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Proton nuclear magnetic resonance investigation of structural changes associated with cooperative oxygenation of human adult hemoglobin*

(allosteric model/sequential model/mechanism of oxygenation of hemoglobin)

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ABSTRACT **The structural changes associated with cooperative oxygenation of human adult hemoglobin as a function of oxygen saturation in aqueous media at neutral pH and at 25–27°C have been investigated by high-resolution proton nuclear magnetic resonance spectroscopy at 250 and 360 MHz. By monitoring the intensities of two hyperfine shifted proton resonances (at about −12 and −18 ppm from H_2O) and two exchangeable proton resonances (at about −6.4 and −9.4 ppm from H_2O) as a function of oxygenation, the amount of oxygen bound to the α and β chains of a hemoglobin molecule can be determined and the relationship between tertiary and quaternary structural changes under a given set of experimental conditions can be investigated. These results suggest that: (*i*) in the absence of organic phosphates, there is no preferential O_2 binding to the α or β chains; (*ii*) in the presence of organic phosphates, the α hemes have a higher affinity for O_2 as compared to the β hemes; (*iii*) the ligand-induced structural changes in the hemoglobin molecule are not concerted; and (*iv*) some cooperativity must be present within the deoxy quaternary state during the oxygenation process. The variations of the exchangeable proton resonances as a function of oxygenation strongly suggest that the breaking of one or more inter- or intrasubunit linkages of a ligated subunit can affect similar linkages in unligated subunits within a tetrameric hemoglobin molecule. Thus, the present results show that two-state allosteric models are not adequate to describe the cooperative oxygenation of hemoglobin. In addition, the present results provide direct correlation to the ligand-induced structural changes (such as in the heme pockets and subunit interfaces) observed to occur in the crystals of deoxy- and oxy-like hemoglobin molecules and in the solution state.**

Despite considerable biochemical and biophysical studies devoted to the hemoglobin (Hb) molecule, the detailed molecular mechanism for the cooperative oxygenation of Hb is not fully understood. For example, it has not been possible, as yet, to correlate quantitatively various structural changes during the oxygenation process with the energetics of the process. A successful solution of the Hb problem is important not only because it would allow us to understand the molecular mechanism of a respiratory transport process, but also because it may give insights into the action of other more complex systems on which metabolic regulation depends. For recent reviews on the structure–function relationship in Hb, refer to refs. 1–5.

Comparing the atomic models of deoxyhemoglobin and oxyhemoglobin-like methemoglobin, Perutz (6) has proposed a stereochemical mechanism for the cooperative oxygenation of Hb. In its original form, his model emphasized the link between the cooperativity and the transition between the two quaternary structures (deoxy quaternary structure is symbolized by T and oxy quaternary structure by R). Perutz's mechanism allows tertiary structural changes to take place each time a subunit is oxygenated, but a single concerted quaternary structural transition (T ⇌ R) is responsible for the cooperativity of the oxygenation process. Thus, the affinity for O_2 of an unligated subunit is not influenced by the state of ligation of its neighbors within a given quaternary structure. The basic conceptual framework of Perutz's scheme shares many features of a two-state allosteric model, such as the one proposed by Monod *et al.* (7). Perutz's model has not only given new insight into the Hb problem, but has also suggested several amino acid residues as excellent markers to monitor both tertiary and quaternary structural changes of Hb upon oxygenation.

By choosing appropriate human mutant hemoglobins as well as chemically modified hemoglobins and by making use of Perutz's atomic models of hemoglobin, we have assigned a number of proton resonances, obtained through high-resolution proton nuclear magnetic resonance (NMR) spectroscopy, to specific amino acid residues in the Hb molecule. Of special interest to this work are the following resonances: the hyperfine shifted proton resonances at about −18 and −12 ppm from H_2O due to protons from the β and α heme, respectively (8, 9); the exchangeable proton resonance at about −9.4 ppm from H_2O due to the intersubunit hydrogen bond between α42(C7) tyrosine and β99(G1) aspartic acid in the $\alpha_1\beta_2$ subunit interface, a characteristic feature of the deoxy quaternary structure (2, 10); and the exchangeable proton resonance at about −6.4 ppm from H_2O due to the intrasubunit hydrogen bond between β98(FG5) valine and β145(HC2) tyrosine, an important feature in the deoxy tertiary structure (11). Hence, by monitoring the intensities of the two hyperfine shifted resonances as a function of oxygenation, we can determine the amounts of O_2 bound to α and β chains as carried out previously in ^2H_2O (12–14,§); and by monitoring the intensities of the two exchangeable proton resonances as a function of oxygenation, we can follow tertiary and quaternary structural changes.

