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Cobalt–Cytochrome *c*. II. Magnetic Resonance Spectra and Conformational Transitions†

L. Charles Dickinson and James C. W. Chien*

ABSTRACT: Between pH ~4 and 10 cobaltocytochrome *c* (^{Co}cyt-*c*) gives an electron paramagnetic resonance (EPR) spectrum with $g_{\parallel}$ = 2.035, $g_{\perp}$ = 2.223, $^{Co}A_{\parallel}$ = 61.4 G, $^{Co}A_{\perp}$ = 49.8 G, $^{N}A_{\parallel}$ = 15.3 G, and $^{N}A_{\perp}$ = 12.5 G. Comparisons with the EPR spectra of deoxycobaltomyoglobin, deoxycobaltohemoglobin, and model compounds and together with other evidence showed cobaltocytochrome *c* to have Met-80 and His-18 as its axial ligands. The protons of these ligands are seen as resonances shifted by the ring-current field of the porphyrin in the 300-MHz ^{1}H nuclear magnetic resonance (NMR) spectra of cobalticytochrome *c* (^{Co}cyt-c^{+}). The methyl and γ-methylene protons of Met-80 in this molecule occupy positions with respect to heme *c* which are somewhat different from those in ferrocytochrome *c*. The ^{1}H NMR spectra also showed that the methyl groups of Leu-32, Ile-75, Thr-63, thioether bridges, and the porphyrin ring in the cobalt protein are in the same state as in native enzyme; the same is also true for Tyr-59, His-26, and His-33 and also possibly Tyr-67, Tyr-74, and Phe-82. Above pH 11, ^{Co}cyt-*c* is converted to a five-coordinated form having $g_{\parallel}$ = 2.026, $g_{\perp}$ = 2.325, $^{Co}A_{\parallel}$ = 80 G, $^{Co}A_{\perp}$ ≈ 10 G, $^{N}A_{\parallel}$ = 17.5 G,, and $^{N}A_{\perp}$ not resolved. Below pH 1.0 the EPR spectrum of ^{Co}cyt-*c* is also five-coordinated with $g_{\parallel}$ = 2.014, $g_{\perp}$ = 2.359, $^{Co}A_{\parallel}$ = 93.8 G, and $^{Co}A_{\perp}$ = 38.8 G. The axial ligands in the alkaline and the acidic forms of ^{Co}cyt-*c* are His-18 and Met-80, respectively. New prominent proton resonance peaks are observed in cobalt–cytochrome *c* which are either absent or weak in native cytochrome *c*. These are situated at 3.0, 1.7, and 1.44 ppm, attributable, respectively, to the ε-CH_2, δ-CH_2 + β-CH_2, and γ-CH_2 of lysyl residues in random-coil peptides. From the areas of these peaks, it is estimated that one–two lysyl residues in ^{Co}cyt-*c* have been modified; four–five lysyl residues in ^{Co}cyt-c^{+} have been modified. These alterations of surface charged groups are probably responsible for the lowered reactivity of ^{Co}cyt-*c* with cytochrome oxidase and the lack of reactivity of ^{Co}cyt-c^{+} with several cytochrome reductase systems.

The preparation of cobalt–cytochrome *c* was first reported by Dickinson and Chien (1974). In the preceding paper (part I, Dickinson and Chien, 1975a) we described an improved method of preparation and gave some of the properties of cobalt–cytochrome *c* and its enzymic activities. The optical spectra and half-reduction potential of ^{Co}cyt-*c*[1] suggest that the Co atom is six-coordinated. On the other hand, the visible spectrum of ^{Co}cyt-c^{+} is devoid of absorption in the vicinity of 695 nm. The 695-nm band is a characteristic spectral feature of ^{Fe}cyt-c^{+} and has been taken by many workers to be ligand specific and to originate in the S–Fe coordination (Schechter and Saludjian, 1967; Sreenathan and Taylor, 1971; Folin et al., 1972; Lambeth et al., 1973). In contrast to this interpretation, Shejter and George (1964) and most recently Castro (1974) argued for a protein conformational origin for the 695-nm band. Because of these discordant views, it behooves us to identify the axial ligands in ^{Co}cyt-*c* by independent means such as magnetic resonance techniques.

Native ^{Fe}cyt-*c* is known to have three pH-dependent conformations in acidic, neutral, and alkaline media designated as species I, II, and III, respectively. Species I and III are said to be partially unfolded; however, their state of coordination of Fe seems to be unknown. A second objective of this study is to determine whether ^{Co}cyt-*c* also exhibits these analogous conformational transitions, and to use EPR to elucidate the state of ligation of its conformational isomers.

Finally, cobalt–cytochrome *c* has somewhat lower reactivity with cytochrome oxidase than the native enzyme and no activity whatsoever with cytochrome *c* reductase (part I). A third objective of this work is to discover the causes for the decreased enzymic activities and to learn more about the mitochondrial electron transfer processes.

Experimental Section

Materials. Sigma Type VI horse heart cytochrome *c* was used in this work. ^{Fe}cyt-*c* was prepared from it by reduction with dithionite and chromatography on a Sephadex G-25 column. The ^{Fe}cyt-*c* samples are pH 8.5; the ^{Fe}cyt-c^{+} samples are pH 6.3. Both are virtually salt free.

Unless stated otherwise, the spectra of cobalt cytochrome

† From the Department of Chemistry and the Materials Research Laboratories, University of Massachusetts, Amherst, Massachusetts 01002. *Received March 3, 1975.* This work was supported in part by Grant No. HL-14270 from the U.S. Public Health Service.

[1] Abbreviations used are: ^{Co}cyt-*c*, cobaltocytochrome *c*; ^{Co}cyt-c^{+}, cobalticytochrome *c*; ^{Fe}cyt-*c*, ferrocytochrome *c*; ^{Fe}cyt-c^{+}, ferricytochrome *c*; ^{Co}Mb, deoxycobaltomyoglobin; ^{Co}Hb, deoxycobaltohemoglobin; ^{Co}TPP, cobalto-*meso*-tetraphenylporphyrin; ^{Co}PP IX (DME), cobalt protoporphyrin IX dimethyl ester; $E_{m,7}$, half-reduction potential; EPR, electron paramagnetic resonance; ^{1}H NMR, proton magnetic resonance; DSS, sodium 2,2-dimethyl-2-silapentanesulfonic acid; shfs, superhyperfine splitting.

