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# Conformational Studies of Various Hemoglobins by Natural-Abundance $^{13}C$ NMR Spectroscopy

**(rabbit/human/quaternary)**

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**ABSTRACT** **Studies of variously liganded hemoglobins (both from human and rabbit) by natural-abundance $^{13}C$ NMR spectroscopy have revealed apparent conformational differences that have been interpreted on the basis of two quaternary structures for the $\alpha_2\beta_2$ tetramer, and variable tertiary structures for the individual $\alpha$ and $\beta$ subunits. In solution, rabbit hemoglobins appear to have somewhat more flexibility than human hemoglobins.**

An enormous amount of research has been devoted to hemoglobin (1–5), the oxygen-carrying protein of erythrocytes. However, many of the molecular details of its action remain unknown, for example, the nature and geometry of the iron-oxygen bond (6–8), the exact relationship between the conformations (9, 10), *inter alia*, of deoxyhemoglobin (Hb), oxyhemoglobin ($HbO_2$), carboxyhemoglobin (HbCO), acid (or aquo) methemoglobin (Hi), and cyanmethemoglobin (HiCN), the relative importance of the $\alpha$ and $\beta$ subunits to the allosteric cooperativity of the hemoglobin molecule (11–14) (for example, the possible existence of quaternary—and tertiary—conformations intermediate between the conformations of Hb and $HbO_2$), the nature of the salt bridges between subunits in various quaternary conformations, and the exact mechanisms by which organic phosphates such as 2,3-diphosphoglycerate (2,3-$P_2$Glr) influence hemoglobin oxygen affinity and allostery.

The present paper presents our results with natural-abundance $^{13}C$ NMR studies of normal human and rabbit hemoglobin. The main molecular differences observed by these techniques depend on the relative mobility of various amino-acid side chains that reflect, in turn, the conformational differences between the various forms of hemoglobin.

A point of special importance in analysis of the spectra we obtained is that particular attention has been paid to *changes* observed for differently liganded hemoglobins from a given biological source, rather than detailed interpretation of each of the absorptions of individual spectra; accordingly, *differences* between the various spectra are the major source of the information we consider. Moreover, by using native proteins under physiological conditions and concentrations, one can monitor changes that are unperturbed by introduction of labels or other alterations of the natural protein. Natural abundance studies have a further potential advantage in that observation of the entire $^{13}C$ range should lead to more information about changes in the protein than would be obtained by observation of a specific label which, though sensitive to changes in its immediate environment, may fail to reflect important changes elsewhere in the protein.

Abbreviations: Hb, deoxyhemoglobin; $HbO_2$ oxyhemoglobin; HbCO, carboxyhemoglobin; Hi, acid methemoglobin; HiCN, cyanmethemoglobin, 2,3-$P_2$Glr, 2,3-diphosphoglycerate.

Previous studies of proteins by $^{13}C$ pulsed Fourier transform spectroscopy have been reported for ribonuclease A (15), for lysozyme (16), and for carboxymyoglobin and carboxyhemoglobin (17), though the last work was carried out under conditions of such a low signal to noise ratio that little molecular information was available.

## EXPERIMENTAL METHODS

*Hemoglobin* was prepared from whole blood freshly drawn from humans or New Zealand white rabbits. The erythrocytes were separated from plasma, washed several times with 0.1 M NaCl, and hemolyzed by treatment with water. After removal of the stromata by centrifugation, the hemolysates were extensively dialyzed against 0.1 M NaCl.

*Methemoglobin* was prepared by direct oxidation of oxyhemoglobin with ferricyanide, followed by extensive dialysis against 0.1 M NaCl.

*Cyanmethemoglobin* was prepared from methemoglobin by treatment with sodium cyanide, followed by dialysis against 0.1 M NaCl.

*Oxy-, Carboxy-, and Deoxyhemoglobins* were prepared by extensive treatment of hemoglobin with water-saturated oxygen, carbon monoxide, or nitrogen.

