Studies of Individual Carbon Sites of Hemoglobins in Solution by Natural Abundance Carbon 13 Nuclear Magnetic Resonance Spectroscopy*

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Proton-decoupled natural abundance $^{13}$C NMR spectra of carbon monoxide hemoglobins were recorded at 15.18 MHz by the Fourier transform method, under conditions of spectrometer sensitivity sufficient for detection of individual carbon resonances. The aromatic region of each spectrum contains broad bands of methine carbon resonances, and some relatively narrow peaks arising from nonprotonated carbons. Resonances of heme carbons were detected in spectra of carbon monoxide hemoglobins, but not in spectra of ferrithemoglobin (as a result of paramagnetic effects). Spectra of carbon monoxide hemoglobins from various species yielded only a few well resolved individual carbon resonances, most notably those of $C^0$ of tryptophan residues. A comparison of the spectra of human adult, human fetal, chicken AII, and bovine fetal hemoglobins yielded assignments for all resonances of $C^0$ of tryptophan residues. In the cases of human fetal, chicken AII, and bovine fetal hemoglobins, each tryptophan yielded a completely resolved individual carbon resonance. The chemical shift difference between the resonances of $C^0$ of Trp-130/3 and $C^0$ of Trp-370 is about 6 ppm. The chemical shift difference between Trp A12/14$^a$ and Trp A12/15$^b$ is 1 ppm or less. A comparison of the chemical shifts of analogous tryptophan residues of the four carbon monoxide hemoglobins suggests very similar conformations in solution.

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were collected in an LKB 7000 Ultrorac fraction collector. The main fetal component F, was eluted between pH 7.5 and 7.4. The elution profile was monitored by the optical density at 415 nm. The fetal exchange into a 0.05 M phosphate/O.1 M NaCl buffer, pH 7.0, by carbon monoxide hemoglobin was concentrated under argon in a Diaflo system. This column repetitive concentration in a Diaflo system. The sample was filtered and was re-equilibrated with carbon monoxide gas. The product was homogeneous as determined by cellulose acetate electrophoresis in a Beckman microzone apparatus using a Tris borate EDTA buffer at pH 8.23, as well as in a pH 8.6 barbital buffer. The hemoglobin migrated at the same rate as a standard sample (obtained from Gelman Instrument Co.) which had been converted to the carbon monoxide form. In addition, sodium dodecyl sulfate polyacrylamide gel electrophoresis of the hemoglobin produced during isolation of the protein. The hemoglobin was eluted with a 0.05 M phosphate/0.1 M NaCl buffer, pH 7.5. Then the sample was processed essentially as described for human adult carbon monoxide hemoglobin in H.O (0.1 M NaCl/0.05 M phosphate buffer, pH 7.0, 34°), recorded at 15.18 MHz using 32,768 accumulations, a recycle time of 1.165 s (10 hours total time), and 0.62-Hz digital broadening.

RESULTS AND DISCUSSION

Fig. 1 shows a fully proton-decoupled natural abundance 13C NMR spectrum of a 3.3 mM solution of human adult carbon monoxide hemoglobin at pH 7.0 and 34°. We can divide this spectrum into the unsaturated carbon region (downfield) and the saturated carbon region (upfield). This report is concerned mainly with nonprotonated aromatic carbons. In Fig. 2A we show the unsaturated carbon region of Fig. 1. Carbonyl resonances are in the range 170 to 210 ppm, aromatic carbon resonances in the range 90 to 160 ppm, and the resonances of C\textsuperscript{3} of arginine residues at the downfield edge of the aromatic region (6). The aromatic region contains some broad features and some relatively narrow lines. In order to confirm our

![Fig. 1. Fully proton-decoupled natural abundance 13C Fourier transform NMR spectrum of 3.3 mM (in tetramer) human adult carbon monoxide hemoglobin in H.O (0.1 M NaCl/0.05 M phosphate buffer, pH 7.0, 34°), recorded at 15.18 MHz using 32,768 accumulations, a recycle time of 1.165 s (10 hours total time), and 0.62-Hz digital broadening.](http://example.com/fig1.png)

![Fig. 2. A, unsaturated carbon region from the spectrum of Fig. 1. The vertical gain in the carbonyl region is one-half that of the aromatic region. B, as spectrum A, but recorded under conditions of noise-modulated off-resonance proton decoupling, using 131,072 accumulations (40 hours total time). The vertical gain of the main carbonyl printout is 8.4 times that of the aromatic region. The inset at about 210 ppm has a 4-fold vertical expansion (relative to the aromatic region) and a 2-fold horizontal expansion. The inset at 100 to 105 ppm has a 4-fold vertical expansion.](http://example.com/fig2.png)
expectation, based on studies of smaller proteins (2–6), that all
narrow resonances in the aromatic region arise from non-
protonated carbons, we recorded a spectrum of human adult
hemoglobin under conditions of noise-modulated off-resonance proton decoupling (12). Any narrow
methine-carbon resonances in the fully proton-decoupled spec-
trum (Fig. 2A) would become broad under the inefficient
decoupling conditions of Fig. 2B (6). However, all narrow
resonances of Fig. 2A remain narrow in Fig. 2B, and therefore
are assigned to nonprotonated carbons.

