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^{13}C Magnetic Resonance Studies of the Binding of Carbon Monoxide to Various Hemoglobins†

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ABSTRACT: Carbon monoxide binding to hemoglobins from a variety of sources has been studied by ^{13}C nuclear magnetic resonance. The two resonances have been specifically assigned to ^{13}CO bound to α or to β subunits. The reason for the anomalous shift of ^{13}CO bound to the α chain of rabbit hemoglobin is discussed with particular reference to residue Phe-48 (CD6). The relative facility with which oxygen displaces carbon monoxide, and the relative thermodynamic affinity for carbon monoxide compared to the unliganded state, of the α and β subunits are found to differ.

In normal hemoglobin tetramers, the acquisition of a ligand by the iron atom of a particular heme group depends critically on whether or not the other heme groups in the tetrameric molecule are liganded. This dependence of the ligand affinity of one subunit on the existing degree of ligation of other subunits accounts for the positive cooperativity with which most normal hemoglobins bind molecules such as oxygen or carbon monoxide (Antonini and Brunori, 1970). Major conformational differences exist between unliganded deoxyhemoglobin and fully liganded forms of the protein (Muirhead *et al.,* 1967; Perutz *et al.,* 1968; Muirhead and Greer, 1970; Bolton and Perutz, 1970) but exactly how the ligand affinity of a given heme is modulated by events elsewhere in the molecule is not presently known. Some of the unanswered questions are to what extent these effects may be caused by steric factors operating to exclude ligands from a heme in one protein conformation while in another confirmation providing for unobstructed ligand approach (Perutz, 1970) or to what extent these effects may reflect electronic factors resulting from changes in the interaction between the iron atom of the heme and the proximal histidine (F8) or by changes in the interaction between substituents about the porphyrin ring (such as the vinyl groups) and the π cloud of the porphyrin. Both families of factors probably contribute and the rigorous dissection into steric or electronic effects undoubtedly oversimplifies the actual situation. For example, the "electronic" effects, though ultimately manifested at the iron atom, probably have their origin in conformational changes of the polypeptide chain which then influence the proximal histidine. Similarly changes in the orientation of polypeptide chains near the periphery of the porphyrin ring can eventually manifest themselves as "electronic" effects at the iron.

To gain insight into some of these questions provides the focus of this work whose ultimate objective is to resolve the origin of the allosteric cooperativity of hemoglobin. In particular we sought to identify the environmental differences experienced by carbon monoxide when bound to the different subunits of various hemoglobins (Moon and Richards, 1972) with some attention to the relative thermodynamic ease of displacement of carbon monoxide by oxygen from α or β subunits and the relative affinities of the unliganded subunits for carbon monoxide. We used the ^{13}C nucleus of ^{13}C-enriched carbon monoxide as the probe in this work.

Experimental Section

Materials and Methods. Hemoglobin was prepared from freshly drawn, citrated whole blood of human, bovine, mouse

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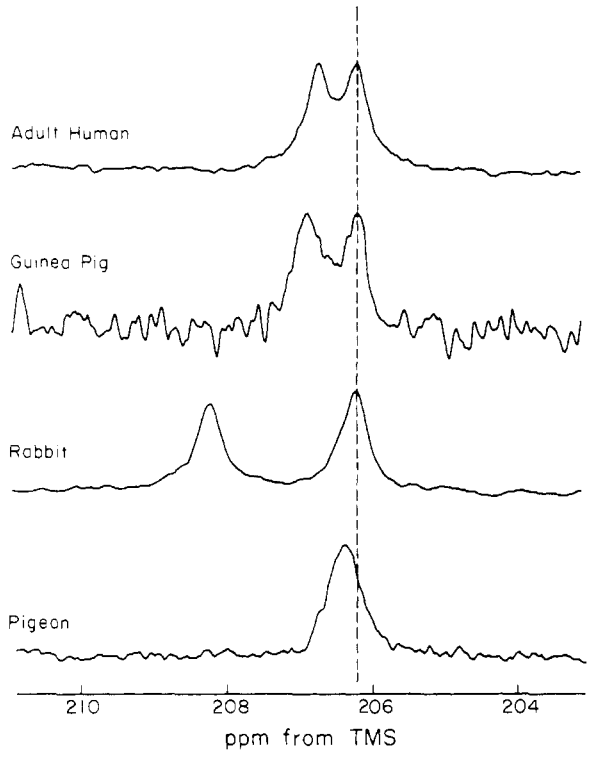

FIGURE 1: Representative spectra of the ^{13}CO resonances from human adult, guinea pig, rabbit, and pigeon carboxyhemoglobins at 25.17 MHz.

(BALB/c), guinea pig (Campbell-Trapani American albino, closed colony), rabbit (New Zealand white), chick (white Leghorn, 3 days old), bullfrog, or wild pigeon origin.

