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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 ferrihemoglobin (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^{γ} of tryptophan residues. A comparison of the spectra of human adult, human fetal, chicken AII, and bovine fetal hemoglobins yielded specific assignments for all resonances of C^{γ} 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^{γ} of Trp-130β and C^{γ} of Trp-37β is about 6 ppm. The chemical shift difference between Trp A12[14]α and Trp A12[15]β 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.

Recent instrumental developments in our laboratory have increased the sensitivity of ^{13}C Fourier transform NMR to the point that it is now practical to detect single carbon resonances of small proteins in solution (1). We have shown (2–6) that the resonances of nonprotonated aromatic carbons are attractive probes of specific sites of small native proteins in solution, because of a favorable combination of factors. First, these resonances are quite narrow, while those of protonated aromatic carbons are relatively broad (2–5). Second, the range of chemical shifts of aromatic carbons is large (about 50 ppm) for a relatively small fraction of all of the carbons of a protein (2–6). Third, folding of a protein into its native conformation produces very significant changes in the chemical shifts of many aromatic carbons (2, 3, 6). We have reported the observation of numerous resolved single carbon resonances in the aromatic regions of the natural abundance ^{13}C NMR spectra of aqueous solutions of hen egg white lysozyme (2), horse heart ferro-, ferri-, and cyanoferricytochrome *c* (3, 6, 7), *Candida krusei* ferricytochrome *c* (6), horse carbon monoxide myoglobin (6), and cyanoferrimyoglobins from horse and sperm whale (6). In this report we show that it is now practical to detect some individual carbon sites of much larger native proteins. Specifically, we report the observation of narrow resonances corresponding to individual carbon sites in the aromatic region of the proton-decoupled natural abundance ^{13}C NMR spectra of hemoglobins.

Natural abundance ^{13}C Fourier transform NMR spectra of hemoglobins have been reported recently by several groups (8–11), but, because of low signal to noise ratios, only general spectral features have been apparent. It should be noted that the inherent symmetry of the hemoglobin tetramer should facilitate the detection of individual carbon sites. Our reference to an individual carbon site implies a contribution of 2 equivalent carbons to the resonance. We show that natural abundance ^{13}C NMR spectra of hemoglobins yield only a few resolved individual carbon resonances, most notably those of C^{γ} of tryptophan residues. We assign these resonances to specific tryptophan residues of the α and β chains. For this initial study we chose the diamagnetic carbonyl derivative of hemoglobin, carbon monoxide hemoglobin.

EXPERIMENTAL PROCEDURE

Human Fetal Carbon Monoxide Hemoglobin (F_0)—Human cord blood (1 volume) was mixed with deionized water (1 volume) at 26° for 30 min to lyse the erythrocytes. The cord blood hemolysate was then reacted with carbon monoxide. The course of the reaction was monitored on a Cary 14 spectrophotometer. Erythrocyte ghosts were removed by centrifugation at 30,000 × *g* at 4° for three 1-hour periods. The hemolysate (5 g of hemoglobin) was dialyzed twice in Spectrapor membrane tubing No. 132680 against 3.5 liters of 0.05 M Tris buffer, pH 8.2, for 12 hours at 4°. The hemolysate was then applied to a DEAE-Sephadex (A-50-120) column (50 g) which had been equilibrated with a 0.05 M Tris buffer at pH 8.0. The column was eluted with

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a pH 8.0 to pH 7.1 linear gradient of 0.05 M Tris, and 20-ml fractions were collected in an LKB 7000 Ultrorac fraction collector. The main fetal component F_0 was eluted between pH 7.5 and 7.4. The elution profile was monitored by the optical density at 415 nm. The fetal carbon monoxide hemoglobin was concentrated under argon in an Amicon Diaflo ultrafiltration system, using an XM-50 membrane. Tris buffer was removed by passing the sample through a Sephadex G-25 (medium) column equilibrated with 0.1 M NaCl at pH 7.5. This column had been layered with a band of sodium dithionite to reduce any ferrihemoglobin produced during isolation of the protein. The hemoglobin was eluted with 0.1 M NaCl and was then recarbonylated and exchanged into a 0.05 M phosphate/0.1 M NaCl buffer, pH 7.0, by repetitive concentration in a Diaflo system. The sample was filtered through a Millipore membrane (0.8 μm) to remove suspended particles, 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 in a pH 8.7 Tris/borate/EDTA buffer, carried out after the NMR experiments, indicated that the product was homogeneous and that no decomposition had occurred.

