Titration Behavior and Tautomeric States of Individual Histidine Residues of Myoglobins

APPLICATION OF NATURAL ABUNDANCE CARBON 13 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY*

(Received for publication, February 22, 1977)

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The titration behavior of individual histidine residues of myoglobins has been studied by observing the pH dependence of the chemical shifts of the nonprotonated aromatic carbon resonances in natural abundance $^{13}$C Fourier transform NMR spectra (at 15.18 MHz and 37°) of horse ferrimyoglobin, horse cyanoferriimaglobin, and red kangaroo cyanoferriimaglobin. In the case of the cyanoferriimaglobins, all nonprotonated side chain carbons of aromatic amino acid residues yield detectable resonances, but only 24 of the 28 carbons of this type yield detectable resonances in the case of horse ferrimyoglobin. Eight of the 11 histidine residues of horse cyanoferriimaglobin (and 7 of the 10 histidines of the kangaroo protein) exhibit titration behavior (pK values in the range 4.4 to 6.6). The imidazole form of each titrating histidine is predominantly (or entirely) in the N$_6$-H tautomeric state. Two of the titrating resonances of the cyanoferriimaglobins (with pK values of 5.3 and about 4.5) do not yield detectable signals in spectra of horse ferrimyoglobin. These two resonances are assigned to C$_7$ of His-64 and His-97 (not on a one-to-one basis). Five of the six titrating resonances of horse ferrimyoglobin have pK values (5.5, 5.7, 6.5, 6.6, and 6.6) which are consistent with those of five of the six reported pK values that were obtained from proton NMR spectra. The sixth pK (<5), observed in our $^{13}$C NMR spectra of horse ferrimyoglobin and the cyanoferriimaglobins, does not have a detected counterpart in the reported proton NMR data. Also, the “high” pK (about 7.4 to 8.0) reported in the proton NMR studies of ferrimyoglobins has no counterpart in our $^{13}$C NMR spectra of horse ferrimyoglobin and the cyanoferriimaglobins from horse, kangaroo, and sperm whale.

One of the three nontitrating histidine C$_7$ resonances of the cyanoferriimaglobins from horse and kangaroo (not observed in spectra of horse ferrimyoglobin) is assigned to the coordinated His-93. Our results indicate that the two uncoordinated nontitrating histidine residues are either in the imidazolium or in the N$_6$-H imidazole state (or a mixture of the two states). The crystal structure of myoglobin suggests that these are His-24 and His-36. We also identify the resonances of C$_1$, C$_8$, and C$_9$ of the two tryptophan residues, and the resonance of C$_5$ of the nontitrating Tyr-146.

Proton NMR spectroscopy has been the method of choice for observing the environment and protonation state of titratable histidine residues of proteins, because the resonances of H$_6$ (and sometimes H$_7$) of these residues (Fig. 1) can be usually observed as resolved single hydrogen peaks with pH-dependent chemical shifts (for a review, see Ref. 1). However, natural abundance $^{13}$C NMR spectra of small native proteins yield numerous narrow single carbon resonances of nonprotonated aromatic carbons (2–4), and the titration behavior of the resonances of C$_7$ of histidine residues can be readily observed (4). We believe that, when sensitivity limitations are overcome by the use of large amounts of protein (5), $^{13}$C Fourier transform NMR is an attractive alternative (or complement) to $^1$H NMR for the study of histidine residues, especially when dealing with a protein which contains many histidines. It is noteworthy that $^{13}$C NMR can yield information about the tautomeric state of the imidazole form of each histidine residue (6–8). If the imidazolium form (Fig. 1A) of a histidine residue deprotonates at N$_6$ to yield the N$_6$-H imidazole tautomer (Fig. 1B), then the C$_7$ resonance should undergo a downfield shift of about 6 ppm (6–8); if deprotonation yields the N$_6$-H tautomer (Fig. 1C), then the C$_7$ resonance should move about 2 ppm upfield (6–8). In contrast, the proton resonance of H$_6$ moves upfield when the imidazolium form of the residue is deprotonated either at N$_6$ or at N$_7$ (compare the $^{13}$C NMR study of azurin in Ref. 8 with the $^1$H NMR results of Ref. 9).

