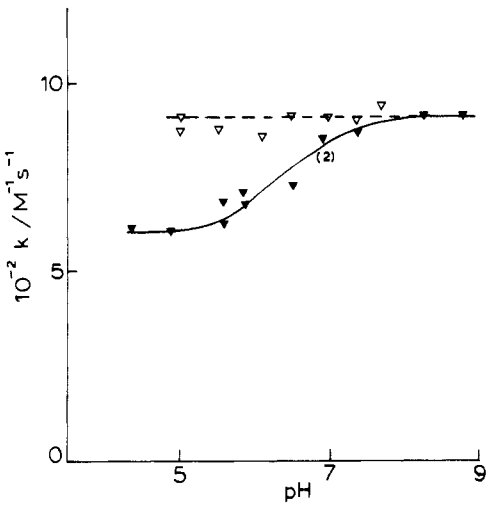


Figure 3. Variation of second-order rate constants, k(25 °C), for the $[Co(terpy)_2]^{3+}$ oxidation of native (▼) and DEPC-modified (▽) horse cytochrome c(II)'s (I = 0.10 M (NaCl)).

$$\text{cyt } c(\text{II})\ H^+ \overset{K_a}{\rightleftharpoons} \text{cyt } c(\text{II}) + H^+ \quad (4)$$

$$\text{cyt } c(\text{II}) + \text{oxidant} \xrightarrow{k_1} \text{products} \quad (5)$$

$$\text{cyt } c(\text{II})\ H^+ + \text{oxidant} \xrightarrow{k_2} \text{products} \quad (6)$$

the expression (7) can be derived. For $[Co(phen)_3]^{3+}$ with horse

$$k = \frac{k_1K_a + k_2[H^+]}{K_a + [H^+]} \quad (7)$$

cytochrome c(II), a fit to (7) using all k values at pH >5.5 gave pK_a = 6.9 ± 0.2, and with *C. krusei* cytochrome c(II), pK_a = 6.9 ± 0.1. With $[Co(terpy)_2]^{3+}$ as oxidant for horse cytochrome c(II), a dependence on pH is also observed with pK_a = 6.6 ± 0.2 (Figure 3). Rate constants k_1 (pH 8.0) and k_2 (pH 5.0) are listed in Table

Table VI. Summary of Rate Constants (k(25 °C)) and Acid Dissociation pK_a Values Here Assigned to His33, I = 0.10 M (NaCl)

reacn	pH	k/M^{-1} s^{-1}	pK_a
Horse Cytochrome *c*			
cyt *c*(II) + $[Co(phen)_3]^{3+}$	8.0	1.8×10^3	6.9[a]
	5.0	1.1×10^3	
cyt *c*(II) + $[Co(terpy)_2]^{3+}$	8.0	9.2×10^2	6.6[a]
	5.0	6.3×10^2	
cyt *c*(II) + $[Fe(CN)_6]^{3-}$	5–8	9.0×10^5	
cyt *c*(II) + $[Co(dipic)_2]^-$	5–8	1.0×10^4	
$[Co(terpy)_2]^{2+}$ + cyt *c*(III)	5–8	1.0×10^3	
$[Ru(NH_3)py]^{2+}$ + cyt *c*(III)	5–8	4.1×10^3	
$[Co(sep)]^{2+}$ + cyt *c*(III)	5–8	3.4×10^5	
Tuna Cytochrome *c*			
cyt *c*(II) + $[Co(phen)_3]^{3+}$	5–8	1.5×10^3	
C. krusei Cytochrome *c*			
cyt *c*(II) + $[Co(phen)_3]^{3+}$	8.0	2.8×10^3	6.9[a]
	5.0	2.4×10^3	

[a] pK_a obtained from variation of rate constants with pH.

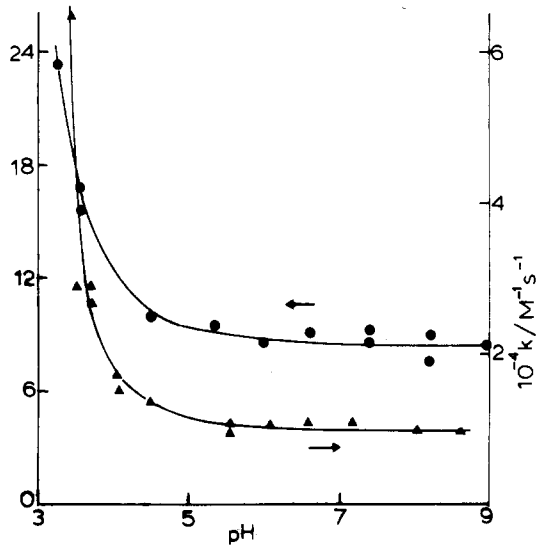


Figure 4. Variation of second-order rate constants, k(25 °C), for the $[Fe(CN)_6]^{3-}$ (●) (left-hand scale) and $[Co(dipic)_2]^-$ (▲) (right-hand scale) oxidation of horse cytochrome *c*(II) (I = 0.10 M (NaCl)).

