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^{1}H NMR and ESR Studies of Oxidized Cytochrome c_{551} from *Pseudomonas aeruginosa*†

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ABSTRACT: Near neutral pH, Fe(III) cytochrome c_{551} exhibits an ESR absorption due primarily to a single species with g values of 3.24, 2.06, and 1.48. These g values are somewhat different from those of horse heart cytochrome c and can be interpreted by the generalizations of Brautigan et al. [(1977) *J. Biol. Chem. 252*, 574] to be due to Fe binding by the imidazole anion of histidine rather than by neutral imidazole. The NMR spectrum of Fe(III) cytochrome c_{551} exhibits a number of hyperfine-shifted peaks whose pattern shows similarities to but many differences from that of horse heart cytochrome c. Variation in shifts of some of the peaks in the pH range 5–9 is ascribed to ionization of a somewhat buried propionic acid side chain (pK = 5.8) and to ionization of the N-terminal NH_3^+ group (pK = 7.7). At alkaline pH >9.4, as shown by a variety of optical and ESR spectral changes, the Met-61 S ligand is replaced by other ligands.

The cytochrome c_{551} of the bacterium *Pseudomonas aeruginosa* (Ambler, 1963) is a primitive version of the "mammalian" cytochrome c of eukaryotes (Dickerson et al., 1976). For example, both horse heart cyt c and cyt c_{551}[1] have a single heme group covalently bound through two thioether linkages to cysteines of the amino acid chain, and both have a histidine and a methionine as fifth and sixth ligands, respectively (Almassy and Dickerson, 1978). However, cyt c_{551} has only 82 amino acids in contrast to horse heart cyt c which has 104 (cf. Table I for a comparison of properties of these two cytochromes).

Antonini and co-workers (1970) discovered a remarkable electron-transfer reaction between cyt c_{551} and a companion copper protein, azurin from *Pseudomonas*, which proceeds in each direction with rate constants $\sim 10^6$ M^{-1} s^{-1}. The redox potentials of horse heart cyt c and cyt c_{551} are both 286 mV, and that of azurin is 314 mV. We expect, therefore, that the biological role of both cyt c_{551} and azurin is as a soluble electron carrier. In order to understand the electron-transfer kinetics of these two proteins (Rosen & Pecht, 1976; Coyle & Gray, 1976), we have studied azurin by emission spectroscopy (Ugurbil & Bersohn, 1977; Ugurbil et al., 1977a), by ^{1}H NMR (Ugurbil & Bersohn, 1977), and by ^{13}C NMR (Ugurbil et al., 1977b). Toward the same goal we have now studied cyt c_{551} by ^{1}H NMR and by ESR.

Experimental Procedures

There were two cyt c_{551} preparations. One was made by Ugurbil whose isolation procedure, previously described (Ugurbil & Bersohn, 1977), is a modification of Ambler's procedure (Ambler, 1963); the other was purchased from Microbiological Research Establishment, Porton, Salisbury, U.K. The NMR sample usually had a concentration of ~3 mM with 0.1 M NaCl and 0.05 M phosphate buffer. A severalfold excess of $K_3Fe(CN)_6$ was added to ensure that cyt c was completely oxidized. NMR measurements were made on a Varian 220-MHz spectrometer. ESR measurements were made on a Varian E-9 X-band spectrometer. In the low-temperature range (<50 K) a carbon resistor was used as a thermometer. Before each electrophoresis on polyacrylamide gel the protein was boiled with 1% $NaDodSO_4$ for 3 min.

ESR Spectra and Assignments. In order to slow down the rapid spin–lattice relaxation rates, the ESR spectra were taken at ~11 K. The spectrum at pH 4.5 (Figure 1) contains contributions from four paramagnetic species. The smallest peak at g = 4.3 is due to a rhombic high-spin Fe(III) complex which has been found in a number of cytochromes (Brautigan et al., 1977) as a small impurity. The sharp peak at g = 2.07 is the envelope of the perpendicular hyperfine components of a Cu impurity whose concentration, as measured by atomic absorption spectrophotometry, is ~1% of the total protein concentration; this is probably azurin (Brill et al., 1968) which, in any case, remains with cyt c_{551} until the last stage of purification. The small peak at g = 5.74 most likely belongs to an irreversibly denatured high-spin cyt c_{551} (see the next section for details). The absorption-like peak at g = 3.24, the broad derivative peak spread over ~700 G at g = 2.06, and the small, barely visible and often undetectable shoulder at g = 1.48 are all assigned to the dominant low-spin species of cyt c_{551}. The minor high-spin components probably have greater amplitude in the observable spectrum because of their narrow line width.