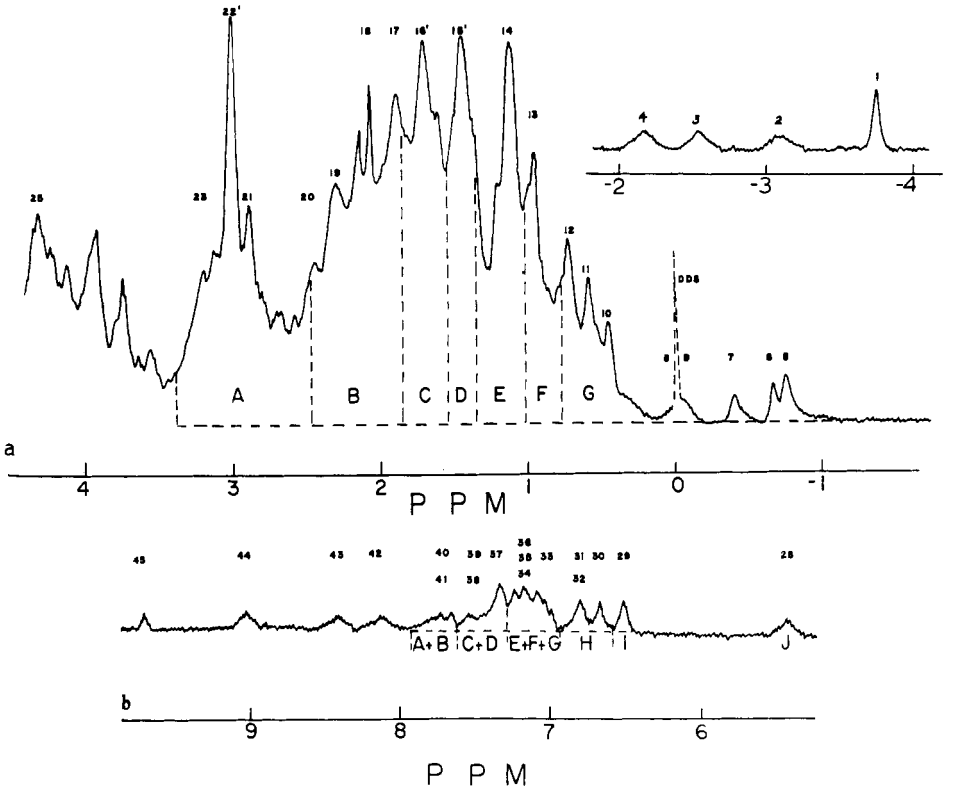


FIGURE 1: Fourier transform 300-MHz ^{1}H NMR spectra of cobalticytochrome *c;* 10 kHz spectral width, 0.4-sec acquisition time, 0.7-sec pulse delay, 55-μsec pulse width, 3000 transients: (a) aliphatic region; (b) aromatic region.

c were run on the A fractions as prepared by the procedure described in part I. For ^{1}H NMR measurements, salt-free samples of ^{Co}cyt-c^+ were concentrated by ultrafiltration. Residual water was removed by rinsing twice with 99.8% D_2O. The sample was then concentrated to about 15%, lyophilized, and redissolved in 99.97% D_2O (Wilmad Glass, Buena, N.J.). The sample was transferred into an NMR tube containing 0.10 mg of DSS (Na salt) and deuterated phosphate buffer salts to yield 0.05 *M* pD 7.0 solutions.

The reduced ^{Co}cyt-*c* is readily autoxidized. The sample preparation differed from that described above by using anerobic conditions throughout. It was not lyophilized but rinsed three times with 99.97% D_2O by ultrafiltrations. The NMR tube was sealed and the solution contained a slight excess of dithionite.

EPR. EPR spectra were obtained on a Varian E-9 X-band spectrometer. A dual cavity was used with solid DPPH as a reference signal of $g = 2.0036$. The spectra were recorded at −195°.

The EPR spectra of the metal-substituted cyt-*c* all have axial symmetry. Since experimentally the hyperfine splittings were found unequal, the average Hamiltonian parameters were determined (Chien, 1971) with the application of second-order perturbation theory and are expressed by the Hamiltonian (Bleaney, 1951):

$$H = \hbar\omega_0 = g\beta H(m) + \hbar Am + \hbar^2(A_\parallel^2 + A^2)\frac{A_\perp^2}{4A^2}\left(\frac{I(I+1) - m^2}{g\beta H(m)}\right) \quad (1)$$

where I is the nuclear spin angular momentum, m is the nuclear spin quantum number, and all other quantities have the usual significance.

Proton Magnetic Resonance. ^{1}H NMR spectra were run on the Varian 300-MHz spectrometer through the service of the NMR Center of the University of Akron.

Results and Discussion

Axial Ligands for ^{Co}cyt-c^+ (II). The axial ligands for ^{Co}cyt-c^+ between pH 4 and 12 were identified with ^{1}H NMR spectra (Figure 1). This is facilitated by comparison with the spectra of ^{Fe}cyt-*c* (Figure 2). The two molecules are isoelectronic. They are both diamagnetic so the most important factor that causes shifts of resonance positions is the ring-current field. Other factors are hydrogen bonds, polarity of the environment, and influence of charged residues.

The protons of the axial ligands are significantly upfield shifted by the ring-current field of heme *c*. In the case of ^{Fe}cyt-*c*, the methyl resonance of Met-80 is found at −3.30 ppm (McDonald et al., 1969; Gupta and Redfield, 1970a). The resonance at −3.77 ppm was assigned by Gupta and Redfield (1970b) to the γ proton of Met-80. Two other single-proton resonances at −2.59 and −1.87 ppm are assigned by McDonald and Phillips (1973) to be the β and the γ protons of Met-80. Figure 1a showed ^{Co}cyt-c^+ to exhibit a similar group of resonances at −3.74, −3.08, −2.53, and −2.15 ppm with relative intensities of 3:1:1:1. Taken together, this group of protons can only be attributed to Met-80. The −3.74 peak is undoubtedly its methyl protons. It lies to the higher field of the corresponding methyl in ^{Fe}cyt-*c* implying a slight difference in the spatial positions of the Met-80 residues in the two molecules. Examinations of the X-ray model suggest that the difference may be due to a stronger shielding influence of ring 1 of the porphyrin[2] on the Met-

[2] Numbering of the ring in porphyrin is given by Falk (1964).

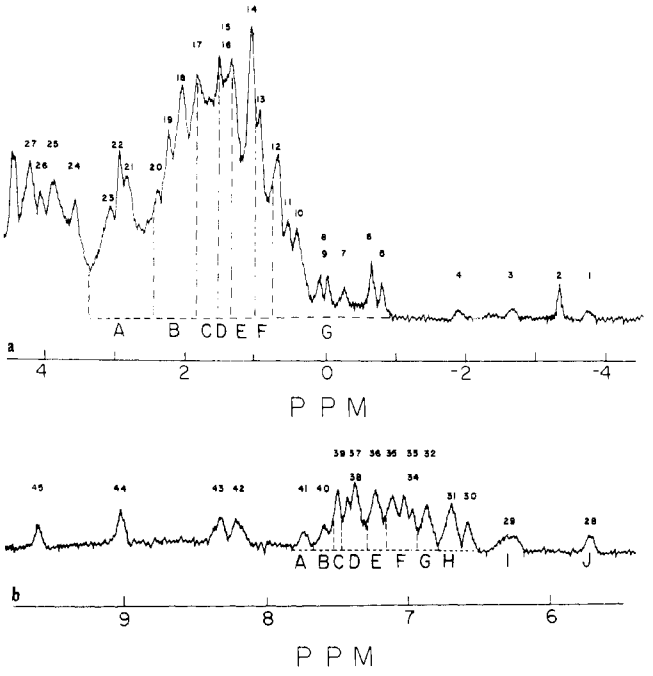


FIGURE 2: Continuous wave 300-MHz ^{1}H NMR spectra of ferrocytochrome *c*: (a) aliphatic region; (b) aromatic region; b has 2× frequency scale and 1.67× amplitude.