Protein was concentrated either with an Amicon ultrafiltration apparatus or by ultracentrifugation. Final concentrations were adjusted spectrophotometrically to 3.0 mM; a molecular weight of 68,000 was assumed. The pH of all samples was adjusted, where necessary, by dialysis against 0.1 M NaCl in 0.01 M Tris buffer of appropriate pH. Sample pH was varied within the range 6.8–7.5.

*$^{13}C$ NMR Spectra* were obtained by use of the pulsed Fourier transform technique on a Varian Associates XL-100-15 spectrometer equipped with a Varian 620 i computer. Samples were contained in a 10-mm diameter tube, concentrically held within a 12-mm tube containing deuterium oxide, which was used as a field-frequency lock. All spectra were proton noise decoupled and were obtained under identical conditions at 34°C. A 50° pulse of 60-$\mu$sec duration was used, with an acquisition time of 0.2 sec. A total of 130,000–175,000 transients were obtained per spectrum, requiring data accumulation times of 8–10 hr.

All spectra used in subsequent discussion obtained under any given set of conditions were highly reproducible. Moreover, no significant changes in relative intensities were observed when longer acquisition times were used for rabbit hemoglobin.

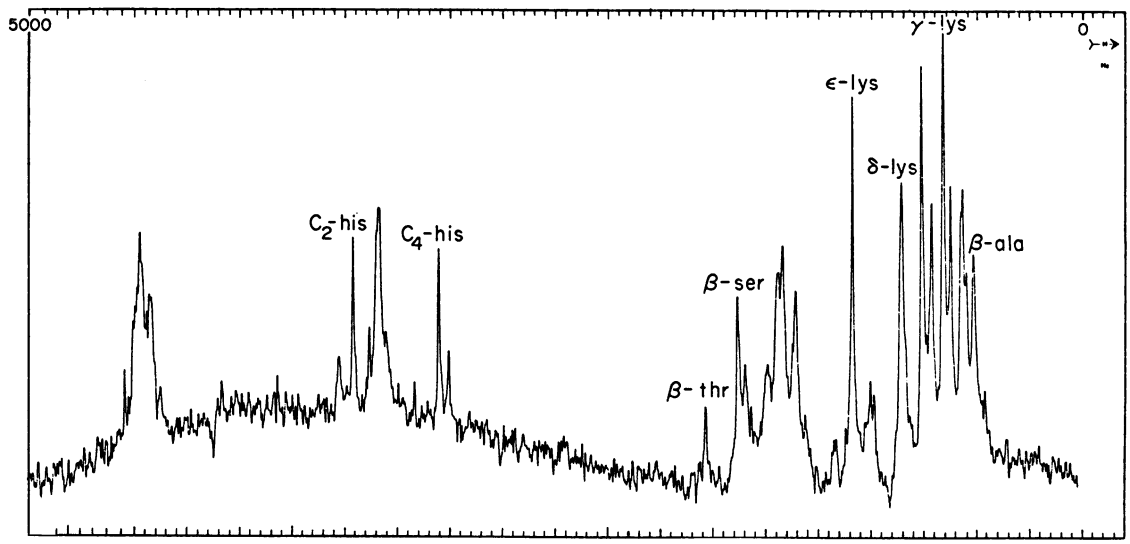


FIG. 1. Acid-denatured $\alpha\beta$-globin from rabbit, pH 0.75.

## RESULTS

Representative spectra of the many observed are collected in Figs. 1–3. Fig. 1 shows acid-denatured $\alpha\beta$ globin from rabbit, Fig. 2 collects typical spectra of various human hemoglobins ($HbO_2$, Hi, Hb, and a sample of Hb in the presence of 2,3-$P_2$Glr), and Fig. 3 shows typical results with rabbit hemoglobins ($HbO_2$, HbCO, HiCN, Hi, and Hb).