VI. On DEPC modification the dependence on pH is no longer observed (Figures 2 and 3).

No dependence on pH (5–9) is observed for the $[Fe(CN)_6]^{3-}$ and $[Co(dipic)_2]^-$ oxidations for horse cytochrome *c*(II) (Figure 4). Similarly with the three reductants $[Co(terpy)_2]^{2+}$, $[Ru(NH_3)_5py]^{2+}$, and $[Co(sep)]^{2+}$, no dependence on pH is observed over the range 5.5–8.0 (Figure 5). At pH >8.4 biphasic kinetics are observed, which may be related to the lysine for methionine change in ligation at the heme Fe.[46]

All reactions studied at pH <5 gave an increase in rate constants as the pH is decreased, in accord with previous studies.[1,2]

Discussion

Table VI summarizes rate constants and the effects of pH observed. Of particular interest are the pK_a's of between 6 and 7 for the 3+ oxidants. None of the inorganic complexes exhibit pK_a's in this region. Also, the two heme propionic acids of cytochrome *c* do not ionize within the pH range 4.5–9.0.[47,48] The pK_a's observed can therefore be assigned to histidine residues on the protein.

The use of three different cytochrome *c*'s has helped in making firm assignments. Not relevant is His18, which is axially coordinated in all three cytochromes and cannot therefore exhibit a pK_a of the magnitude observed. Horse cytochrome *c* has in addition two other histidines (His26 and His33), tuna cytochrome *c* one (His26), and *C. krusei* cytochrome *c* three (His26, His33, and His39). No kinetic pK_a is obtained for tuna cytochrome *c* with $[Co(phen)_3]^{3+}$. Also, from NMR studies on cytochrome *c*(II) it has been shown that His26 has pK_a's which are out of range, and for horse cytochrome *c*, pK_a = 3.5, and for tuna cytochrome *c*, pK_a < 3.5.[49] It is unlikely that His26 contributes. The only possible assignment therefore is His33. This residue gives NMR pK_a's for horse (6.54) and *C. krusei* cytochrome *c*'s (6.74) (Table VII). Although His39 of *C. krusei* cytochrome *c* like His33 undergoes DEPC modification and has a pK_a of 6.56, it would be surprising if it contributed alongside His33 in determining the reaction pattern, since it is located on the rear face of the molecule, well away from the exposed heme edge and the reaction site. We note that while all three cytochrome *c*'s contain Glu21, *C. krusei* cytochrome *c* has no Lys25 or Lys22, and this could contribute to the 2-fold faster rate constant for *C. krusei* cytochrome *c* with $[Co(phen)_3]^{3+}$. Rate constants for horse and *C. krusei* cytochrome *c*'s DEPC modified at His33 do not change as the pH is varied in the range 5–8. Protonation of the imidazole cannot occur for the DEPC-modified protein, consistent with the above interpretation. Also, the intramolecular rate constant for horse cytochrome *c* Ru modified at His33 has been reported to be independent of pH.[35e]

Rate constants for the reactions of $[Co(phen)_3]^{3+}$ and $[Co(terpy)_2]^{3+}$ decrease with pH by 25–30% of the value at high pH

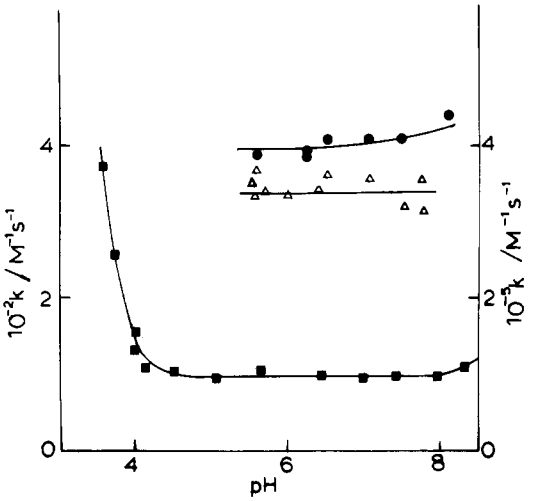


Figure 5. Variation of second-order rate constants, k(25 °C), for the $[Co(terpy)_2]^{2+}$ (■), $[Ru(NH_3)_5(py)]^{2+}$ (●) (both left-hand scale), and $[Co(sep)]^{2+}$ (▲) (right-hand scale) reductions of horse cytochrome *c*(III) (I = 0.10 M (NaCl)). The reaction with $[Co(terpy)_2]^{2+}$ was also investigated from pH 8.4 to 9.5 when a biphasic treatment was required; at pH >9.5 the reaction reverts to an essentially uniphasic process.