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[1] Abbreviations used: cyt c_{551}, *Pseudomonas aeruginosa* ferricytochrome c_{551}; NMR, nuclear magnetic resonance; ESR, electron spin resonance; TSP, sodium 3-(trimethylsilyl)propionate.

0006-2960/79/0418-0774$01.00/0

Table I: Properties of Horse Heart cyt *c* and *Ps.* cyt c_{551}

	horse heart cyt *c*	cyt c_{551}
no. of amino acids	104	82
isoelectric point	10.4	4.7
no. of histidines	3	1
NMR peak positions of porphyrin ring methyls	34.0, 31.3, 10.3, 7.2[a]	31.9, 27.4, 15.4, 13.4[b]
NMR peak position of iron-bonded methionine methyl	−23.4[a]	−17.6[b]
NMR peak positions of heme meso protons	−4.4, −6.6[a]	−1.3, −1.6, ~−3.4[b]
g values measured by ESR	low-spin form (major): 3.06, 2.25, 1.25[c] low-spin form (minor): 3.2, 2.08[c]	low-spin form (major): 3.24, 2.06, 1.48

[a] Data from Redfield & Gupta (1972). [b] NMR and ESR data of cyt c_{551} measured at pH 4.1. [c] Data from Brautigan et al. (1977).

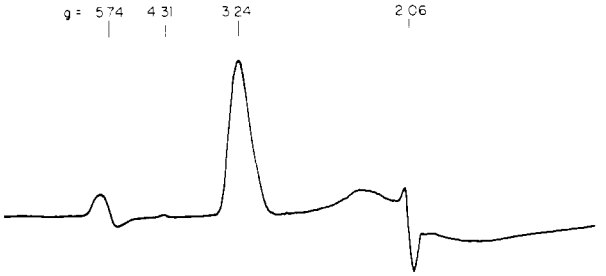

FIGURE 1: ESR spectrum of cyt c_{551} at 10.4 K, pH 4.5, protein concentration 1 mM.

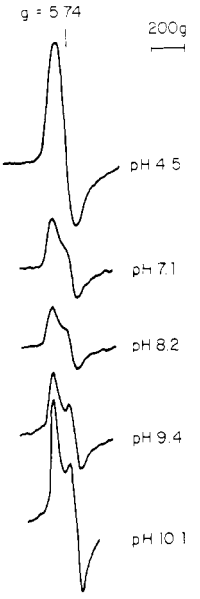

FIGURE 2: pH dependence of the cyt c_{551} low-temperature (11 K) ESR peak at $g \sim 6$; the gain and modulation amplitude settings are the same for all the measurements.

Furthermore, other things being equal, the relative ESR intensity for a transition within the $M_S = \pm^1/_2$ doublet of an $S = {}^5/_2$ manifold (the minor high spin component) varies as $3g$ while that for a transition within an $S = {}^1/_2$ system (the major component) varies as g (Aasa, 1970; Aasa & Vänngard, 1975).

When the pH was varied from 4.5 to 10.1, the main change in line shape and intensity is of the peak at $g = 5.74$. Figure 2 shows that, as the pH is increased from 4.5 to 8.2, the intensity of the axially symmetric high-spin species decreases, and a rhombic high-spin species (Peisach et al., 1971) begins to appear. With further increase of the pH from 9.4 to 10.1, there is a large increase of the rhombic signal with a concomitant decrease of the intensity of the low-spin signal at g = 3.24 (not shown in Figure 2). The g values of this new rhombic component are $g_x = 6.16$ and $g_y = 5.54$. As discussed in a later section this new high-spin species which originates from the main low-spin form can be identified as a cyt *c* in which the methionine sulfur atom has been displaced by a new iron ligand. All the ESR observable paramagnetic species and their g values are listed in Table II. The x, y, and z principal axes of the g tensor of the main low-spin species are defined in Figure 3; the specific assignments of g values to principal axes are explained in the section on NMR spectra.