## DISCUSSION

### Analysis of spectra

The spectrum of denatured rabbit hemoglobin of Fig. 1 shows the resonances anticipated for a polypeptide of appropriate amino-acid composition in a random coil conformation with extensive segmental motion, which results in the observed, relatively sharp absorptions. The general features of this and other spectra used in this work, including the detailed justifications of assignments of particular resonances to particular amino acids, will be discussed more extensively elsewhere.

In the various forms of human hemoglobins shown in Fig. 2, the main changes of note among the series $HbO_2$, Hi, Hb, and Hb in the presence of 2,3-$P_2$Glr are (*i*) a steady decrease in the resonance due to the $\epsilon$-carbon of lysine residues, (*ii*) a somewhat less obvious decrease in the resonances due to the $\delta$ and $\gamma$ carbons of lysine, and (*iii*) an increase (2-fold over the entire series) in intensities of resonances due to the methyl groups ($\beta$ carbons) of the alanine residues.

The decrease in intensities of the resonances of the side-chain carbons of lysine residues arises, in our view, because of immobilization of these side chains as the protein conformation becomes tighter, and lysines that are free in solution in oxyhemoglobin become involved in salt bridges as the structure changes to that of deoxyhemoglobin. As these side chains become immobilized, their relaxation times decrease (18) and their absorptions broaden and are removed from the sharp resonances, characteristic of mobile side chains, and are added to the broad, unresolved absorptions.

This interpretation is in accord with studies of the conformational and segmental motion of ribonuclease A (19), and of the resulting relaxation times, that led to the general conclusion that backbone carbons and carbons in side chains that experience little if any motion independent of their stretch of polypeptide chain have spin-lattice relaxation times less than 0.1 sec. Two major exceptions to this generalization are the peptide carbonyl carbons, which have $T_1$ about 0.416 sec, and the $\epsilon$-carbons of lysine side chains, which have $T_1$ about 0.330 sec, due to their relative freedom in ribonuclease A. Using the technique of progressive saturation, we have independently determined that the relaxation times characteristic of the $\epsilon$-carbons of "mobile" lysine side chains has an average $T_1 = 0.4 \pm 0.1$ sec.

A similar explanation can account for the increase in intensity of the methyl groups, though in this case, the increase in intensity depends on the saturation behavior of these absorptions. These methyl groups, which are likely to experience free-spin rotation, will have $T_1 > 0.5$ sec. The spectra were observed with acquisition times of 0.2 sec, so that under our conditions these resonances will be largely saturated. They do not, therefore, appear with their full intensity in the observed spectra of oxyhemoglobin. As the protein becomes progressively more rigid, as in conversion to Hi, Hb, or Hb in the presence of 2,3-$P_2$Glr, these groups will become increasingly immobilized, their relaxation times will decrease, and their apparent intensity will increase because the absorptions have become less saturated under our conditions of observation.

Fig. 3 shows the natural-abundance spectra of the various rabbit hemoglobins ($HbO_2$, HbCO, HiCN, Hi, and Hb). In general, the observed variations between the differently liganded species agree well with those observed for human hemoglobins. Again one of the prominent differences focuses on the signal from the $\epsilon$-carbons of lysine residues. Between $HbO_2$ and HbCO, little change is observed. There is a slight decrease in HiCN relative to $HbO_2$ or HbCO, and a very significant decrease for Hi. Deoxyhemoglobin shows the most marked decrease. About half the total decrease observed between $HbO_2$ and Hb has occurred in acid (or aquo) methemoglobin (Hi).

The spectra of similarly liganded hemoglobins from rabbits and humans show further interesting differences, which seem to indicate a generally looser structure for rabbit than for human hemoglobins. Thus, the spectra of rabbit $HbO_2$ and HbCO show more detail than that of human $HbO_2$.