In this report, we study the effect of pH on the chemical shifts of the nonprotonated aromatic carbon resonances of horse ferri- and cyanoferriimaglobin and of kangaroo cyanoferriimaglobin. We assign some of these resonances to specific residues in the sequence, and we present information about the ionization behavior (and tautomeric state) of the histidine residues. Most (but not all) of our results are consistent with reported interpretations of the pH dependence of proton NMR spectra of myoglobins (10–13).

EXPERIMENTAL PROCEDURES

Most materials and methods have been described (14). Some of the spectra obtained with the use of commercial horse myoglobin (14)
were compared with spectra of a myoglobin sample that we extracted from horse meat by the procedure used previously for the preparation of kangaroo myoglobin (14). The two horse myoglobins gave indistinguishable results. Unless otherwise indicated, myoglobin solutions in H$_2$O were used. Protein solutions in D$_2$O were prepared as follows. About 15 ml of the protein solution (initially in H$_2$O) was diluted to 60 ml with D$_2$O and concentrated back to about 15 ml (by ultrafiltration) a total of six times. Also, after the sixth dilution, KCl (added to a concentration of 0.1 M), the pH was adjusted to the desired value with NaOD, and the solution was incubated at 36°C for 24 h.

Carbon 13 NMR spectra were obtained at 15.18 MHz by means of the Fourier transform method. Chemical shifts were obtained from spectra recorded essentially as described (14), and are reported in parts per million downfield from the 13C resonance of Me$_3$Si. They were measured digitally relative to a trace of internal dioxane (at 67.86 ppm). For the measurement of the temperature dependence of chemical shifts, spectra of horse ferrimyoglobin in H$_2$O (16 mM, pH 6.5 at room temperature) were recorded at 8, 18, and 33°C, spectra of horse cyanoferrimyoglobin in H$_2$O (13 mM, pH 8.1 at room temperature) were recorded at 20, 36, and 47°C, and spectra of horse cyanoferrimyoglobin in D$_2$O (16 mM, pH meter reading 8.2 at room temperature) were obtained at 17, 27, 38, and 50°C.

Partially relaxed Fourier transform (PRFT) spectra of horse ferrimyoglobin (19 mM in D$_2$O, pH meter reading 8.1) and cyanoferrimyoglobin (16 mM in D$_2$O, pH meter reading 8.2) were recorded at 35°C, essentially as described (4). Selectively proton-decoupled spectra of horse ferrimyoglobin (19 mM in D$_2$O, pH meter reading 8.1), horse cyanoferrimyoglobin (16 mM in D$_2$O, pH meter reading 8.2), and kangaroo cyanoferrimyoglobin (13 mM in H$_2$O, pH 8.4) were obtained at 27°C, as described (4).

We used the convolution-difference method (15) for improving the resolution of nonprotonated aromatic carbon resonances and for eliminating the broad methine carbon features from our 13C NMR spectra of myoglobins, as follows. Two separate Fourier transform spectra were obtained from each accumulated time-domain spectrum (stored on a disk), one with a small digital broadening (typically 1 Hz) and one with a large digital broadening (typically 10 to 20 Hz), and then the second spectrum was subtracted digitally from the first one. Details have been given elsewhere (4).

