CARBON-13 NMR STUDIES OF ^{13}CO BINDING TO HUMAN HEMOGLOBIN*

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Summary - In the ^{13}C NMR spectrum of hemoglobin A carbonylated with ^{13}CO, separate resonances can be distinguished at 207.04 ppm and 206.60 ppm (with respect to the ^{13}C resonance of external tetramethylsilane) for ^{13}CO bound to the α and β chains of the hemoglobin tetramer. A study of the ^{13}CO derivatives of the isolated α and β chains, and of the abnormal hemoglobin M_{IWATE} which contains α chains which are in the met [Fe(III)] form and do not bind CO, has permitted an assignment of the high field (206.60 ppm) resonance to the β chain ^{13}CO and the low field one to the α chain ^{13}CO. The identification of these ^{13}CO resonances permits a study of the differences in the chemistry of the α and β heme units in intact hemoglobin. Some results on the differences in the redox behavior of these chains are included.

INTRODUCTION

An elegant sterochemical model based on X-ray analyses of horse and human methemoglobin and on difference map analyses of various mutant hemoglobins and deoxyhemoglobin, has been proposed by Perutz[1,2] to account for heme cooperativity and the Bohr effect in tetrameric hemoglobins. Two questions about the Perutz model have recently been subjected to experimental tests by magnetic resonance and spin labeling methods: 1) Do the α and β subunits have different intrinsic binding affinities for ligands at the heme oxygen binding site?, and 2) Do oxyhemoglobin and methemoglobin have the same conformation? Because separate resonances can be distinguished for ^{13}CO bound to the heme units of the α and β hemoglobin chains[3,4,5],

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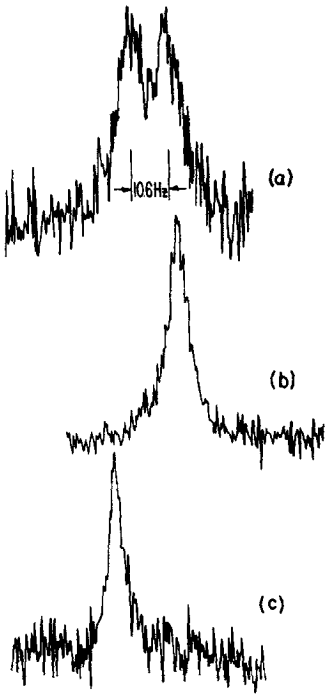

Fig. 1. Proton Noise Decoupled ^{13}C FT-NMR Spectra in the ^{13}CO Region of: (a) Carbonyl HbA, 22,000 pulses; (b) Carbonylated β Chains, 16,000 pulses; and (c) Carbonylated α chains, 8,500 pulses. In each case the sweep width was 200 Hz and the spectra were accumulated as 512 points in the frequency domain.

new insights regarding these questions should result from a study of the ^{13}C NMR spectroscopy of carbonyl hemoglobins. In contrast to other labels which have been used in hemoglobin studies[6,7,8,9] ^{13}CO is an innocuous reporter group which is bound directly to an "active site" and which bears a close similarity to oxygen as a ligand toward hemoglobin. We have obtained high resolution ^{13}C NMR spectra of some carbonylated normal and abnormal hemoglobins and report the results here.

Methods and Materials

Normal whole human hemoglobin was obtained from C. T. Gregg of The Los Alamos Scientific Laboratory. Isolated α and β chains were obtained from

normal blood in the form of p-hydroxy mercuribenzoate derivations. Hb M_{IWATE} ($\alpha_2^{87(F8)His\ Tyr}\beta_2$)[10] was characterized by peptide mapping and amino acid analysis, as well as by its spectral properties, its reactivity to cyanide[11], and its oxygen equilibrium. The Hb M_{IWATE} was isolated from whole blood hemolysate by chromatography at room temperature on Bio Rex 70 with 0.13 M sodium phosphate buffer at pH 6.42[12]. A sample of carbon-13 enriched CO (90% ^{13}C) was supplied by B. B. McInteer, T. R. Mills, and M. Goldblatt of The Los Alamos Scientific Laboratory. Carbonylated hemoglobin samples were prepared by exposure to a positive pressure of ^{13}CO in a closed system for at least 60 min.

Proton decoupled pulse ^{13}C NMR Fourier transform spectra were obtained at 25.2 MHz with a Varian XL-100-15 spectrometer interfaced to a Data General Supernova Computer using the deuterium resonance (15.4 MHz) of external D_2O as a lock. Free induction decays of 100 μ sec rf pulses were accumulated as 1024 points in the time domain and transformed into a 512 point real spectrum of 200 Hz with a data acquisition time of 2.56 sec. All chemical shifts are reported in ppm downfield with respect to external neat TMS. The NMR measurements were obtained with solutions at their natural pH which was within the range 6.6 - 7.3. No attempt was made to rid the normal human hemolysate of residual 2,3-diphosphoglycerate. In studies in progress, we have found that the ^{13}CO resonances of human carbonyl hemoglobins are affected only when the pH is greater than 7.8 or less than 6, and when the diphosphoglycerate concentration is above 50 mM (for a heme concentration of 7 mM).