Chicken Carbon Monoxide Hemoglobin (AII)—Chicken blood (250 ml) was obtained after decapitation of one large rooster and was immediately heparinized, and then washed with a 1% NaCl solution, and centrifuged at 4000 × *g* for 20 min at 4°. The sample was resuspended and the process was repeated three more times. The sample was then treated in the same manner as the human fetal hemolysate with the exception that a linear gradient of 0.05 M Tris buffer, pH 8.15 to pH 6.9, was used for elution of hemoglobins AI and AII. Purity was established by means of cellulose acetate electrophoresis of our sample and of standard AI and AII samples kindly provided by F. R. N. Gurd, Department of Chemistry, Indiana University.

Bovine Fetal Carbon Monoxide Hemoglobin—Blood obtained from a 4-day-old Charolais calf was immediately citrated. The sample was washed, and a concentrated, carbonylated, membrane-free hemolysate was prepared. Five per cent NaCl was used to facilitate membrane sedimentation. Small molecule impurities were removed by chromatography on a Sephadex G-25 (medium) column equilibrated in a 0.05 M phosphate/0.1 M NaCl buffer, pH 7.5, essentially as described for the human fetal hemoglobin. The sample was then exchanged into a 0.05 M phosphate/0.1 M NaCl buffer, pH 6.8, using an XM-50 membrane in a Diaflo ultrafiltration apparatus. Cellulose acetate electrophoresis indicated the presence of two hemoglobins (3:2 ratio), neither of which migrated with bovine adult hemoglobin.

Human Adult Carbon Monoxide Hemoglobin—Erythrocytes were washed with 1% NaCl as described for chicken hemoglobin, and the sample was then lysed, carbonylated, and clarified as described for the human fetal hemoglobin. Small ions were removed on a Sephadex G-25 (medium) column equilibrated in a 0.05 M phosphate/0.1 M NaCl buffer, pH 7.5. Then the sample was processed essentially as described for the human fetal carbon monoxide hemoglobin. We determined by electrophoresis that this sample contained approximately 90% hemoglobin A_0.

Human Adult Ferrihemoglobin—Hemolysates were prepared as described for human adult carbon monoxide hemoglobin. Ferrihemoglobin was prepared by the oxidation of oxyhemoglobin with potassium ferricyanide at pH 7.0. Small ions were either removed by desalting on Sephadex G-25 (fine) or by exhaustive dialysis against buffers of high ionic strength. Purity was monitored by cellulose acetate electrophoresis.

Carbon 13 NMR—Proton-decoupled natural abundance ^{13}C Fourier transform NMR spectra were recorded at 15.18 MHz, with the use of 20-mm spinning sample tubes. Details have been given elsewhere (1-3, 5). Each spectrum was obtained with the use of 90° radiofrequency pulse excitation, 8,192 time domain addresses, and a spectral width of 4,000 Hz. Fourier transformation was done on 16,384 time domain points, by placing 8,192 addresses with a zero value at the end of each block of 8,192 accumulated data points. Chemical shifts were measured digitally, and are reported in parts per million downfield from Me_4Si. Estimated accuracy is ±0.2 ppm for hemoglobin and ±0.1 ppm for myoglobin. Dioxane (at 67.8$_6$ ppm) was used as an internal standard, except for human fetal hemoglobin, which was referenced to external ethylene glycol (at 64.3 ppm).

RESULTS AND DISCUSSION

Fig. 1 shows a fully proton-decoupled natural abundance ^{13}C 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. 2*A* 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 Cζ 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

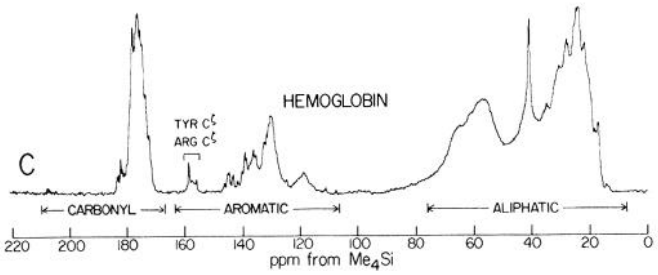

FIG. 1. Fully proton-decoupled natural abundance ^{13}C Fourier transform NMR spectrum of 3.3 mM (in tetramer) human adult carbon monoxide hemoglobin in H_2O (0.1 M NaCl/0.05 M phosphate buffer, pH 7.0, 34°), recorded at 15.18 MHz using 32,768 accumulations, a recycle time of 1.105 s (10 hours total time), and 0.62-Hz digital broadening.

