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Cytochrome *c*. Observation of Numerous Single-Carbon Sites of the Reduced and Oxidized Species by Means of Natural-Abundance ^{13}C Nuclear Magnetic Resonance Spectroscopy

(horse heart/protein/heme/electron transfer/nonprotonated aromatic carbons)

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ABSTRACT **Proton-decoupled, natural-abundance ^{13}C nuclear magnetic resonance spectra (obtained at 15.18 MHz by the Fourier transform method) of aqueous ferrocytochrome *c*, ferricytochrome *c*, and mixtures of both species were recorded. The 18 nonprotonated aromatic carbons of amino-acid residues and the 16 nonprotonated aromatic carbons of the heme yielded 22 narrow single-carbon resonances and 6 narrow two-carbon resonances in the spectrum of ferrocytochrome *c*. Only some of these resonances were detected in the spectrum of ferricytochrome *c*.**

Fast electron transfer between ferrocytochrome *c* and ferricytochrome *c* produced chemical exchange effects in spectra of mixtures of the two species: 16 nonprotonated aromatic carbons yielded narrow exchange-averaged resonances as a consequence of their small natural linewidths in both redox states and the small changes in their chemical shifts (relative to the reciprocal of the lifetime between electron exchange) when going from the reduced to the oxidized species. These peaks were assigned to carbons situated far from the iron atom. Their fast exchange behavior was used to establish a one-to-one correspondence between resonances in spectra of the two redox states. The other 18 nonprotonated aromatic carbons yielded exchange-broadened resonances as a consequence of large chemical-shift differences between the diamagnetic and paramagnetic species, and/or large paramagnetic broadening of the resonances of ferricytochrome *c*. We assigned these resonances (only one of which was identified in the spectrum of ferricytochrome *c* alone) to carbons that are near the iron atom: C^{ζ} of Tyr 67, C^{γ} of His 18, and the 16 nonprotonated carbons of the porphyrin ring. Tentative specific assignments for C^{ζ} of Tyr 67 (in the spectra of both redox forms) and for C^{γ} of His 18 (in the spectrum of ferrocytochrome *c*) are also presented.

The complete structure of crystalline ferricytochrome *c* (from horse hearts and bonito) and of ferrocytochrome *c* (from tuna hearts) have been reported (1, 2). Our goal is to apply ^{13}C Fourier transform (FT) nuclear magnetic resonance (NMR) to the study of these proteins in solution. We present here the proton-decoupled, natural-abundance ^{13}C NMR spectra of oxidized and reduced horse-heart cytochrome *c*. These spectra were obtained with the use of our recently developed 20-mm probe (3), which facilitates the detection of single-carbon resonances of proteins (4).

MATERIALS AND METHODS

Horse-heart cytochrome *c* was obtained from Sigma Chemical Co., St. Louis, Mo. (Type VI) and from Calbiochem, La Jolla, Calif. (A grade). Sigma cytochrome *c* was either used as received or it was first purified on a column of Sephadex G-25 and then concentrated in a Diaflo ultrafiltration apparatus fitted with a UM-10 membrane (Amicon Corp.). No differences could be detected between the spectra of purified and unpurified samples. Cytochrome *c* from Calbiochem was purified on a column of Sephadex G-25 or G-100 and then concentrated as above. Partial reductions were made with sodium borohydride or ascorbic acid. Complete reductions were made with sodium dithionite. Oxidized samples were obtained by reaction of ferrocytochrome *c* with excess potassium ferricyanide. Small ions were removed on a column of Sephadex G-25. Concentrations of oxidized and reduced species were determined (on a Cary 14 spectrometer) as described by Margoliash and Walasek (5), with the use of the extinction coefficients of Margalit and Schejter (6). Samples of 6.2–14.4 mM cytochrome *c* at pH 6.7 were used. The pH was adjusted and measured at room temperature as described (4).

^{13}C NMR spectra were obtained at 15.18 MHz by the FT method. The apparatus has been described (3, 4). All spectra were recorded at 41° ± 1°C. 90° Radiofrequency pulses were used for ^{13}C excitation. For the fully proton-decoupled spectra, the ^{1}H irradiation (0.6–0.7 G at 60.37 MHz) was centered at about 6 ppm downfield from the ^{1}H resonance of tetramethylsilane (TMS), and had a random-noise modulation bandwidth of about 10 ppm. For the noise-modulated off-resonance decoupling experiments (7), the ^{1}H irradiation was centered at 8–10 ppm upfield from TMS and had a modulation bandwidth of about 5 ppm.