Abbreviations: Hb, hemoglobin; Hb A, normal human adult hemoglobin; T and R, symbols for the deoxy and oxy quaternary structures of Hb, respectively, in the Perutz stereochemical model for the oxygenation of Hb; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; P_2-glycerate, 2,3-diphosphoglycerate; Ins-P_6, inositol hexaphosphate.

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§ G. Viggiano, N. T. Ho, and C. Ho, unpublished data.

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EXPERIMENTAL SECTION

Materials

Hb was prepared in the usual manner from fresh whole human blood obtained from the local blood bank (13). Phosphates were removed from the Hb samples by passage through a column of Sephadex G-25 (Pharmacia) equilibrated with 0.01 M Tris buffer containing 0.1 M NaCl at pH 7.5 (15). Deoxy Hb samples for ^{1}H NMR studies were prepared by standard procedures used in this laboratory (13). All chemicals were reagent grade obtained from commercial suppliers and were used without further purification.

Partial O_2 saturations for NMR studies were obtained by mixing oxy and deoxy Hb samples in a specially designed NMR sample system. This set-up consists of a standard 5-mm precision NMR sample tube and a precision coaxial inner tube. The bottom of the coaxial inner tube is cut open and attached to a nylon insert with a narrow channel. Deoxy Hb solution is transferred into the sample tube under nitrogen atmosphere. The coaxial tube is then put inside the 5-mm NMR sample tube and oxy Hb solution is transferred into the coaxial inner tube. Raising the inner tube allows a given amount of oxy Hb to flow into the lower chamber. In this manner, Hb samples at appropriate O_2 saturations can be prepared. The O_2 saturation accuracy is better than 5% for samples prepared by this mixing technique. Details will be published elsewhere.§

Methods

High-resolution ^{1}H NMR spectra at 250 MHz were obtained by using the MPC-HF 250-MHz superconducting spectrometer at an ambient temperature of 27°C. Chemical shifts are referenced to the water proton signal, which is 4.83 ppm downfield from the proton resonance of a standard, 2,2-dimethyl-2-silapentane-5-sulfonate, at 27°C. A negative sign in the chemical shift indicates that the resonance is downfield from the water resonance. Signal-to-noise ratios were improved by signal averaging (about 1600 scans) using the NMR correlation spectroscopy technique with a Sigma-5 computer interfaced to the MPC-HF 250 NMR spectrometer (16). The sweep time for the spectral region from −1400 to −7400 Hz downfield from H_2O was 0.6 s. The chemical shift measurements are accurate to ±0.1 ppm.

In order to obtain a better base line for the exchangeable proton resonances at −9.4 and −6.4 ppm downfield from H_2O, a higher frequency spectrometer was used in some of our studies. The 360-MHz ^{1}H NMR spectra were obtained by using a Bruker HXS-360 NMR spectrometer located at the Stanford Magnetic Resonance Laboratory. This spectrometer was also operated in the correlation mode, and the temperature inside the probe was 25°C. The sweep time for the spectral region from −1900 to −3600 Hz downfield from H_2O was 1 s, with a delay of 0.3 s per scan, for approximately 800 scans.

The areas of the −18 ppm β-heme resonance and the −12 ppm α-heme resonance were measured by digital integration of the spectrum, taking into account only the downfield portion of the −18 ppm peak and the upfield portion of the −12 ppm peak in order to reduce the contributions of other overlapping hyperfine shifted resonances. Details on the analysis of the hyperfine shifted proton resonances will be published elsewhere.§ The areas of the exchangeable proton resonances at −9.4 and −6.4 ppm were determined by weighing the peaks cut out from the spectrum recorded on chart paper.