Table VII. Summary of Histidine pK_a Values for Different Cytochrome *c*'s

cyt *c*	His	pK_a
horse Fe(II)	26	2.5
	33	6.54,[a] 6.7[b]
horse Fe(III)	26	
	33	6.41[a]
tuna Fe(II)	26	>3.5
C. krusei Fe(II)	26	
	33	6.74,[a] 6.9[b]
	39	6.56[a]
His		6.04[c]

[a] References 50 and 54. [b] This work. [c] *Handbook of Chemistry and Physics*, 58th ed.; Weast, R. C., Ed.; CRC: Boca Raton, FL, 1977; p D-148.

(46) Gadsby, P. M. A.; Peterson, J.; Foote, N.; Greenwood, C.; Thomson, A. J. *Biochem. J.* **1987**, *246*, 43.
(47) (a) Angstrom, J.; Moore, G. R.; Williams, R. J. P. *Biochem. Biophys. Acta* **1982**, *703*, 87. (b) Moore, G. R. *FEBS Lett.* **1983**, *161*, 171.
(48) Chung, H. K.; Warshel, A. *Biochemistry* **1986**, *25*, 1675.
(49) Cohen, J. S.; Fisher, W. R.; Schechter, A. N. *J. Biol. Chem.* **1974**, *249*, 1113.

(Figures 1 and 2). In other words, protonation of His33 inhibits reaction, which is consistent with the electrostatic effect of H^+ on reactions of cytochrome *c*(II) with positively charged oxidants. What is surprising is that there is no similar effect of pH with $[Co(terpy)_2]^{2+}$ as reductant for cytochrome *c*(III) or with two other reductants $[Ru(NH_3)_5py]^{2+}$ and $[Co(sep)]^{2+}$. All three complexes are sufficiently inert/stable for this factor not to be relevant. It has been shown here and elsewhere that the reduction potential of cytochrome *c* is independent of pH in the range 5–7.5,[2] the values obtained in the present studies being 273 mV.[50] Similarly, the protein self-exchange rate constant is independent of pH.[51]

From the rate constants for the reactions of $[Co(terpy)_2]^{3+}$ and $[Co(terpy)_2]^{2+}$ at pH 8, the reduction potential for the horse cytochrome *c*(III/II) couple is greater than that for the $[Co(terpy)_2]^{3+/2+}$ couple by 2 mV. If the latter has a potential of 270 mV (Table I), then the cytochrome *c*(III/II) couple is 272 mV. At pH 5 the rate constants suggest a 12-mV difference, and the "apparent" cytochrome *c*(III/II) value is 284 mV. The question that has to be addressed is why the reaction of $[Co(terpy)_2]^{2+}$ does not exhibit an $[H^+]$ dependence in effect cancelling that observed for $[Co(terpy)_2]^{3+}$. It would seem that there are local effects centering around His33, which are not reflected in the independently measured reduction potentials.

From previous studies reactions of proteins with inorganic complexes have been shown to occur in two steps involving association of the reactants (K) followed by electron transfer (k_{et}).[52] When K is not sufficiently large to give rise to saturation kinetics, as in the present studies at I = 0.10 M, the second-order rate constant is equal to $k_{et}K$. The pH effect observed in the reaction with positively charged oxidants most likely stems from the K component of the rate constant and not k_{et}, since the independently determined reduction potential does not vary with pH. The binding of positively charged reaction partners is thus affected by the state of protonation of His33 in the case of the reduced protein, but not with the oxidized protein, when any effect is below the limits of detection.

We have considered the possibility that there is some sort of conformational change contributing to the reactions of the positively charged complexes. One possibility is that Glu21, which is close to the center of reaction, becomes more remote from His33 in the case of cytochrome *c*(III), with the result that the reductants are no longer influenced by protonation at His33. In their NMR studies, Moore and Williams[54] have not detected any changes stemming from the protonation of His33. The most notable conformational changes on cytochrome *c* with change in oxidation state are believed to be around the lower part of the exposed heme edge (Figure 1) encompassing residues 42–54.