The red cells were separated from plasma by centrifugation at 2000*g* and washed several times with 0.1 M sodium chloride. The packed erythrocytes were then lysed with two volumns of distilled water at room temperature for 20–30 min. After removal of the stromata by centrifugation at 30,000*g*, the hemolysates were stripped of organic phosphates by extensive dialysis against 0.1 M sodium chloride as described by Bunn *et al.* (1971). (This method has been shown to reduce the concentration of organic phosphates to a value less than 0.1 mM in a 2 mM hemoglobin solution.) The hemoglobin solutions were then concentrated by ultrafiltration (Amicon, UM-10 membrane) and adjusted spectrophotometrically to a final hemoglobin concentration of 2.0 mM in 0.1 M sodium chloride.

The α and β subunits of normal adult and sickle cell hemoglobin were separated and purified as the *p*-hydroxymercuribenzoate (HMB)[1] derivatives as described by Geraci *et al.* (1969). Hemoglobin was allowed to react with HMB overnight at pH 6.15 ± 0.05 and 4°. The crude reaction product was centrifuged to remove precipitated material and was then applied to a column of Sephadex G-25 equilibrated with 0.01 M phosphate buffer at pH 8.0. The HMB–hemoglobin thus eluted was then concentrated by ultrafiltration. The α and β subunits were separated on a single column (2.5 × 30 cm) of DE 52-cellulose (Whatman). The HMB α chains were eluted first with 0.01 M phosphate buffer (pH 8.0), followed by residual hemoglobin, eluted with 0.02 M phosphate buffer (pH 7.5), and then HMB β subunits were eluted with 0.1 M phosphate buffer (pH 7.0). The isolated HMB subunits were dialyzed against 0.1 M sodium chloride, and concentrated by ultrafiltration. They were checked for purity by polyacrylamide disc gel electrophoresis. The HMB β subunits contained no detectable impurities while the HMB α subunits typically contained 1–2% (but never more) hemoglobin tetramer.

Extensive treatment with water-saturated carbon monoxide (enriched in ^{13}C by 90–92%) converted the purified, unliganded hemoglobins or separated HMB subunits to their carboxy derivatives. The presence of methemoglobin was measured spectrophotometrically prior to addition of CO by removal of 10-μl aliquot which was diluted to 10 ml of 0.15 M sodium chloride which had been previously saturated with air. The ratios of absorptions at 415, 420, and 430 nm (Benesch *et al.*, 1965) were then immediately determined. In those cases where only the relative chemical shifts of the bound ^{13}CO signals were being monitored, the methemoglobin content was typically 2–3% and never exceeded 10% in any of the various samples studied. No increase in methemoglobin content was detected after determination of any cmr spectrum. In those experiments where the relative degree of ligation of the two subunits was measured (see below), the amount of methemoglobin never exceeded 3% and did not change detectably during the course of the experiments.

Quantitative treatment of rabbit deoxyhemoglobin with nonsaturating amounts of ^{13}CO was accomplished in a specially constructed, closed atmosphere sample tube which has an attached uv cell. This tube was held concentrically in a 12-mm tube containing D_2O which served as a field frequency lock. Before addition of carbon monoxide, the samples were deoxygenated in the cell by extensive flushing with water-saturated nitrogen. Complete deoxygenation was determined by the method of Benesch *et al.* (1965) using the criterion that the ratio $OD_{670}/OD_{730} \geq 2.3$. Water-saturated ^{13}CO was injected into the sample tube with a gas-tight syringe through a rubber sleeve covering the access port. The extent of hemoglobin saturation with ^{13}CO was followed spectrophotometrically at 650 nm.

^{13}C nuclear magnetic resonance (nmr) spectra were obtained using the pulse Fourier transform technique on a Varian Associates XL-100-15 spectrometer equipped with a Varian 620/i computer (16K memory). Samples were contained in a 12-mm tube with a 5-mm tube containing D_2O inserted concentrically to serve as a field frequency lock. All spectra were obtained under identical conditions at a probe temperature of 34° using a 90° pulse of 150-μsec duration with a spectrum width of 2500 Hz.

Spin-lattice relaxation time (T_1) measurements were made with proton noise decoupling using the progressive saturation intensity ratio technique as described by Freeman and Hill (1971). These authors have also investigated the effect of a finite H_1 on progressive saturation experiments of the type used here. They found that, when $\Delta F/H_1$ is less than 0.8, the error in the measured value of T_1 is less than 5%. In our case, H_1 was 1666 Hz (τ_{90} = 150 μsec) and ΔF for the ^{13}CO resonances was about 500 Hz, which, applying the analysis of Freeman and Hill, leads to an error of less than 1% in the measured value of T_1 caused by the finite pulse strength (or long pulse duration). The values reported are based on numerous (7–8) independent measurements of oxygen-free samples. Uncertainties are standard mean derivations of the mean values reported. (The observed T_1 values did not vary detectably as a function of relative methemoglobin concentration over the range 2–60%). The pulse intervals (0.2–2.4 sec) used in these determinations were varied randomly so as to eliminate systematic errors during the long data acquisition periods (1–10 hr) necessary to achieve adequate signal-to-noise.