In rabbit $HbO_2$ and HbCO, the $\gamma$ and $\delta$ carbons of some glutamic acid residues are visible, whereas these are only barely, if at all, detectable in the spectrum of human $HbO_2$. The intensity of these resonances due to the $\gamma$ and $\delta$ carbons of glutamic acid correspond to their intensity in denatured rabbit hemoglobin. There are four more glutamic acid residues in rabbit than in human hemoglobin, but this fact alone is insufficient to account for the marked spectral differences in this regard between rabbit and human hemoglobin, which must arise from the considerably greater mobility of the $\gamma$ and $\delta$ carbons of glutamic acid residues in rabbit compared to human $HbO_2$.

Whereas the intensity of the resonance due to the $\epsilon$-carbon of lysine residues shows little change between rabbit $HbO_2$ and HiCN, the glutamic acid resonances are appreciably reduced in rabbit HiCN relative to $HbO_2$.

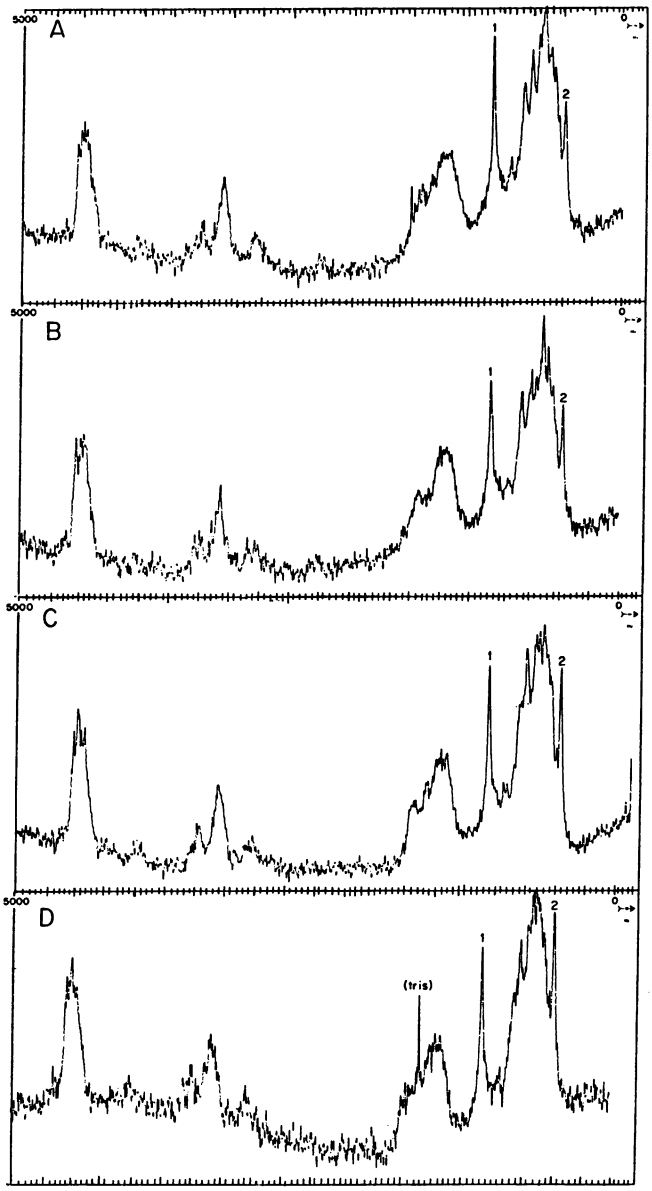


FIG. 2. Typical spectra of human hemoglobins: (*A*) = $HbO_2$, pH 7.25; (*B*) = Hi, pH 7.12; (*C*) = Hb, pH 7.41; (*D*) = Hb + 2,3-$P_2$Glr (5 mM), pH 7.38. The resonances of $\epsilon$-lysine and $\beta$-alanine carbons are indicated (*1* and *2*, respectively).

The greater general flexibility of rabbit compared to human hemoglobins seems to be further confirmed by the absence of an increase in the intensity of the alanine methyl groups in the conversion of rabbit $HbO_2$ to Hb; presumably in both forms these methyl carbons have sufficiently long relaxation times that they are saturated in our experiments, which use acquisition times of 0.2 sec. Moreover, the intensity of this resonance in the native proteins corresponds to its relative intensity in either acid- or base-denatured rabbit $\alpha\beta$ globin.

