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^{1}H NMR Spectroscopy of *Paracoccus denitrificans* Cytochrome *c*-550†

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ABSTRACT: The ^{1}H NMR spectra of ferri- and ferrocytochrome *c*-550 from *Paracoccus denitrificans* (ATCC 13543) have been investigated at 300 MHz. The ferricytochrome *c*-550 shows hyperfine-shifted heme methyl resonances at 29.90, 29.10, 16.70, and 12.95 ppm and a ligand methionyl methyl resonance at −15.80 ppm (pH 8 and 23 °C). Four pH-linked structural transitions were detected in spectra taken as a function of pH. The transitions have been interpreted as loss of the histidine heme ligand ($pK \leq 3$), ionization of a buried heme propionate ($pK = 6.3 \pm 0.2$), displacement of the methionine heme ligand by a lysyl amino group ($pK \simeq 10.5$), and loss of the lysyl ligand ($pK \geq 11.3$). The temperature behavior of hyperfine-shifted resonances was determined. Two heme methyl resonances (at 16.70 and 12.95 ppm) showed downfield hyperfine shifts with increasing temperature. The cyanoferricytochrome had methyl resonances at 23.3, 20.1, and 19.4 ppm. NMR spectroscopy did not detect the formation of a complex with azide. The second-order rate constant for electron transfer between ferric and ferrous forms was determined to be 1.6×10^4 M^{-1} s^{-1}. Heme proton resonances were assigned in both oxidation states by cross-saturation and nuclear Overhauser enhancement experiments. Spin-coupling patterns in the aromatic region of the ferrocytochrome spectrum were investigated.

Cytochromes *c* are found widely distributed in all eukaryotic species as well as in most prokaryotic species. As a general trend, these cytochromes *c* show species specificity for electron-transfer reactions with coupled proteins. Notable exceptions have been electron-transport proteins from *Paracoccus denitrificans*, which have been shown to cross-react with components of the mitochondrial electron-transport chain (Smith et al., 1976; Timkovich et al., 1982). On the basis of these and other metabolic comparisons, it has been proposed that *Paracoccus* may be closely related to the prokaryote that evolved into the mitochondrion of higher organisms (John & Whatley, 1975). The respiratory electron-transport chain of *Paracoccus* has been intensively studied (John & Whatley, 1977), especially the properties of the main soluble *c*-type cytochrome, termed cytochrome *c*-550. Its crystal structure has been determined (Timkovich & Dickerson, 1976) as has its amino acid sequence (Timkovich et al., 1976; Ambler et al., 1981). Detailed investigations have been made of the kinetic properties and immunological cross-reactions (Kuo et al., 1983) of this protein.

The purpose of this paper is to extend the structural characterization of this cytochrome by ^{1}H NMR spectroscopy. ^{1}H NMR is a valuable technique to monitor the electronic environment in the vicinity of the heme, because the hyperfine chemical shifts induced by the iron paramagnetism of the ferric state are highly sensitive to distances and orientations (the pseudocontact interaction) and electron-density distribution (the contact interaction). In this regard, it compliments X-ray crystallography, which is insensitive to the distribution of outer shell electrons. In the ferrous state, high-field NMR detects narrow lines of heme and amino acid side-chain protons. Precise chemical shifts, line widths, and, in some cases, spin-coupling patterns provide information on conformation. For example, a detailed NMR comparison of horse and tuna cytochromes *c* (Moore & Williams, 1980b) has elucidated fine structural differences in the solution state not readily apparent in the respective crystal structures. Prokaryotic cytochromes *c* show greater structural diversity among themselves and in comparison to eukaryotic cytochromes than is found among just eukaryotic cytochromes considered as a subclass. As additional prokaryotic cytochromes are studied by ^{1}H NMR, a library of spectra and assignments is growing. This expanding library will create new avenues for the comparison of protein structures.

Materials and Methods

Paracoccus denitrificans (ATCC 13543) was cultured and cytochrome *c*-550 was isolated as described by Scholes et al. (1971). A final chromatography step on hydroxyapatite was included in the purification (Ambler et al., 1981). The purity ratio (absorbance at the Soret maximum to absorbance at 280 nm for the oxidized form) of the final material was 5.2. This is equivalent to the value that may be calculated from the spectrum of pure cytochrome given in Scholes et al. (1971). In some experiments, material with a purity ratio of 4 was employed. Spectra of this material did not differ in the critical spectral regions of interest from fully purified cytochrome. Samples for NMR were dialyzed vs. 50 mM ammonium bicarbonate, pH 7.8, lyophilized, and redissolved in deuterated buffer. This solvent was 99.8% deuterium oxide, 10 mM potassium phosphate, and 100 mM sodium chloride, adjusted to the appropriate pH. Values labeled pH* represent the direct reading of a glass combination electrode in deuterium oxide after the electrode had been calibrated in protic reference solutions. Adjustments of pH for titration studies were made by the addition of aliquots of ^{2}HCl or NaO^{2}H. Potassium cyanide and sodium azide were added to protein samples from concentrated stock solutions in ^2H_2O adjusted to the same pH* as the protein. Cytochrome reduction was accomplished by the addition of a minimum amount of solid sodium dithionite.

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Table I: Resolved Resonances of *Paracoccus* Ferricytochrome *c*-550