Table II: Various Spin Forms of Ferricytochrome c_{551}

g values	species
$g_x = 1.48$[a] $g_y = 2.06$[b] $g_z = 3.24$	low-spin cyt c_{551} (major species)
$g_x \simeq g_y = 5.74$	high-spin Fe(III) species at pH 4.5 (minor species)
$g = 4.31$	rhombic Fe(III) species (minor impurity)
$g_{max} = 2.06$[c]	copper(II) azurin (≤1% of total protein concn)
$g_x = 6.16$ $g_y = 5.54$	high-spin Fe(III) cyt c_{551} species at 11 > pH > 8

[a] $g = 1.48$ peak is not a well-assigned peak. [b] The broad peak in Figure 1 with line width ~700 G. [c] The sharp derivative peak in Figure 1.

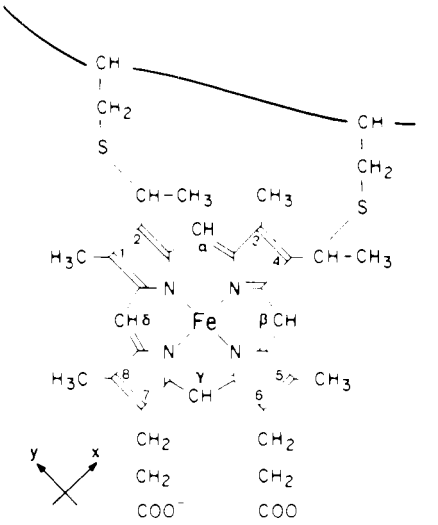

FIGURE 3: Labeling of porphyrin positions and location of principal axes x and y of the g tensor.

Gel Electrophoresis Pattern of cyt c_{551} Preparations. Figure 4 shows the polyacrylamide gel electrophoresis pattern of an $NaDodSO_4$ solution of cyt *c*. The electrophoresis pattern of

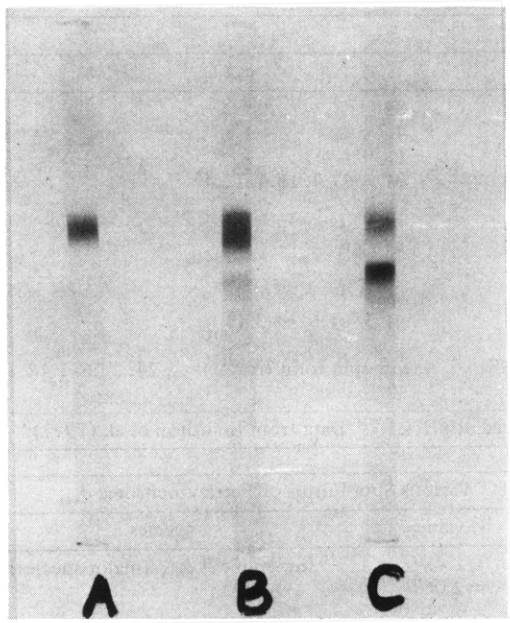

FIGURE 4: Polyacrylamide gel electrophoresis pattern of cyt c_{551}. (A) Freshly prepared, highly purified cyt c_{551}; (B) an old, extensively manipulated cyt c_{551}; (C) fresh cyt c_{551} with a small amount of azurin added.

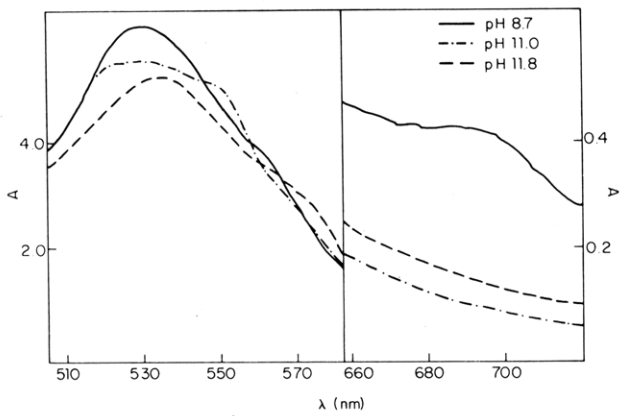

FIGURE 5: Visible spectrum of cyt c_{551}. Protein concentration, 0.53 mM; cell length, 1 cm.