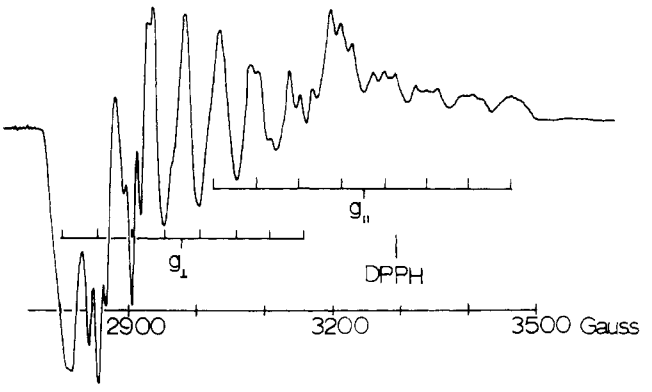


FIGURE 3: EPR spectrum of 0.1% cobaltocytochrome *c* in 0.02 *M* pH 8 sodium phosphate as reduced by minimal soldium dithionite: ν = 9.260 GHz; microwave power, 10 mW; 4-G modulation at 100 kHz; 77K.

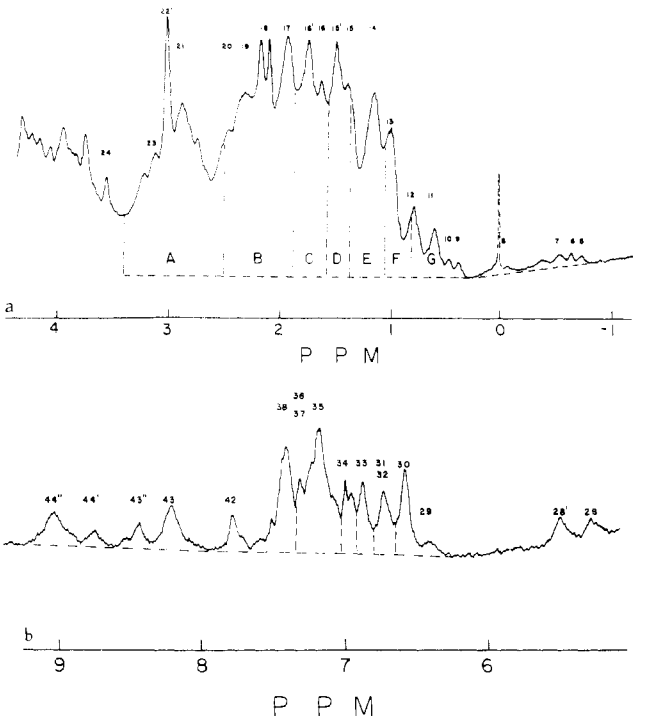


FIGURE 4: Fourier transform 300-MHz ^{1}H NMR spectra of cobaltocytochrome *c*; 5-kHz spectral width, 0.8-sec acquisition time; 0.4-sec pulse delay; 50-μsec pulse width; 3000 transients: (a) aliphatic region; (b) aromatic region.

80 methyl in ^{Co}cyt-c^+. This would necessitate a decrease of shielding for one of the γ protons but leave the remaining protons of this residue more or less unaffected. We tentatively assign the resonances of ^{Co}cyt-c^+ at −3.08, −2.53, and −2.15 ppm to the γ, β, and γ protons, respectively.

McDonald and Phillips (1973) assigned the resonance in ^{Fe}cyt-*c* at 0.13 ppm to the C_4 proton of His-18. This represents a shift of about 6.84 ppm from the "natural" position by the porphyrin ring-current field as compared to the shift of the high-field γ proton of Met-80 of 6.36 ppm by the same field. A similar peak was observed at +0.08 ppm in ^{Co}cyt-c^+. Therefore, the ^{1}H NMR evidence suggests ^{Co}cyt-c^+ to have both Met-80 and His-18 as axial ligands.

Axial Ligands for ^{Co}cyt-c (II). The form of ^{Co}cyt-*c* which exists between pH 4 and 12 is referred to as state II. It is paramagnetic and its state of ligation can be studied with EPR techniques. An EPR spectrum of ^{Co}cyt-*c* (II) at pH 5–10.5 is shown in Figure 3. It is characteristic for an axial system. The more intense low-field signals are the perpendicular lines; the weak high-field signals are the parallel lines. On a theoretical basis (Freeman and Watson, 1965) the axial principal value of the anisotropic hyperfine tensor is negative. The ^{59}Co (I = 7/2) hyperfine lines have m_I = −7/2, −5/2, −3/2, . . . +7/2 in the order of increasing applied field. In the spectrum shown in Figure 3 the four high-field perpendicular lines overlap with the four low-field parallel lines. The principal values of the g and ^{Co}A tensors are given in Table I, column 2. The signs of the hyperfine splittings are undetermined; the magnitude symbols are omitted according to general practice.

In addition to the cobalt hyperfine splittings, there are also ligand superhyperfine splittings seen as the 1:1:1 splittings on the m = ±1/2 and ±3/2 parallel lines. This is most reasonably attributed to interaction of the unpaired electron with an axial nitrogen atom with $^{N}A_{\parallel}$ = 15.3 G. A similar superhyperfine structure was also partially resolved for the perpendicular resonances with m = −5/2, −3/2, and −1/2. The value of $^{N}A_{\perp}$ thus obtained is 12.5 G. The analysis of the spectrum of ^{Co}cyt-*c* (II) is facilitated by comparison with the EPR spectra of ^{Co}Mb (Chien and Dickinson, 1972), ^{Co}Hb (Dickinson and Chien, 1973), and model compounds (Walker, 1970; Dickinson and Chien, 1975b). The five- and six-coordinated complexes differ primarily in their hyperfine anisotropy. The ^{Co}A anisotropy is large for the five-coordinated complexes whereas the six-coordinated complexes have comparable parallel and perpendicular values for ^{co}A. For axial nitrogenous ligands, the five-coordinated complexes have larger ^{N}A shfs than the six-coordinated complexes.

State II of ^{Co}cyt-*c* has EPR spectral parameters all within the range observed for six-coordinated complexes (Table I, columns 2 and 7). Therefore, between pH 4 and 12, ^{Co}cyt-*c* must have two axial ligands. The ^{14}N shfs requires one of these to be a nitrogenous coordinating group; His-18

Table I: EPR Spectral Parameters.