Nuclear Overhauser effects were determined both by direct

[1] Abbreviations used are: HMB, *p*-hydroxymercuribenzoate; PMB, *p*-mercuribenzoate.

TABLE I: Hemoglobin ^{13}CO Chemical Shifts.[a]

Source	δ (α subunit)	δ (β subunit)	pH
Human adult	206.75	206.19	6.35–8.00
Human fetal[b]	206.75	206.23	6.80
Human sickle cell	206.77	206.22	6.49–7.03
Bovine[b]	206.69	206.23	7.00
Mouse[b]	206.67	206.03	6.21
Guinea pig[b]	206.87	206.13	6.92
Rabbit	208.18	206.18	6.94–7.39
Frog[b]	206.63	206.19	6.82
Chick	206.73	206.20	7.05–8.70
Pigeon	(single peak: 206.36)		6.68–7.28
PMB α (adult)	206.55		7.21–8.78
PMB β (adult)		206.17	7.50–9.49
^{13}CO (solution)	184.60		

[a] Chemical shifts in ppm from external Me_4Si ± 0.04. [b] Chemical shift based on a single sample of blood.

integration of resonances with and without proton noise decoupling and by comparison of intensity ratios using continuous and gated noise decoupling techniques (Freeman *et al.*, 1972).

Results

Figure 1 displays representative spectra of the ^{13}CO resonances of various carboxyhemoglobins, and Table I collects the chemical shift data for all proteins studied. In general, the carboxyhemoglobins show two distinct resonances of equal intensity which presumably originate from carbon monoxide bound either to the α or β subunits. In contrast, pigeon carboxyhemoglobin gave a single, rather sharp resonance centered at 206.36 ppm from external Me_4Si. Also, the resonances generally occur at very similar absolute chemical shifts with a separation of 0.5 ppm. Pigeon carboxyhemoglobin lacks the resonance at lower field and rabbit carboxyhemoglobin, though having the upfield peak at the usual position, shows a low-field peak shifted considerably further downfield than normal (the separation between the peaks is 2.0 ppm).

The positions of the resonances of carboxyhemoglobin in intact rabbit, human adult, human fetal, and sickle cell erythrocytes agree well with those for the corresponding carboxyhemoglobins in solution. The two resonances of human hemoglobins in intact erythrocytes were not resolved, presumably due to magnetic field inhomogeneity and increased viscosity within the cells; the position of the broadened resonances was not, however, shifted. The resonances of carboxyhemoglobin within rabbit erythrocytes were still resolved and occurred at the same position as those in solution. Swelling of rabbit red cells in hypotonic salt solutions led to decreased line widths for the resonances probably due to decreased intracellular viscosity.

All the ^{13}CO resonances were pH independent in the range pH 6–8. The addition of a 2:1 or 5:1 molar excess of 2,3-diphosphoglycerate at pH 7.0–7.4 did not affect the chemical shifts of human or rabbit carboxyhemoglobins. Moreover, extensive carbamylation of the ϵ-amino groups of the lysines and of the α-amino groups of the terminal valine residues in human adult, sickle cell, or rabbit hemoglobin had no effect on the chemical shifts for the resulting carbamylated carboxyhemoglobin.

Studies of spin–lattice relaxation times (T_1) for the ^{13}CO resonances, determined by progressive saturation, yield a value of $T_1 = 0.3 \pm 0.1$ sec for both resonances of a single rabbit carboxyhemoglobin sample at pH 7.0 and 3 mM protein concentration. More extensive determinations of T_1 for the ^{13}CO resonances of several carbamylated sickle cell carboxyhemoglobin samples (containing amounts of methemoglobin from 3–60%) all give a uniform value of $T_1 = 0.60 \pm 0.16$ sec. As no systematic deviations in T_1 as a function of the relative amount of methemoglobin was observed, we conclude that the presence of this paramagnetic species does not significantly affect the relaxation of carbon monoxide bound to other subunits or molecules. Line widths for individual ^{13}CO resonances were 7 ± 1 Hz.