Time-domain data were accumulated in 8192 addresses of a Nicolet-1085 computer, using a 4000 Hz spectral width and a recycle time of 1.105 sec. A spectrum of ferrocytochrome *c* recorded with a recycle time of about 2 sec indicated that a recycle time of 1 sec yielded resonances that had at least 80% of their equilibrium intensities, except for some carbonyl resonances with relatively long spin-lattice relaxation times. 16,384 accumulations (5 hr total time) were used for recording most spectra. In order to increase the signal-to-noise ratio, the accumulated time-domain signal was multiplied by an exponential function with a negative time constant (corresponding to a line-broadening of 0.62 Hz for most spectra). In order to improve spectral definition, Fourier transformation was done on 16,384 time-domain points, by placing 8192 addresses with a zero value at the end of each block of 8192 accumulated data points. In this way there was one point every 0.488 Hz in the frequency-domain spectrum. Chemical shifts were measured digitally and are reported relative to external CS_2.

Abbreviations: FT, Fourier transform; ppm, parts per million; TMS, tetramethylsilane; NMR, nuclear magnetic resonance.

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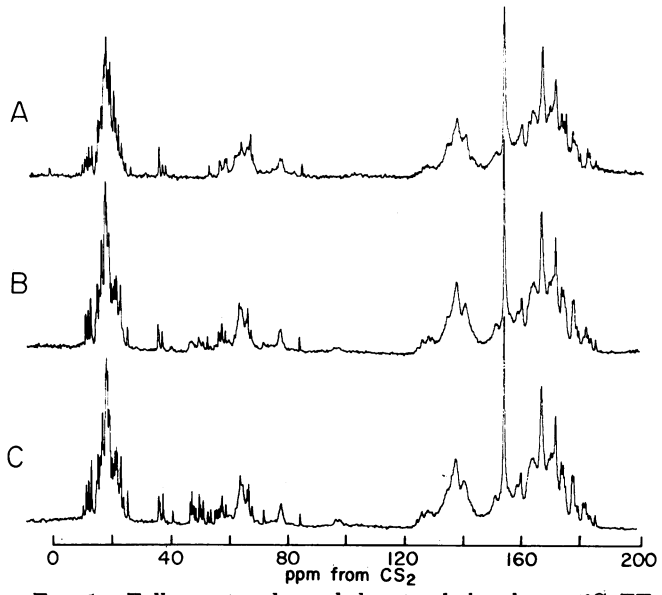

FIG. 1. Fully proton-decoupled natural-abundance ^{13}C FT NMR spectra of horse-heart cytochrome *c* [in 50 mM phosphate buffer (pH 6.7) 41°C], recorded at 15.18 MHz using 16,384 accumulations (5 hr) per spectrum. (*A*) 8.4 mM ferricytochrome *c*. (*B*) A solution 1.2 mM in ferricytochrome *c* and 10.4 mM in ferrocytochrome *c*. (*C*) 11.6 mM ferrocytochrome *c*.

RESULTS AND DISCUSSION

In Fig. 1 we show the fully proton-decoupled natural-abundance ^{13}C NMR spectra of ferricytochrome *c* (Fig. 1*A*), of ferrocytochrome *c* (Fig. 1*C*), and of a solution in which 90% of the cytochrome *c* was in the reduced state (Fig. 1*B*). We can divide the resonances of amino-acid residues into the carbonyl region (10–25 ppm upfield from CS_2), the region of aromatic carbons, which also contains C^ζ of the arginine residues (35–85 ppm upfield from CS_2), and the region of saturated carbons (120–185 ppm upfield from CS_2) (4). In addition, we must consider the ^{13}C resonances of the heme. On the basis of known ^{13}C chemical shifts of porphyrins (8) and diamagnetic metalloporphyrins (9), the carbonyl groups, the nonprotonated aromatic carbons, and the saturated side-chain carbons of the heme of *ferro*cytochrome *c* should contribute resonances to the three corresponding regions of the polypeptide spectrum. However, the *meso* carbons should resonate in the range 90–100 ppm upfield from CS_2 (8, 9), a region free of peptide carbon resonances. For ferricytochrome *c*, we expect large paramagnetic shifts (10, 11) and perhaps considerable broadening (12) of the porphyrin carbon resonances.