The two peaks at the downfield end of the carbonyl region (at
207.4 ppm and 206.9 ppm) have been observed in 13C NMR
spectra of carbon monoxide hemoglobin prepared with 80 to
90% 13C-enriched carbon monoxide (13–15), and have been
assigned to carbon monoxide bound to the hemes of the α
chains and β chains, respectively (13–15). Our hemoglobin was
prepared with carbon monoxide of natural isotopic composition
(1.1% 13C), so that our peaks truly represent individual
carbon sites in the natural abundance 13C NMR spectrum of
carbon monoxide hemoglobin. The intensity of these reso-
cances can be used to estimate how many carbons contribute
to other peaks in Fig. 2 (5).

There are 55 nonequivalent nonprotonated aromatic carbons
of amino acid residues and 32 nonequivalent nonprotonated
aromatic carbons of the hemes in a molecule of human adult
hemoglobin (16). If we also include the resonances of Cτ of the 6
nonequivalent arginine residues, we hope to find, ideally, 93
resolved narrow resonances (arising from nonprotonated car-
bons) in the aromatic region of Fig. 2. Clearly, few resolved
individual carbon resonances can actually be observed. We
have shown (6) that the convolution-difference method of
Campbell et al. (17) can improve the resolution of non-
protonated aromatic carbon resonances of small proteins (6).
We have applied the convolution-difference method to our 13C
NMR spectra of hemoglobins. The resulting spectra (not
shown) may be useful in future studies, but they are not
necessary for extracting the information presented below. It
should be noted that we use a magnetic field strength of only
14.2 kG. The greater resolution of a higher magnetic field
strength would be very desirable. Nevertheless, we show below
that even at 14.2 kG some resonances assignable to individual
carbon sites can be observed in the aromatic regions of 13C
NMR spectra of carbon monoxide hemoglobin.

On the basis of their chemical shifts (2, 3, 6), the resonances
at the downfield edge of the aromatic region (155 to 159 ppm in
Fig. 2) can be assigned to Cτ of the 6 nonequivalent tyrosine
and the 6 nonequivalent arginine residues. The peaks in the
range 141 to 147 ppm (Fig. 2) are not detected in the spectrum
of the paramagnetic ferrihemoglobin (Fig. 3). On the basis of
this behavior (3, 6) and chemical shift considerations (3, 6), we
assign these resonances to nonprotonated aromatic carbons
of the hemes. Most of the 16 nonprotonated aromatic carbons
of the heme of horse carbon monoxide myoglobin yield resonances
in the range 140 to 146 ppm (6). It is likely that the region 141
to 147 ppm (Fig. 2) contains most but not all of the resonances
of the 32 nonequivalent nonprotonated aromatic carbons of the
hemes of human adult carbon monoxide hemoglobin.

We tentatively assign the peak at 124.7 ppm (Fig. 2) to Cτ
of the 4 proximal histidine residues (two individual carbon sites).
We base this tentative assignment on three observations. First,
there are no nonprotonated carbon resonances with chemical
shifts comparable to that of this peak in the 13C NMR spectra
of proteins without a heme group (6). Second, this resonance is
not present in a spectrum of the paramagnetic ferrihemoglobin
(Fig. 3). Third, in the case of horse heart ferrocytochrome c, Cτ
of the histidine coordinated to the iron (His-18) has a chemical
shift of 122.7 ppm (6).

On the basis of their chemical shifts (6), the resonances at
111.1 and 107.8 ppm (Fig. 2) must arise from Cτ of the
peak at 107.8 ppm is an individual carbon resonance. The
specific assignment of this resonance to Trp C3[37]β is
presented below.