Results and Discussion

The ^{13}C NMR spectra in the ^{13}CO region are reproduced in Fig. 1 for carbonylated samples of hemoglobin A (HbA) (a), and isolated normal β (b) and α (c) chains. The spectra strongly suggest that the high field (206.61 ppm) signal in the HbA-^{13}CO spectrum is due to the carbonylated β chain whereas the low field signal (207.04 ppm) arises from ^{13}CO bound to the heme unit of the α chain. Supporting evidence for this suggestion, also offered in a recent report by Antonini et al.[5], is necessary because we have found that[13]:

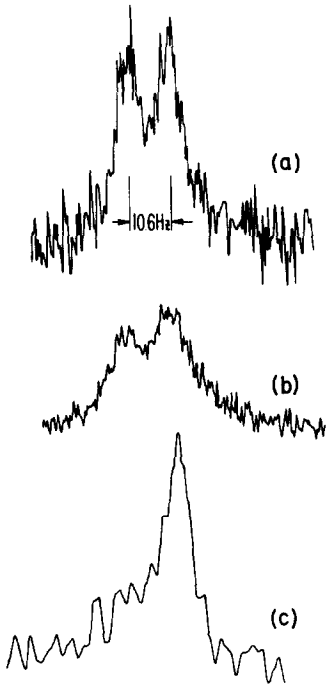

Fig. 2. Proton Noise Decoupled ^{13}C FT-NMR Spectra in the ^{13}CO Region of: (a) Carbonyl HbA, 22,000 pulses; (b) Carbonylated Hemolysate containing 75% HbA and 25% Hemoglobin M_{IWATE} (met heme units in the α chains) 22,000 pulses; and (c) Hemoglobin M_{IWATE} (met α-chains) treated with an eleven-fold excess of sodium dithionite and ^{13}CO, 21,000 pulses.

(a) the ^{13}CO resonances of single chain heme-protein-carbonyl complexes (e.g. lamprey hemoglobin and myoglobin) span a large chemical shift range of 2 ppm; and (b) some heme-protein-carbonyl resonances are strongly shifted by the perturbation of the protein, viz. one α-carbonyl resonance of rabbit hemoglobin shifts by more than 1 ppm when the pH of the medium is lowered from 7 to 5 [4,10] and the separated hemoglobin single chains were studied as the p-hydroxy mercuribenzoate derivatives. The supporting evidence was obtained from studies of Hb M_{IWATE}, a variant of human hemoglobin in which

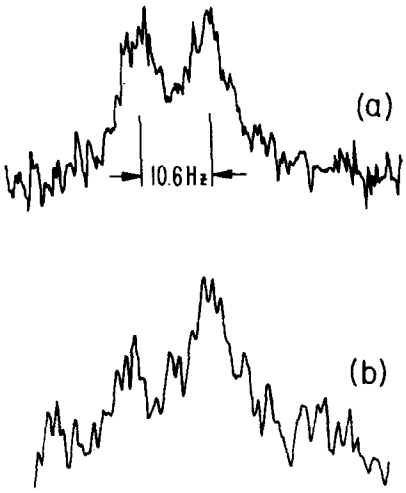

Fig. 3. Proton Noise Decoupled ^{13}C FT-NMR Spectra in the ^{13}CO Region of: (a) Carbonyl HbA (7 mM in heme), 21,000 pulses; and (b) Met HbA (7 mM in heme) treated with 3.8 mM Fe(II) citrate and ^{13}CO, 22,000 pulses.

the heme units of the α chains are in the ferric form and do not bind CO (Fig. 2). In the spectrum of the hemolysate, which contained 25% Hb M_{IWATE} and 75% Hb A, the downfield peak was ∿ 75% as large as the upfield peak. The spectrum of the isolated abnormal hemoglobin exhibited a single peak with a chemical shift of 206.36, corresponding most closely to the upfield peak of Hb A (Table 1). The addition of a large excess of sodium dithionite resulted in only a partial reactivity of the α chain hemes for ^{13}CO, with the appearance of a small peak downfield from that of the β chain.

The identification of these ^{13}CO resonances allows one to study the differences in the chemistry of the α and β heme units in intact hemoglobin. The spectrum shown in Fig. 3b is of the ^{13}CO region of normal met HbA which had been treated with one-half equivalent of Fe(II) citrate and then treated with ^{13}CO. Similar spectra were obtained when sodium dithionite was used as the reductant. It is clear that the β heme units of normal met HbA are more readily reduced than the α hemes. While it is possible that the preferential reduction observed is due merely to the greater accessibility of the reducing

TABLE 1

^{13}C Chemical Shifts and Line Widths of ^{13}CO in Carbonyl Hemoglobins

	α		β	
	Chem. Shift(a)	Line Width(b)	Chem. Shift(a)	Line Width(b)
Isolated Chains (7 mM heme)	206.98	5.3	206.60	6.6
Hemoglobin A (7 mM heme)	207.04	9.9	206.61	10.7
Hemoglobin A (20 mM heme)	207.15	14.0	206.77	16.0
Hemoglobin M_{IWATE}			206.36	17.0
Hemoglobin A (1/2 in the form of met Hemoglobin)	206.98	10.0	206.39	13.0
Hemoglobin A (7 mM heme and 8 M in urea)	207.18	8.5	206.69	9.0

(a) Downfield with respect to external TMS, in ppm ± 0.03.