We must also consider the effect of the heme on the ^{13}C chemical shifts of adjacent peptide carbons. His 18 and Met 80 are the fifth and sixth ligands to the iron in both redox states (1, 2, 13, 14). The ^{13}C chemical shifts of these residues in ferrocytochrome *c* may be affected to some extent by coordination to the iron. For ferricytochrome *c*, large chemical shift changes and perhaps a broadening of the resonances is expected for amino-acid carbons near the iron atom.

The aromatic region of the spectrum presents the greatest opportunity for observing numerous resolved single-carbon resonances (4). In Fig. 2 we show an expanded view of the

TABLE 1. *Chemical shifts of nonprotonated aromatic carbons and of C^ζ of the arginines of horse-heart cytochrome c*

Assignment[b]	Chemical shift[a] Reduced[c]	Chemical shift[a] Oxidized[d]	Assignment[b]	Chemical shift[a] Reduced[c]	Chemical shift[a] Oxidized[d]
2 Arg-ζ	35.7(1)[e]	35.9(2)[f]	Porph.	53.3(16)	[g]
Tyr-ζ	36.0(2)		Porph.	54.8(17)	[g]
Tyr-ζ	37.2(3)[e]	37.0(3)	Porph.	55.3(18)	[g]
Tyr-ζ		38.1(4)	[h]	56.1(19)	56.4(7)
Tyr 67-ζ[i]	40.4(4)	31.3(1)	[h]	56.6(20)	56.2(6)
Porph.	46.4(5)	[g]	[h]	57.1(21)	57.9(8)
Porph.	46.5(6)	[g]	[h]	57.2(22)	58.1(9)
Porph.	46.9(7)[e]	[g]	[h]	58.4(23)	58.6(10)
Porph.	47.3(8)	[g]	[h]	61.7(24)	61.2(11)
Porph.	47.6(9)	[g]		63.2(25)	63.4(12)
				65.7(26)	67.3(15)
Porph.	48.3(10)	[g]	Tyr-γ		
Porph.	49.4(11)[e]	[g]	Trp 59-δ_2	66.1(27)[e]	66.2(13)
				67.3(28)	66.5(14)[e]
Porph.	49.7(12)	[g]			
Porph.	50.1(13)	[g]			
Porph.	50.9(14)[e]	[g]	His 18-γ[i]	71.3(29)	[g]
[h]	52.4(15)	52.6(5)	Trp 59-γ	83.6(30)	83.9(16)

[a] In ppm upfield from external CS_2. Absolute values are accurate to ±0.3 ppm. Relative values within each spectrum are accurate to ±0.1 ppm.

[b] Greek letter is standard IUPAC-IUB carbon designation. *Porph.*, porphyrin.

[c] Measured digitally from the spectrum shown in Fig. 2*C*. Number in *parentheses* is the peak number in Fig. 2*C*.

[d] Measured digitally from the spectrum shown in Fig. 2*A*. Number in *parentheses* is the peak number in Fig. 2*A*. The resonances of ferricytochrome *c* are listed side by side with the corresponding resonances of ferrocytochrome *c* (see *text* and Fig. 4).

[e] Two-carbon resonance.

[f] Three-carbon resonance.

[g] Not observed (see *text*).

[h] Phe-γ, or Trp 59-ϵ_2, or His 26-γ, or His 33-γ.

[i] Most probable assignment to a specific residue (see *text*).