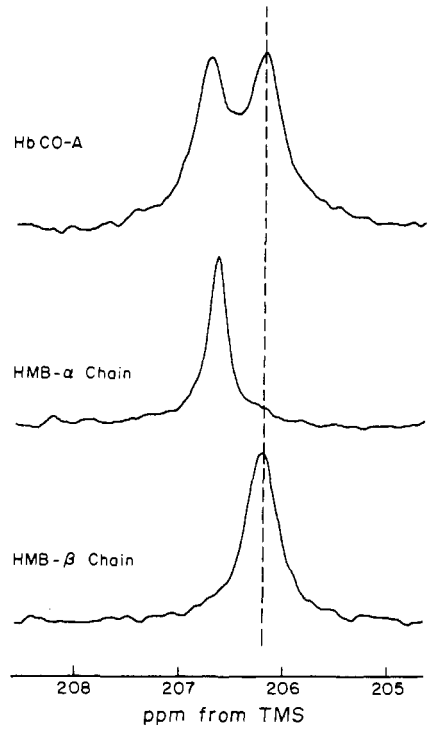

FIGURE 2: Spectra of the ^{13}CO resonances of a sample of intact human adult hemoglobin and the isolated HMB derivatives of its α and β subunits.

The observed nuclear Overhauser effects are: for rabbit carboxyhemoglobin, NOE = 1.1 ± 0.1 (for both resonances), and for carbamylated sickle cell carboxyhemoglobin, NOE = 1.00 ± 0.08 (for both resonances).

The subunits were separated as their *p*-hydroxymercuribenzoate derivatives and Figure 2 shows the single sharp ^{13}CO resonances of isolated carboxy-HMB α and β subunits. The chemical shift for ^{13}CO bound to HMB β subunits corresponds exactly with that of the higher field resonances of adult and sickle cell carboxyhemoglobin. The chemical shift of the ^{13}CO bound to the HMB α subunits was consistently observed 0.2 ppm upfield of the lower field resonances of adult and sickle cell carboxyhemoglobin.[2] The positions of the ^{13}CO resonances did not vary over the pH ranges listed in Table I. Accordingly we assign the lower field resonance (206.7 ppm) to ^{13}CO bound to the α subunits and the higher field resonance (206.2 ppm) to ^{13}CO bound to the β subunits of adult and sickle cell carboxyhemoglobins.

Antonini *et al.* (1973) have made similar assignments based on separation of HMB derivatized chains, removal of the HMB, and nmr examination of the resulting mercurial-free ^{13}CO α and ^{13}CO β chains. The mercurial-free ^{13}CO β chains

[2] While this manuscript was in the review process, similar results were reported for normal adult carboxyhemoglobin by Vergamini *et al.* (1973).

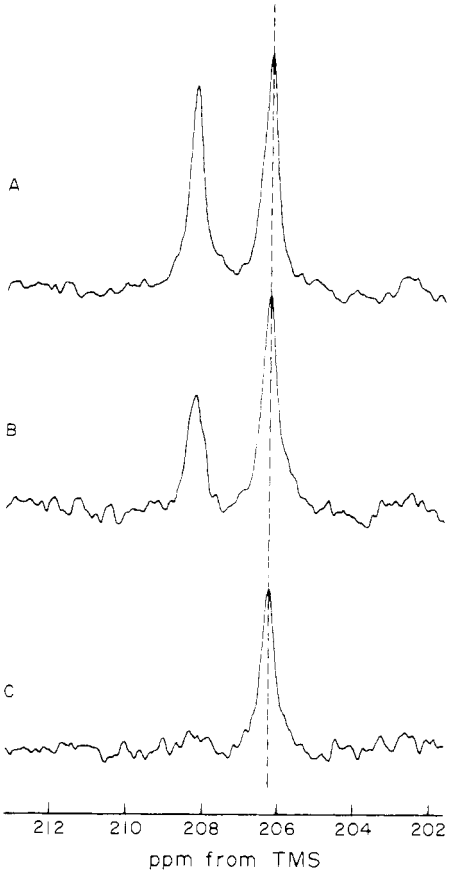

FIGURE 3: The ^{13}CO resonance spectra of rabbit hemoglobin as a function of increasing exposure to air. The spectra were plotted in the absolute intensity mode to show the rapid decrease in the intensity of the α-subunit resonance (low field) relative to the slower decrease in the intensity of the β-subunit resonance (high field). A, air excluded; B, brief exposure to air; C, more extensive exposure to air.