From the standpoint of spectral resolution, the resonances of
Cτ of tryptophan residues appear to be the most promising
individual carbon probes in natural abundance 13C NMR
spectra of hemoglobins. We have studied the 13C NMR spectra
of hemoglobins from various species, chosen on the basis of
interesting substitutions of tryptophan residues. In Fig. 4 we

![Fig. 3. Unsaturated carbon region in the natural abundance 13C Fourier transform NMR spectrum of 3.6 mM (in tetramer) human adult ferrihemoglobin in H2O (0.1 M NaCl/0.05 M phosphate buffer, pH 6.9, 34°C), recorded at 15.18 MHz under conditions of noise-modulated off-resonance proton decoupling, with 29,422 accumulations, a recycle
time of 2.055 s (17 hours total time), and 1.24 Hz digital broadening. The vertical gain of the carbonyl region is one-third that of the aromatic region.](https://www.jbc.org/content/282/44/6405)

![Fig. 4. Unsaturated carbon region in natural abundance 13C Fourier transform NMR spectra of human adult and human fetal carbon monoxide hemoglobins in H2O (0.1 M NaCl/0.05 M phosphate buffer, pH 7.0). Inset: Vertical scale expansions are shown for the region of Cτ of tryptophan residues. Contributions from broad methine-carbon bands were removed digitally in each inset. A, human adult hemoglobin, from Fig. 1A. The inset is from the same data set as Fig. 2B, but with a digital broadening of 0.93 Hz. B, human fetal (Fet) hemoglobin, 2.3 mM in tetramer, at 36°C, fully proton
decoupled, after 251,167 accumulations with a recycle time of 1.105 s (77 hours total time). Digital broadening was 0.62 Hz for the main spectrum and 0.93 Hz for the inset.](https://www.jbc.org/content/282/44/6405)
compare the unsaturated carbon regions of natural abundance 13C NMR spectra of human adult and human fetal hemoglobins, both in the carbon monoxide form. The differences in the region of C\textsuperscript{\gamma} of tryptophan residues are discussed below. The only other difference between these spectra that we can explain at this time is the presence of a peak at 169 ppm, just upfield of the main carbonyl band, in the spectrum of human fetal hemoglobin (Fig. 4B). We assign this peak to the carbonyl of the NH\textsubscript{\gamma}-terminal glycine residues of the \gamma chains. This assignment is based on the close similarity of the chemical shift of this resonance to that of NH\textsubscript{\gamma}-terminal glycine residues in small peptides (18-20) and horse myoglobin (6), and on the observation that this resonance titrates approximately 1 ppm downfield when the pH is raised from 7.0 to 7.6. A more detailed justification for this assignment is identical with that presented for the assignment of the resonance at 168.5 ppm (at pH 6.7) in the spectrum of horse carbon monoxide myoglobin to the carbonyl of Gly-1 (6). The pK\textsubscript{\textgamma} of Gly-1\textgamma of human fetal hemoglobin is not known. However, on the basis of the measured apparent pK\textsubscript{\textgamma} of NH\textsubscript{\gamma}-terminal glycine residues in some myoglobins (21), we expect a pK\textsubscript{\textgamma} in the range 6.5 to 8.5.

The carbonyl resonance of Val-1 of sperm whale myoglobin is not sufficiently removed from the main carbonyl band to permit an easy identification (6). Similarly, Fig. 4A indicates that the carbonyl resonances of Val-1\alpha and Val-1\beta of human adult hemoglobin are not resolved from the main carbonyl band. The same should be true for Val-1\alpha of human fetal hemoglobin.

In Fig. 5 we show the region of C\textsuperscript{\gamma} resonances of tryptophan residues in the spectra of human adult, human fetal, chicken AI\textsubscript{1}, and bovine fetal hemoglobins, and the corresponding region in the 13C NMR spectrum of horse carbon monoxide myoglobin (6). Human adult hemoglobin has 3 nonequivalent tryptophan residues: A\textsubscript{12}[14], A\textsubscript{12}[15], and C\textsubscript{33}[37]. On the basis of the crystal structure (22), we would expect the C\textsuperscript{\gamma} resonances of Trp A\textsubscript{12}[14] and A\textsubscript{12}[15] to have very similar chemical shifts. Indeed, there is a 2-carbon resonance at 111.1 ppm (Fig. 5A), which we now tentatively assign to these 2 residues. By elimination, the resonance at 107.8 ppm is assigned to C\textsuperscript{\gamma} of Trp C\textsubscript{33}[37]. These assignments are confirmed below.