label	chemical shifts[a]				area (protons)	T_1 (ms)	temp coeff[c]	
	pH* 4.0	pH* 8.0	pH* 10.5	horse *c*[b]			*a*	$b \times 10^{-3}$
a	29.50	29.90	29.90	35.29	3	49.0	17.47	3.68
b	29.65	29.10	29.00	32.39	3	49.0	14.17	4.42
c	25.05	25.80	25.80	24.06	1	~1.0	-13.76	11.71
d	16.95	16.70	17.00	9.98	3	69.0	24.44	-2.29
e	14.95	15.20	15.20	14.49	1	25.0	-12.27	8.13
f	13.30	12.95	12.95	7.20	3	77.0	24.61	-3.45
h	12.45	12.50	12.50	12.55				
i	13.75	11.95	11.95		~1	63.0		
j	11.60	11.80			~1	63.0	13.22	-0.42
k	9.60	9.60	9.60		1	49.0	-2.49	3.58
l	-0.35	-0.35	-0.35		~1	90.0	-0.35	0
m	-0.55	-0.55	-0.55		~3	34.0	-1.87	0.39
n	-0.60	-0.65	-0.65	-0.60	~1	115.0	-0.65	0
o	-1.00	-1.05	-1.10	-0.94	~1	64.0		
p	-1.20	-1.20	-1.20		~1	97.0	3.26	-1.32
q	-1.85	-2.15	-2.25	-2.72	2	25.0	2.85	-1.48
r	-2.50	-3.55	-3.55	-4.26	1	8.0	35.03	-11.42
s	-15.00	-15.80	-15.80	-24.29	3	~4.0	27.04	-12.68
t	-19.20	-24.25	-24.25		1	~2.0	0.04	7.19
u	-24.10	-25.15	-25.10	-28.00	1	~3.0		
1			22.7		3			
2			21.4		3			
3			18.8		3			
4			16.5					
5			12.6					
6			11.2					
7			10.8					
8			10.3					
9			-2.9					
10			-4.2					
11			-5.0					

[a] Chemical shifts are in ppm from DSS. No entry in any column means the data were unobservable or unreliable, usually as a result of overlap with other resonances. [b] Comparative values of chemical shifts for horse ferricytochrome *c* at pH* 8 as determined in this study. Resonances are matched with the homologous *Paracoccus* resonances. Homology was judged on the basis of similarity in chemical shift, area, and line width. [c] For all resonances, the observed temperature behavior between 280 and 307 K could be fit within experimental error to a function linear in reciprocal temperature. The coefficients reported correspond to the best fit to $\omega = a + b/T$, where ω is the chemical shift in ppm from DSS, T is the temperature in K, and a and b are the best fit parameters. In principle, a should represent the chemical shift extrapolated to infinite temperature. However, because of the limited temperature range explored, it is only appropriate to consider it as an empirical parameter that allows one to calculate shifts in the range of 280–307 K. For resonances downfield of DSS, a positive value of the slope parameter b is expected for resonances following simple Curie's Law behavior. For resonances upfield of DSS, a negative slope is expected for Curie's Law behavior.

Reduced samples were sealed in 5-mm NMR tubes under an argon atmosphere. Partially reduced samples were prepared by splitting a protein sample into two portions, reducing one portion with dithionite, shaking it in air to eliminate excess dithionite, recombining the portions, deoxygenating under alternate cycles of argon and vacuum, and sealing the tube under argon. Percent reduction was confirmed by comparing the relative intensities of the oxidized-state heme methyl resonances and the reduced-state meso proton resonances. In some experiments, the mixed sample was transferred to optical cells of 1-mm path length and the percent reduction further confirmed by optical spectroscopy.

Spectra were obtained on a Nicolet spectrometer operating at 300 MHz in the Fourier-transform mode and equipped with a variable-temperature probe. Measurements of T_1 relaxation times were accomplished by an inversion–recovery pulse sequence with alternating transmitter phases. Spectra of the ferrocytochrome were resolution enhanced by application of a rising–falling double-exponential apodization weighting function to the free-induction decay. These latter two techniques are standard features of the Nicolet software. Cross-saturation of resonances between the ferrous and ferric forms followed the technique of Redfield & Gupta (1971). Measurements of nuclear Overhauser enhancements (Keller & Wutrich, 1978a,b) and *J*-modulated spin decoupling (Campbell & Dobson, 1975) have been described. Peak areas were measured by cutting and weighing plotted spectra or by the Nicolet software. Chemical shifts are reported in parts per million from sodium 4,4-dimethyl-4-silapentanesulfonate (DSS). Horse cytochrome *c* from heart muscle was obtained from the Sigma Chemical Co. and was used without further purification. The NMR spectrum of horse cytochrome *c* was obtained under identical conditions used for the *Paracoccus* cytochrome to facilitate comparisons. The horse cytochrome *c* spectrum was essentially identical with previously published results (Wuthrich, 1969; McDonald et al., 1969; Redfield & Gupta, 1971). Chemical shifts for horse cytochrome *c* mentioned in comparisons were taken from our spectra.

Results and Discussion

Ferricytochrome Spectra. The structure of the heme prosthetic group and a conventional numbering scheme are given in the insert to Figure 1. The spectrum of *Paracoccus* ferricytochrome *c*-550 is shown in Figure 1 and resolved resonances are listed in Table I. The three proton resonances labeled a, b, d, and f are assigned to heme methyl substituents on the basis of their intensity and by chemical shift homology to other cytochromes. The pattern of these heme methyl resonances is qualitatively similar to that observed in horse cytochrome *c* (see Table I) in that the four methyls are grouped into two pairs with one pair further downfield. This has been interpreted (Redfield & Gupta, 1971) for horse cytochrome *c* as due to an asymmetric distribution of unpaired electron density with greatest density on two of the four heme pyrroles. Qualitatively, the resonance pattern of heme methyls in *Paracoccus* c-550 would indicate that in this cytochrome,