RESULTS AND DISCUSSION

**General Considerations** The 13C NMR spectrum of a dinuclear magnetic protein can be divided (2-4) into the region of carbonyl resonances (about 170 to 185 ppm downfield from Me$_3$Si), the region of aromatic resonances (about 100 to 160 ppm), and the region of aliphatic resonances (about 10 to 75 ppm). The downfield edge of the aromatic region also contains the resonances of C' of arginine residues (normally at about 158 ppm) (4). In this paper, we are concerned mainly with the relatively narrow resonances of nonprotonated aromatic carbons (4). When dealing with a heme protein in a diamagnetic state, all nonprotonated aromatic carbons, including those of the heme, yield narrow resonances (4). However, the 16 nonprotonated aromatic carbons of the heme in a paramagnetic protein have not yet yielded clearly identified resonances in the aromatic region (4). Presumably, these carbons yield resonances that are significantly broadened or shifted (or both) by the paramagnetic center (16). Carbon 13 NMR studies of cytochrome c (4, 17) suggest that the resonances of only a few of the nonprotonated aromatic carbons of amino acid residues of myoglobins in paramagnetic states should be significantly affected by the paramagnetic center.

In Fig. 2, we show the regions of aromatic carbons (and C' of arginine residues) in the convolution-difference proton-decoupled 13C NMR spectra of horse ferrimyoglobin (at pH 7.7, Fig. 2A), and the cyanoferrimyoglobins from horse (at pH 8.3, Fig. 2B), red kangaroo (at pH 7.9, Fig. 2C), and sperm whale (at pH 8.3, Fig. 2D). Peaks are numbered consecutively from left to right (at pH ~ 8), with one number assigned to each carbon and not to each peak. The carbon count (number of carbons per peak) was obtained not just from the intensities in the spectra of Fig. 2 but from a detailed study of the pH dependence of the chemical shifts of all resonances (Figs. 3 to 6). We do not include Peak a of Fig. 2, B and C, in our peak numbering system, because it appears that this is a heme carbon resonance (see below). Peak 28 of horse cyanoferromyoglobin is relatively broad and not clearly detectable in Fig. 2B, but it is observed in spectra with somewhat higher signal-to-noise ratios (Fig. 7).

There are seven types of nonprotonated aromatic carbons of amino acid residues: C' of phenylalanines, C' of tyrosines, C' of histidines, and C', C8, and C6 of tryptophans. For convenience, we shall include C' of arginine residues in our discussion of nonprotonated aromatic carbons of amino acid residues. In the case of horse myoglobin, the 2 arginines, 2 tyrosines, 7 phenylalanines, 11 histidines, and 2 tryptophans (18) contribute a total of 30 nonprotonated side chain carbons. Only 26 of these carbons yield detectable resonances in spectra of horse ferrimyoglobin (Figs. 2A and 3), but all 30 can be observed in spectra of horse cyanoferromyoglobin (Figs. 2B, 4, and 7). When going from horse to red kangaroo myoglobin, Tyr-103 and Leu-149 become phenylalanines, His-113 and His-116 become glutamines, and Asn-140 becomes a histidine (18). The arginines and aromatic residues of kangaroo myoglobin contribute 29 nonprotonated side chain carbons, all of which can be detected in spectra of the cyanoferromyoglobin (Figs. 2C and 5). When going from horse to sperm whale myoglobin, Phe-151 becomes a tyrosine, Asn-12 becomes a histidine, and the number of arginines increases to 4 (18). As a result, the arginines and aromatic residues contribute 34 nonprotonated side chain carbons, 33 of which yield detectable resonances in spectra of the cyanoferromyoglobin. The exception is C' of the coordinated His-85 (see below).

**Temperature Dependence of Chemical Shifts** – Implicit in the discussion of the previous subsection was the assumption that all the numbered peaks of Figs. 2 and 7, but not Peaks a, b, and c, of the cyanoferrimyoglobins (see Figs. 7 and 8), arise from nonprotonated side chain carbons of aromatic amino acid residues (and C' of arginine residues). In order to rule out the possibility that some of the peaks under consideration are resonances shifted into the aromatic region by the paramagnetic center.
Fig. 2. Region of aromatic carbons (and C$^\alpha$ of arginine residues) in the convolution-difference natural abundance $^{13}$C NMR spectra of myoglobins (about 12 mM protein in H$_2$O, 0.1 M KCl) recorded at 15.18 MHz, under conditions of noise-modulated off resonance proton decoupling, with 32,768 accumulations per spectrum, and a recycle time of 0.555 s (for horse ferrimyoglobin and kangaroo cyanoferrimyoglobin) or 1.05 s (for horse and sperm whale cyanoferrimyoglobins). A, horse ferrimyoglobin, pH 7.7, 36°; B, horse cyanoferrimyoglobin, pH 8.3, 39°; C, kangaroo cyanoferrimyoglobin, pH 7.9, 37°; D, sperm whale cyanoferrimyoglobin, pH 8.3, 36°.