	^{Co}cyt-*c*						
	State II	State III	State I	^{Co}Mb[c]	^{Co}TPP·L[d]	^{Co}TPP·2L[d]	^{Co}PPIX·MTE[e]
$g_{\parallel}$	2.035	2.026	2.014	2.028	2.02–2.03	2.02–2.07	2.016
$g_{\perp}$	2.223	2.325	2.359	2.325	2.31–2.33	2.21–2.30	2.360
$^{Co}A_{\parallel}$, G	61.4	80	93.8	79.0			95.6
(cm^{-1})	(0.00583)	(0.00757)	(0.00882)	(0.00748)	(0.00764–0.00812)	(0.0060–0.0080)	(0.00901)
$^{Co}A_{\perp}$, G	49.8	(10)[a]	38.8	6.0			35.9
(cm^{-1})	(0.00517)	(0.00109)	(0.00427)	(0.00065)	(0.0010–0.0013)	(0.0023–0.0062)	(0.00377)
$^{N}A_{\parallel}$, G	15.3	17.5	0	17.5			0
(cm^{-1})	(0.00152)	(0.00166)		(0.00166)	(0.0013–0.0016)	(0.0009–0.0010)	
$^{N}A_{\perp}$, G	12.5	*b*	0	*b*	*b*	*b*	0
(cm^{-1})	(0.00130)						
ΔE, cm^{-1}	10870	7437	6728	7437	7324–7800	8061–11555	6728
P, cm^{-1}	0.0192	0.0202	0.0309	0.0191			0.0301
K	0.273	0.107	0.235	0.019			0.221

[a] Estimate based on inadequately resolved features. [b] Not resolved. [c] Chien and Dickinson (1972). [d] Walker (1970); ranges of values covering all the derivatives and ligands reported. [e] Dickinson and Chien (1975b).

seems to be the most likely candidate. It will be shown in the discussion below that the acidic species I has a sulfur axial ligand. However, attempts to identify the sixth ligand using ^{1}H NMR were unsuccessful (see below). The ^{1}H NMR spectra of ^{Co}cyt-*c* and ^{Fe}cyt-*c*$^+$ are given in Figures 4 and 5, respectively.

All the protons of the prosthetic group and ligands in ^{Fe}cyt-*c*$^+$ are greatly shifted by contact interaction; pseudocontact interaction also contributes. The contact shift (σ_i^c) and pseudocontact shift (σ_i^p) are given as[3] (McConnell and Chestnut, 1958; Jesson, 1967; Kurland and McGarvey, 1970):

$$\sigma_i^c = -[A\bar{g}\beta S(S+1)/(\gamma_H/2\pi)3kT] \quad (2)$$

$$\sigma_i^p = -[\beta^2 S(S+1)/9kT]\,(g_{\parallel}^2 - g_{\perp}^2)\,[(3\cos^2\theta - 1)/r^3] \quad (3)$$

For instance, the positions of the Met-80 residue are: −24.20 ppm for the methyl group (Wüthrich, 1969, 1970; Gupta and Koenig, 1971), and −25.1 and −28.1 ppm for the γ protons (McDonald and Phillips, 1973). Peaks 66 and 67 in Figure 5a at −2.44 and −2.68 ppm are the hemethioether bridge methyls. Careful searches for peaks at large shifts, up to ±30 ppm from either extrema of the normal spectral region, failed to detect any resonances in ^{Co}cyt-*c*.

Even though ^{Co}cyt-*c* and ^{Fe}cyt-*c*$^+$ are both $S = \frac{1}{2}$ paramagnetic molecules, the former has a single electron in the $3d_{z^2}$ orbital (vide infra), whereas the latter has its unpaired electron in a $3d\pi$ orbital. Delocalization is possible in all bonding directions in ^{Fe}cyt-*c*$^+$ but mostly only along the axial direction in ^{Co}cyt-*c*. This makes it difficult to estimate the probable positions for the resonances of Met-80 protons in ^{Co}cyt-*c*. However, we can estimate with a fair degree of confidence the resonance position of the heme meso protons. LaMar and Walker (1973a,b) made a ^{1}H NMR and EPR investigation of low-spin ^{Co}TPP complexes. They found for the meso proton a pseudocontact shift of 15.0 ppm and a contact shift of 4.0 ppm both in the downfield direction. The magnetic anisotropy of ^{Co}TPP is $g_{\parallel}^2 - g_{\perp}^2 = -6.8$. With the smaller magnetic anisotropy of ^{Co}cyt-*c*$^+$, we estimate a pseudocontact downfield shift of 2 ppm for the meso proton assuming a distance of 4 Å reported by Scheidt (1974a,b) for ^{Co}TPP(pip)$_2$. The contact shift for the meso protons should be about 4.0 ppm downfield. If we assume the ring-current field effect to be the same for ^{Co}cyt-*c* and ^{Co}cyt-*c*$^+$, then these protons should resonate at about 14–16 ppm. No resonance was observed in these regions or their vicinity. Other considerations[4] led us to conclude that the electron spin-lattice relaxation time is quite long for ^{Co}cyt-*c*, and that the protons of its prosthetic group are

[3] In eq 2, $A = (1/3)(A_{\parallel} + 2A_{\perp})$ is the isotropic hyperfine constant, $\bar{g} = (1/3)(g_{\parallel} + 2g_{\perp})$, and other quantities have the usual significance. Equation 3 is applicable to the case of a single populated spin level with negligible contribution from second-order Zeeman contribution for a "polycrystalline" state with $|\Delta g\beta Hh^{-1}| \gg \tau_r^{-1}$ where τ_r is the tumbling time of the protein molecule in solution. For ^{Co}cyt-*c*, $\Delta g = 0.18$ (Table I) and τ_r is about 10^{-7} sec. Therefore, $|\Delta g\beta Hh^{-1}| = 10^{11}$ sec^{-1} $\gg \tau_r^{-1} = 10^7$ sec^{-1}. In this equation θ is the angle between the proton–metal vector and the tetragonal axis of the heme.

[4] Let us estimate how much broader the methine proton resonances of ^{Co}cyt-*c* would be as compared to ^{Co}TPP (LaMar and Walker, 1973a), which is about 9 ppm. Among the paramagnetic relaxation processes discussed by Lewis and Morgan (1968), the Raman and the spin-rotational processes are likely to be relevant. The relaxations times are given by Kivelson (1966) for the Raman process:

$$T_1^{-1} = 32\,(\xi/\Delta)^2\,(\phi q_0/r_0\Delta)^4\,\tau_c^{-1} \quad (A)$$

and by Atkins and Kivelson (1966) for the spin-rotational process:

$$T_1^{-1} = (\Delta g_{\parallel}^2 + 2\Delta g_{\perp}^2)\,(kT/12\pi\eta a_0^3) \quad (B)$$