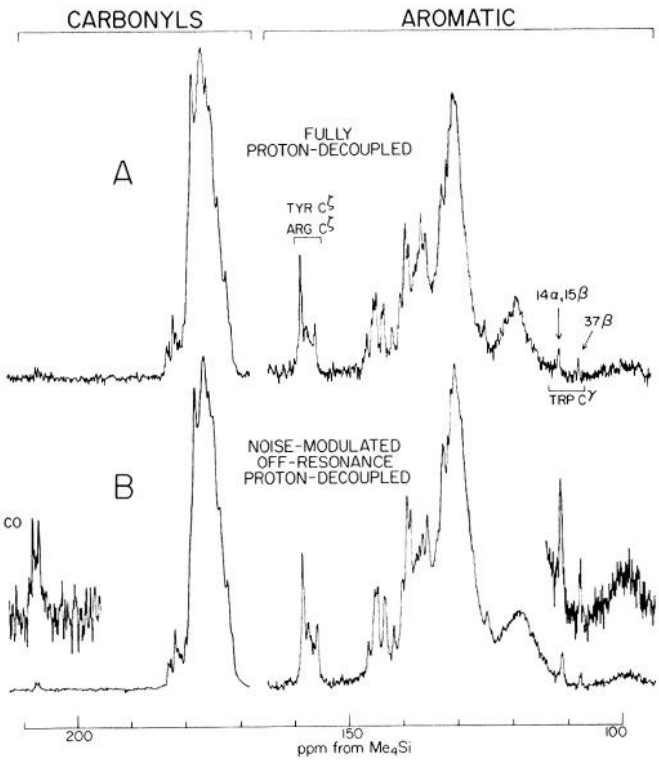

FIG. 2. *A*, unsatuated carbon region from the spectrum of Fig. 1. The vertical gain in the carbonyl region is one-half that of the aromatic region. *B*, as spectrum *A*, but recorded under conditions of noise-modulated off-resonance proton decoupling, using 131,072 accumulations (40 hours total time). The vertical gain of the main carbonyl printout is 0.4 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.

expectation, based on studies of smaller proteins (2–6), that all narrow resonances in the aromatic region arise from nonprotonated carbons, we recorded a spectrum of human adult carbon monoxide hemoglobin under conditions of noise-modulated off-resonance proton decoupling (12). Any narrow methine-carbon resonances in the fully proton-decoupled spectrum (Fig. 2*A*) would become broad under the inefficient decoupling conditions of Fig. 2*B* (6). However, all narrow resonances of Fig. 2*A* remain narrow in Fig. 2*B*, 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 ^{13}C NMR spectra of carbon monoxide hemoglobin prepared with 80 to 90% ^{13}C-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% ^{13}C), so that our peaks truly represent individual carbon sites in the natural abundance ^{13}C NMR spectrum of carbon monoxide hemoglobin. The intensity of these resonances 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 Cζ of the 6 nonequivalent arginine residues, we hope to find, ideally, 93 resolved narrow resonances (arising from nonprotonated carbons) 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 nonprotonated aromatic carbon resonances of small proteins (6). We have applied the convolution-difference method to our ^{13}C 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 ^{13}C NMR spectra of carbon monoxide hemoglobins.

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 Cζ 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 C$^{\gamma}$ 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 ^{13}C 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*, C$^{\gamma}$ 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 C$^{\gamma}$ of the tryptophan residues: A12[14]α, A12[15]β, and C3[37]β. 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 C$^{\gamma}$ of tryptophan residues appear to be the most promising individual carbon probes in natural abundance ^{13}C NMR spectra of hemoglobins. We have studied the ^{13}C NMR spectra of hemoglobins from various species, chosen on the basis of interesting substitutions of tryptophan residues. In Fig. 4 we

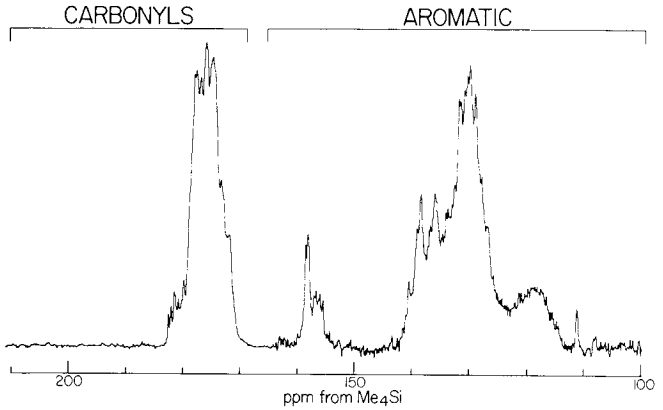

FIG. 3. Unsaturated carbon region in the natural abundance ^{13}C Fourier transform NMR spectrum of 3.6 mM (in tetramer) human adult ferrihemoglobin in H_2O (0.1 M NaCl/0.05 M phosphate buffer, pH 6.9, 34°), recorded at 15.18 MHz under conditions of noise-modulated off-resonance proton decoupling, with 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.