The remaining resonances (Peaks 1 to 27, 29, 30, a, b, and c) arise from a total of 32 carbons, 29 of which must be nonprotonated side chain carbons of aromatic and arginine residues. The chemical shifts (at 39°) and linewidths of Peaks a, b, and c suggest that these resonances arise from nonprotonated porphyrin carbons. The chemical shifts of Peaks a, b, and c extrapolated to 1/T = 0 (16 mM horse cyanoferrimyoglobin in D$_2$O, pH meter reading 8.2) are about 140, 98, and 94 ppm, respectively. The small temperature dependence of these chemical shifts is surprising. The peculiar effect of pH on the chemical shifts of these resonances is shown in Fig. 8 (Peak a is not detected in our spectra of sperm whale cyanoferrimyoglobin).

We did not examine the temperature dependence of the chemical shifts of the cyanoferrimyoglobins from red kangaroo and sperm whale. By analogy with the horse cyanoferrimyoglobin, it is reasonable to conclude that Peaks 1 to 29 of Fig. 2C and Peaks 1 to 33 of Fig. 2D arise from nonprotonated side chain carbons of aromatic and arginine residues. Thus, all 29 of these carbons of kangaroo cyanoferrimyoglobin can be observed in our spectra, but one of the 34 carbons of this type in sperm whale cyanoferrimyoglobin is not detected. We show below that the "missing" resonance is probably that of C$^\gamma$ of the coordinated His-93 residue.

Identification of Resonances of Titratable Histidine Residues - Assignment of resonances to specific amino acid residues in the sequence is facilitated if we first determine which type or types of carbons give rise to each peak. The resonances...
of nonprotonated aromatic carbons of proteins are clustered in
three regions (4). Chemical shift considerations (see Fig. 5
of Ref. 4) indicate that the downfield region (about 155 to 159
ppm in Fig. 2) contains only the resonances of $c^1$ of tyrosine
and arginine residues, and that the upfield region (about 109
to 112 ppm in Fig. 2) contains only the resonances of $c^1$ of
the two tryptophan residues. The central region (about 124 to 141
ppm in Fig. 2) contains the remaining resonances of nonpro-
tonated aromatic carbons (4). The pH dependence of only the
central region is shown in Figs. 3 to 6. The effect of pH on the
chemical shifts of $c^1$ of the tyrosine and arginine residues has
been presented elsewhere (14). The chemical shifts of $c^1$ of
the two titratable residues are essentially independent of pH.

The effect of pH on chemical shifts can be used to identify
the resonances of $c^1$ and $c^1$ of titratable tyrosine residues (14)
and those of $c^1$ of titratable histidine residues (4). In a pre-
vious publication (14), we showed that Tyr-103 (horse and
sperm whale) and Tyr-101 (sperm whale) show titration be-
havior at pH $\geq 6$, and that Tyr-146 (horse, kangaroo, and
sperm whale) does not titrate. We also presented specific
assignments for the resonances of $c^1$ and $c^1$ of the titratable
tyrosine residues (14). The $y$ carbon of Tyr-103 gives rise to
Peaks 11 and 20 of horse ferrimyoglobin (Figs. 2A and 3), Peak 27 of
sperm whale cyanoferrimyoglobin (Figs. 2B and 4), and Peak 31 of
sperm whale cyanoferrimyoglobin (Figs. 2D and 6) (14). Peak
30 of Figs. 2D and 6 arises from $c^1$ of Tyr-151 (14).