Human fetal hemoglobin (F\textsubscript{\textgamma}) has the same \alpha chain as human adult hemoglobin (A\textsubscript{\textalpha}). The amino acid sequence of the \gamma chain has 39 substitutions relative to the \beta chain of hemoglobin A\textsubscript{\textbeta} (16). However, the only substitution involving a tryptophan residue is the replacement of Tyr-130\textbeta by Trp-130\textgamma. Therefore, we expect an additional resonance in the region of C\textsuperscript{\gamma} of tryptophan residues in the spectrum of human fetal hemoglobin. The experimental result is shown in Fig. 5B. There is a new resonance at 114.3 ppm, which we assign to C\textsuperscript{\gamma} of Trp-130\textgamma. The resonance at 107.8 ppm in the spectrum of human adult hemoglobin, tentatively assigned above to C\textsuperscript{\gamma} of Trp-37\textbeta, is still present, with about the same chemical shift, in the spectrum of human fetal hemoglobin (Fig. 5B). The unresolved 2-carbon resonance at 111.1 ppm in the spectrum of human adult hemoglobin, tentatively assigned to C\textsuperscript{\gamma} of Trp-14\textalpha and Trp-15\textbeta, becomes slightly split in the spectrum of human fetal hemoglobin (Fig. 5B), as a result of an upfield shift (about 0.5 ppm) of one component. The other component has not moved appreciably. We do not expect a large change in the chemical shift of C\textsuperscript{\gamma} of Trp-14\textalpha when going from adult to fetal hemoglobin. However, some of the 39 substitutions when going from the \beta chains to the \gamma chains may cause a difference between Trp-15\textbeta and Trp-15\textgamma. On this basis, we tentatively assign the relatively unshifted peak at 111.3 ppm (Fig. 5B) to C\textsuperscript{\gamma} of Trp-14\textalpha, and the peak at 110.5 ppm to C\textsuperscript{\gamma} of Trp-15\textgamma. An examination of the spectra of chicken AI\textsubscript{1} and bovine fetal hemoglobins confirms these assignments.

Chicken AI\textsubscript{1} hemoglobin has no tryptophan residues in the \alpha chains (16) and 4 tryptophan residues in the \beta chains (23), at positions 3, 15, 37, and 130. Therefore, on the basis of the above assignments, we predict resonances at about 114.3 ppm, about 110.5 ppm, and about 108 ppm, for C\textsuperscript{\gamma} of Trp-130\textbeta, Trp-15\textbeta, and Trp-37\textbeta, respectively. Hopefully, the fourth resonance (C\textsuperscript{\gamma} of Trp-37\textbeta) will not overlap with any of the above ones. The spectrum yields four resonances, at 114.6 ppm, 110.4 ppm, 109.5 ppm, and 108.1 ppm (Fig. 5C). Clearly, the resonance at 109.5 ppm is the new one (Trp-37\textbeta), while the other three have...
chemical shifts in excellent agreement with the predicted ones.

Bovine fetal hemoglobin has 2 nonequivalent tryptophan residues, Trp-14α and Trp-36β (16). The latter is analogous to Trp-37β of the other hemoglobins (16). On the basis of our assignments, we predict that the region of Cα of tryptophan residues in the 13C NMR spectrum of bovine fetal hemoglobin will have only two resonances, at about 111.3 ppm (Trp-14α) and 108 ppm (Trp-36β). The spectrum (Fig. 5D) yields resonances at 111.4 ppm and 107.8 ppm.

On the basis of the structural similarity of myoglobin and the subunits of hemoglobin (24, 25), we speculated that the chemical shift of Cα of Trp A12[14] of myoglobin might be very similar to the corresponding chemical shifts of Trp A12[14]α and Trp A12[15]β of hemoglobin. A spectrum of horse carbon monoxide myoglobin yields resonances at 109.7 ppm and 111.1 ppm (Fig. 5E). We tentatively assign the peak at 111.1 ppm to Cα of Trp-14.

Within experimental error, the four hemoglobins we have examined yield the same chemical shift for Cα of the invariant Trp-37β (Trp-36β in the case of chicken All hemoglobin). This chemical shift (108.0 ± 0.2 ppm) is about 2 ppm upfield from the corresponding value in denatured hen egg white lysozyme (2). The Cα chemical shift of Trp-130β in human fetal hemoglobin is, within experimental error, the same as that of Trp-130β in chicken All hemoglobin, and is more than 4 ppm downfield from the corresponding value in denatured lysozyme (2). The Cα chemical shift of Trp A12[14]α is also quite invariant when comparing human adult, human fetal, and bovine fetal hemoglobin (Fig. 5), and is about 1 ppm downfield from the corresponding value in denatured lysozyme (2). Trp A12[15]β of chicken All hemoglobin and Trp A12[15]β of human fetal hemoglobin yield Cα resonances with nearly identical chemical shifts. However, there is a small downfield shift (about 0.5 ppm) when going to Trp A12[15]β of human adult hemoglobin. On the whole, the Cα chemical shifts of analogous tryptophan residues of the four hemoglobins we have studied are remarkably similar. This result, taken together with the fact that the chemical shifts change by as much as 6 ppm when going from one tryptophan to another within a hemoglobin molecule, suggests a great conformational similarity, in solution, between these various hemoglobins, in the regions of their tryptophan residues.

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