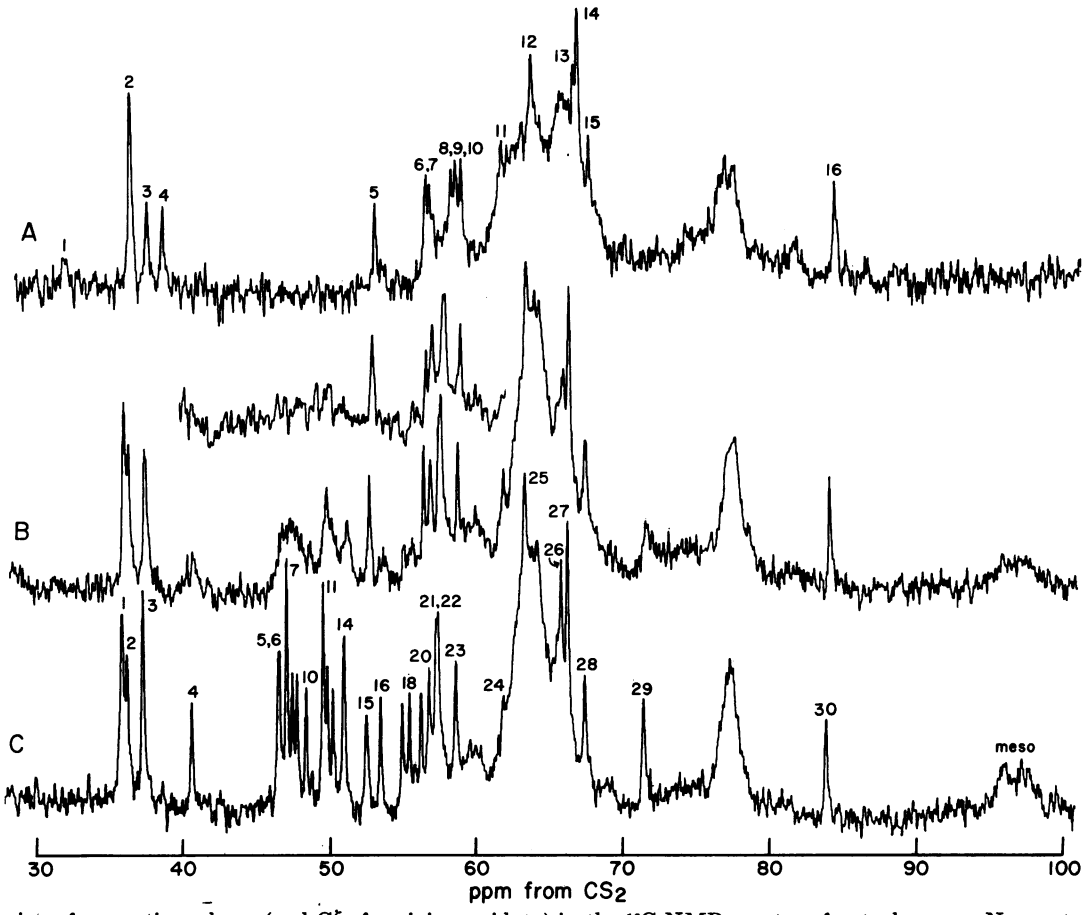

FIG. 2. Region of aromatic carbons (and C^{ζ} of arginine residues) in the ^{13}C NMR spectra of cytochrome c. Nonprotonated carbon peaks in spectra A and C are numbered consecutively from left to right. (A) Ferricytochrome c, from Fig. 1A. It was necessary to use Fig. 3A, with its higher signal-to-noise ratio, to identify peaks 1 and 11. (B) 9:1 mixture of ferrocytochrome c and ferricytochrome c, from Fig. 1B. (C) Ferrocytochrome c, from Fig. 1C. Numerous methine carbons contribute to the broad feature in the range 60–70 ppm. Some nonprotonated carbon resonances in this range are easier to identify in the noise-modulated off-resonance proton-decoupled spectrum (Fig. 3B). The insert between A and B is taken from a spectrum of 10.1 mM cytochrome c which had a 3:1 ratio of reduced to oxidized species, no phosphate buffer, and 1 N KCl.

aromatic region of the spectra of Figs. 1A–C and of a small portion of the spectrum of a 75% reduced sample. As for hen egg-white lysozyme (4), the aromatic region of the ^{13}C spectrum of ferrocytochrome c (Fig. 2C) contains numerous narrow resonances and some broad peaks. Theoretical considerations (15) can be invoked (4) to predict that aromatic *methine* carbons should give rise to the broad peaks, while all nonprotonated carbons (in a diamagnetic protein) should yield narrow resonances. This expectation can be verified experimentally (4) be noise-modulated, off-resonance proton-decoupling (7). Any *narrow* methine carbon resonances in the fully proton-decoupled spectrum (Fig. 2C) would become broad in the noise-modulated, off-resonance, proton-decoupled spectrum (4). All the narrow resonances in the aromatic region of the fully proton-decoupled spectrum (Fig. 2C) remain narrow in the noise-modulated, off-resonance, proton-decoupled spectrum (Fig. 3B), and, therefore, must be assigned to nonprotonated aromatic carbons. By the same method, we have shown that the narrow peaks in the aromatic region of the spectrum of ferricytochrome c (Fig. 2A) are also nonprotonated carbon resonances (see Fig. 3A). However, in this case the nonprotonated carbons of the heme and C^{γ} of His 18 will not necessarily yield narrow resonances, because of possible paramagnetic broadening (12). Chemical shifts of all observed nonprotonated aromatic carbon resonances are given in Table 1. The assignments of these resonances are based on evidence presented below, and on known ^{13}C chemical shifts of amino acids and peptides (4, and references cited therein).