At pH $\leq 6$, six resonances of horse ferrimyoglobin show
titration behavior (Fig. 3). The values of the chemical shifts of
these resonances at high pH, and the direction of the titration
shifts as the pH is lowered, are characteristic of $y$ carbons of
titrating histidine residues whose imidazole form is predomi-
nantly (or entirely) in the N$^i$-H tautomeric state (6-8). This
tautomeric state is the predominant one of histidine residues
in small peptides (6), but the N$^i$-H tautomeric state has been
encountered in some titratable histidine residues of proteins
(8, 21). Application of the usual Henderson-Hasselbalch treat-
ment to the chemical shifts of Fig. 3 yields the pK values, the
chemical shifts of the imidazole state ($\delta_{im}$), and the titration
shifts ($\Delta_{tit}$) given in Table I.

In the case of horse cyanoferrimyoglobin, we find 8 titrating
histidine residues, all of which have the imidazole form predomi-
nantly (or entirely) in the N$^i$-H tautomeric state. Fig. 4
yields the values of pK, $\delta_{im}$, and $\Delta_{tit}$ given in Table I. It is
apparent from Table I (and by visual inspection of Figs. 3 and
4) that each of the 6 observed titrating histidines of horse
ferrimyoglobin (Fig. 3) has a readily identifiable counterpart
in the spectrum of the cyanoferrimyoglobin (Fig. 4). The addi-
tional 2 observed titrating histidines of the cyanoferrimyglo-
bin are those which give rise to Peaks 11 and 20 of Figs. 2B and
4. We conclude that these peaks arise from titrating histidine

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**Fig. 3.** Effect of pH on the chemical shifts of some nonprotonated aromatic carbons of horse ferrimyoglobin at 36°. Peak numbering system and typical sample and spectral conditions are given in Fig. 2A. $\bullet$, $\circ$, $\triangle$, $\square$, indicate peaks that arise from 1, 2, 3, and 4 carbons, respectively. ---, best fit (single pK) theoretical titration curves.

**Fig. 4.** Effect of pH on the chemical shifts of some nonprotonated aromatic carbons of horse cyanoferrimyoglobin at 36°. Peak numbers are those of Figs. 2B and 7. The meaning of symbols and curves is given in the legend of Fig. 3. The titration curve of Peak 27 ($c^1$ of Tyr-103) is a two-pK fit (14). Typical sample and spectral conditions are those of Fig. 7 for Peak 28 and Fig. 2B for all other peaks.

**Fig. 5.** Effect of pH on the chemical shifts of some nonprotonated aromatic carbons of red kangaroo cyanoferrimyoglobin at 36°. Peak numbers and typical sample and spectral conditions are those of Fig. 2C. The meaning of symbols is given in the legend of Fig. 3. ---, best fit (single pK) theoretical titration curves.
titrating histidine resonances (Peaks 14, 19, and 21 of horse cyanoferrimyoglobin and Peaks 15 and 21 of kangaroo cyanoferrimyoglobin). There is no resonance in the spectrum of the kangaroo protein that could possibly correspond to Peak 19 of the horse protein ($\delta_{\text{Me}$-$\text{Si}$} = 3.6$ ppm, $\delta_\alpha = 133.6$ ppm, pK = 5.5). Thus, Peak 19 of horse cyanoferrimyoglobin must arise from His-113 or His-116. However, Botelho (13) has unambiguously established by means of proton NMR titration studies of human myoglobin (which has Gln-113 and Gln-116), California sea lion myoglobin (which has His-113 and Gln-116), and other myoglobins (with His-113 and His-116), that His-113 and His-116 have pK values of about 5.5 and 6.5, respectively. Therefore, we assign Peak 19 of horse cyanoferrimyoglobin to His-113.

Peaks 14 and 21 of horse cyanoferrimyoglobin (both with pK values of about 6.4) have $\delta_\alpha$ and $\Delta_\text{A}$ values similar to (but not identical with) those of Peaks 15 and 21, respectively, of the kangaroo protein (Table I). We conclude that His-116 of horse cyanoferrimyoglobin and His-140 of kangaroo cyanoferrimyoglobin give rise either to Peak 21 of each protein or to Peak 14 of horse and Peak 15 of kangaroo cyanoferrimyoglobin.