It has been demonstrated from NMR studies that the His33 pK_a for horse cytochrome *c*(II) (6.54) it not very different from that for cytochrome *c*(III) (6.41).[53] Also in DEPC modification experiments (eq 2) the rate constants obtained under identical conditions are for cytochrome *c*(II) (5.9 M^{-1} s^{-1}) and *c*(III) (4.6 M^{-1} s^{-1}). This suggests that His33 has similar acid–base properties and that the accessibility is the same in both oxidation states. However, a study of the temperature dependence of the Trp33 resonances in tuna cytochrome *c* has shown that this residue is mobile with some oscillation of its side chain.[55] The X-ray structure of horse cytochrome *c* reveals that His33 is on the surface of the molecule with its imidazole ring pointing away from the protein, and one would not expect the latter residue to be immobile either. It has moreover been reported that there is an unusual dynamic conformation process on horse cytochrome *c*(III) that is influenced by a pK_a of 6.8,[56] where His33 is the only group known to have a pK_a of this magnitude. It is possible that solvation of the protein in this locality is different for the two oxidation states. Such a remote (external) effect would not of itself be expected to influence the reduction potential at the exposed heme edge or Fe active site. It could however influence the approach of a redox partner and selection of a site for electron transfer.

We note as a side issue that in our studies on DEPC modification (using a 30-fold excess of DEPC) a preference for His33 followed by His39 modification is observed, with no tendency to modify His26. These findings are not in agreement with previous studies on the reaction of horse cytochrome *c* with DEPC in 90-fold and 150-fold excess, when modification of two histidines was reported to occur at first His26 and then His33.[57] This assignment was based on the findings of Cooper et al.,[58] who, following DEPC modification (24-fold excess) and analysis of proteolitically cleaved protein fragments by using secondary ion mass spectroscopy, concluded that there was only one modification (38%) at His26. However, Leitch et al.[59] have reported that in the case of cytochrome c_{551} the DEPC-modified products are relatively unstable, preventing the isolation of modified peptides after proteolysis. It is known that other modifications can occur at high DEPC concentrations.[45] We observe no reaction of DEPC with tuna cytochrome *c*, which has only His26. Together with the kinetic results discussed above, this leads us to believe that our assignment of the site of DEPC modification on horse cytochrome *c* as His33 is correct.

Finally, the pH independence of self-exchange rate constants for horse cytochrome *c*(II/III)[51] suggests that the His33 site is not implicated in the self-exchange process. The three reductants used in the present study have different reduction potentials ($[Co(terpy)_2]^{3+/2+}$ (270 mV), $[Ru(NH_3)_5py]^{3+/2+}$ (273 mV), and $[Co(sep)]^{3+/2+}$ (−260 mV)) and self-exchange rate constants (M^{-1} s^{-1}) of 400, 4.7×10^5, and 5, respectively.[60,61,42] The variations in rate constants (Table VI) are as might be expected for reactions occurring at the same binding site in each case.

Acknowledgment. We thank the U.K. SERC for postgraduate support (to R.T.H.).

(50) Reduction potentials of 260–265 mV (invariant with pH 5.0–7.5) have also been reported, e.g. p 12 of ref 2. The variations observed presumably relate to different experimental procedures and conditions.
(51) (a) Gupta, R. K.; Koenig, S. H.; Redfield, A. G. *J. Magn. Reson.* **1972**, *7*, 66. (b) Gupta, R. K. *Biochim. Biophys. Acta* **1973**, *292*, 291.
(52) Moore, G. R.; Harris, D. A.; Leitch, F. A.; Pettigrew, G. W. *Biochim. Biophys. Acta* **1984**, *764*, 331.
(53) Cohen, J. S.; Hayes, J. *J. Biol. Chem.* **1974**, *249*, 5472.
(54) Moore, G. R.; Williams, R. J. P. *Eur. J. Biochem.* **1980**, *103*, 513.
(55) Moore, G. R.; Williams, R. J. P. *Eur. J. Biochem.* **1980**, *103*, 533.
(56) Burns, P. D.; La Mar, G. N. *J. Am. Chem. Soc.* **1979**, *101*, 5844.
(57) Konopka, K.; Waskell, L. *Biochim. Biophys. Acta* **1988**, *954*, 189.
(58) Cooper, H. M.; Jemmerson, R.; Hunt, D. F.; Griffin, P. R.; Yates, J. R.; Shabanowik, J.; Zhu, N.-Z.; Paterson, Y. *J. Biol. Chem.* **1987**, *262*, 11591.
(59) Leitch, F. A.; Moore, G. R.; Pettigrew, G. W. *Biochemistry* **1984**, *23*, 1831.
(60) Farina, R.; Wilkins, R. G. *Inorg. Chem.* **1968**, *7*, 514.
(61) Brown, G. M.; Krentzien, H. J.; Abe, M.; Taube, H. *Inorg. Chem.* **1979**, *18*, 3374.