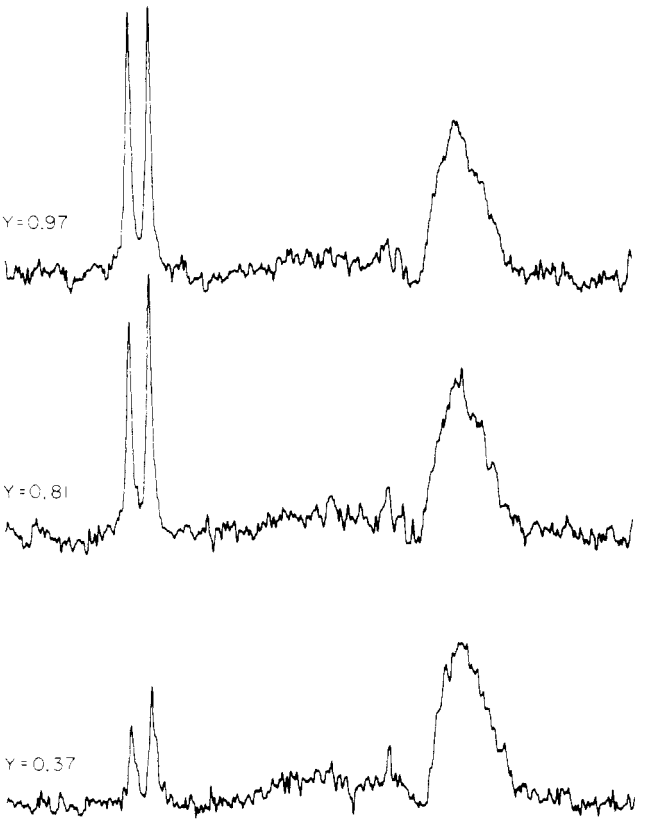

FIGURE 4: Spectra of the ^{13}CO and natural abundance peptide carbonyl resonances of rabbit hemoglobin treated with nonsaturating amounts of ^{13}CO. The sample contained 5.0 ml of 2.0 mM hemoglobin in 0.15 M sodium chloride–0.05 M Tris buffer (pH 7.0) in a closed atmosphere sample tube of 76-ml volume. The lower spectrum indicates the distribution of ^{13}CO on the α and β subunits after equilibration against 600 μl of ^{13}CO. Integration of the ^{13}CO and carbonyl (656 carbons at natural abundance) resonance regions indicates that the hemoglobin is about 37% saturated; 44% of the ^{13}CO resides on the α hemes and 56% of the ^{13}CO on the β hemes. The center spectrum shows the same sample after a total of 1500 μl of ^{13}CO have been injected into the tube (81% saturated; α 44%, β 56%), and the upper spectrum shows the sample after a total of 2100 μl of ^{13}CO have been injected (97% saturated; α 50%, β 50%).

were observed to absorb upfield of the upfield resonance in the tetramer. The mercurial derivatives of both the α and β chains and the mercurial-free α chains exist as monomers whereas the mercurial-free β chains exist largely as tetramers (Yip *et al.*, 1972) which may explain the difference in these results for separated, largely tetrameric β chains compared to ours. Also, the signal-to-noise and resulting resolution of the reported spectra seem to make difficult the precise assignment of chemical shifts.

Addition of small amounts of air to rabbit carboxyhemoglobin causes the lower field resonance to decrease more than the higher field resonance (Figure 3). Similar though much less dramatic effects are observed when solutions of adult and sickle cell carboxyhemoglobins are exposed to limited amounts of oxygen. Reequilibration of such partially oxygenated samples with saturating amounts of ^{13}CO leads to regeneration of equal intensities for the two ^{13}CO resonances (as in spectrum A of Figure 3), thus eliminating the possibility that the preferential decrease in the resonance at lower field is the result of preferential oxidation of the α subunits to the methemoglobin state (Mansouri and Winterhalter, 1973).

Addition of low concentrations of ^{13}CO to solutions of rabbit deoxyhemoglobin at pH 7.0 in the absence of organic phosphates in closed atmosphere sample tubes shows more intense absorption of the higher field than of the lower field resonance; further, addition of saturating concentrations of ^{13}CO to these same samples gives high and low field absorptions of equal intensity (Figure 4).

Discussion

The results of this work confirm that carbon monoxide interacts differently with the heme group of α and β subunits in a wide variety of hemoglobins (Moon and Richards, 1972; Matwiyoff and Needham, 1972; Matwiyoff *et al.*, 1973; Vergamini *et al.* 1973). Other studies have also revealed differences between the two types of subunits. For example, proton magnetic resonance studies (Lindstrom *et al.*, 1972) show that subtle differences exist in the protein conformation around the α and β heme groups of various human carboxyhemoglobins. Kinetic differences in the reactivities of the α and β subunits have been reported for the binding of nitric oxide (Henry and Cassoly, 1973) and *n*-butyl isocyanide (Olson and Gibson, 1972). Quantitative studies using a spin-labeled analog of ATP led Ogata and McConnell (1972a,b) to conclude that a quantitative description of hemoglobin ligation requires modification of the simple two-state allosteric model (Monod *et al.*, 1965) by the explicit recognition of the nonequivalence of the α and β subunits.