(b) Full width, in Hz, at half-maximum intensity.

agent to the β hemes, it is also possible that preferential reduction occurs because the conformational changes associated with the transition, $(\alpha^{met}\beta^{met})_2 \rightarrow (\alpha^{met}\beta^{deoxy})_2$, are more favorable than for the process, $(\alpha^{met}\beta^{met})_2 \rightarrow (\alpha^{deoxy}\beta^{met})_2$. In this regard, we note that a major difference between the α and β chains in the region around the heme pockets is that there is a strong interaction between the γ methyl groups of valine E 11 of the β chain and the H_2O bound to Fe(III) of the heme unit[1,2]. In the transition to the deoxy Fe(II) form of the β unit, the water molecule, and the steric perturbation, is removed.

Spectra similar to those shown in Fig. 3b are obtained when carbonyl

HbA is treated with fractional equivalents of the oxidizing agent $K_3Fe(CN)_6$. In this case, it is the α-heme carbonyl units which are more easily oxidized. Since the intermediate which loses the electron in the oxidation process presumably is the deoxyheme[14,15] (or decarbonylated heme) our results suggest that ^{13}CO is dissociated more facilely from the α- than from the β-hemes. Indeed our preliminary ^{13}C NMR studies of carbonyl HbA solutions flushed with argon gas for different time intervals indicate that the α heme units do lose ^{13}CO more readily than the β hemes. The results of these latter experiments may be a complex function of rate and equilibrium effects because of the relatively long signal averaging times (∿ 3-4 hrs.) required to obtain acceptable signal strengths from which integrated relative intensities are obtained. Particularly for the unusual rabbit hemoglobins, we have found[4,13] that the kinetically controlled decarbonylated product $(\alpha^{deoxy}\beta^{CO})_2$ obtained from the treatment of carbonyl Hb with argon slowly rearranges [$t_{1/2}$ ∿2 hrs. to a mixture of the main product $(\alpha^{deoxy}\beta^{CO})_2$ and small amounts of $(\alpha^{CO}\beta^{deoxy})_2$, or $(\alpha^{deoxy}\beta^{deoxy})_2$ and $(\alpha^{CO}\beta^{CO})_2$]. In contrast to these preliminary results, Olson, et al.[16] found that the rate of dissociation of oxygen from the α chains of HbA is much lower than the rate for the β chains. However, Gibson[17] has concluded from a comparison of the dissociation rate constants of oxy-[17] and carbonyl[18] hemoglobins that the rate limiting step in the dissociation process may be different from O_2 and CO ligands.

The chemical shift and line width data obtained in this study are summarized in Table 1. Antonini, et al.[5] who studied single chains free of paramercuribenzoate found that the ^{13}CO resonances of the isolated β chains occurred 0.2 ppm upfield of the β chain resonances in HbA. Our results, which were obtained under conditions of much higher resolution and sensitivity, (but with the single chains as paramercuribenzoate derivatives) indicate that, at equivalent heme concentrations, it is the α chain resonance which is shifted slightly downfield in HbA compared to the isolated chains. At higher hemoglobin concentrations both resonances shift downfield and broaden appreciably. Treatment of the carbonyl HbA with 8 M urea, which splits the

tetramer into $\alpha\beta$ dimers[19,20] leads to a downfield shift of the α chain resonance . Whether the shift results from the breaking of salt bridges at the ($\alpha\beta$) - ($\alpha\beta$) interface is not clear. In addition to dissociation, urea probably induces a partial unfolding of the chains.

For both the abnormal hemoglobin and the mixed met-carbonyl HbA, there is a pronounced upfield shift of the β ^{13}CO resonance which suggests that the α^{met} chain has a different conformation than the $\alpha^{13}CO$ chain. Similar observations have been made by Huestes and Raftery[8] who found, by ^{19}F NMR spectroscopy of HbA trifluoroacetonylated at cysteine (β-93) that partially liganded HbA β chains can exist in conformations different from those found in oxy- and deoxyhemoglobin. These suggestions are not inconsistent with crystal structure data -- in the met form the Fe(III) atoms are $\sim$ 0.3 Å out of the plane of the porphyrin ring, whereas in the oxy and carbonyl form the Fe(II) atoms are in the porphyrin plane[1]. More ^{13}C NMR work is in progress on the spectra of partially liganded hemoglobins, including the effects of disphosphoglycerate and pH.

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