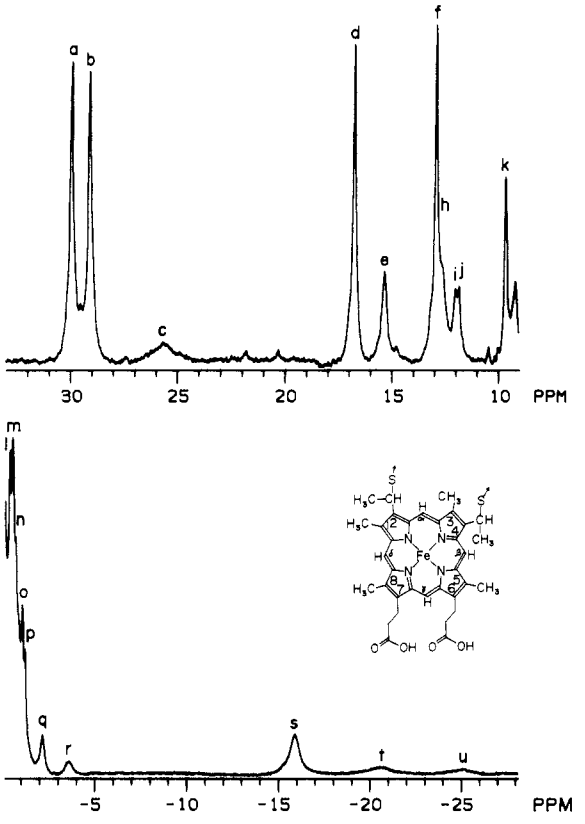

FIGURE 1: ^{1}H NMR spectrum of the extreme downfield and upfield regions of *Paracoccus* ferricytochrome c-550. The sample was 1 mM in 99.8% deuterium oxide containing 10 mM sodium phosphate and 100 mM sodium chloride at pH* 7.6, 23 °C. Peaks are labeled for subsequent discussion in the text and tables. The insert shows the structure of the heme prosthetic group of *Paracoccus* cytochrome *c*-550. Meso protons and pyrrolic substituents are labeled according to a conventional scheme. The arrows from the sulfur atoms indicate attachment to the remaining portion of the cysteine residues. In this orientation, the axial histidine is below and the axial methionine is above the plane of the ring.

unpaired density was also asymmetrically distributed but to a slightly lesser extent than that in horse cytochrome *c*. Although the qualitative pattern is similar, the exact shifts have significant quantitative differences. The most striking are resonances labeled d and f in Table I, where there are relative differences of 7.02 and 5.75 ppm, respectively. Ferricytochromes *c* that have been studied by NMR do show a range of shifts for these heme methyl resonances. Excluding *Alcaligenes facaelis* ferricytochrome *c*-554, which may be a special case (Timkovich & Cork, 1984), a survey of cytochromes indicates that the most downfield pair of methyl resonances falls between 38 and 27 ppm and the relatively upfield pair between 17.5 and 7.2 ppm. The relatively upfield pair in horse cytochrome *c* at 9.98 and 7.20 ppm corresponds more closely to comparable resonances found in *Crithidia oncopelti* cytochrome *c*-557 at 12.9 and 8.6 ppm (Keller et al., 1973) than to the *Paracoccus* resonances d and f at 16.70 and 12.95 ppm, while these correspond more closely to a pair in *Thermus thermophilus* cytochrome *c*-552 at 17.5 and 13 ppm (Hon-Nami et al., 1980). The precise structural origins for these shifts remain obscure, but the observations highlight the fact that the hyperfine shifts are extremely sensitive to small structural changes.

Other hyperfine-shifted resonances in *Paracoccus* *c*-550 bear a similarity to resonances observed in horse cytochrome *c* (see Table I). The basis for the similarity is comparable, but not equivalent, chemical shifts and approximately equal areas. Such data are suggestive, but not conclusive. Speculative assignments have been given for some of these (Redfield & Gupta, 1971). It is interesting to note that *Paracoccus* *c*-550 contains homologous residues in its sequence corresponding to the assignments proposed by Redfield & Gupta (1971) for horse cytochrome *c*. This adds some credence to the proposed assignments, but one could not claim rigorous proof. (The cited reference contains an unfortunate error in discussing an assignment to protons of Val-32. Position 32 is a leucyl residue in both horse and *Paracoccus* cytochromes. This is a heme contact residue in both proteins.) The observed similarities in the hyperfine-shifted spectra of horse cytochrome *c* and *Paracoccus* *c*-550 point toward homology in structure and electronic properties.

This conclusion was also reached by a comparison of the X-ray crystal structures of the proteins (Timkovich & Dickerson, 1976). The present NMR comparison underscores the similarities and indicates that the homology would extend to the solution state and to the precise electronic environment about the heme. The crystal structures of cytochrome c_2 from *Rhodospirillum rubrum* and to a lesser extent that of cytochrome *c*-551 from *Pseudomonas aeruginosa* also show similarities to horse cytochrome *c*, yet their hyperfine NMR spectra are quite distinctive from that of horse cytochrome *c* or *Paracoccus* *c*-550. So the NMR correspondence between horse and *Paracoccus* cytochromes could not have been predicted solely on the basis of the crystal structures.

The effects of temperature on the spectrum of ferricytochrome *c*-550 are summarized in Table I. Most of the resonances move with temperature in a manner consistent with Curie's law. Some of the resonances, d, e, and j, move downfield with increasing temperature. Similar abnormal behavior has been noted for select resonances in other cytochromes (Wutrich, 1969; Smith, 1979; Chao et al., 1979). No universally accepted explanation exists for this behavior.

Spin–lattice relaxation times for resolved resonances are reported in Table I. Certain similarities may be found to data from horse cytochrome *c* (Redfield & Gupta, 1971), although there is far from quantitative agreement. In principle, these relaxation times contain information about conformational freedom and distances to other spin centers, but to date, such interpretations have not been made. The difficulty has been assessing the relative importance of different mechanisms, especially electron–dipole and dipole–dipole, to the total relaxation rate. Nevertheless, short T_1's indicate proximity to the paramagnetic heme iron with smaller values indicating shorter distances. By this criterion, resonance c should be among the closest protons to the heme iron in *c*-550. From the crystal structure, the closest proton is the C_2 H of the fifth ligand histidine. A broad peak at ca. 25 ppm is observable in a variety of cytochromes that have histidine as a sixth ligand. These include horse cytochrome *c* at 24.1 ppm, *R. rubrum* c_2 at 24.5 ppm [Figure 5 in Smith & Kamen (1974)], and *Pseudomonas* *c*-551 at 25.1 ppm (observable only in inversion–recovery spectra because of overlap with another resonance; R. Timkovich, unpublished data). It is reasonable that the histidine C_2 proton would have a nearly constant chemical shift in these cytochromes, since the ligand geometry has been shown by crystallography to be highly conserved. Further correlations and assignment evidence are necessary to unambiguously identify this peak, but the present hypothesis is consistent with available data. This resonance could become a valuable marker for histidine coordination in other cytochromes.