the recently made commercial sample showed a sharp band with no recognizable azurin contamination (Figure 4A). Figure 4B is the gel pattern of a 3-year-old local preparation. The main protein band is much broader than that of the fresh preparation, and a small band of a high molecular weight species also appears. Moreover, the ESR spectrum of the old preparation exhibits a much higher concentration of the high-spin species with $g = 5.74$. These results suggest that the $g = 5.74$ peak comes from an irreversibly denatured cyt c species. In Figure 4B the band below the cyt c band belongs to an azurin contaminant as is confirmed by comparison of it with the pattern of azurin (Figure 4C).

pH Dependence of the Visible Spectrum of Oxidized cyt c_{551}. The visible absorption spectrum of cyt c_{551} has been reported previously (Horio et al., 1960; Fanger et al., 1967), and the band at 695 nm characteristic of the porphyrin Fe–Met S band has been shown to disappear around pH 11 (Vinogradov, 1970). In the pH range 4.5–8.0 the visible spectrum of cyt c_{551} is invariant, which is consistent with the ESR findings that, as the pH is increased in this range, the major low-spin component of the spectrum is invariant and only the minor high-spin component decreases in intensity. However, in the pH range 8.7–11.8 the visible spectrum does

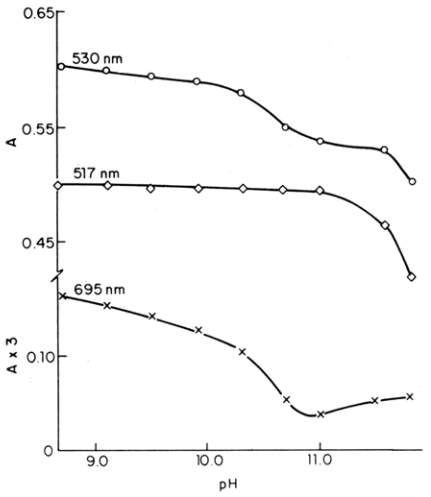

FIGURE 6: pH dependence of cyt c_{551} optical absorption at 517, 530, and 695 nm. Protein concentration, 0.53 mM; cell length, 1 cm. The scale of absorption for 695 nm is different from that at 517 and 530 nm, as shown on the left side of the figure.

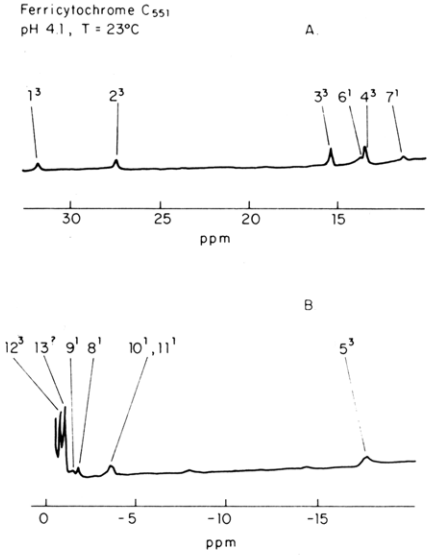

FIGURE 7: Parts of the 220-MHz NMR spectrum of cyt c_{551} at 23 °C and pH 4.1. TSP was used as an internal standard. Downfield from TSP (Figure 7A) is positive and upfield (Figure 7B) is negative. The superscript by each number indicates the number of protons per molecule contributing to each resonance.

change with pH, and indeed Figures 5 and 6 can be interpreted by the conversion of the neutral species into a new species at pH ~10.6, followed in the pH range 11.0–11.8 by conversion into yet another form. Absorption at 530 and 695 nm exhibits a pK of 10.6 (Figure 6). The pH range 11.0–11.8 is characterized by the simultaneous appearance of a new visible spectrum and a new high-spin rhombic ESR spectrum. In horse heart cyt c at pH 9.4, the S of Met-80 at the sixth position is replaced by the ϵ-NH_2 of Lys-79; in cyt c_{551} there is no corresponding lysine.