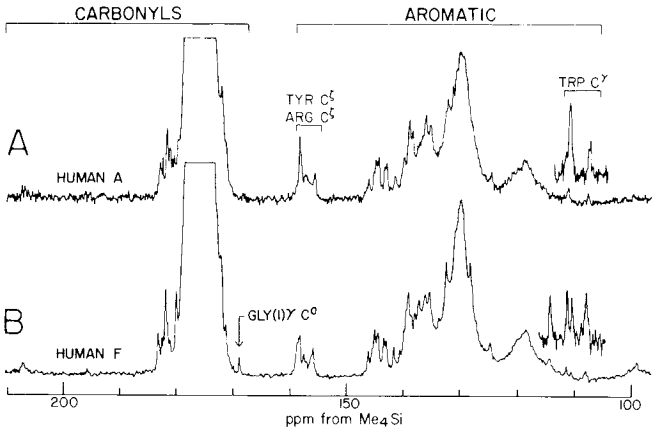

FIG. 4. Unsaturated carbon region in natural abundance ^{13}C Fourier transform NMR spectra of human adult and human fetal carbon monoxide hemoglobins in H_2O (0.1 M NaCl/0.05 M phosphate buffer, pH 7.0). *Insets* with vertical scale expansions are shown for the region of C$^{\gamma}$ of tryptophan residues. Contributions from broad methine-carbon bands were removed digitally in each *inset*. *A*, human adult hemoglobin, from Fig. 1*A*. The *inset* is from the same time domain data as Fig. 2*B*, but with a digital broadening of 0.93 Hz. *B*, human fetal (F_0) hemoglobin, 2.3 mM in tetramer, at 36°, 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*.

compare the unsaturated carbon regions of natural abundance ^{13}C NMR spectra of human adult and human fetal hemoglobins, both in the carbon monoxide form. The differences in the region of C^{γ} 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. 4*B*). We assign this peak to the carbonyl of the NH_2-terminal glycine residues of the γ chains. This assignment is based on the close similarity of the chemical shift of this resonance to that of NH_2-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_a of Gly-1γ of human fetal hemoglobin is not known. However, on the basis of the measured apparent pK_a of NH_2-terminal glycine residues in some myoglobins (21), we expect a pK_a 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. 4*A* indicates that the carbonyl resonances of Val-1α and Val-1β of human adult hemoglobin are not resolved from the main carbonyl band. The same should be true for Val-1α of human fetal hemoglobin.

In Fig. 5 we show the region of C^{γ} resonances of tryptophan residues in the spectra of human adult, human fetal, chicken AII and bovine fetal hemoglobins, and the corresponding region in the ^{13}C NMR spectrum of horse carbon monoxide myoglobin (6). Human adult hemoglobin has 3 nonequivalent tryptophan residues: A12[14]α, A12[15]β, and C3[37]β. On the basis of the crystal structure (22), we would expect the C^{γ} resonances of Trp A12[14]α and A12[15]β to have very similar chemical shifts. Indeed, there is a 2-carbon resonance at 111.1 ppm (Fig. 5*A*), which we now tentatively assign to these 2 residues. By elimination, the resonance at 107.8 ppm is assigned to C^{γ} of Trp C3[37]β. These assignments are confirmed below.

Human fetal hemoglobin (F_0) has the same α chain as human adult hemoglobin (A_0). The amino acid sequence of the γ chain has 39 substitutions relative to the β chain of hemoglobin A_0 (16). However, the only substitution involving a tryptophan residue is the replacement of Tyr-130β by Trp-130γ. Therefore, we expect an additional resonance in the region of C^{γ} of tryptophan residues in the spectrum of human fetal hemoglobin. The experimental result is shown in Fig. 5*B*. There is a new resonance at 114.3 ppm, which we assign to C^{γ} of Trp-130γ. The resonance at 107.8 ppm in the spectrum of human adult hemoglobin, tentatively assigned above to C^{γ} of Trp-37β, is still present, with about the same chemical shift, in the spectrum of human fetal hemoglobin (Fig. 5*B*). The unresolved 2-carbon resonance at 111.1 ppm in the spectrum of human adult hemoglobin, tentatively assigned to C^{γ} of Trp-14α and Trp-15β, becomes slightly split in the spectrum of human fetal hemoglobin (Fig. 5*B*), 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^{γ} of Trp-14α when going from adult to fetal hemoglobin. However, some of the 39 substitutions when going from the β chains to the γ chains may cause a difference