Chemical Shift Environment. The studies on separated HMB chains indicate that the lower field resonance arises from ^{13}CO bound to α subunits while the higher field resonance represents ^{13}CO bound to β subunits in adult and sickle cell hemo-

globin. By analogy we make similar assignments for the other species of hemoglobin studied including rabbit hemoglobin, the upfield resonance of which falls at the same position as that of other ^{13}CO β subunits. In our view the low-field resonance represents ^{13}CO bound to rabbit α subunits which, we conclude, must be anomalous. These assignments agree with those made previously (Moon and Richards, 1972) on the basis of a rather circuitous analogy to kinetic studies of the relative rates of oxygen displacement by carbon monoxide in α as compared to β subunits (Olson *et al.*, 1971).

The environment of ^{13}CO seems to be essentially the same when bound to the tetramer as when bound to the isolated, monomeric HMB α or β subunit. (The chemical shift for the ^{13}CO β resonance in the tetramer is identical with that for the ^{13}CO HMB β subunit, while that for the ^{13}CO α resonance in the tetramer is 0.2 ppm downfield of that for the ^{13}CO HMB α subunit). This observation suggests that, in the completely liganded state, interactions between subunits influence little, if at all, the environments of the ligands; such interactions appear to play their dominant role in the unliganded or partially liganded states of the "tight" quaternary conformations of the hemoglobin tetramer.

We observe that the ^{13}CO chemical shifts of carboxyhemoglobins do not depend on pH in the range pH 6–8. Other studies do suggest changes in this pH range. For example, Ogawa and Shulman (1971) reported that the heme-proton chemical shifts of cyanmethemoglobin and several "mixed state" hemoglobins are markedly sensitive to pH over this range, a sensitivity they attributed to changes in the quaternary conformation of the molecule. Olsen and Gibson (1973) observed a pH and ionic strength dependence of the relative affinities of the α and β subunits for *n*-butyl isocyanide; the behavior of the two subunits in this regard becomes almost equivalent at high pH. If any similar changes do occur with carboxyhemoglobins they are not manifest in the environment of the bound carbon monoxide or in the nature of the bond between the heme iron and the ligand as measured by chemical shifts of the bound ^{13}CO.

What are the origins for the differences between the environments experienced by carbon monoxide bound to α or to β subunits? We interpret the evidence presently at hand to suggest that these chemical shift differences reflect principally effects transmitted from the polypeptide helices through the porphyrin ring or the proximal histidine (F8) to the heme iron and thence to the ligand. These effects may have significant stereochemical contributions arising, for example, from the precise orientation of the heme group within its binding cleft.

More specifically, changes in the region of the C/D helices seem to play an especially dominant role. Three major arguments support this conclusion: (i) absence of a pH dependence of the chemical shifts; (ii) absence of differences in chemical shifts for ^{13}CO bound to α-type or β-type subunits with a wide variety of amino acid substitutions contrasted to a generally consistent difference between ^{13}CO bound to α-type subunits *vs.* ^{13}CO bound to β subunits; (iii) the anomalously low field resonance for ^{13}CO bound to rabbit α subunits.

(i) Though evidence cited earlier suggests that minor differences, particularly in the quaternary structure, may exist as a function of pH and can be reflected within the heme pocket, such differences are not manifest in the observed ^{13}CO chemical shifts. We believe this indicates that small conformational changes may occur within the pocket where the bound carbon monoxide resides without influencing the chemical shift of this ligand because the bond between it and the heme iron has not been altered.

(ii) In fetal hemoglobin, there are 39 amino acid substitutions in the γ subunits compared to the β subunits of normal adult hemoglobin (Schroeder *et al*, 1963). Two of these substitutions (E14 and E15) occur within 4 Å of the heme (Perutz, 1969) and have been observed to cause changes in the ^1H resonances of the methyl protons of the valine residue (E11) to which they are adjacent (Lindstrom *et al.*, 1972). Moreover, valine E11 directly contacts the carbon monoxide ligand in the insect hemoglobin erythrocruorin (Huber *et al.*, 1970) and therefore is presumably at least very near the carbon monoxide in β-type chains of other hemoglobins as well. Yet carbon monoxide has the same chemical shift when bound either to human adult β or to fetal γ subunits. We therefore conclude that the chemical shift does not sensitively reflect such changes in the pocket within which the ligand dwells.

Similar comments apply to α chains as a wide range of α chains of most species exhibit an unchanging resonance for bound ^{13}CO though many amino acid substitutions occur.

However, there is a generally consistent difference between ^{13}CO bound to α compared to β subunits which we believe reflects the major structural difference between α-type and β-type subunits. Subunits of the β-type possess a D helix; subunits of the α-type lack a D helix.