Spectra of the ferricytochrome obtained as a function of pH* have revealed pH-linked structural transitions. System-

atic titration studies were performed only in 10 mM phosphate buffer with 100 mM NaCl, but spectra at critical values of pH* were checked in solutions buffered with 50 and 5 mM phosphate without NaCl. No ionic strength effects were detected. Below pH* 4, peaks broadened and decreased in intensity. Because of the poor quality of spectra, an accurate transition pK could not be measured, but an approximate estimate would be a p$K \leq 3$. This transition presumably reflects an acid isomerization coupled to the loss of histidine coordination as has been observed in mammalian cytochromes *c* [Dickerson & Timkovich (1975) and references cited therein]. At high pH* values, above 8.5, spectra showed decreases in intensity, especially for the heme and methionine methyls, and the appearance of new resonances listed in Table I. At still higher values of pH*, there was loss of intensity for all resonances and disappearance of the hyperfine-shifted spectrum by pH* 12. These effects are interpreted in terms of two alkaline isomerizations with pK transition values separated by less than 2 pK units. The intensity decrease of the heme methyls indicated a transition pK of 10.5. This value correlates with a commonly observed alkaline isomerization in other cytochromes in which the sixth ligand methionine is replaced by another strong field ligand, most likely a lysine amino group (Dickerson & Timkovich, 1975; Smith, 1979; Chao et al., 1979). The new resonances that appeared at 22.7, 21.4, and 18.8 ppm are believed to be heme methyls in the lysine-ligated form. Their maximum intensity never attained three protons per mol of cytochrome, but it is believed that this was due to the onset of the second alkaline transition in which all intensity was lost. Between pH* 8.5 and 10.5, the increase in intensity of the new resonances approximately paralleled the decrease of the normal heme methyl resonances. The second alkaline transition was estimated to have p$K \geq$ 11.3. This transition may represent loss of the lysine ligand (Dickerson & Timkovich, 1975).

Another structural transition was detected near neutrality. This had more subtle effects on the spectrum, affecting only chemical shifts but not intensities. Chemical shifts as a function of pH* are shown in Figure 2 for select resonances and summarized in Table I for all resolved resonances. The shift changes were fit by a one-proton titration curve with a transition pK of 6.3 ± 0.2. This change may be the deprotonation of a heme propionic acid side chain (Moore et al., 1980).

Ferricytochrome *c*-550 was not stable when stored in its NMR buffer at 4 °C, although the ferrous form appeared quite stable and could be stored for months without detectable changes. The cyano complex of the ferricytochrome was examined in the presence of 100 mM KCN at pH* 7.7, 23 °C. The hyperfine-shifted methionyl methyl resonance at −15.80 ppm was absent in the spectrum of the cyano complex. Methyl resonances were detected at 23.3, 20.1, and 19.4 ppm. Single proton resonances were detected at 24.5 and 12.5 ppm. NaN_3 at a concentration of 100 mM, pH* 7.6, 23 °C, had no effect on the native ferricytochrome spectrum. Although both are strong field ligands, it has been observed for eukaryotic cytochromes that the association constant for cyanide may be much larger than that for azide (Margoliash & Schejter, 1966; Gupta & Redfield, 1970).

Mixed Oxidation States. Mixtures of ferric and ferrous cytochrome *c*-550 gave NMR spectra that were the simple superposition of a pure ferric and a pure ferrous component. The rate of electron self-exchange in mixed oxidation state samples was determined by the method of Gupta et al. (1972). The bimolecular rate constant at 25 °C, pH 7.5, in 100 mM

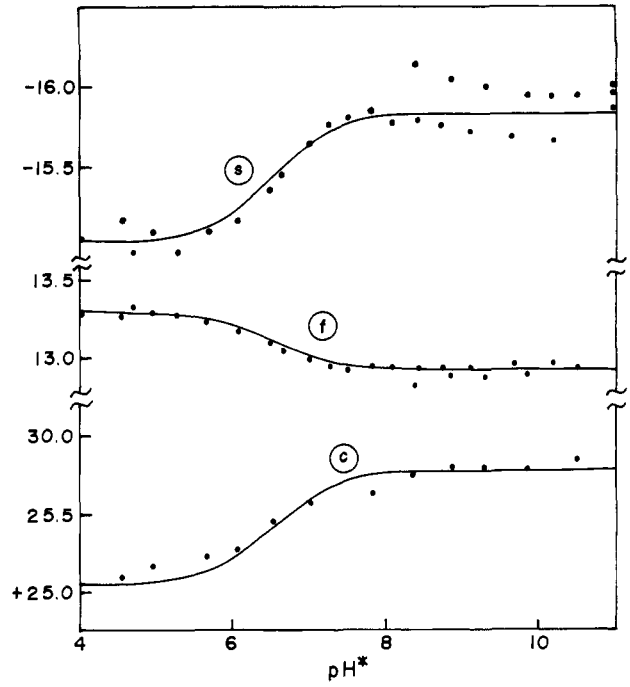

FIGURE 2: Effect of pH* on chemical shifts in *Paracoccus* ferricytochrome *c*-550. All spectra were obtained at 23 °C. Resonance labels correspond to Figure 1 and Table I. For clarity, only three select resonances are plotted. Other effects are summarized in Table I and supplementary Figure S2. Data points are indicated by circles; the solid lines correspond to theoretical one-proton titration curves with pK = 6.3.

NaCl was 1.6×10^4 M^{-1} s^{-1}. This value may be compared to other electron-transfer rates reported for cytochromes. Self-exchange rates of 2×10^3 M^{-1} s^{-1} (30 °C, pH 7, 0.1 M KCl) and 2×10^4 M^{-1} s^{-1} (30 °C, pH 10, 0.1 M KCl) have been reported for horse cytochrome *c* (Gupta et al., 1972) and 1×10^2 M^{-1} s^{-1} (40 °C, neutral pH, 0.1 ionic strength) for *Candida kursei* cytochrome *c* (Gupta, 1973). The self-exchange rate of cobalt-substituted cytochrome *c* has been reported to be less than 1.33×10^2 M^{-1} s^{-1} (25 °C, pH 7, 0.1 M NaCl; Chien et al., 1978). Electron transfer in a complex of horse cytochrome *c* and ferrocyanide has been reported to be 2.08×10^4 s^{-1} (25 °C, neutral pH, 0.1 M ions) (Stellwagen & Shulman, 1973). These rates determined by NMR techniques may be contrasted to rate constants directly determined by stopped-flow kinetics for reactions such as the reduction of horse cytochrome *c* by ferrous EDTA (ethylenediaminetetraacetic acid) (2.57×10^4 M^{-1} s^{-1}, 25 °C, pH 7, 0.1 M NaCl; Hodges et al., 1974). Not all cytochromes possess rates on this order. The self-exchange rate for *Pseudomonas aeruginosa* cytochrome *c*-551 has been measured to be 1.2×10^7 M^{-1} s^{-1} (42 °C, pH 7, 0.05 M phosphate; Keller et al., 1976), and the rate of *Alcaligenes faecalis* cytochrome *c*-554 has been estimated to be 3×10^8 M^{-1} s^{-1} (40 °C, pH 8, 0.1 M NaCl; Timkovich & Cork, 1984).