The aromatic region of the spectrum of ferrocytochrome c (Fig. 2C) contains 24 narrow single-carbon resonances (peaks 2, 4–6, 8–10, 12, 13, 15–26, and 28–30) and six narrow two-carbon resonances (peaks 1, 3, 7, 11, 14, and 27). Thus, we detect 36 nonprotonated carbons in the aromatic region. Our division of peaks into single-carbon and two-carbon types requires the assumption that the nuclear Overhauser enhancement (16) is about the same for all the above resonances. We have made preliminary measurements which indicate that peaks 4, 15, and 16 are single-carbon resonances with no detectable nuclear Overhauser enhancement. 18 nonprotonated side-chain carbons of the 12 aromatic residues (4 Tyr, 4 Phe, 3 His, and 1 Trp), 16 nonprotonated aromatic carbons of the heme, and two ζ-carbons of the arginines (a total of 36 nonprotonated carbons) should contribute to the spectrum of Fig. 2C. Every nonprotonated aromatic carbon has been detected.

Peak 30 of Fig. 2C is easily assigned (4) to C^{γ} of Trp 59. Assignment of the other nonprotonated carbon resonances of

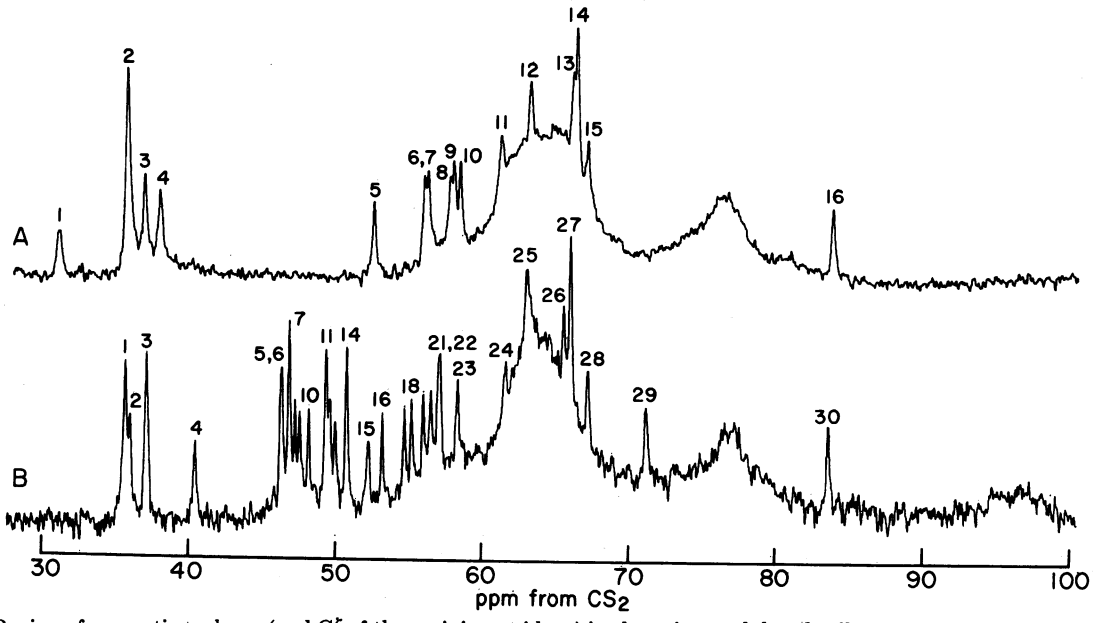

FIG. 3. Region of aromatic carbons (and C^{ζ} of the arginine residues) in the noise-modulated, off-resonance, proton-decoupled, natural-abundance ^{13}C NMR spectra of horse-heart cytochrome *c* [in 50 mM phosphate buffer (pH 6.7) 41°C], recorded at 15.18 MHz. (*A*) 14.4 mM ferricytochrome *c*, after 46,000 accumulations (14 hr). Peak designations are those of Fig. 2*A*. (*B*) 11.5 mM ferrocytochrome *c*, after 16,384 accumulations (5 hr). Peak designations are those of Fig. 2*C*.