RESULTS

Representative ^{1}H NMR spectra of Hb A as a function of O_2 saturation in aqueous media at pH 7 are shown in Fig. 1 at 250 MHz and in Fig. 2 at 360 MHz. The 250-MHz spectra display both the hyperfine shifted and exchangeable proton resonances over the spectral region from −6 to −28 ppm downfield from the water proton resonance. The 360-MHz spectra are limited to the spectral region from −5 to −10 ppm downfield from H_2O, where the exchangeable proton resonances are detected, in order to take full advantage of the improved resolution at higher frequency, especially for the resonance at about −6.4 ppm. We have found that the linewidths of the hyperfine shifted proton resonances of deoxy Hb A increase with the square of the applied magnetic field, in agreement with our earlier findings (17). Thus, for hyperfine shifted proton resonances, there is no improvement in the resolution in going from 250 to 360 MHz. The exchangeable proton resonance at −9.4 ppm lies over a much broader hyperfine shifted resonance. A

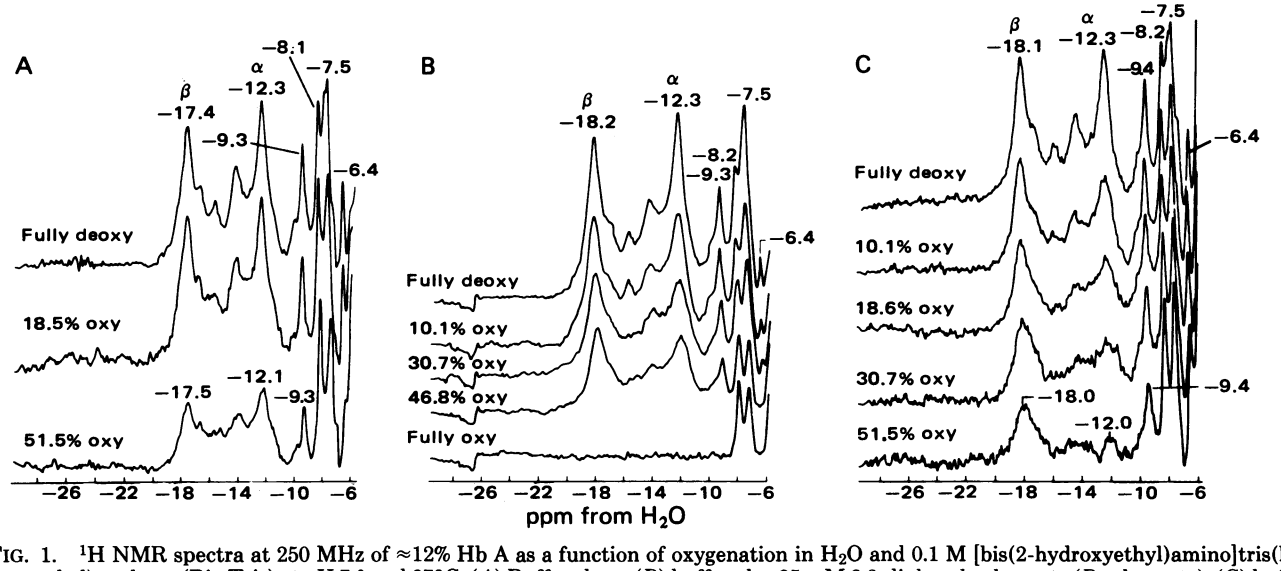

FIG. 1. ^{1}H NMR spectra at 250 MHz of ≈12% Hb A as a function of oxygenation in H_2O and 0.1 M [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane (Bis-Tris) at pH 7.0 and 27°C. (*A*) Buffer alone; (*B*) buffer plus 35 mM 2,3-diphosphoglycerate (P_2-glycerate); (*C*) buffer plus 10 mM inositol hexaphosphate (Ins-P_6).