Another general feature of all the spectra is the steady decrease in the overall aliphatic region of the spectra, measured relative to the carbonyl and $\alpha$-carbon resonances (which show very little change with changes in ligands) as one moves from $HbO_2$ to Hb either in the rabbit or human series. These general changes correlate with the more discrete

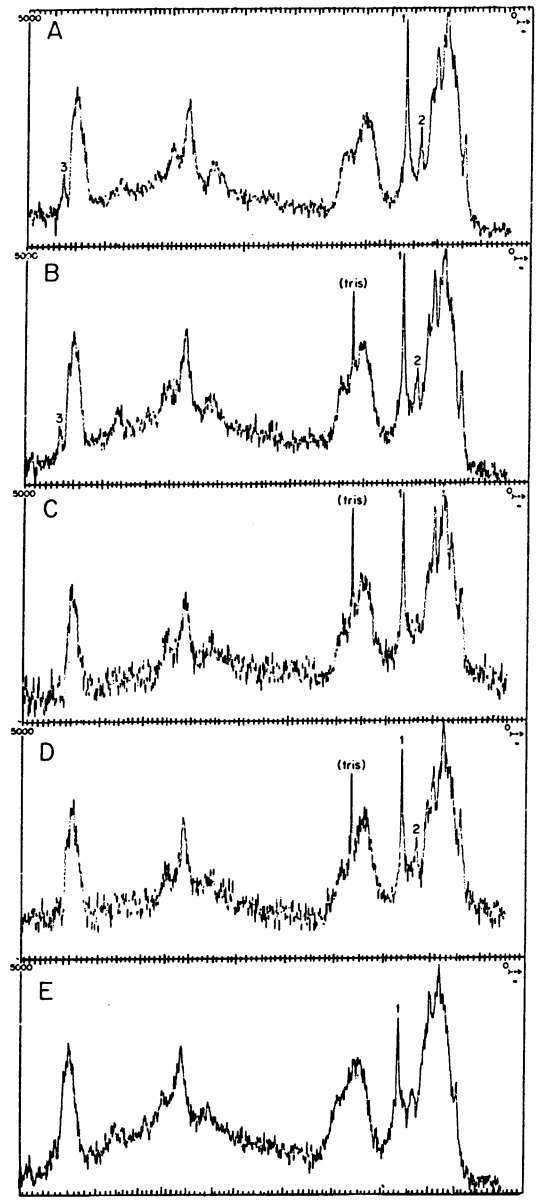


FIG. 3. Typical spectra of rabbit hemoglobins: (*A*) = $HbO_2$, pH 6.90; (*B*) = HbCO, pH 7.29; (*C*) = HiCN, pH 7.45; (*D*) = Hi, pH 7.41; (*E*) = Hb, pH 7.02. The resonances of $\epsilon$-lysine, $\gamma$-glutamic acid, and $\delta$-glutamic acid carbons are indicated (*1*, *2*, and *3*, respectively).

differences already discussed (for example, resonances due to the $\epsilon$-carbon of lysines).

**Conformational differences**

*$HbO_2$ Compared to Hb.* The most striking spectral change on deoxygenation of either rabbit or human hemoglobin is the decrease in intensity of the resonance from the $\epsilon$ carbons of lysine. This difference is enhanced in the presence of 2,3-$P_2$Glr and results from immobilization of lysine side chains. Though we can not, in the absence of a better quantitative knowledge of the $T_1$ and $T_2$ values for the resonances in question, decide unequivocally how many lysine residues are immobilized in the conversion of $HbO_2$ to Hb, we can estimate that about 25% of those residues that are free in human $HbO_2$ are immobilized on deoxygenation, while 33% of the free lysine residues are immobilized on deoxygenation of rabbit hemoglobin.