^1H *NMR Spectra and Assignments.* (*a*) *Temperature and pH Dependence of the NMR Spectrum; Methyl Group Assignments.* Low-spin ferric heme containing proteins exhibit proton resonances hyperfine-shifted to fields below or above the normal range for proteins. The individual resonances are sensitive to the nature of the iron ligands and heme structure (Shulman et al., 1971). Figure 7 shows the hyperfine-shifted peaks of the NMR spectrum of oxidized cyt c_{551} (pH 4.1, 18 °C); this spectrum has been previously reported (Keller et al., 1976). The various peaks are labeled by numbers with the

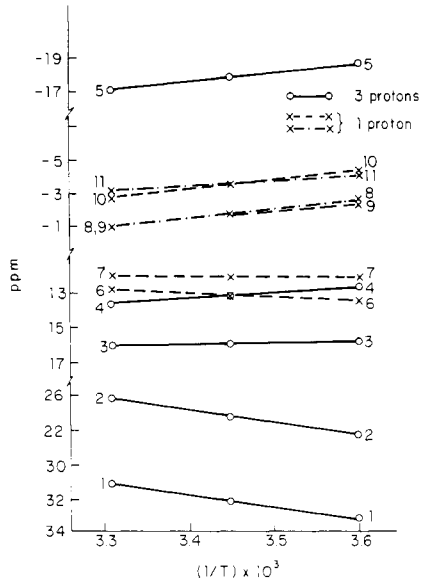

FIGURE 8: Temperature dependence of the chemical shift of various NMR peaks of cyt c_{551}. The peaks are numbered as in Figure 7.

superscripts indicating the numbers of protons in each peak. In addition, there are a few rather broad one-proton peaks in the upfield region (−5 to −15 ppm and <−17.5 ppm) which are probably due to methylene protons of Met-61.

The positions of the peaks numbered 1–13 were studied as a function of both pH and temperature (Figure 8). Five intense three-proton peaks stand out in the spectrum: peaks 1–4 at 31.9, 27.4, 15.4, and 13.4 ppm (downfield) and peak 5 at −17.6 ppm (upfield). There are just five methyl groups whose protons could feel so strongly the magnetic field of the unpaired electron, and they are in Met-61 and at positions 1, 3, 5, and 8 of the porphyrin ring. In analogy with the spectrum of mammalian cyt *c* (Redfield & Gupta, 1972; Keller and Wüthrich, 1978), peaks 1–4 are assigned to porphyrin methyls and peak 5 to the CH_3 of Met-61. Perhaps the strongest argument that can be given for this assignment is that the *g* tensor of the unpaired spin of mammalian cyt *c* has *x* and *y* principal axes passing through diagonally opposite pyrrole groups. Thus the porphyrin methyl resonances should occur in pairs. The pair structure is exhibited not only by the similar positions of 1,2 and 3,4 but also by the fact that each member of the pair shows the same temperature dependence which is quite different for the two pairs (Figure 8). We have not in fact measured the ESR spectrum of a single crystal of cyt c_{551}. However, it has been shown (Hori, 1971) that the location of the principal *g* axes is closely related to the orientation of the plane of the Fe-bonded histidine. The X-ray structures (Dickerson et al., 1976) show that the orientation of the Fe-bonded histidine with respect to the porphyrin plane is very similar in the two proteins, and, hence, we assume that the principal *g* axes are identically situated in the respective porphyrin planes. The *x* and *y* axes lie (within 5°) along the lines joining the nitrogen atoms of rings A and C and rings B and D, respectively (Figure 3). A calculation (Shulman et al., 1971) shows that the unpaired electron, although mainly on the iron atom, will have a greater density on the pyrroles lying along the *y* axis as compared to those on the *x* axis. The downfield shifts of methyl protons attached to rings B and D should therefore be larger. Peaks 1 and 2 are therefore assigned to the methyls at positions 3 and 8 and the less shifted peaks to the methyls at 1 and 5.