Assignment of Some Resonances of Nontitrating Residues—Consider first the C$^\alpha$ resonances of tyrosine and arginine residues. The effect of pH yielded the identification of the resonances of C$^\alpha$ of titrating tyrosine residues (14). Selective proton decoupling can be used to distinguish the resonances of C$^\alpha$ of all tyrosine residues from those of C$^\alpha$ of arginine residues, as follows (4). Single frequency $^1$H decoupling is applied at or near the resonance frequencies of the arginine CH$_2$ protons (about 3.2 ppm downfield from the $^1$H resonance of Me$_4$Si), with low enough power to prevent decoupling of the aromatic protons of the tyrosines (6.8 to 7.2 ppm). The arginine C$^\alpha$ resonances remain sharp, while the tyrosine C$^\alpha$ resonances broaden considerably (4). In order to avoid the possible complicating effect of observable scalar coupling of the arginine $\zeta$ carbons to slowly exchanging NH protons of the guanidinium groups, these selective decoupling experiments are usually done on protein solutions in D$_2$O. Peaks 2 and 3 of horse ferrimyoglobin and cyanoferrimyoglobin, and Peaks 1 and 2 of kangaroo cyanoferrimyoglobin are just as narrow under conditions of h total time). The convolution-difference method was not used, in order not to degrade the detectability of broad resonances (such as Peak 28). Instead, a digital broadening of 0.9 Hz was applied. Peak numbers and typical sample and spectral conditions are those of Fig. 2D. The meaning of symbols and curves is given in the legend of Fig. 3. The titration curve of Peak 31 (C$^\alpha$ of Tyr-103) is a two-pK fit (14).
selective proton decoupling as when full proton decoupling is applied. Thus, these peaks are assigned to Cλ of the 2 arginine residues of these proteins. Peaks 1 and 4 of horse ferrimyoglobin and Peak 3 of kangaroo cyanoferrimyoglobin, which undergo broadening when selective decoupling is used, are assigned to Cλ of tyrosine residues. Thus, Peak 3 of kangaroo cyanoferrimyoglobin must arise from Tyr-146. Since Peak 4 of horse ferrimyoglobin and Peak 1 of horse ferrimyoglobin have been assigned to the titratable Tyr-103 (14), Peak 1 of the cyanoferrimyoglobin and Peak 4 of the ferrimyoglobin must be assigned to Tyr-146.

Peak 4 of horse ferrimyoglobin (Fig. 2A) is relatively broad and has a slightly temperature-dependent chemical shift. 155.4 ppm at 33°C and about 158 ppm at 1/T = 0 (at pH 6.5). Both effects are consistent with the proximity of Cλ of Tyr-146 to the iron. With the use of the crystal coordinates of sperm whale myoglobin (19), we calculate that the distances from the iron to the z carbons of Tyr-146, Tyr-103, and the arginines are about 10, 12, and more than 15 Å, respectively. Note that dipolar paramagnetic broadening is proportional to the inverse sixth power of the distance to the paramagnetic center (16). We can use the measured paramagnetic contribution to the linewidth of the Cλ resonance of Tyr-146 of horse ferrimyoglobin to estimate the expected paramagnetic (dipolar) broadening of other resonances. Peak 4 of horse ferrimyoglobin (Fig. 2A) has a paramagnetic broadening of about 5 Hz (measured from spectra obtained without the use of the convolution-difference method). From this result, we estimate that the paramagnetic (dipolar) broadening should be less than 2 Hz for all remaining nonprotonated side chain carbons of aromatic (and arginine) residues of horse ferrimyoglobin, except the y carbons of the following residues (numbers in brackets indicate the estimated paramagnetic dipolar broadening, in hertz): His-93 (760), His-97 (180), His-64 (61), Phe-43 (29), Tyr-103 (4), Phe-46 (3), and Phe-138 (3). Therefore, all resonances except those of Cλ of His-93, His-97, His-64, and Phe-43 should be readily detectable in our spectra of horse ferrimyoglobin.