In these equations, ξ is the spin-orbit coupling constant, Δ is the energy of the lowest excited orbital state, $\phi q_0/r_0$ is the amplitude of the time-dependent electric field gradient, $\Delta g_{\parallel} = g_{\parallel} - 2.0023$, $\Delta g_{\perp} = g_{\perp} - 2.0023$, and the other quantities in eq B have the same significance as in the Stoke's equation. According to either process ^{Co}cyt-*c* should have greatly increased T_1 and therefore broader ^{1}H NMR line width. In the spin-rotational processes, the parameters for ^{Co}cyt-*c* are: $\Delta g_{\parallel} = 0.0327$, $\Delta g_{\perp} = 0.2127$ (part I), and $a_0 \sim 25$ Å. The values for ^{Co}TPP are: $\Delta g_{\parallel} \approx -0.14$, $\Delta g_{\perp} \approx 1.10$ (Walker, 1970), and $a_0 \sim 15$ Å (Fleischer, 1970). At the same solution viscosity and temperature, one finds $T_1(^{Co}\text{TPP})/T_1(^{Co}\text{cyt-}c) \approx 8 \times 10^{-3}$. For the Raman process, it is not possible to estimate the field gradient except to say that it should be much smaller for ^{Co}cyt-*c* than for ^{Co}TPP because the former is fully ligated. The other parameters for ^{Co}cyt-*c* are: $\Delta = 11{,}280$ cm^{-1} (part I) and $\tau_C \sim 10^{-7}$ sec. For ^{Co}TPP they are: $\Delta = 1250$ cm^{-1} (Assour, 1965) and $\tau_c = 2 \times 10^{-10}$ sec (LaMar and Walker, 1973a). Assuming the same spin-orbit coupling constants for both molecules, we estimate a ratio of $T_1(^{Co}\text{TPP})/T_1(^{Co}\text{cyt-}c) \approx 4 \times 10^{-9}$. Since the ^{1}H NMR line width in a paramagnetic molecule is directly dependent on T_1 (Solomon, 1955), the methine proton resonance in ^{Co}cyt-*c* is orders of magnitude greater than that of ^{Co}TPP and cannot be detected.

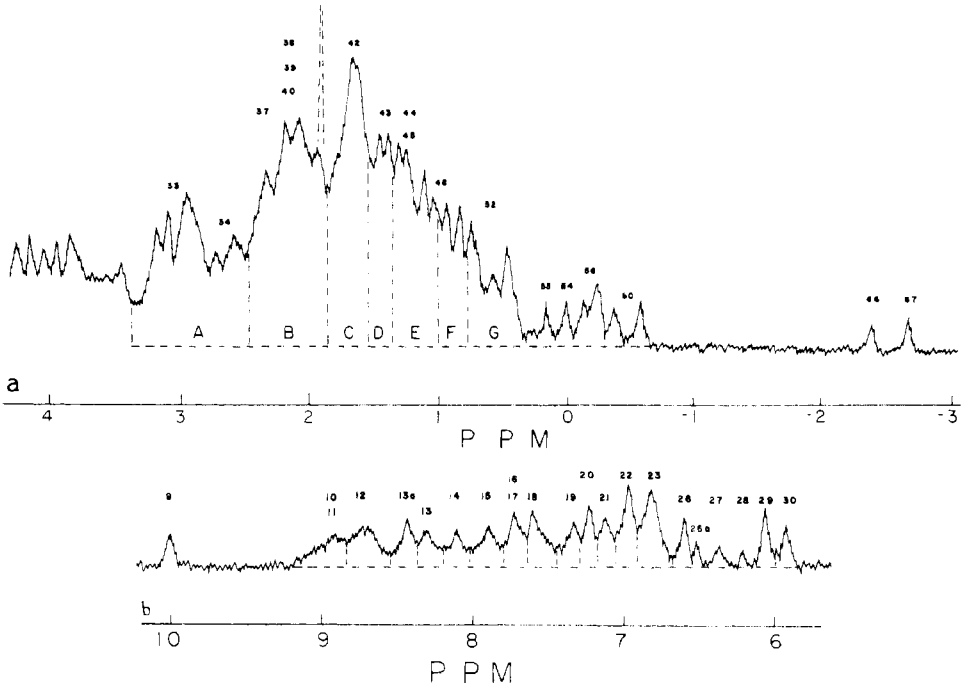


FIGURE 5: Continuous wave 300-MHz ^{1}H NMR spectra of ferricytochrome *c*: (a) aliphatic region; (b) aromatic region, 1.67 × amplitude. The very large contact-shifted resonances are not included.

broadened to beyond detection as the result of it.

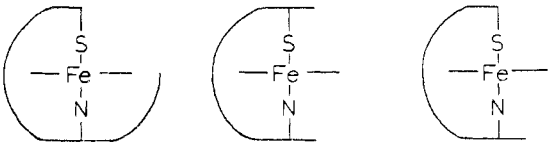
pH-Dependent Structural Transformations. When the pH of a ^{Co}cyt-*c* solution is increased to 11.2, its EPR begins to change to a new spectrum; the change is complete at pH 12.9 (Figure 6). The spectrum is that of the alkaline state III of ^{Co}cyt-*c*. State III is much more readily autoxidized than state II. Even in the absence of oxygen, the overall signal intensity at pH 12.9 is decreased by 20% and dithionite must be added to maintain adequate signal-to-noise in further titration. The sample can be titrated back to pH 8 with 2 *N* HCl to give primarily state II so the conformational transition is largely reversible. The EPR parameters for ^{Co}cyt-*c* (III) are given in Table I, column 3.

Several titrations to low pH resulted in the total disappearance of the EPR signal. It seems likely that the molecule undergoes another conformational transition to state I with $pK_a \sim 3$. There are two possible reasons for the nonobservance of EPR. The Co could be in a high-spin configuration which would have very short spin–lattice relaxation time (Kennedy et al., 1972). EPR of high-spin Co(II) complexes can be seen only at liquid He temperature. Secondly, ^{Co}cyt-*c* in state I may be very susceptible to autoxidation. To resolve these possibilities, we added 0.4 ml of salt-free ^{Co}cyt-*c* to 0.1 ml of 2 *N* HCl containing Pt asbestos and methyl viologen in an atmosphere of H_2. Under these conditions Figure 7 was observed. This spectrum differs from the other spectra in the absence of nitrogen shfs. At least three of the perpendicular hyperfine splittings were resolved. The spectral parameters of this acidic state ^{Co}cyt-*c* (I) are given in Table I, column 4.

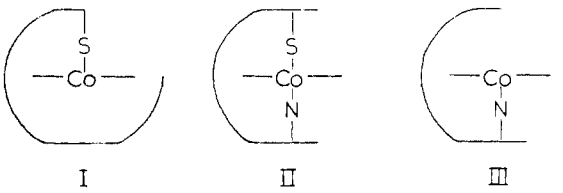
The EPR spectral characteristics of ^{Co}cyt-*c* (III) closely resemble all the other five-coordinated Co complexes as can be readily seen by comparing column 3 in Table I with columns 5 and 6. The ^{14}N shfs showed that His-18 is still coordinated. Therefore, the S–Co bond of Met-80 must be severed in highly alkaline medium.

The EPR spectrum of the acidic state I of ^{Co}cyt-*c* is quite unusual and does not resemble the spectra of any cobalt hemoproteins and model systems. The spectrum is devoid of ^{14}N shfs and the cobalt hyperfine anisotropy is intermediate of the five- and six-coordinated complexes with nitrogenous ligands. A spectrum nearly identical with Figure 7 can be obtained by adding to a toluene solution of ^{Co}PP IX dimethyl ester a stoichiometric amount of methylthioethanol (Dickinson and Chien, 1975b; also compare columns 4 and 8 of Table I). Therefore, we can conclude that ^{Co}cyt-*c* (I) is five-coordinated with Met-80 as the axial ligand; His-18 having a pK_a of 2.5 (Babul and Stellwagen, 1972) is ionized and not coordinated to Co at low pH.