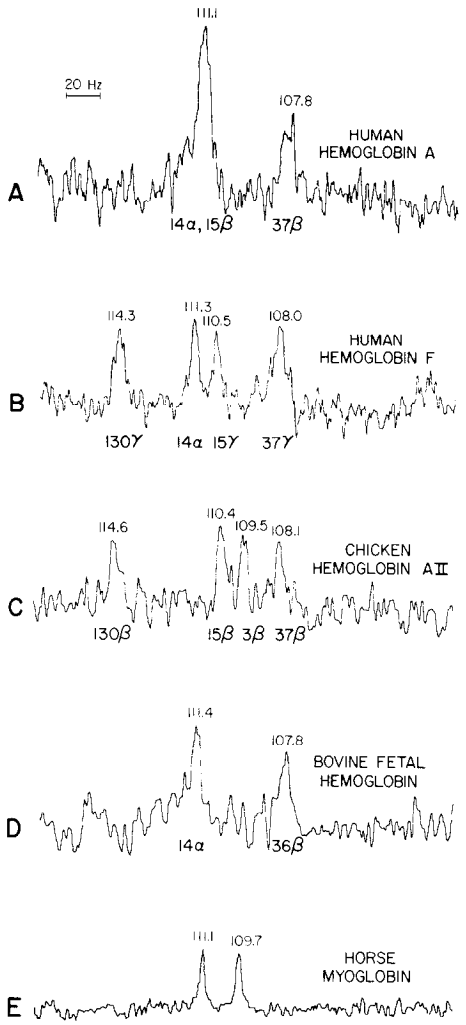

FIG. 5. Region of C^{γ} resonances of tryptophan residues in natural abundance ^{13}C Fourier transform NMR spectra of some carbon monoxide hemoglobins and horse carbon monoxide myoglobin in H_2O (0.1 M NaCl/0.05 M phosphate buffer). Contributions from broad methine-carbon bands were removed digitally from the hemoglobin spectra. Chemical shifts are given in parts per million downfield from Me_4Si. *A*, same as *inset* of Fig. 4*A*. *B*, same as *inset* of Fig. 4*B*. *C*, chicken (AII) hemoglobin, 2.6 mM in tetramer, pH 7.0, 36°, fully proton-decoupled, after 131,072 accumulations with a recycle time of 1.105 s (40 hours total time), and a digital broadening of 1.55 Hz. *D*, bovine fetal hemoglobin, 3.3 mM in tetramer, pH 7.0, 36°, fully proton-decoupled, after 98,304 accumulations with a recycle time of 1.105 s (30 hours total time), and a digital broadening of 1.55 Hz. *E*, horse carbon monoxide myoglobin, 9.7 mM, pH 6.7, 36°. Spectral conditions are given in Fig. 1*B* of Ref. 5.

between Trp-15β and Trp-15γ. On this basis, we tentatively assign the relatively unshifted peak at 111.3 ppm (Fig. 5*B*) to C^{γ} of Trp-14α, and the peak at 110.5 ppm to C^{γ} of Trp-15γ. An examination of the spectra of chicken AII and bovine fetal hemoglobins confirms these assignments.

Chicken AII hemoglobin has no tryptophan residues in the α chains (16) and 4 tryptophan residues in the β 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^{γ} of Trp-130β, Trp-15β, and Trp-37β, respectively. Hopefully, the fourth resonance (C^{γ} of Trp-3β) 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. 5*C*). Clearly, the resonance at 109.5 ppm is the new one (Trp-3β), 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 C^{γ} of tryptophan residues in the ^{13}C 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. 5*D*) 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 C^{γ} 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 hemoglobins. A spectrum of horse carbon monoxide myoglobin yields resonances at 109.7 ppm and 111.1 ppm (Fig. 5*E*). We tentatively assign the peak at 111.1 ppm to C^{γ} of Trp-14.

Within experimental error, the four hemoglobins we have examined yield the same chemical shift for C^{γ} of the invariant Trp-37β (Trp-36β in the case of chicken AII hemoglobin). This chemical shift (108.0 $\pm$ 0.2 ppm) is about 2 ppm upfield from the corresponding value in denatured hen egg white lysozyme (2). The C^{γ} chemical shift of Trp-130γ in human fetal hemoglobin is, within experimental error, the same as that of Trp-130β in chicken AII hemoglobin, and is more than 4 ppm downfield from the corresponding value of denatured lysozyme (2). The C^{γ} 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 of denatured lysozyme (2). Trp A12[15]β of chicken AII hemoglobin and Trp A12[15]γ of human fetal hemoglobin yield C^{γ} 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 C^{γ} 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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