In mixed oxidation state samples, cross-saturation experiments (Redfield & Gupta, 1971; Keller & Wutrich, 1978a) were performed to map the positions of the ferric hyperfine-shifted heme methyl resonances to their corresponding position in the ferrous cytochrome. It proved possible to correlate all four heme methyl resonances; the results are summarized in Table II. *Paracoccus* *c*-550 shares with horse cytochrome *c* an unusual shift of one of the ferrous heme methyl resonances: 2.27 ppm in *c*-550 and 2.19 ppm in horse cytochrome *c*. The primary position for methyl substituents on diamagnetic heme porphyrins is expected to be between 3 and 4 ppm (Scheer & Katz, 1975), and indeed, other cytochromes follow

Table II: Assigned Resonances of *Paracoccus* Ferrocytochrome *c*-550

assignment[a]	chemical shift[b]
Met-100	
C_β	−1.32
C_β	−3.47
C_γ	−1.50
C_γ	−2.46
S–CH_3	−2.84 (−15.80)
Thr-101 C_γ	−1.52
meso	
α	9.15
β	9.61
γ	9.54
δ	9.10
heme methyl	
1	3.47 (12.95)
3	3.27 (29.10)
5	3.57 (16.70)
8	2.27 (29.90)
2-methine	5.34
4-methine	6.36
2,4-methyl	2.35
Trp	
C2	7.37 or 6.70
C4/C7	7.29
C7/C4	7.21
C5/C6	5.64
C6/C5	5.25
Phe-102	7.56
unknown aromatic[c]	7.68, 7.07
unknown aromatic[c]	7.23, 6.97
unknown aromatic[c]	7.02, 6.87

[a] Amino acid numbering scheme is that of Ambler et al. (1981). [b] Chemical shifts are in ppm from DSS. Values in parentheses for select resonances are the corresponding shifts in the ferricytochrome. [c] These shifts correspond to coupled multiplets in the aromatic region that were uncovered in spin-decoupling experiments. It was not possible to determine the full coupling pattern to other potentially coupled spin systems, so that assignment to a particular type of aromatic side chain was not accomplished.

this expectation (Keller & Wutrich, 1978a; Ulrich et al., 1982). The primary position in porphyrins is downfield of the usual aliphatic methyl location because of the large ring current of the prophyrin plane. In following paragraphs, evidence will be presented that assigns the 2.27 ppm resonance of *c*-550 to the 8-substituent position. The 2.19 ppm resonance in horse cytochrome *c* has also been assigned to the 8 position. The chemical shift homology and the homology between the crystal structures of these two cytochromes afford a rationalization for the observed shifts. In both structures, the 8-methyl is located above the plane of a tryptophan residue, Trp-59 in the horse cytochrome *c* sequence and Trp-71 in the *c*-550 sequence. The orientation is such as to produce an upfield shift due to the Trp aromatic ring current. This tryptophan is invariant in all *c*-type cytochromes of known sequence including *Euglena* *c*-552 and *Pseudomonas* *c*-551, which do not demonstrate a relatively upfield heme methyl resonance (Keller & Wutrich, 1978a; Schejter & Wutrich, 1980). A tipping of the orientation of the heme tryptophan plane or a small displacement in these cases could obliterate the upfield-shift contribution from the tryptophan ring. The crystal structure of *Pseudomonas* *c*-551 indeed shows that the analogous tryptophan, Trp-56 in *Pseudomonas* *c*-551 numbering, is displaced relative to the orientation in horse or *Paracoccus* cytochromes so that it should contribute no ring current effect to any heme methyl. The crystal structure of *Euglena* *c*-552 is not known, but the NMR correlation proposed here would predict that its tryptophan analog is oriented in a conformation distinct from that of horse or *Paracoccus* cytochromes.

Ferrocytochrome c-550: Assignment of Heme Resonances. In deuterium-exchanged ferrocytochrome *c*-550, resonances with an area corresponding to four protons are observed between 9 and 10 ppm. These are assigned to the meso protons on the basis of homology with other cytochromes and porphyrins. A resonance representing less than one proton was observed at 10.7 ppm and is tentatively assigned to a slowly exchanging amide proton. The chemical shifts of the meso protons were somewhat sensitive to temperature. At 20 °C, four resonances were observable at 9.63, 9.54, 9.15, and 9.10 ppm. At higher temperatures, the line widths of all ferrocytochrome resonances became sharper, but the meso protons overlapped. At 60 °C, the meso spectrum consisted of two nonresolved peaks of two protons each at 9.60 and 9.14 ppm. Because of this behavior, nuclear Overhauser enhancement (NOE) experiments (vide infra) were performed at 20 °C, while other investigations of the ferrocytochrome spectrum took advantage of the narrower lines at 60 °C.

The assignment strategy for ferrocytochrome heme resonances was to observe negative NOE's between the meso protons and neighboring heme protons (see Figure 3). The logic behind this structural analysis and successful precedents have been given by previous workers (Keller & Wutrich, 1978a; Ulrich et al., 1982). The present discussion will focus on results for *Paracoccus* *c*-550. The validity of assignments deduced by these techniques may be questioned, because they rely solely on NOE data and chemical shift homology to other cytochromes. Definitive, rigorous assignments could require further data, such as selective deuteration of specific sites. Such companion studies have been beyond the scope of this paper and other investigations of cytochrome ^1H NMR. Fortunately, the magnitude of the negative NOE's observed in these small cytochromes can be quite large. Magnitudes on the order of 50–60% have been reported previously for irradiation of a meso proton and observation of nearby heme protons (Keller & Wuthrich, 1978a,b). In less favorable cases, when the rate of relaxation of the detected resonance may be large or when there may be efficient magnetization transfer to other spin systems neither irradiated nor detected, much smaller NOE's on the order of 5% may be observed (Keller et al., 1980). Observed magnitudes for the present study are given with the appropriate figure.