Analogous to the transformation described above for ^{Co}cyt-*c*, ^{Fe}cyt-*c* also exhibit two transitions with nearly the same p*K* values. Castro and Bartnicki (1975) have proposed that these structural isomers of ^{Fe}cyt-*c* differ only in their protein conformations but not their state of ligation. The authors referred pictorially to them as having the short C (acidic), C (4 < pH < 12), and G geometries:



The results given above suggest the following structures for ^{Co}cyt-*c* at various pH ranges:



If ^{Fe}cyt-*c* and ^{Co}cyt-*c* are alike as indicated by many of

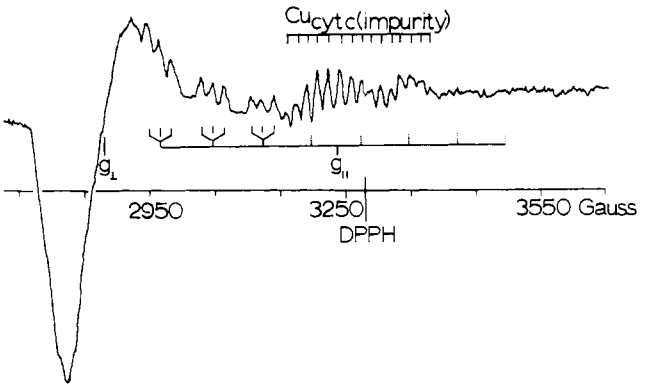


FIGURE 6: EPR spectrum of cobaltocytochrome *c* at pH 12.9, ionic strength ~0.02. Spectrometer settings except gain are identical with those for Figure 3.

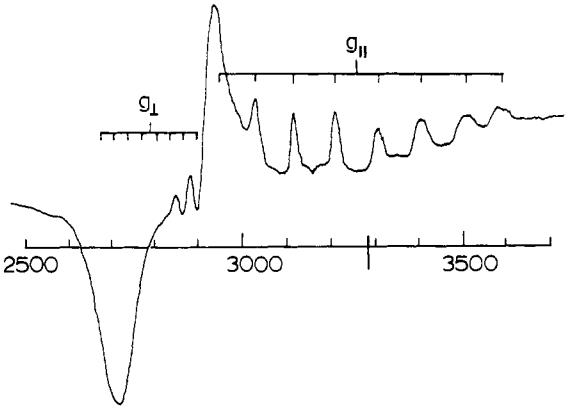


FIGURE 7: EPR spectrum of cobaltocytochrome *c* at pH 0.5. Spectrometer settings except gain are identical with those for Figure 1.

their properties, then the structures of ^{Fe}cyt-*c* are probably analogous to those given by I, II, and III instead of the ones proposed as G, C, and short C.

Electronic Configuration. The absence of measurable superhyperfine interaction of the unpaired electron with the in-plane nitrogen atoms showed that the unpaired electron is largely in a d_{z^2} orbital. This is supported by the strong dependence of $g_\perp$ and ^{Co}A on the ligands and the presence of superhyperfine splittings with the axial ligands. According to the first-order perturbation theory, the EPR spectra should have:

$$g_\parallel = 2.0023 \quad (4)$$

$$g_\perp = 2.0023 + 6\xi/\Delta E \quad (5)$$

where ξ is the spin-orbit coupling coefficient and $\Delta E = E(d_{xz},d_{yz}) - E(d_{z^2})$. The spectra of ^{Co}cyt-*c* have $g_\parallel$ greater than the free-spin value so the first-order theory is not adequate, nor would a second-order theory give consistent values of $\xi/\Delta E$. However, we will discuss the spectra on this basis. An analysis of the spectra according to third-order perturbation theory will be published elsewhere.

The spin-orbit coupling constant of free Co^{2+} ion is 530 cm^{-1}; however, in covalent complexes the magnitude of ξ is reduced by about 20 to 40%. Choosing ξ = 400 cm^{-1}, and using eq 5, we find ΔE = 6728, 7437, and 10,870 cm^{-1} for states I, II, and III, respectively.

It is of interest to compare these energy separations with those found in model compounds as shown in Table I. For the five-coordinated complexes with nitrogenous axial ligand, the values of ΔE range from 7324 to 7800 cm^{-1}. Both ^{Co}cyt-*c* (III) and ^{Co}Mb fall in this range. The ΔE value is as small as 1250 cm^{-1} for ^{Co}TPP without any axial ligand (Assour, 1965). Another additional axial ligand increases ΔE further. Thus, ^{Co}cyt-*c* (II) and ^{Co}TPP with two axial nitrogenous ligands have $\Delta E \sim$ 10,000 cm^{-1}. There is theoretical basis for the increase of ΔE with axial ligation. Zerner et al. (1966) calculated values of ΔE of 6000 and 11,000 cm^{-1}, respectively, for low-spin ferrous cases of ^{Fe}TPP·H_2O and ^{Fe}TPP·$2H_2O$. The increase in ΔE with ligation is largely due to the increase of energy of the d_{z^2} orbital without greatly affecting the energy of the $e_g(d\pi)$ orbitals.

It is of interest to note that ΔE is smaller for the acidic state of ^{Co}cyt-*c* than the alkaline state. Therefore, Met-80 poses a weaker ligand field on the cobalt atom than His-18.

We can also learn something about the delocalization of the unpaired electron population from cobalt to the ligands using the first-order perturbation theory. Maki et al. (1964) derived the relationships:

$$A_\parallel = P[-K + (4/7) - (1/7)(g_\perp - 2.0023)] \quad (6)$$

$$A_\perp = P[-K - (2/7) + (45/42)(g_\perp - 2.0023)] \quad (7)$$

where $P = 2\beta_e\beta_n\gamma_{Co}(3d_{z^2}|r^{-3}|3d_{z^2})$, β_e and β_n are the electron and nuclear Bohr magneton, γ_{Co} is the gyromagnetic ratio for cobalt, and K is the Fermi contact term. For free cobaltous ion, P_0 = 0.023 cm^{-1}. On a theoretical basis $^{Co}A_\parallel$ and $^{Co}A_\perp$ should have opposite signs. Using eq 6 and 7 we obtained values of P and K for the various conformation states of ^{Co}cyt-*c* (Table I). Based on the values of P, there is about 15% delocalization of the unpaired electron to the axial ligands of ^{Co}Mb and of states II and III of ^{Co}cyt-*c*. On the other hand, P for the acidic state of ^{Co}cyt-*c* is greater than 0.023 indicating no electron delocalization or net donation of electron from the axial ligand. These differences are understandable since histidine is a π-acceptor ligand whereas methionine is not capable of accepting π electrons.

Protein Structures of Cobalt-Cytochrome c. Detailed analysis of the ^{1}H NMR spectra of ^{Co}cyt-c^+ should reveal how the protein tertiary structure is altered by metal substitution. Seven methyl groups appear to be so situated that they are subject to strong high-field shifts as also are the four porphyrin methyl groups. The comparison of the position of these resonances in ^{Co}cyt-c^+ and ^{Fe}cyt-*c* along with the assignment by McDonald and Phillips (1973) are summarized in Table II.