(iii) Many amino acid substitutions have just been shown to be possible without perturbing the chemical shift of ^{13}CO bound to β-type chains or bound to α-type chains. However, rabbit α chains have markedly anomalous properties not only with respect to the chemical shift of bound carbon monoxide but also with regard to the unusually facile oxidation of the heme iron of rabbit α chains (Matwiyoff *et al.*, 1973). Between rabbit and human α chains there are a total of 25 amino acid substitutions (Ehrenstein, 1966). Three of these seem to us of especial significance: Val-29 (B10), Phe-48 (CD6) and Thr-49 (CD7) compared to the human α chain pattern of Leu-29, Leu-48, and Ser-49 (braunitzer *et al.*, 1961). In two of these three residues (29 and 49) substitutions are unique to rabbits. In the third (48) species variability has been observed but always between homologous amino acids (Val or Leu) (Dayhoff, 1972); only in rabbits is the change to an aromatic residue such as Phe seen. Rabbits also have a unique substitution in Ser-63 (Ehrenstein, 1966) but as the side chain of this residue points away from the heme pocket, and as it does not change in the other rabbit hemoglobin allele (see following paragraph), we have not deemed it especially significant compared to the three substitutions on which we should like to focus attention (B10, CD6, and CD7). In the tertiary structure of the α chain residue B10 lies in close proximity to CD6 and CD7 which are in turn directly adjacent to the region occupied by the D helix in β chains. (In fact, because of its uniquely large size, we feel that the substitution of the aromatic residue, Phe-48, at CD6 may well be the dominant change.)

Matwiyoff *et al.* (1973) recently reported that three resonances are observed for ^{13}CO bound to hemoglobins from some Dutch lop rabbits, two of which (those at highest and lowest field) occur at the positions we have consistently observed for our rabbit carboxyhemoglobins and a third (at intermediate field) which occurs at a position characteristic for ^{13}CO bound to α subunits of human and most other hemoglobins. The sum of the intensity of the α-^{13}CO resonances at intermediate and lowest fields equals the intensity of the high field β-^{13}CO resonance. This suggests that these rabbits have two types of α subunits and Hunter and Munro (1969) have shown that two α chain alleles are, in fact, present in Dutch lop rabbits. One allele has the normal rabbit α chain sequence at 29, 48, and 49, while the other allele has amino acids at these positions found in most other α chains (Leu-29, Leu-48, and Ser-49). More-

over, all other known variations of the normal rabbit α chain (Val-29, Phe-48, Thr-49) involve reversions back to residues common to α chains of hemoglobins from humans and other species (Leu-29, Leu-48, Ser-49).

These observations suggest that changes in the region of the C/D helices can profoundly influence the bound ligand. Deletion of the D helix alters the angle between the E and F helices which in turn should modify interactions between the protein and heme (as well, possibly, as interaction between the iron and proximal histidine F8). Thus, the absence of the D helix in α chains may largely account for the way in which these chains bind ligand differently than β chains. In a similar way, in α chains themselves, changes in the C/D region can affect interaction between the protein and the heme, in turn modifying the interaction between the iron of these hemes and ligands such as carbon monoxide.

Relative Affinities (Oxygen vs. Carbon Monoxide). The results of this work give some indication of the relative thermodynamic affinities of the α and β subunits for oxygen relative to carbon monoxide—particularly for rabbit hemoglobin. In general, hemoglobins have a higher affinity for carbon monoxide relative to oxygen by factors of the order of 230-260:1 (Joels and Pugh, 1958). We find that admission of small quantities of oxygen to samples of rabbit hemoglobin previously saturated with carbon monoxide and in the absence of organic phosphates leads first to a decrease in the intensity of the low field α chain ^{13}CO resonance indicating displacement of carbon monoxide by oxygen from the α chains. (To ensure that this observation is not caused by preferential oxidation or denaturation of the α chains, we demonstrated that subsequent removal of oxygen and resaturation with ^{13}CO regenerate the original spectrum; monitoring the electronic spectrum at 418 nm also shows no evidence for formation of methemoglobin.) Accordingly we conclude that the α chains are less discriminating than β chains for carbon monoxide as compared to oxygen as a ligand. Though this more facile displacement of carbon monoxide by oxygen from the α chains is especially marked in rabbit hemoglobin, it is also seen to a lesser degree in human and other hemoglobins. Based on the time scale of these experiments (minutes to hours) we identify these differential preferences as thermodynamic and not kinetic effects.

Order of Ligation of Carbon Monoxide. Some disagreement exists on the preferences of the two kinds of subunits for carbon monoxide relative to their unliganded states. Based on studies of the binding of *n*-butyl isocyanide and carbon monoxide to hemoglobin, Gray and Gibson (1971) identified the β subunit as being more reactive (kinetically) to carbon monoxide. On the other hand, Heustis and Raftery (1972) concluded that the α subunit has the higher equilibrium affinity for carbon monoxide based on their studies of a hemoglobin derivative with a trifluoroacetonyl group attached to cysteine-93β. Our results (Figure 4) demonstrate that for rabbit hemoglobin at moderate saturation with carbon monoxide ($Y \sim 0.37$) more ligand is bound to the β than to the α subunits ($\beta^{CO}/(\alpha^{CO} + \beta^{CO}) \sim 0.56$).