The positions of peak 5 (Met-61 methyl protons) and peaks 6 and 8–11 depend linearly on $1/T$ (Figure 8). The pairs of

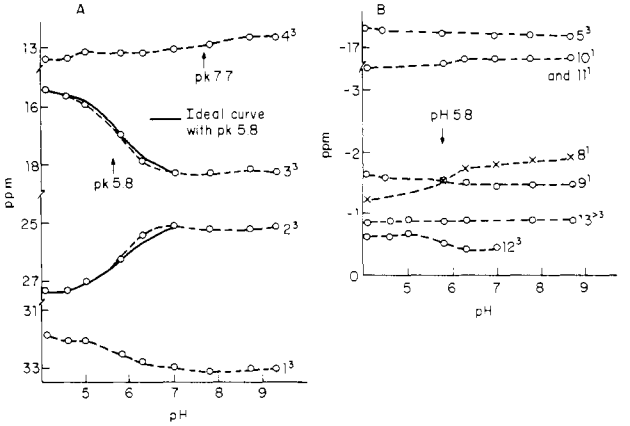

FIGURE 9: pH dependence of various NMR peaks of cyt c_{551}. Labeling of each peak is the same as in Figure 7.

peaks 8,9 (−1.3 and −1.6 ppm) and 10,11 (−3.4 ppm) are assigned to the pairs of ring meso protons α,γ and β,δ, respectively. These assignments, to be justified later on, are based on the temperature dependence, the pair structure, and a theoretical calculation of the pseudocontact shift (Shulman et al., 1971). Peak 6 at 14.08 ppm has a small but evident temperature dependence; no definite assignment to a heme proton is made. Peak 7 at 11.36 ppm has no temperature dependence and is probably due to a ring current shifted N–H proton (Stellwagen & Shulman, 1973).

The positions of some of the hyperfine-shifted peaks vary with pH with clear pK_a values of 5.8 and 7.7. At pH >9.8 they decrease in intensity with no evident broadening or shift. A titration of 3 mM cyt c_{551} in 0.1 M NaCl shows that there are only two titrable groups within the pH range 5.1–8.3 and these two groups have pK_a values of 7.6 and 5.8, consistent with the NMR findings. A terminal NH_3^+ group will usually be titrated at pH ~7.8 (Garner et al., 1973; Wilbur & Allerhand, 1977) so we can assign the titrating group with pK = 7.7 to the α-amino group of Glu-1. The other pK, 5.8 (Figure 9), is assigned to one of the two propionic acid side chains of the porphyrin. It is known that buried carboxylic acids can have pK values higher than solvent-exposed carboxylic acids by as much as 2–3 pK units (Tanford et al., 1959). Moreover, the X-ray structure of other cyt *c*'s (Timkovich & Dickerson, 1976) shows that the outer propionic acid group at position 6 is somewhat exposed to the solvent, whereas the inner propionic acid group at position 7 is buried. Thus the pK of 5.8 exhibited by both the NMR peaks and the pH meter is assigned to the propionic acid group at position 7.

Arguments were given above to show that methyl group protons attached to diagonally opposite pyrrole rings had similar spin densities. The members of the two pairs are not influenced in the same way by changes of pH (Figure 9). The titration of the propionic acid side chain is a local effect altering the shifts of only two of the ring methyls (peaks 2 and 3) and not the Met-61 methyl resonance. One of the two strongly affected resonances 2 and 3 must correspond to the methyl at position 8 ortho to the titrating group. As we already know that peaks 1 and 2 correspond to positions 3 and 8, we must assign peak 1 to position 3 and peak 2 to position 8. Peaks 3 and 4 correspond to methyls at positions 1 and 5; however, ring A is tightly held by a thioether linkage to the protein, and its methyl is therefore less likely to be affected by the titration than ring C which is on the exposed heme edge. Moreover, the amino-terminal titration is more likely to affect rings A and B to which it is closer rather than the relatively

remote C and D rings. The resonance of the CH_3 group at position 1 should therefore be unshifted at pH 5.8 and be shifted at pH 7.7. Hence we arrive at the final assignments: peaks 1–4 are due to methyl groups at positions 3, 8, 5, and 1, respectively. Actually, besides peaks 2 and 3, peak 1 also exhibits a small shift near pH 5.8, but none of these three peaks varies near pH 7.7. In contrast, peak 4, due to the deeply buried methyl at position 1, shows no pH dependence near pH 5.8 but exhibits a small titration curve at pH 7.7. We infer that the amino terminus of the protein which is solvent exposed below this pH bends back and hydrogen bonds to the rest of the protein above this pH.