### Table 1

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- Assigned to Cλ of His-97 or His-64 (see Footnote g and text).
- Estimated uncertainty is ±0.1 ppm.
- Either Peak 15 or Peak 21 of kangaroo cyanoferrimyoglobin (Figs. 2c, 5) is assigned to His-116 (see text).
- Estimated uncertainty is ±0.1 ppm.
- Estimated uncertainty is ±0.3 ppm.
These resonances are clustered into two groups (see Figs. 3 to 6); the non-titrating resonances in the range 124 to 131 ppm can arise from $\delta^N$ of non-titrating tyrosine residues (4), $\delta^C$ of tryptophan residues (4), and $\delta^C$ of non-titrating histidine residues in the imidazolium (4) or $\delta^N$-$\delta^H$ imidazole state (6–8); the non-titrating resonances in the range 134 to 141 ppm can arise from $\delta^N$ of phenylalanine residues (4), $\delta^C$ of tryptophan residues (4), and $\delta^C$ of non-titrating histidine residues in the $\delta^N$-$\delta^H$ imidazole form (4, 6–8).

Theoretical and experimental results presented elsewhere (3, 4) indicate that the resonances of $\delta^N$ and $\delta^C$ if $D_2O$ is the solvent of tryptophan residues can be identified on the basis of their long spin-lattice relaxation times ($T_1$), relative to the $T_1$ values of $\gamma$ carbons of tyrosine, histidine, and phenylalanine residues (3). Whenever two classes of carbons have different $T_1$ values, PRFT spectra can be used to distinguish their resonances (4, 22). We have applied the PRFT method to ferrimyoglobin and cyanoferriyoglobin from horse. In the spectrum of the cyanoferriyoglobin, two components of Peak 8–11 arise from $\delta^C$, and Peak 25–26 arises from $\delta^C$ of the tryptophan residues. Because of limited signal-to-noise ratios, our PRFT spectrum of the ferrimyoglobin did not yield the assignment of $\delta^C$ of the tryptophan residues. Two components of Peaks 22 to 24 (Figs. 2A and 3) arise from $\delta^C$ of these residues.

By elimination, Peaks 19, 21, and one component of Peaks 22 to 24 of horse ferrimyoglobin (Figs. 2A and 3) must arise from $\delta^C$ of Tyr-146 and 2 non-titrating histidine residues (but not the coordinated His-93). Also by elimination, the counterparts of these resonances in the spectrum of horse cyanoferriyoglobin are Peaks 22, 23, and 24 (Fig. 4). In the spectrum of kangarooy ferrimyoglobin (Fig. 5), Peaks 22 to 26 have practically the same chemical shifts as Peaks 22 to 26 of horse cyanoferriyoglobin (Fig. 4). Thus, the assignments presented above for Peaks 22 to 26 of the horse protein are probably applicable to the corresponding peaks of the kangaroo protein. By elimination, Peak Z of kangaroo cyanoferriyoglobin (Fig. 5) is tentatively assigned to $\delta^C$ of His-93. Consider now Peaks 25 to 31 of sperm whale cyanoferriyoglobin. Peaks 30 and 31 have been assigned to $\delta^C$ of Tyr-151 and Tyr-103, respectively (14). It is apparent from a comparison of Figs. 6 and 4 that Peaks 28 and 29 of the sperm whale protein (Figs. 2D and 6) probably arise from the same carbons ($\delta^N$ of the tryptophan residues) as Peaks 26 and 26 of horse cyanoferriyoglobin (Figs. 2B and 4). Peak 27 of Figs. 2D and 6 probably corresponds to Peak 24 of Figs. 2B and 4. Peaks 25 and 26 of sperm whale cyanoferriyoglobin probably correspond to Peaks 22 and 23 of horse cyanoferriyoglobin (not necessarily on a one-to-one basis). The relatively broad Peak 28 of the horse protein (Fig. 7), assigned above to $\delta^C$ of His-93, does not have a clearly detectable counterpart in our spectra of sperm whale cyanoferriyoglobin.