From Perutz's x-ray results (5), two lysines ($40\alpha$ and $127\alpha$) seem to be strongly immobilized on conversion of human $HbO_2$ to Hb. Immobilization of an additional lysine ($82\beta$) should occur in the presence of 2,3-$P_2$Glr. On this basis, we would estimate that about half of the total lysines (11 residues) are always immobilized in intact hemoglobin, whatever the nature of its ligands.

As the intensities of the $\epsilon$-carbons of lysine are about the same in rabbit and human hemoglobin, we guess that both hemoglobins have about the same number of bound lysines. Deoxygenation of rabbit $HbO_2$ shows a greater decrease in the intensity of the $\epsilon$-carbon of lysine than for deoxygenation of human $HbO_2$, suggesting that deoxygenation of rabbit $HbO_2$ causes immobilization of one or two more lysine residues than in the case of deoxygenation of human $HbO_2$.

*Rabbit Compared to Human Hemoglobin.* As noted above, the variously liganded rabbit hemoglobins seem generally to have more flexibility and mobility than their human counterparts. Comparison of the amino-acid compositions of these two types of hemoglobins provides a possible rationalization of these results; rabbit hemoglobin contains (*i*) more amino acids with larger side chains than does human hemoglobin and (*ii*) fewer proline residues.

Specifically, of the 25 amino-acid substitutions between rabbit and human $\alpha$-chains, 18 of these involve sterically larger residues and only six involve sterically smaller residues in rabbit, as compared to human, hemoglobin. There are 14 amino-acid substitutions between rabbit and human $\beta$ subunits. Eight of these are sterically larger and two are sterically smaller in rabbit, compared to human, $\beta$-chains. Moreover, rabbit $\beta$-subunits lack three proline residues present in normal human hemoglobin.

These substitutions could cause rabbit hemoglobin to have a significantly less compact, looser structure than human hemoglobin. Additionally, the larger number of proline residues in human hemoglobin should increase its rigidity as compared to rabbit hemoglobin.

Charge and polarity differences between the two kinds of hemoglobin may contribute to the differences in segmental motion and flexibility between them, but, in our view, the steric factors discussed above best explain the observed results.

*Rabbit HbCO and HiCN Compared to $HbO_2$, Hi, and Hb.* Though previously assumed to have conformations equivalent to that of human $HbO_2$, human HbCO and HiCN seem to differ subtly from $HbO_2$, as monitored by a fluorine label attached to Cys $93\beta$ (20). Our data on rabbit hemoglobins suggest that the conformational differences between $HbO_2$, HbCO, and HiCN are small compared to the larger differences between these liganded species and the weakly- or nonliganded forms Hi and Hb. Our data also show that significant difference between $HbO_2$ and HbCO, if any, is unobservable, suggesting that bonding of either oxygen or of carbon monoxide causes very similar changes in the tertiary structures of the subunits, and leads to essentially equivalent quaternary structures for these hemoglobins. Moreover, the conformation of HiCN differs significantly from that of $HbO_2$ or HbCO. This evidence argues against the "metsuperoxide" bonding description (21, 6) (as a metsuperoxide should have the same structure as HiCN), and favors either the original suggestion of Pauling and Coryell (22), or that of Gray (23), in which oxygen forms two bonds to iron and adopts a geometry similar to that, for example, of ethylene in many metal–organic complexes. In these model cases, the bonding of both ethylene and carbon monoxide, though having different geometries, causes very similar spectral changes.

The differences between $HbO_2$ and HiCN appear largely manifest in greater mobility of the glutamic acid residues in $HbO_2$; they seem to be much more restricted in HiCN (as well as in Hi and Hb, though in these two forms the lysine residues are also immobilized; they are not in HiCN). Thus, only the glutamic acid side chains show marked differences between $HbO_2$ and HiCN. Though the molecular origin of these changes is uncertain, an intriguing possibility is that the side chains of glutamic acid are chiefly involved in intrasubunit H-bonding interactions and are, therefore, particularly sensitive to changes in subunit tertiary structure. Lysine residues may, on the other hand, be principally involved in intersubunit interactions, and changes in lysine resonances may largely reflect changes in quaternary structure of the hemoglobin.