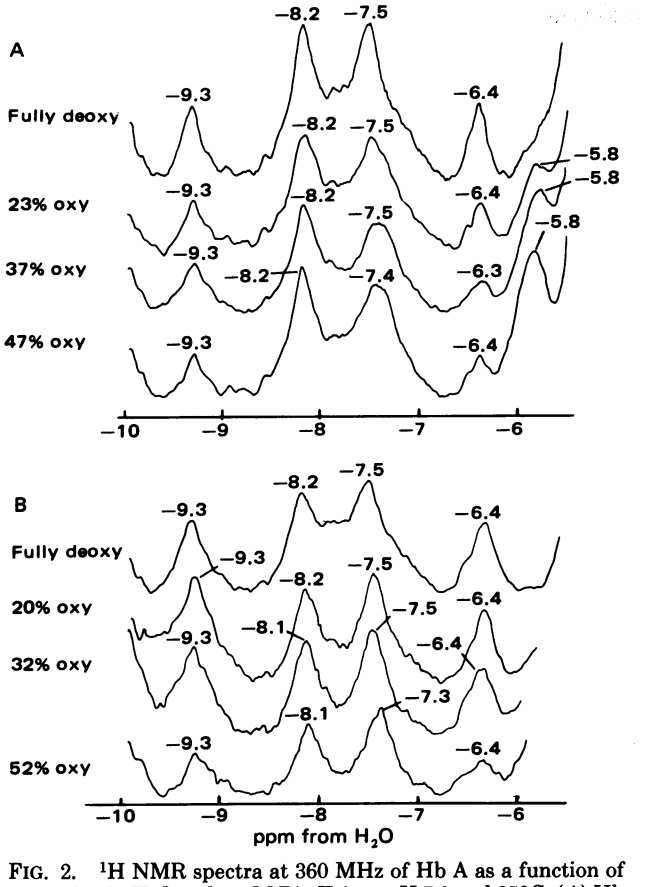

FIG. 2. ^1H NMR spectra at 360 MHz of Hb A as a function of oxygenation in H_2O and 0.1 M Bis-Tris at pH 7.0 and 25°C. (*A*) Hb A, 13.5%; (*B*) Hb A, 12%, plus 10 mM Ins-P_6.

comparison of the 250 MHz spectra obtained in ^2H_2O and in ^1H_2O allows an easy separation of these two types of resonance at around −9.4 ppm. At 360 MHz, the exchangeable proton resonance at −9.4 ppm is not greatly affected by the hyperfine shifted resonance underneath it as it is in the 250-MHz spectrum. This is because, at higher frequency, the hyperfine shifted resonance becomes even broader and is thus essentially reduced to the base line level.

The areas of these four resonances (at about −18, −12, −9.4, and −6.4 ppm) were measured as a function of oxygenation in the presence and absence of organic phosphates. The variations of these four resonances under different experimental conditions are summarized in Figs. 3–5.

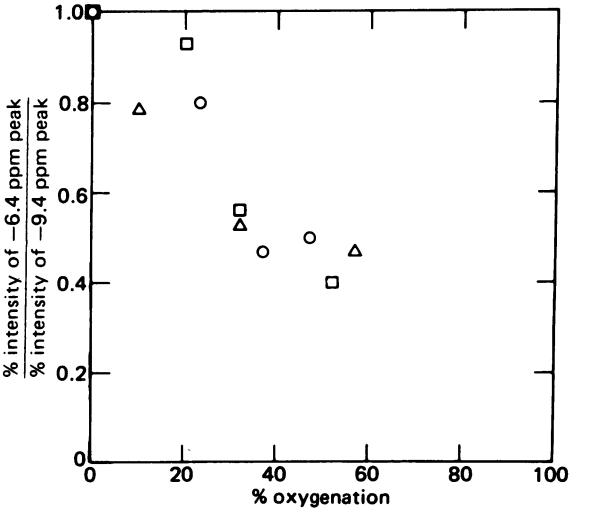

FIG. 4. Variation of the ratio of the tertiary structure probe at −6.4 ppm to the quaternary structure probe at −9.4 ppm as a function of oxygenation of Hb A in H_2O and 0.1 M Bis-Tris at pH 7.0. ○, 13.5% Hb A in buffer at 25°C (obtained from 360-MHz spectra); △, 12.5% Hb A in buffer plus 35 mM P_2-glycerate at 27°C (obtained from 250-MHz spectra); □, 12% Hb A in buffer plus 10 mM Ins-P_6 at 25°C (obtained from 360-MHz spectra).