From the cross-saturation experiments, heme methyl substituents had been observed at 2.27, 3.27, 3.47, and 3.57 ppm but were not yet assigned to a specific position. Selective irradiation at 9.54 ppm did not produce appreciable NOE's to any resonance, in particular to none of the ring methyls. This is evidence that the 9.54 ppm peak is the γ-meso proton. Irradiation at 9.10 ppm gave a strong enhancement at both 3.47 and 2.27 ppm, supporting the assignment of the meso proton as δ and these as the ring methyls 1 and 8, but without identifying which was which. A small enhancement was also noted at −2.88 ppm, the chemical shift of the methionyl methyl. In the crystal structure, this group is 4.09 Å from the δ proton, 4.41 Å from the α proton, and at least 6 Å from the next nearest meso proton. This lends credence to the δ assignment. Irradiation at 9.15 ppm gave an enhancement at 3.27 ppm, correlating with only one of the ring methyls. This indicates that the meso proton may be either α or β and the ring methyl either 3 or 5. Additional enhancements were observed at 7.56, 5.34, 2.33, and 1.62 ppm, and a very weak effect was observed at −2.84 ppm. The latter would suggest that the α meso has been irradiated, but the effect was only marginally observable (<5%) and was not taken as sufficient evidence for assignment. Irradiation at 9.61 ppm gave an enhancement at 3.57 ppm

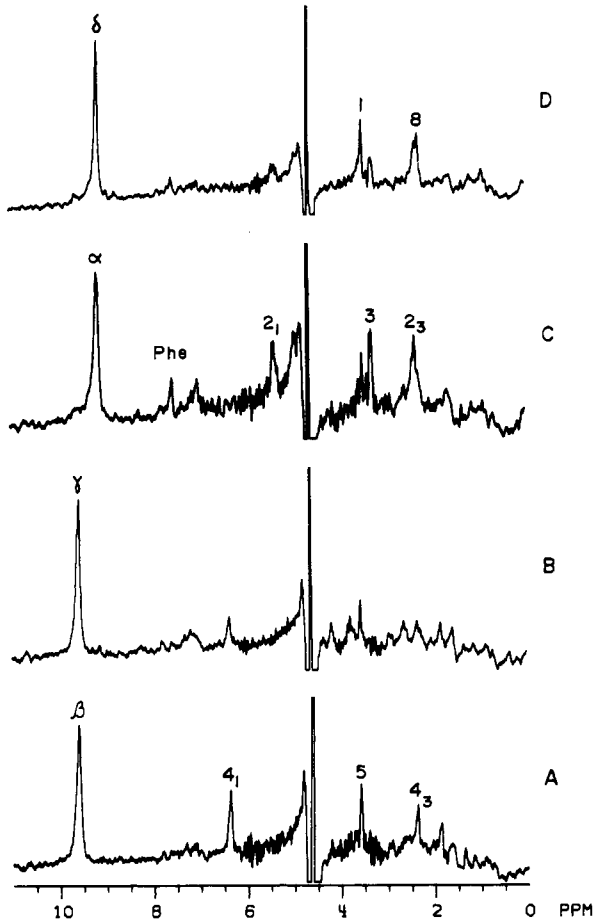

FIGURE 3: Nuclear Overhauser enhancements produced by preirradiation of the heme meso protons in *Paracoccus* ferrocytochrome *c*-550. Experiments were conducted at 23 °C, pH* 7.6. When one considers these experiments, it should be stressed that the meso protons are only partially resolved for the pair at 9.61 and 9.54 ppm and the pair at 9.15 and 9.10 ppm. Since irradiation is never perfectly selective for such overlapping resonances, weak NOE's due to the nearest-neighbor meso proton are present in some spectra. The dominant NOE's to be discussed were judged to be the primary effects from their magnitude, from experiments involving different combinations of irradiation power and duration, and from experiments in which the irradiation was moved partially off-resonance. All observed NOE's were negative, and magnitudes reported in parentheses were determined by the relative area of the difference peaks compared to the area of the meso protons, which were taken as internal standards, each corresponding to one proton. The reported magnitudes may be inaccurate because of noise in the difference spectra, overlap with other small peaks, and effects of spin diffusion. (A) Preirradiation of the meso proton at 9.61 ppm (labeled β) produced a negative enhancement at 6.36 (labeled 4_1, corresponding to the 4-methine substituent, 40%), at 2.35 (labeled 4_3, corresponding to the 4-methyl substituent, 9%), and at 3.57 ppm (labeled 5, corresponding to the heme 5-methyl substituent, 12%). (B) Preirradiation of the meso at 9.54 ppm (γ) did not produce any new enhancements. (C) Preirradiation at 9.15 ppm (α) produced enhancements at 5.34 (2_1, 38%), 2.35 (2_3, 13%), 3.27 (3, 14%), and 7.56 ppm (Phe, 20%). (D) Preirradiation at 9.10 ppm (δ) produced new major enhancements at 3.47 (1, 20%) and 2.27 ppm (8, 19%).

correlating with one ring methyl and suggesting the meso as β or α and the ring methyl as 5 or 3. Additional enhancements were observed at 6.36, 2.35, and 1.84 ppm, but nothing at −2.84 ppm.