Table II summarizes the limited number of assignments of non-titrating resonances that are presented in this report.

### Table II

<table>
<thead>
<tr>
<th>ASSIGNMENT</th>
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<td>Tyr-146</td>
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<td>$\delta^C$</td>
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</table>

a Not assigned.

b The signal-to-noise ratio in the PRFT spectrum of horse ferrimyoglobin was not sufficient to rule out the possibility that the assignments of Peaks 22 and 24 should be interchanged.

c Not detected (see text).
d Tentative assignment.

### Table III

**Comparison of our pK values for histidines of horse ferrimyoglobin with reported ones**

We do not necessarily imply a one-to-one correspondence between values on the same line.

<table>
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<td>5.0</td>
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</tr>
</tbody>
</table>

a At 36°C in H2O.

b At 16°C in D2O, with "pH" taken as uncorrected pH meter reading.
c At 20°C in D2O, with "pH" taken as uncorrected pH meter reading.

d Tentative assignment.

Studies are in agreement with respect to the presence of 5 titrating histidines with pK values in the range 5.5 to 6.6. However, our results indicate the presence of a histidine residue with a pK < 5, not detected by 1H NMR. Furthermore, when going to horse cyanoferriyoglobin, we observe 2 additional titrating histidine residues, with pK values of 5.3 and about 4.4 (see Table I), which were not detected in 1H NMR spectra of sperm whale cyanoferriyoglobin (13). Most peculiar is the fact that we do not observe the histidine with a high pK (7.4 to 8.0) that has been reported in the 1H NMR studies of ferrimyoglobin (12, 13). This high pK has been assigned first to His-64 (23, 24) and then to His-36 (12, 13). One way to reconcile the 1H and 13C NMR results is to invoke the presence of a tautomeric equilibrium between the $\delta^N$-$\delta^H$ and $\delta^N$-$\delta^H$ imidazole forms, which are expected to have $\delta^C$ chemical shifts about 6 ppm downfield and about 2 ppm upfield, respectively, from the $\delta^C$ chemical shift of the imidazolium form of the residue (6–8). If there is fast exchange between appropriate proportions of the two imidazole tautomers, then it is possible for the imidazole form of the residue to have a $\delta^C$ chemical shift that is very similar to that of the imidazolium form. In such a case a titrating histidine would yield a "nontitrating" $\delta^C$ resonance, but a titrating behavior in the 1H NMR spec-
trum (see introductory section). However, the 'H NMR spectra of carbon monoxide myoglobin from horse (10) and sperm whale (13) and of oxymyoglobin and cyanoferrimyoglobin from sperm whale (13) do not yield evidence for the presence of a histidine with the high pK. In the case of sperm whale azidoferrimyoglobin, Hayes et al. (12) reported the presence of the high pK, while Botelho (13) did not observe it. Additional work seems in order.

Our results indicate the presence of only 2 nontitrating histidine residues (other than His-93) in cyanoferrimyoglobins (see Table II). These histidines have chemical shifts consistent with assignments to imidazolium or N^\text{Me}-H imidazole states (8). Strictly speaking, we cannot rule out the possibility that one or both of these histidines titrate, but with the imidazole form being a mixture of the N^2-H and N^\text{Me}-H tautomers, in proportions that yield a chemical shift essentially indistinguishable from that of the imidazolium form (see above). The crystal structure of myoglobin (19) indicates that His-24 and His-36 are likely candidates for the N^2-H tautomeric state.

REFERENCES
Titration behavior and tautomeric states of individual histidine residues of myoglobins. Application of natural abundance carbon 13 nuclear magnetic resonance spectroscopy.
D J Wilbur and A Allerhand


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