*$HbO_2$ Compared to Hi.* From x-ray diffraction studies of structures of single crystals, Perutz has suggested (24) that $HbO_2$ and Hi have similar structures, whereas this work would suggest that in solution the conformations of these hemoglobins differ appreciably, and that at physiological pH both rabbit and human Hi have structures roughly intermediate between those of $HbO_2$ and Hb. These conclusions agree with those obtained by use of a fluorine label on Cys $93\beta$ that show, at least in the vicinity of the label, strong similarities between human Hb and Hi (20). This similarity is further supported by the observation that Hi binds ATP (25); such binding is normally a property of Hb, but not of $HbO_2$.

In this regard, the effect of ATP on the electron paramagnetic resonance spectrum of Hi deserves comment. In the absence of ATP, two resonances are observed (26), one attributed to high-spin and the other to low-spin Fe III; these forms are in equilibrium. Addition of ATP causes the virtual elimination of the signal due to low-spin Fe III, and a concomitant increase in the signal due to high-spin Fe III. Though no explanation of these results was offered, one possibility is that the forms in equilibrium are acid methemoglobin (Hi) with high-spin Fe III and alkaline methemoglobin (HiOH) with low-spin Fe III. The HiOH structure should approximate that of HiCN, for which separation between the

β-subunits should be small. Accordingly, addition of ATP should shift the equilibrium toward the high-spin, acid methemoglobin form. On this basis, one should anticipate that elevation of this pH should cause the $^{13}$C spectrum of Hi to appear increasingly similar to that of HiCN and, in fact, this is a result that we have observed.

*General Comments.* Because the actual crystals used in the determination of the structure of "oxyhemoglobin", though crystallized as $HbO_2$, are generally understood to have undergone considerable oxidation to methemoglobin and because the solution conformation of Hi appears to differ appreciably from that of $HbO_2$, some question has been raised as to whether oxyhemoglobin actually, in solution, has the structure assigned to it*.

This may, however, not be so serious an objection. One should distinguish clearly between differences in the energies of various species, on the one hand, and differences in their conformations on the other. There is no requirement that these two properties be simply related. Thus, some conformational changes may represent relatively large energy differences, while other conformation changes, which appear to be as extensive, may occur with relatively much smaller changes in the energy of the system. Lattice effects in a crystal that was formed as $HbO_2$ and in which considerable oxidation of $HbO_2$ to Hi may have occurred may well cause the Hi to retain a conformation very similar to that of $HbO_2$, in which case the reported structure of oxyhemoglobin may be essentially correct.

## CONCLUSION

The use of natural-abundance $^{13}$C NMR spectroscopy, which focuses particularly on differences between the spectral characteristics of closely related proteins (as in the sequence $HbO_2$, HbCO, HiCN, Hi, and Hb) has been used to study the conformation of protein molecules in solution.

The similarity between rabbit $HbO_2$ and HbCO suggests that both these forms have nearly identical quaternary conformations for the $\alpha_2\beta_2$ tetramer, as well as for the individual α and β subunits. The differences between these forms ($HbO_2$ and HbCO) and rabbit HiCN suggest to us that, though HiCN has the quaternary structure of $HbO_2$ or HbCO, there have been subtle conformational changes in the tertiary structures of the α and β subunits in rabbit HiCN.

The quaternary structure of Hi seems to be between that of the strongly liganded hemoglobin and deoxyhemoglobin. We do not suggest an intermediate quaternary structure for Hi, but interpret our results in terms of an equilibrium between two limiting quaternary structures (that for strongly liganded hemoglobins on the one hand and deoxyhemoglobin on the other).

Finally, human hemoglobins seem to have tighter, less mobile structures in solution than do the analogous rabbit hemoglobins.

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