DISCUSSION

The four resonances that we have investigated in this paper are sensitive probes for monitoring the cooperative oxygenation of Hb A. We shall discuss these preliminary results in terms of the molecular mechanism for the oxygenation process.

Variation of hyperfine shifted proton resonances as a function of oxygenation

Fig. 3 summarizes the changes in the areas or intensities of the β-heme resonance at −18 ppm and of the α-heme resonance at −12 ppm upon oxygenation. There are two major conclusions that can be drawn from these results. First, the ratio of the intensities of the α- and β-heme resonances remains essentially constant upon oxygenation in 0.1 M Bis-Tris at pH 7.0 and 27°C as shown in Fig. 3*A*. However, the same ratio decreases upon oxygenation in the presence of P_2-glycerate and Ins-P_6, the effect of the latter being much greater, as shown in Fig. 3 *B* and *C*. The present results have confirmed and extended the earlier findings reported by this laboratory on ^1H NMR studies of oxygenation of Hb A carried out in ^2H_2O media; namely, (*i*) there is no preference for the binding of O_2 to α and β chains,

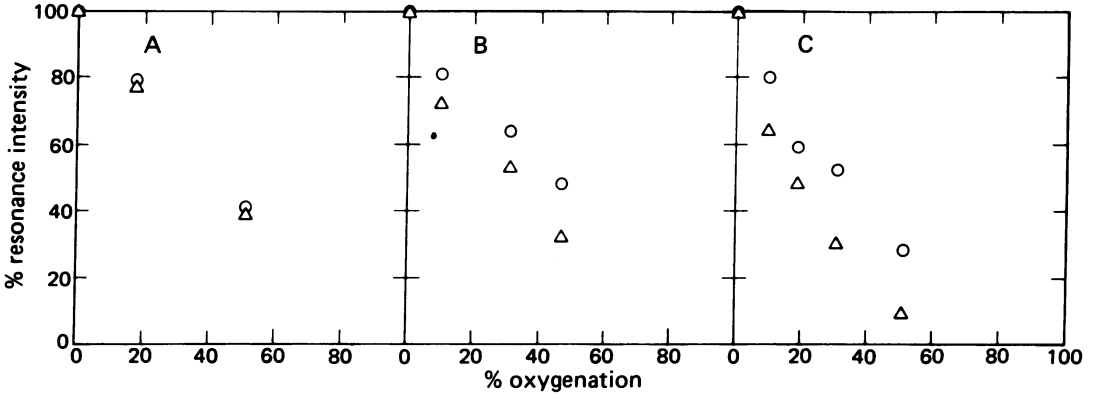

FIG. 3. Variation of hyperfine shifted proton resonances of the β heme at −18 ppm (○) and the α heme at −12 ppm (△) as a function of oxygenation of ≈12% Hb A in H_2O and 0.1 M Bis-Tris at pH 7.0 and 27°C. (*A*) Buffer alone; (*B*) buffer plus 35 mM P_2-glycerate; (*C*) buffer plus 10 mM Ins-P_6. The experimental results were obtained from 250-MHz NMR spectra.