ferrocytochrome *c* is facilitated by an examination of the spectral changes that occur when the percentage of ferricytochrome *c* is gradually increased, starting from pure ferrocytochrome *c* (Fig. 2). The two redox states of cytochrome *c* undergo fast electron transfer in solution, with a rate that is very dependent on ionic strength (17). The results of Gupta *et al.* (17) can be used to estimate that the rate constant for the electron transfer process at 40°C is about 7×10^3 M^{-1} sec^{-1} at an ionic strength of 0.2 (Fig. 2*B*) and about 6×10^4 M^{-1} sec^{-1} at an ionic strength of 1 (*insert* above Fig. 2*B*). The transfer produces a fluctuation in the chemical shifts of the ^{13}C resonances between the values in the two redox states. The effect on the observed spectra can be analyzed with well-known equations for chemical exchange processes in NMR (18, 19). It is useful to consider the cases of fast and slow exchange, defined by $2\pi|\nu_0 - \nu_r| \ll kC_t$ and $2\pi|\nu_0 - \nu_r| \gg kC_t$, respectively (19). Here ν_0 and ν_r are the resonance frequencies in Hz of the oxidized and reduced species, respectively, and C_t is the total molar concentration of cytochrome *c*.

Peaks 1–3, 15, 19–28, and 30 of Fig. 2*C* (pure ferrocytochrome *c*), which arise from 18 nonprotonated carbons, remain narrow upon addition of ferricytochrome *c*, and undergo a change in position that is proportional to the mole fraction of the oxidized species (Fig. 4). This fast exchange behavior (19) is a consequence of the small values of $|\nu_0 - \nu_r|$ of all these carbons, and it can provide a one-to-one correlation between the resonances of the two forms of cytochrome *c* (Fig. 4). The small values of $|\nu_0 - \nu_r|$ and the lack of any paramagnetic broadening of these resonances in the spectrum of ferricytochrome *c* (Figs. 2*A* and 3*A*) indicate that we are dealing here with carbons that are far from the iron atom, namely C^{ζ} of the two arginine residues and 16 of the 18 nonprotonated carbons of aromatic residues (Table 1). The two excluded amino-acid carbons are C^{γ} of His 18 and C^{ζ} of Tyr 67, which are relatively close to the iron atom (1).

In contrast to the above behavior, some narrow resonances

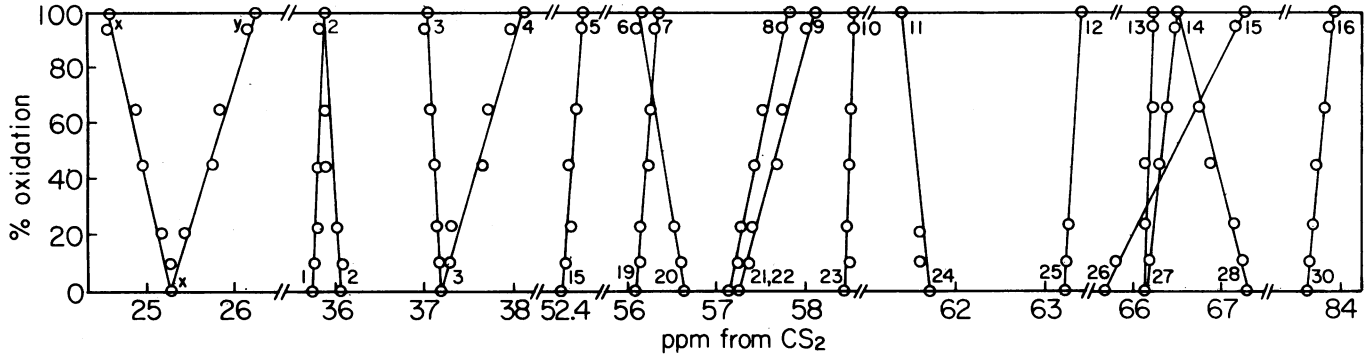

FIG. 4. Chemical shifts of some resonances that have fast-exchange behavior in ^{13}C NMR spectra of mixtures of the two redox forms of horse-heart cytochrome *c*, as a function of percentage of ferricytochrome *c*. Resonances are identified by their peak designations in Figs. 2 and 3, except for peaks *x* and *y*, which are carbonyl signals.