The agreement between the methyl resonances (peaks 5 to 25 in Table II) is remarkably good for the two molecules. These resonances are shifted by the various ring-current fields of His-18, Tyr-67, Tyr-74, Phe-82, and Trp-59 (McDonald and Phillips, 1973). In addition, the heme meso proton peaks 43, 44, and 45 are also situated at the same positions for ^{Co}cyt-c^+ and ^{Fe}cyt-*c*. Their shifts are thought to be influenced by the ring-current fields of Tyr-67 and Phe-82. Therefore, these methyl groups and meso protons in ^{Co}cyt-c^+ occupy the same spatial positions relative to the above aromatic residues as they are in the native enzyme. Conversely and by inference, the segments of the ^{Co}cyt-c^+ molecule containing residues Cys-14, Cys-17, His-18, Trp-59, Tyr-67, Tyr-74, and Phe-82 are in the native conformation.

Some of the protons of the aromatic residues which have been tentatively assigned by McDonald and Phillips (1973) also have the same shifts for the Co and the Fe proteins.

Table II: Comparison of Key ^{1}H NMR Spectral Features of ^{Co}cyt-c^+ and ^{Fe}cyt-c.

Peak no.	Chemical Shifts (ppm) ^{Co}cyt-c^+	^{Fe}cyt-c	Assignment
5	−0.75	−0.76	Leu-32 (032CD2)[a] methyl
6	−0.65	−0.65	Ile-75 (075CG1) methyl
7	−0.40	−0.45	Thr-63 methyl
9	0.29	0.30	Thioether bridge methyl
15	1.35	1.4	Thioether bridge methyl
18	2.1	2.1	Porphyrin ring methyl
24	3.52	3.4	Porphyrin ring methyl
24	3.69	3.5	Porphyrin ring methyl
25	3.88	3.8	Porphyrin ring methyl
33	6.98	6.98	Try-59 C-2
34	7.07	7.05	His-26 C-4
35	7.17	7.12	His-33 C-4
40	7.65	7.61	His-33 C-2
42	8.10	8.0	His-26 C-2
43	8.39	8.31	Heme meso protons
44	9.02	9.02	Heme meso protons
45	9.69	9.61	Heme meso protons

[a] Coordinate nomenclature of Takano et al. (1971).

These are peaks 33 to 42. However, there are unattributed aromatic protons whose shifts are different for the two molecules. The most notable ones are peaks 28, 29, and 42 in Figures 1b and 2b. There are also differences in the distribution of the remaining aromatic protons between 6.7 and 7.7 ppm. Therefore, there are structural differences between the two species either because they belong to different oxidation states or are the result of metal substitution.

Not many definitive conclusions can be drawn from the ^{1}H NMR spectra of ^{Co}cyt-c about its protein conformation due to lack of references for comparison. There is pseudocontact shift in ^{Co}cyt-c but not in ^{Co}cyt-c^+. Rein et al. (1968) reported the g values for ^{Fe}cyt-c^+ to be 3.0, 2.26, and 2.0 at pH 7 which gives the magnetic anisotropy value $(g_\parallel^2 - g_\perp^2)$ of about +4.45. The value is −0.81 for ^{Co}cyt-c. Therefore, the pseudocontact shifts for the two proteins are in opposite directions (cf. eq 3) and the shifts are greater for ^{Fe}cyt-c^+. Consequently the ^{1}H NMR spectra of ^{Co}cyt-c rather resemble more ^{Co}cyt-c^+ than ^{Fe}cyt-c^+. Comparisons of Figures 1a and 4a showed that the latter has fewer protons in zones G and F and more in zones B and A than the former.

Structure and enzymic activities. The ability of modified redox metalloenzymes such as cobalt-cytochrome c to undergo enzymic reactions is governed by three considerations. The thermodynamic requirement must be met by having a suitable $E_{m,7}$ as discussed in part I. Secondly, an electron-transport pathway must be available and finally it must have the proper enzyme binding sites. Thermodynamically, cobalt-cytochrome c should be able to react with cytochrome oxidase and some high potential cytochrome c reductase systems. Thus, the slower rate for the former reaction and the failure to react in the latter reactions must be due to the other two factors.

Various electron-transport pathways have been proposed; two of these are quite specific. In the Dickerson-Winfield mechanism (Takano et al., 1973) reduction takes place by means of a free-radical pathway (Winfield, 1965) involving the surface residue Tyr-74 and the internal residues Tyr-67 and Trp-59. An oxidation mechanism of comparable detail has not been proposed by these authors.

Alternatively, Salemme et al. (1973) proposed a mechanism in which electron addition to and withdrawal from the heme takes place by an essentially reversible reaction involving direct interaction of the electron donating or withdrawing group of the enzymes with the exposed edge of the heme of cyt-c accompanied by the perturbation of a hydrogen bond network involving Tyr-46, Tyr-48, Thr-49, Asn-52, Trp-59, Tyr-67, Thr-78, and Phe-82, with the last residue playing a key role. In the previous section, we have presented ^{1}H NMR evidence showing that the stereochemistry of Trp-59, Tyr-67, Tyr-74, and Phe-82 in cobalt-cytochrome c does not differ markedly from the native state. Furthermore, we have measured the rates of electron transfer between ^{Co}cyt-c^+ and ^{Fe}cyt-c and between ^{Co}cyt-c and ^{Fe}cyt-c^+ and found them to be comparable to those between the reduced and oxidized states of the native enzyme. Therefore, regardless of which alternative electron-transfer mechanism is closer to the truth, the observed enzymic activities of cobalt-cytochrome c do not originate from any modification of the electron-transfer pathways. The only viable explanation which remains is the alteration of the enzyme binding sites.

It is now fairly firmly established that both the oxidase and reductase bind to cytochrome c with the help of lysyl residues on the cytochrome c molecule. Poly(L-lysine) is a competitive inhibitor to the reactions (Davies et al, 1964; Mochan et al., 1973; Davis et al., 1972). Oxidase and reductase have different binding sites on cytochrome c as shown by antibody-binding studies (Smith et al., 1973, 1974).

There are altogether 19 lysyl residues in horse heart cytochrome c. With the possible exception of Lys-5 and -7, the remaining residues are all situated at the surface of the molecule. The "natural" resonance positions for lysyl protons in random coiled proteins have been given by McDonald and Phillips (1969) at 3.02 ppm (ϵ-CH_2), 1.68 (δ-CH_2 + β-CH_2), and 1.43 ppm (γ-CH_2). In the 300-MHz ^{1}H NMR spectrum of ^{Fe}cyt-c (Figure 2a), there are small peaks at 2.95 ppm (22), at 1.55 ppm (16), and at 1.35 ppm (15). They have relatively broad line widths, and these shifts do not correspond exactly with the "natural" resonance positions. There are also peaks in these vicinities in the spectrum of ^{Fe}cyt-c^+ (Figure 5a) which may be attributed to lysyl protons.