(*b*) *New Proton Assignments.* The dominant hyperfine shift for low-spin Fe(III) heme meso protons is caused by the pseudocontact interaction (Shulman et al., 1971); the latter is a result of the dipolar interaction of the nucleus with an electron with an anisotropic g value. The explicit expression for the shift (Kurland & McGarvey, 1970) is

$$\Delta\nu_{pc}/\nu = [\beta^2 S(S+1)/9kTr^3]K\{[g_z^2 - \tfrac{1}{2}(g_x^2 + g_y^2)] \times (1 - 3\cos^2\Omega) + \tfrac{3}{2}(g_y^2 - g_x^2)\sin^2\Omega\cos 2\Psi\} \quad (1)$$

where β is the Bohr magneton, S, the electronic spin, is $^1/_2$, and r, Ω, and Ψ are the spherical polar coordinates of the proton in question with respect to the x, y, and z axis system whose origin is the center of the Fe atom. K is a constant, $\sim$0.75, which takes into account the fact that the g tensor is virtually isotropic when the electron is not on the Fe atom. For the meso protons which lie in the porphyrin plane, r = 4.5 Å and $\Omega = \pi/2$, but the value of Ψ is uncertain. First of all, the x and y principal axes of the g tensor lie within 5° of the N_A–N_C and the N_B–N_D axes, respectively, in horse heart cyt c (Mailer & Taylor, 1972) and, we assume, also for cyt c_{551}. If the corresponding axes were all coincident, for the methine protons Ψ would be $(2n-1)\pi/4$ (n = 1–4 for α, γ, β, and δ) and $\cos 2\Psi = 0$ leaving all four protons with the same pseudocontact shift. Substituting T = 295 K, g_x = 1.48, g_y = 2.06, g_z = 3.24, and the constants quoted above into eq 1, one finds

$$\Delta\nu_{pc}/\nu = 1.42 \times 10^{-6}\{7.28 + 3.08\cos 2\Psi\} \quad (2)$$

Letting $\Psi = (2n-1)\pi/4 + \epsilon$ where $|\epsilon| = 5°$, one obtains

$$\Delta\nu_{pc}/\nu = 10^{-6}[10.3 + (-1)^n 4.37\sin 2\epsilon] \quad (3)$$

Equation 3 predicts that the β and δ protons (n = 1,3) and also the α,γ pair (n = 2,4) will have the same pseudocontact shift but that the difference in pseudocontact shifts between the two pairs of methine protons could be 1.52 ppm (if ϵ were as much as 5°). Keller et al. (1976) found that in ferrocytochrome c_{551} the methine protons were located between 9.24 and 9.86 ppm downfield. If the effect of oxidation on the methine protons is just to switch on the pseudocontact shift, then we expect to find the methine protons in or near the range 0–2 ppm upfield.

One can go further. The theory of Shulman et al. (1971) predicts that the closer a ring carbon atom is to the y axis the larger will be its spin density. Referring to Figure 3, a rotation of the y axis away from N_B–N_D and toward the C_3–C_7 axis (a negative ϵ) would cause a larger spin density at C_3 and a smaller density at C_8. This is in accord with the fact that peak 1 from the C_3–CH_3 lies at a lower field than peak 2 from the C_8–CH_3. If ϵ is negative, then the β,δ pair will lie upfield of the α,γ pair. The β,δ pair is assigned to peaks 10,11 appearing at −3.4 ppm, the α proton to peak 9 at −1.6 ppm, and the γ proton to peak 8 at −1.3 ppm. The last assignment is made because the γ proton is located between two propionate-bearing pyrrole rings and peak 8 changes most rapidly near pH 5.8. Our assignment of peaks to meso protons differs somewhat from that of Keller et al. (1976) which is in part why the above discussion was rather detailed. Table III summarizes the assignments of the hyperfine-shifted peaks.