in the spectrum of fully reduced cytochrome *c* (peaks 4–14, 16–18, and 29 in Fig. 2*C*), which also arise from a total of 18 nonprotonated carbons, undergo considerable broadening when going to a 9:1 mixture of reduced and oxidized species (under conditions that yield $k \approx 7 \times 10^3\ M^{-1}\ sec^{-1}$, Fig. 2*B*). These resonances become too broad for detection (under our instrumental conditions) when the ratio of reduced to oxidized species in lowered to 3:1 and the ionic strength is raised to yield $k \approx 6 \times 10^4\ M^{-1}\ sec^{-1}$ (*insert* above Fig. 2*B*). Only one of these resonances seems to "reappear" in the spectrum of fully oxidized cytochrome *c* (peak 1 in Figs. 2*A* and 3*A*), at about 9 ppm downfield from the nearest resonance (of the ones under discussion) in the spectrum of ferrocytochrome *c* (peak 4 of Figs. 2*C* and 3*B*). For the carbon that gives rise to peak 1 in the spectrum of ferricytochrome *c*, the broadening in the spectra of mixtures of the two redox states (Fig. 2*B* and *insert* above it) results from $2\pi|\nu_0 - \nu_r| \gtrsim kC_t$. For the 17 nonprotonated aromatic carbons that did not yield detectable resonances in the spectrum of ferricytochrome *c*, (even under the conditions of high signal-to-noise ratio of Fig. 3*A*), the broadening in the spectra of mixed species is probably also a consequence of large values of $|\nu_0 - \nu_r|$ (10, 11). However, there may be a contribution to the broadening from the presumably large values of the linewidths of the corresponding resonances of ferricytochrome *c* (19). In any case, we conclude that all the resonances that become broad in the spectra of mixtures of the two redox states arise from carbons that undergo large paramagnetic shifts and/or broadenings when going to ferricytochrome *c*. We have assigned these resonances to carbons near the iron atom: C^ζ of Tyr 67, C^γ of His 18, and the 16 nonprotonated carbons of the porphyrin. Within this category, we have assigned the resonances in the range 46–55 ppm upfield from CS_2 in the spectrum of ferrocytochrome *c* (peaks 5–14 and 16–18 of Figs. 2*C* and 3*B*), which arise from 16 carbons, to the 16 nonprotonated carbons of the porphyrin. This assignment is based on reported chemical shifts (in the range 45–57 ppm upfield from CS_2) of analogous carbons of some diamagnetic metalloporphyrins (9). By elimination, the resonances at 40.4 ppm (peak 4 of Figs. 2*C* and 3*B*) and 71.3 ppm (peak 29) were assigned to C^ζ of Tyr 67 and C^γ of His 18. The specific assignment of the peak at 40.4 ppm to C^ζ of Tyr 67 is based on known chemical shifts of C^ζ of tyrosine residues in other systems (4). Although we believe that it is unlikely, we cannot exclude the possibility that our assignment for C^ζ of Tyr 67 and/or C^γ of His 18 may have to be interchanged with a porphyrin carbon assignment. In the spectrum of ferricytochrome *c*, we have tentatively assigned peak 1 (Figs. 2*A* and 3*A*) to C^ζ of Tyr 67. This carbon is further away from the iron atom, and is therefore expected to undergo less paramagnetic broadening than C^γ of His 18 and the porphyrin carbons.

Our assignments for all detected nonprotonated aromatic carbon resonances, and C^ζ of the arginine residues, of ferrocytochrome *c* and ferricytochrome *c* are summarized in Table 1.

The peaks at about 96 and 97 ppm in the spectrum of ferrocytochrome *c* (Fig. 2*C*) can be assigned to the protonated *meso* carbons of the porphyrin ring (8, 9). The peak at about 77 ppm (Fig. 2*C*) arises from the eight protonated ϵ-carbons of the tyrosine residues (4). It is not certain that Tyr 67 contributes to the corresponding peak in the spectrum of ferricytochrome *c* (Fig. 2*A*). The other broad bands in Fig. 2*C* are also assigned to aromatic methine carbons (4).

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