One explanation of this apparent discrepancy may lie in the quaternary conformations of the partially liganded hemoglobins which may conceivably be different in our work and that of Gray and Gibson on the one hand compared to that of Huestis and Raftery on the other. To make the argument, we first note that the oxygen affinity of dissociated β chains has been found to exceed somewhat that of α chains. Indeed, Ogata and McConnell (1972b) incorporated these observations into their generalized concerted transition model where they assume that, in the oxy (relaxed, R) conformation, the β chains have a higher affinity for oxygen than do the α chains. Conversely, in the deoxy (tight, T) quaternary state, the α chains seem to have the higher oxygen affinity. (The assumption that the equilibrium ligand affinity of the separated chains reflects that of the chains in the R tetramer finds support in our observation, previously discussed, that the chemical shifts of ^{13}CO are virtually identical whether this ligand is bound to the intact, completely liganded and, therefore, R tetramer or to the separated HMB subunits.)

Accordingly, if, in the T state, α subunits have a higher affinity while, in the R state, β subunits have the higher affinity, one might well have situations where, at low total ligation with the tetramer predominantly in the T state, the α chains are preferentially liganded, whereas at moderate total ligation, with the tetramer predominantly in the R state, the β chains have more bound ligand.

Moreover, if the relative ligand affinities of the two types of subunits differ more with carbon monoxide than with oxygen as a ligand as some studies suggest (Olson and Gibson, 1973; Ogata and McConnell, 1972b), this effect (more ligand on α chains at low saturation, more ligand on β chains at moderate saturation) might be accentuated with carbon monoxide as ligand.

These relative affinities are undoubtedly also strongly influenced by the presence of organic phosphates, and this condition varied within the group of studies cited.

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Carbon-13 Nuclear Magnetic Resonance Studies of Structure and Function in Thyrotropin-Releasing Factor. Determination of the Tautomeric Form of Histidine and Relationship to Biology Activity†

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ABSTRACT: The pH dependence of the ^{13}C chemical shifts of histidine in thyrotropin releasing factor (TRF) <Glu-His-Pro-NH_2 demonstrates that the N^{τ}-H tautomer is the predominant form of the imidazole ring in histidine in basic solution. Protonation of the imidazole ring of His in TRF causes changes in the chemical shifts of the His residue itself as well as in the chemical shifts of the β carbon of <Glu and in the β and δ carbons of Pro, possibly as a result of slight changes in steric constraints on the peptide. Spin-lattice relaxation times (T_1) of the carbons in TRF are not affected by protonation of the imidazole ring of His. This implies that there is no large change in the rate of overall molecular reorientation nor in the relative rates of reorientation of the individual residues, hence no measurable change in conformation upon protonation of the imidazole ring of His. The ^{13}C chemical shifts of the highly biologically active N^{τ}-methylimidazole TRF are more similar to TRF than those of the almost inactive N^{π}-methylimidazole derivative. Protonation of the *N*-methylimidazole derivatives causes chemical shift changes in the spectra of these compounds and results in spectra which resemble the protonated form of TRF. The chemical shift changes which occur upon protonation of histidine in TRF, the N^{π}- and the N^{τ}-methylhistidine TRF derivatives, are of similar magnitude to the changes found in histidine monomer, N^{π}- and N^{τ}-methylhistidine, respectively. The pK_a values of His and *N*-methylhistidine in the above peptides reflect the pK_a values of free His and the *N*-methylhistidine monomers. The N^{τ} tautomer of His in TRF is postulated to be that which interacts with the receptor. The interaction at the receptor would be a nonbonded, van der Waals type. This would explain the higher activity of the N^{τ}-methylimidazole TRF.

Thyrotropin-releasing factor (TRF)[1] controls the secretion of thyrotropic hormone from the anterior pituitary gland (Schally *et al.*, 1968). The sequence of TRF is <Glu-His-Pro-NH_2[2] (Figure 1) (Folkers *et al.*, 1969; Burgus *et al.*, (1969a). Structure-function studies on TRF have been carried out *via* two

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[1] Abbreviation used is: TRF, thyrotropin-releasing factor.

[2] We use the convention that N^{π} in histidine is the N atom closest to the point of attachment of the imidazole ring to the β carbon. This is in accordance with the recently suggested IUPAC-IUB Commission on Biochemical Nomenclature (*Biochemistry 11,* 1726 (1972)). All amino acids are of the L configuration.