On the other hand, strong resonances are seen in the spectrum of ^{Co}cyt-c which corresponds to the "natural" proton resonance positions for lysyl residues. These are 22′ at 3.00 ppm, 16′ at 1.70 ppm, and 15′ at 1.44 ppm. The first one is particularly narrow in line width indicating short rotational correlation time appropriate for a freely rotating side-chain group. It seems that some of the lysyl residues, which are hydrogen bonded to other charged residues such as Asp, Glu, and others in the native enzyme, are now freed and may be extended into the aqueous environment. The number of such lysyl residues involved was estimated by subtracting peak 22′ in ^{Co}cyt-c from an area comparable to peak 22 in ^{Fe}cyt-c; the difference corresponds to three-four protons. Therefore, the surface modification involves one to two lysyl residues. The oxidase binding site probably involves about four lysyl residues; the most important of these are Lys-13 and Lys-22. Acetylation of these residues decreases oxidase activity by 74%; trinitrophenylation of the same residues lowers the activity to 39% (Wada and Okunuki, 1968). Of the two residues, Lys-13 is apparently more critical. Wada and Okunuki (1969) observed that a monosubstituted derivative of horse heart cyt-c trinitrophenylat-

Table III: Some Bond Lengths in Metalloporphyrin Complexes.[a]

	M–N_e[b]	M–N_a[c]
CoTPP$^+$ Im_2	1.983[d]	1.926[d]
FeTPP$^+$ Im_2	1.989[e]	1.974[e]
CoTPP pip_2	1.987[d,h]	2.436[d,h]
FeTPP pip_2	2.004[f]	2.127[f]
CoTPP$^+$$pip_2$	1.979[d,g]	2.060[d,g]

[a] All entries are average values in angstroms. [b] Metal to equatorial porphyrin nitrogen bond distance. [c] Metal to axial ligand nitrogen bond distance. [d] Ibers et al., 1974. [e] Collins et al., 1972. [f] Radonovich et al., 1972. [g] Scheidt et al., 1973. [h] Scheidt, 1974a.

ed at Lys-13 was 50% as active as the native protein in the bovine cytochrome oxidase system. Margolish et al. (1973) derivatized this residue with 4-nitrobenz-2-oxa-1,3-diazole and found the same decrease in oxidase reactivity. Our results are consistent with a change in Lys-13 which perturbs the surface charge distribution leading to a lowering of ^{Co}cyt-c's affinity for cytochrome oxidase.

Much more extensive modification must occur at the reductase binding site to account for complete inhibition of the reaction. We return now to examine the ^{1}H NMR spectrum of ^{Co}cyt-c^+ (Figure 1a). The peak 22′ is even more prominent than that for ^{Co}cyt-c. This is also accompanied by new resonances 16′ and 15′ at 1.68 and 1.43 ppm, respectively. The interpretation is the same as above except it is estimated that the environment of four to five lysyl residues on the surface of ^{Co}cyt-c^+ has been altered.

Reaction of cytochrome c with reductase is seriously impaired when one or two lysines were modified with pyridoxal phosphate (Aviram and Schejter, 1973). The location of these residues has not been pinpointed. A preliminary study of three pyridoxal phosphate containing peptides suggests Lys-5, -7, -8, -72, -73, -86, -87, and -88 as possible candidates. Some of these are likely to be the ones in ^{Co}cyt-c^+ which resonate at the "natural" positions. The extensive modification of lysyl residues in reduced cobaltocytochrome c is probably not a result of the preparation of porphyrin–cytochrome c or the metal insertion process. Iron-reconstituted cytochrome c^+ was found to react half as rapidly as the native cytochrome c^+ with cytochrome c reductase. We cannot, however, entirely discount the possibility that cobalt ion can be more deleterious than iron in the insertion process (part I). More experiments are underway to help resolve this point.

If indeed the above structural differences result from the substitution of the Fe atom with the Co atom, can one rationalize it on the basis of nuclear charges and orbital occupancies. Recently, there have been several X-ray studies of low-spin six-coordinated tetraphenylporphyrin complexes of cobalt and iron in both oxidized and reduced states. Some reported bond lengths are collected in Table III.

Let us first consider Co(III) and Fe(II) complexes which are isoelectronic and indeed isostructural for the piperidine case. There is a small contraction of the M–N_e distance and a larger decrease of the M–N_a distance for cobalt attributable to the greater nuclear charge. The axial contraction together with a possible tilting of the axial ligand with respect to the heme normal sometimes seen in low-spin cobalt complexes (Scheidt, 1974a,b) could account for the observed shift of Met-80 resonances in ^{Co}cyt-c^+ as compared to ^{Fe}cyt-c. Nevertheless we wonder how a change of 0.13 Å in the axial bond distance could result in the changes observed for the lysyl residues. The M–N_a distance is also shorter for Co(III) than Fe(III) by 0.1 Å. Compared to all other complexes, Co(II) has greatly increased M–N_a distance. This is obviously due to the presence of d_{z^2} electron. Oxidation of Co(II) would pull in the axial ligands with a total change of 0.8 Å. Oxidation of Fe(II) would cause less than one-tenth of this change. These electronic effects could be partly the sources for the observed structural modifications in cobalt-substituted cytochrome c.

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The Association of Bovine β-Casein. The Importance of the C-Terminal Region†

Gillian P. Berry and Lawrence K. Creamer*

ABSTRACT: Bovine β-casein exists in the monomer form in solution (pH 6.5, 0.1 *M* NaCl, 0.5% w/v) at low temperatures, but associates to form polymers at higher temperatures. Gel filtration chromatography at 36° showed that the polymer is large with a hydrodynamic size greater than that of a globular protein with a mol wt of 1.34×10^6. Removal of two C-terminal amino acids per molecule decreased the proportion of polymer in the solution, although the chromatographic behavior of the modified β-casein monomers and polymers was retained. Removal of a 20 amino acid peptide from the C terminus of the β-casein completely destroyed its ability to form polymers and removed the 8-anilino-1-naphthalenesulfonate binding site. However, deletion of segments of the protein from the N terminus did not decrease the ability of the modified β-casein to associate, nor did it affect the 8-anilino-1-naphthalenesulfonate binding site greatly. It seems likely that all, or some, of the 20 amino acids at the C terminus are responsible for the associative behavior of β-casein, possibly by the direct participation of their side chains in hydrophobic bond formation. However, removal of the C-terminal peptides may have disrupted the spatial structure of the native protein so that it could no longer associate normally.

The association of proteins has been a subject of interest for a considerable length of time. Studies on chymotrypsin, insulin, hemoglobin, and other crystalline proteins, for example, have shown which amino acids are at the site of association. The association of β-casein of bovine milk is reversible (Sullivan et al., 1955), being dependent on temperature and to a lesser extent on pH, ionic strength, and concentration. A further characteristic of this association is that apparently only the monomer (mol wt 24,000) and a polymer (mol wt ~600,000) co-exist (Waugh et al., 1970; Schmidt and Payens, 1972).

The monomer β-casein consists of 209 amino acids in a single chain with no cystine cross-links (Ribadeau-Dumas et al., 1972). It is a hydrophobic protein whose net charge of −13 at pH 7.0 resides in the N-terminal 50 amino acids. The remainder of the protein contains few hydrophilic residues and there is a high proportion of proline residues in the sequence. The spatial structure of the monomer protein is not known with certainty although optical rotatory disper-

† From the New Zealand Dairy Research Institute, Palmerston North, New Zealand. *Received January 28, 1975.*