Table III: Assignment of Some Hyperfine-Shifted Resonances of Oxidized cyt c_{551}

label of resonance	peak position (ppm) at 22 °C, pH 4.1	assignment
1	31.9	3-CH_3 of ring B
2	27.4	8-CH_3 of ring D
3	15.4	5-CH_3 of ring C
4	13.4	1-CH_3 of ring A
5	−17.6	Met-61 CH_3
8	−1.3	heme meso proton γ
9	−1.6	meso proton α
10,11	−3.4	meso protons β,δ
12	−0.93	thioether bridge CH of ring B

Discussion

From the above results it is possible to follow the behavior of cyt c_{551} as a function of pH.

$$\text{cyt } c_{551}\text{ (low spin)} \xrightleftharpoons{pK_1 = 5.9} \underset{\text{I (>pH 4.0)}}{}$$

$$\text{cyt } c_{551}\text{ (low spin)} \xrightleftharpoons{pK_2 = 7.7} \underset{\text{II}}{}$$

$$\text{cyt } c_{551}\text{ (low spin)} \xrightleftharpoons{pK_3 = 10.6} \underset{\text{III}}{}$$

$$\underset{\text{IV}}{\text{cyt } c_{551}\text{ (high spin)}} \xrightleftharpoons{\text{pH} > 11.0} \underset{\text{V}}{\text{cyt } c_{551}\text{ (low spin)}}$$

The NMR studies were conducted on species I–III and yielded values for constants K_1–K_3. The ESR spectra detected the high-spin species IV with g_x = 6.16 and g_y = 5.54 (Table I). The optical spectra established K_3 and detected species IV and V. Basically the conversion of I to II is accompanied by an ionization of a semiburied propionic acid side chain. The conversion of II to III is accompanied by an ionization of the N-terminal NH_3^+ group. III → IV involves a replacement of the Met-61 S atom by a weak ligand. When IV is converted to V, the weak ligand is replaced by a strong ligand, probably OH^-. The interconversion of the various species I–III suggests a pH dependence of the redox kinetics of cyt c_{551}.

The kinetics of the interconversion of forms I and II can be inferred from the line widths (Figure 10) of the CH_3 protons whose shift is changing the most with pH near pH 5.8. In Figure 10 one sees that the widths of peaks 2 and 3 are greater at pH 5.8 than at 5.0 or 8.7. From the widths at pH 5.8 and the total shifts during the titration, one can by well-known methods (Carrington & McLachlan, 1967) deduce that the rate of interchange is 3×10^3 s^{-1} at pH 5.8. One can show that this line breadth is not due to exchange with the high-spin species with g = 5.74. The intensity of the ESR line of the latter decreases monotonically with pH in the range 4.5–8.3. Were the line breadth due to exchange with this high-spin species, it would be greatest at the lowest pH instead of being maximum at an intermediate pH. Also, if the exchange with the high-spin species were important, we would expect broadening also of peaks 1 and 4. There is slight broadening of peak 1 but none of peak 4, consistent with their different behavior near pH 5.8 (Figure 9).

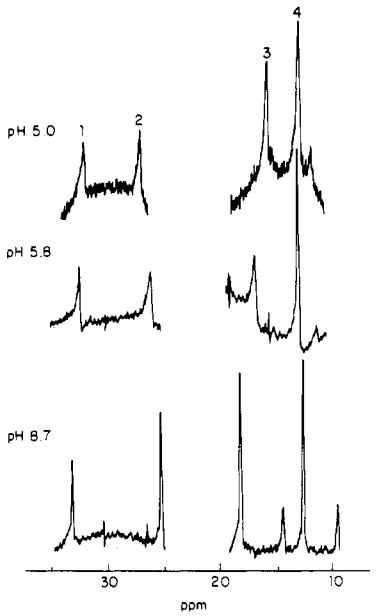

FIGURE 10: pH dependence of the cyt c_{551} NMR spectrum in the low-field region.

Our central finding is that there are substantial differences between the ESR and NMR spectra of cyt c_{551} and mammalian cyt *c*. On the other hand, the iron atom ligands are clearly the same as shown by X-ray crystallography and in other ways. Nevertheless, the spectral differences seem to be much too large to be explained by perturbations caused by differences in the amino acid sequence of two proteins. The most attractive explanation at this time is the hypothesis of Brautigan et al. (1977), namely, that the *g* values of horse heart cyt *c* reflect binding by the imidazole anion (Im^-) and neutral imidazole (Im), respectively, of histidine. If this explanation be true, there is a delicate transition between Im^- and Im binding which depends subtly on the disposition of other charged groups in these small proteins.

Acknowledgments

We thank Gloria Chu for her aid with the electrophoresis.

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