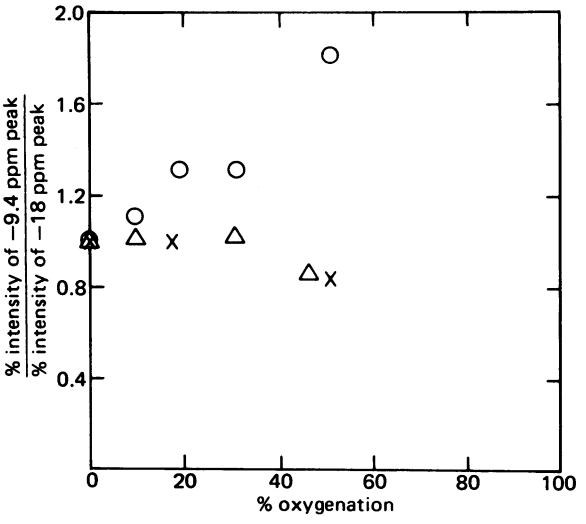

FIG. 5. Variation of the ratio of the quaternary structure probe at −9.4 ppm to the β-heme resonance at −18 ppm as a function of oxygenation of ≈12% Hb A in H_2O and 0.1 M Bis-Tris at pH 7.0 and 27°C. X, Buffer alone; Δ, buffer plus 35 mM P_2-glycerate; O, buffer plus 10 mM Ins-P_6. The experimental results were taken from 250-MHz NMR spectra.

and (*ii*) the α chains have a higher affinity for O_2 as compared to the β chains in the presence of organic phosphates (12–14). Second, the intensities of the α-heme resonance at −12 ppm and of the β-heme resonance at −18 ppm upon oxygenation decrease proportionally more than the total number of deoxy chains as measured by the degree of O_2 saturation of Hb A. This suggests that (*i*) the −12 ppm α peak or the −18 ppm β peak, or both, are characteristic of the Hb molecule with a deoxy-like conformation, and (*ii*) when the tetramer is partially oxygenated, some structural changes occur in such a way that the unligated α or β chains, or both, have a spectrum in which the −12 ppm, the −18 ppm, or both peaks are either missing or altered. These results are in agreement with those carried out in ^2H_2O. Details will be published elsewhere.§

Variation of tertiary and quaternary structural changes with the binding of O_2 to hemoglobin

In the absence of organic phosphate, the intensity of the −9.4 ppm quaternary structure probe maintains a constant ratio to the intensities of both the −18 ppm β-heme resonance and the −12 ppm α-heme resonance upon oxygenation (Figs. 3 and 5), but the −6.4 ppm resonance, sensitive to the tertiary structure of the β-heme pocket, is found to decrease with respect to the −9.4 ppm resonance (Fig. 4). This finding suggests that there is no concerted structural change if O_2 is distributed randomly between the α and β chains (as shown and discussed in the last section). This conclusion is independent of the specific spectral assignment of the −6.4 ppm resonance.

In the presence of organic phosphates, concerted structural changes alone cannot account for the observed spectral features of these four resonances upon oxygenation. The ratio of the intensities of the −18 ppm and −12 ppm resonances is not constant on oxygenation (Fig. 3 *B* and *C*), in contrast to the case in the absence of organic phosphate (Fig. 3*A*). This shows a specific effect of these allosteric effectors on the α and β chains of Hb A. In the presence of Ins-P_6, the ratio of the intensities of resonances at −9.4 ppm (the quaternary structure probe) and at −18 ppm (β heme) increases with oxygenation, almost doubling from 0 to 50% O_2 saturation (Fig. 5). At the same time, the tertiary structure probe of the β chain at −6.4 ppm disappears almost completely. In order to retain the model of a concerted allosteric mechanism, there must be a strong preferential ligation of the β chains. However, this is not in agreement with the relative change in intensities between the −18 ppm β-heme peak and the −12 ppm α-heme peak upon oxygenation as shown in Fig. 3 and discussed in our previous publications (12–14). In the presence of P_2-glycerate (Fig. 5), the ratio of the areas of the −9.4 ppm peak to the −18 ppm peak is constant (up to approximately 40% O_2 saturation), as is the case in the absence of organic phosphate (Fig. 5). However, in the presence of P_2-glycerate, the −12 ppm α-heme peak decreases in intensity with respect to the −18 ppm β-heme peak, as it does in the presence of Ins-P_6 (Fig. 3). Within the accuracy of the present experimental measurements, it is difficult to give an exact quantitative relationship to the variation of the −9.4 and −6.4 ppm resonances as a function of oxygenation. However, it can be concluded that the intensity of the −6.4 ppm tertiary structure probe is found to decrease with respect to the −9.4 ppm peak at any partial O_2 saturation.