The protons observed at 5.34 and 6.36 ppm upon irradiation at the α and β meso candidates are possible thioether bridge methine protons because of their chemical shifts and correlation with corresponding methine protons in other cytochromes (Keller & Wutrich, 1978b; Ulrich et al., 1982). Irradiation at 5.34 ppm gave an enhancement at 9.15, 7.56, and 2.35 ppm. Irradiation at 6.36 ppm gave an enhancement at 9.61, 6.36, and 2.34. The respective effects at 9.15 and 9.61 ppm are the reciprocal NOE's expected for the corresponding meso protons. It is significant that the resonance at 2.35 ppm has appeared upon irradiation at both the α- and β-meso protons and both bridge methine protons. The thioether bridge methyl groups are expected to have a chemical shift in this range (Keller & Wutrich, 1978b). In previous ferrocytochromes, the bridge methyls have had distinguishable chemical shifts. The best interpretation of the *c*-550 data is that in this case both groups have the same shift. Irradiation at 2.35 ppm gave a NOE at 3.27 ppm (previously assigned as either ring methyl 1 or 8). The first effect is attributed to a NOE between bridge methyl 4 and ring methyl 3 and thus would assign 3.27 ppm as ring methyl 3. By correspondence, ring methyl 5 would be assigned to 3.57 ppm, the α meso to 9.15 ppm, and the β meso to 9.61 ppm. The second effect is attributed to a NOE between bridge methyl 2 and ring methyl 1. Thus, 3.47 ppm is assigned to ring methyl 1, and 2.27 ppm is, by elimination, assigned to ring methyl 8. Other aliphatic resonances that appeared upon irradiation of meso protons are unlikely to be thioether bridge methyls because of the small magnitude of the NOE and the lack of an NOE to the bridge methines or ring methyls when the aliphatic candidates were irradiated. The methine proton at 5.34 ppm is assigned to bridge substituent 2 on the basis of its NOE with the α meso and the proton at 6.36 ppm to substituent 4 on the basis of a NOE with the β-meso proton.

Contributory evidence and an additional possible assignment come from the observed NOE between the methine proton at 5.34 ppm and the aromatic proton at 7.56 ppm. In the crystal structure of *c*-550, the only aromatic side chain near to a bridge methine is Phe-102. From the atomic coordinates, the carbon–carbon distance between the methine carbon of substituent 2 and the ring carbon of Phe-102 is 4.38 Å. The respective orientations point the attached protons toward each other. Thus, a substantial NOE is anticipated and was observed at 7.56 ppm. Upon irradiation of the ligand methionyl methyl at −2.84 ppm, weak NOE's were observed at 7.6, 3.47 (methyl 1), 2.27 (methyl 8), and 2.35 ppm (thioether methyl 2) in further support of assignments.

Assignments deduced from the NOE pattern are incorporated into Table II. The magnitude of the hyperfine chemical shift in the *oxidized* state has been interpreted in terms of the unpaired electron spin density over the corresponding pyrrole (Redfield & Gupta, 1971; Keller & Wutrich, 1978a). The fact that the heme methyls 3 and 8 have the largest downfield shifts in the ferric spectrum indicates that the asymmetric distribution of electron density places greatest density over the pyrroles bearing substituents 2/3 and 7/8. This is the same qualitative distribution found in horse cytochrome *c*.

Ferrocytochrome c-550: Assignment of Ligand Methionine Resonances. The methyl resonance at −2.84 ppm in the spectrum of ferrocytochrome *c*-550 is assigned to the methionine methyl on the basis of homology to other cytochromes *c*. In previous studies, this methyl resonance has been found within ±0.3 ppm of −3 ppm [Ulrich et al. (1982), for example). On the basis of homology to other cytochromes (Keller et al., 1980; Senn et al., 1980), C_β and C_γ methionyl protons are expected in this upfield region, since they also experience a large ring current shift from the heme. Truncated driven nuclear Overhauser enhancements (TOE) (Wagner & Wutrich, 1979) were employed to assign the corresponding methionyl C_β and C_γ methylene protons, as shown in Figure 4. In Figure 4B,D, it can be seen that the largest enhancement upon irradiation of the −3.47 ppm resonance was produced at

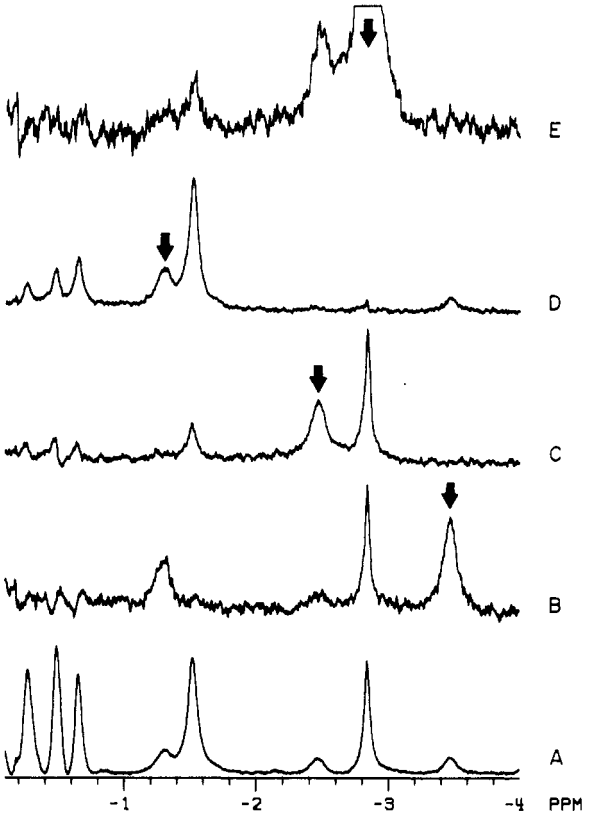

FIGURE 4: Upfield region of the ^1H NMR spectrum of *Paracoccus* ferrocytochrome *c*-550 and related NOE experiments. The sample was at pH* 7.6 and 23 °C. (A) The normal ferrocytochrome spectrum; (B–D) difference spectra obtained between off-resonance and on-resonance irradiation with a low-power selective frequency prior to acquisition. The off-resonance position was −4 ppm, where no features of the spectrum are apparent. On-resonance irradiation is indicated by the bold arrows. The irradiation frequency was applied for 50 ms prior to acquisition in the expreiments shown. The vertical scales are not the same in all difference spectra but have been adjusted for a convenient display. The magnitude of enhancements to be discussed was computed on the basis of the relative areas in the difference spectra when the irradiated peak was assigned an area on the basis of the number of protons to which it corresponds in the normal spectrum. (B) Irradiation of the −3.47 ppm resonance produced negative enhancements of 20% at −2.84, 20% at −2.46, and 60% at −1.32 ppm. (C) Irradiation at −2.47 ppm produced negative enhancements of 40% at −2.84 and 30% at −1.50 ppm but no observable effect at −3.47 ppm. (D) Irradiation at −1.32 ppm produced a negative enhancement of 40% at −3.47 ppm. The large difference peak at −1.52 ppm is not solely an NOE but, in part, may arise from partial saturation of this peak during irradiation by the on-resonance frequency at −1.32 ppm. The radio frequency is not perfectly selective but has a finite band width. The difference peaks downfield of −0.7 ppm may represent NOE's from the partially saturated −1.52 ppm resonance. (E) Irradiation at −2.84 ppm produced negative enhancements of 60% at −2.47 and 20% at −1.50 ppm.

−1.32 ppm. Figure 4D is the converse experiment showing the largest enhancement at −3.47 ppm upon irradiation at −1.32 ppm. This indicates that these resonances are geminal protons. In the ferrocytochrome spectrum, the large resonance at −1.52 ppm integrates to four protons, which we interpret as due to the overlap of a methyl and a single proton resonance. The hypothetical resonances were not resolved by inversion–recovery experiments. Irradiation at −2.46 ppm produced an enhancement at −1.50 ppm. Although this is only a slight difference from −1.52 ppm, we believe that it represents a real difference and that the effect is on the single proton buried beneath the larger methyl resonance. The magnitude of the enhancement observed indicates that the resonances at −2.46 and −1.50 ppm are geminal protons. The enhancements observed in Figure 4B–D at −2.84 ppm indicate that the single

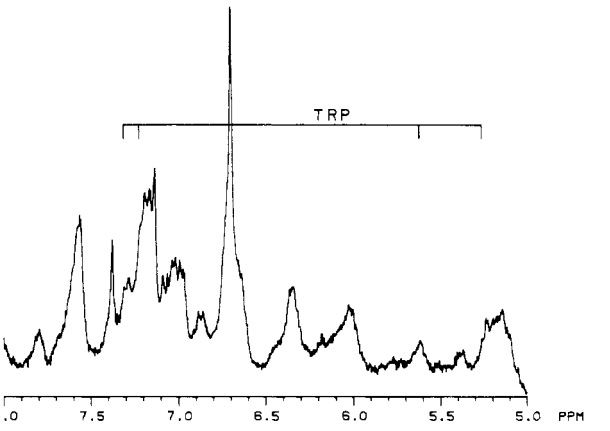

FIGURE 5: ^1H NMR spectrum of the aromatic region of *Paracoccus* ferrocytochrome *c*-550. The sample was at pH* 7.6 and 60 °C. This spectrum has been resolution enhanced by application of a Gaussian-shaped weighting function to the free-induction decay before Fourier transformation. Coupled resonances for the sole assigned tryptophan are indicated by the connecting solid line.

protons are methionyl. Upon irradiation of the methyl resonance at −2.84 ppm (Figure 4E), the largest enhancements observed are at the geminal protons at −2.46 and −1.50 ppm. From the crystal structure of *c*-550 (Timkovich & Dickerson, 1976), it is known that the methionine ligand side chain is in an extended conformation. The magnitude of the enhancements in Figure 4E indicates that −2.46 and −1.50 ppm resonances are C_γ protons, and thus, the other pair are C_β protons. The large enhancement at −1.52 ppm in Figure 4D may be due to two sources. The first is not a true NOE but is due to partial saturation of the −1.52 ppm resonance by the finite band width of the irradiating frequency at −1.32 ppm. However, there might also be a contributing NOE. The crystal structure of *Paracoccus c*-550 shows that a methyl group from Thr-101 is located close to the C_β carbon of the ligand methionine. Such an arrangement would be expected to lead to an NOE. Further, the orientation of this Thr-101 methyl with respect to the heme ring plane is such that the methyl resonance should experience a large upfield ring current shift contribution. On the basis of these points, the −1.52 ppm resonance has been tentatively assigned to the Thr-101 methyl.

Ferrocytochrome c-550: Aromatic Region. The resolution-enhanced spectrum of the aromatic region of *Paracoccus* ferrocytochrome *c*-550 is shown in Figure 5. Some multiplet structure may be discerned in this resolution-enhanced spectrum at 60 °C that is not evident at lower temperatures. There were no irreversible changes in the spectra of *c*-550 as a result of heating to 60 °C. Overlap of resonances at 300 MHz made assignments difficult. It was possible to identify coupled resonances arising from a tryptophan side chain by *J*-modulated spin-echo double-resonance difference spectra (Campbell & Dobson, 1975), as indicated in Figure S8 (see paragraph at end of paper regarding supplementary material) and Table II. Cytochrome *c*-550 contains two tryptophan residues (Ambler et al., 1981), but we were unable to identify which residue gave rise to the observed spin-coupled resonances. Additional coupling of aromatic resonances was observed and is summarized in Table II. We were not able to unambiguously determine the complete coupling pattern of these resonances and so cannot assign them to a specific type of side chain. Two singlets were identified in *J*-modulated spin-echo spectra at 7.37 and 6.70 ppm. The only singlets expected in the aromatic region arise from the C_2 protons of the two tryptophan resonances. The sole histidine residue in *c*-550 is the fifth heme ligand, and resonances arising from this side

chain are expected further upfield because of the ring current of the heme plane (Moore & Williams, 1980a).

Supplementary Material Available

Spectrum of the high pH form of ferricytochrome *c*-550 (Figure S1), chemical shifts as a function of pH for ferricytochrome resonances (Figure S2), chemical shifts as a function of temperature for all resolved ferricytochrome resonances (Figure S3), spectra of the heme meso protons as a function of temperature in the ferrocytochrome (Figure S4), cross-saturation difference spectra showing the identification of heme methyl resonances in the ferrous state (Figure S5), the spectrum of cyanoferricytochrome *c*-550 (Figure S6), difference spectra showing the NOE observed between thioether bridge resonances and heme methyl resonances in the ferrous state (Figure S7), and spin-echo double-resonance difference spectra showing the coupling of the assigned tryptophan residue (Figure S8) (12 pages). Ordering information is given on any current masthead page.

Registry No. Cytochrome *c*-550, 9064-80-6.

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