Implications of the present results for the mechanism of oxygenation of hemoglobin

From the variations of the four proton resonances investigated as a function of oxygenation, the experimental results suggest that when a Hb tetramer is partially oxygenated, some structural changes must occur in the unligated subunits in such a manner that quenching of their respective hyperfine shifted proton resonances occurs. These results clearly show that there are no concerted structural changes as sensed by these two hyperfine shifted proton resonances and suggest that some cooperativity must be present during the oxygenation of the Hb molecule in the T state. These findings are in agreement with the ^1H NMR studies on the oxygenation of Hb M Milwaukee reported by this laboratory (18, 19). The ferric β chains of this naturally occurring valency hybrid detect more than two structures when its two normal α chains bind O_2 in the absence of organic phosphate.

From the variation of the two exchangeable proton resonances at −6.4 ppm and −9.4 ppm as a function of oxygenation, we can conclude that the intrasubunit hydrogen bond between valine at β98(FG5) and the penultimate tyrosine at β145(HC2) can be broken (as manifested by the disappearance of the −6.4 ppm peak) before the ligation of the β chains and also while the intersubunit hydrogen bond between tyrosine at α42(C7) and aspartic acid at β99(G1) (as manifested by the presence of the −9.4 ppm peak) is still intact. The relative intensities of these four resonances exhibit a complex variation as a function of oxygenation, especially in the presence of organic phosphate. It is quite clear that there are no concerted structural changes upon oxygenation of Hb A as manifested by structural changes of these four resonances.

Thus, our experimental results cannot support a model for the oxygenation of hemoglobin based purely on a two-state allosteric description, such as the one proposed by Monod *et al.* (7), but our results do have many features of a sequential-type model, such as the one proposed by Koshland *et al.* (20). Our data show that ligand-induced conformational changes can be propagated to neighboring subunits and thereby could influence their oxygen affinities. However, a strictly sequential-type model would not require a change in the quaternary structure during the ligation as an essential requirement for cooperativity. It is equally clear on the basis of our data as well as that of others that there is a change in the quaternary structure of a Hb molecule in going from the deoxy to oxy state. It is evident that features of both concerted and sequential models have to be incorporated into a more general model for the cooperative oxygenation of hemoglobin. In a new modified stereochemical

model, Perutz (4, 5) has proposed that salt bridges linking the subunits in the T structure break progressively as O_2 is added, and even those salt bridges in the unligated subunits that have not ruptured can be "weakened." He further believes that within the T structure there could be cooperativity. The variations of the four proton resonances upon oxygenation reported in this paper provide strong support for these ideas. Due to the dovetailed nature of the $\alpha_1\beta_2$ subunit interface, Perutz (4) maintains that there can be only two quaternary structures (T and R). However, if one adopts the definition of quaternary structure proposed by Bernal (21)—the relative arrangement of subunits in a multisubunit protein—any stretching or weakening of intersubunit linkages would produce a new quaternary structure. With this definition, our results would suggest that there are more than two quaternary structures when a Hb molecule is going from the deoxy to oxy state.

The present preliminary results strongly suggest that these four proton resonances are excellent structural probes to investigate the detailed molecular mechanism for the cooperative oxygenation of Hb. In addition, these ^1H NMR results from experiments carried out in aqueous media provide direct support to the idea that ligand-induced structural changes observed to occur in crystals of deoxy- and oxy-like Hb molecules do exist in the solution state, at least in the heme pockets and in the $\alpha_1\beta_2$ subunit interface. Thus, it is relevant to correlate the structural changes in Hb crystals to the functional properties under physiological conditions. Further detailed insights into the sequence of events leading to the oxygenation of hemoglobin will require progress in the following areas: (*i*) additional quantitative correlation among spectral changes, functional properties, and energetics of the oxygenation process; (*ii*) assignment of additional structural probes; and (*iii*) improvement in NMR instrumentation and techniques to permit a more accurate measurement of the areas of the resonance and to observe proton resonances closer to the water proton resonance.

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