Proton Nuclear Magnetic Resonance and Biochemical Studies of Oxygenation of Human Adult Hemoglobin in Deuterium Oxide†

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ABSTRACT: The proton nuclear magnetic resonance spectrum of human adult deoxyhemoglobin in D_2O in the region from 6 to 20 ppm downfield from the proton resonance of residual water shows a number of hyperfine shifted proton resonances that are due to groups on or near the α and β hemes. The sensitivity of these resonances to the ligation of the heme groups and the assignment of these resonances to the α and β chains provide an opportunity to investigate the cooperative oxygenation of an intact hemoglobin molecule in solution. By use of the nuclear magnetic resonance correlation spectroscopy technique, at least two resonances, one at ~18 ppm downfield from HDO due to the β chain and the other at ~12 ppm due to the α chain, can be used to study the binding of oxygen to the α and β chains of hemoglobin. The present results using approximately 12% hemoglobin concentration in 0.1 M Bistris buffer at pD 7 and 27 °C with and without organic phosphate show that there is no significant line broadening on oxygenation (from 0 to 50% saturation) to affect the determination of the intensities or areas of these resonances. It is found that the ratio of the intensity of the α-heme resonance at 12 ppm to that of the β-heme resonance at 18 ppm is constant on oxygenation in the absence of organic phosphate but decreases in the presence of 2,3-diphosphoglycerate or inositol hexaphosphate, with the effect of the latter being the stronger. On oxygenation, the intensities of the α-heme resonance at 12 ppm and of the β-heme resonance at 18 ppm decrease more than the total number of deoxy chains available as measured by the degree of O_2 saturation of hemoglobin. This shows the sensitivity of these resonances to structural changes which are believed to occur in the unligated subunits upon the ligation of their neighbors in an intact tetrameric hemoglobin molecule. A comparison of the nuclear magnetic resonance data with the populations of the partially saturated hemoglobin tetramers (i.e., hemoglobin with one, two, or three oxygen molecules bound) leads to the conclusion that in the presence of organic phosphate the hemoglobin molecule with one oxygen bound maintains the β-heme resonance at 18 ppm but not the α-heme resonance at 12 ppm. These results suggest that some cooperativity must exist in the deoxy quaternary structure of the hemoglobin molecule during the oxygenation process. Hence, these results are not consistent with the requirements of two-state concerted models for the oxygenation of hemoglobin. In addition, we have investigated the effect of D_2O on the oxygenation of hemoglobin by measuring the oxygen dissociation curves of normal adult hemoglobin as a function of pH in D_2O and H_2O media. We have found that (1) the pH dependence of the oxygen equilibrium of hemoglobin (the Bohr effect) in D_2O medium is shifted by approximately 0.4 pH unit toward higher pH in comparison to that in H_2O medium and (2) the Hill coefficients are essentially the same in D_2O and H_2O media over the pH range from 6.0 to 8.2. It appears that deuterium oxide does not alter the basic mechanism of cooperative oxygenation of hemoglobin. Hence, the experimental results of hemoglobin obtained in D_2O can be used to gain insight into those carried out in H_2O.

During the past few years experimental evidence from different sources has suggested that the α and β chains of hemoglobin (Hb)[1] are nonequivalent. [For a summary of such experimental evidence, see Johnson & Ho (1974), Baldwin (1975), and Shulman et al. (1975) and the references cited therein.] The X-ray structural analyses of Hb crystals carried out by Perutz and his colleagues clearly show a strong asymmetry between α and β chains that is believed to be important for the structure–function relationship of Hb (Perutz, 1970; Perutz & Ten Eyck, 1971; Fermi, 1975). At the tertiary level, the carboxyl terminal of histidine HC3(146)β of the β chain is linked by a "salt bridge" to the aspartic acid at FG1(94)β (His-146β-Im$^+$···$^-$OOC-Asp-94β) in the deoxy structure (Perutz, 1970) and is one of the groups responsible for the alkaline Bohr effect as suggested by Perutz (1970) and shown by Kilmartin et al. (1973) to occur under the experimental conditions of 0.2 M phosphate plus 0.2 M chloride at 30 °C. At the quaternary level, the intersubunit "salt bridges" stabilizing the deoxy structure are (1) at the $\alpha_1\beta_2$ interface a pair of residues Lys-40α_1-NH_3^+···$^-$OOC-His-146β_2 and Tyr-42α_1-OH···$^-$OOC-Asp-99β_2 and (2) at the $\alpha_1\alpha_2$ interface a pair of residues Lys-127α_1-NH_3^+···$^-$OOC-Arg-141α_2 and Asp-126α_1-COO^-···$^+$Gua-Arg-141α_2 (Perutz, 1970; Perutz et al., 1974). Furthermore, organic phosphates, 2,3-diphosphoglycerate (P_2-glycerate) and inositol hexaphosphate (Ins-P_6), are found to bind in a cavity between the two β chains (Arnone, 1972; Arnone & Perutz, 1974).

Magnetic resonance techniques (Davis et al., 1969, 1971; Lindstrom et al., 1971, 1972; Ogata & McConnell, 1971, 1972; Ogawa & Shulman, 1972; Huestis & Raftery, 1972a,b, 1973; Lindstrom & Ho, 1972; Ho et al., 1973; Johnson & Ho, 1974; Breen et al., 1974; Huang & Redfield, 1976; Asakura & Lau,

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[1] Abbreviations used: Hb, hemoglobin; NMR, nuclear magnetic resonance; P_2-glycerate, 2,3-diphosphoglycerate; Ins-P_6, inositol hexaphosphate; Bistris, [bis(2-hydroxyethyl)imino]tris(hydroxymethyl)-methane; P_{O_2}, partial pressure of oxygen; P_{50}, partial pressure of oxygen at 50% oxygenation; T and R, symbols for the deoxy and oxy quaternary structures of hemoglobin, respectively, in the Perutz stereochemical model for the oxygenation of hemoglobin (Perutz, 1970).

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1978; Viggiano & Ho, 1979) have been found to be very helpful in gaining information at partial ligations of hemoglobin over a range of experimental conditions. Switching between deoxy and oxy structures can occur independent of the ligation in mutant or valency hybrid hemoglobins (Ogawa & Shulman, 1972; Lindstrom et al., 1972; Perutz et al., 1974; Ho et al., 1975; Fung et al., 1976, 1977). In this respect, the α and β chains appear to play a different role in the Hb tetramer. According to the findings of Ogawa & Shulman (1972), oxy quaternary structure (R) is much more favored in the $Hb(\alpha^{II}\beta^{III}CN)_2$ valency hybrid, whereas in $Hb(\alpha^{III}CN\beta^{II})_2$ the two structures are close in energy. Kinetic measurements show biphasic behavior which has been related to differences in the rate of oxygenation of the α and β chains and/or changes of the structure at the quaternary level (Gibson, 1973; Salhany et al., 1975; Sawicki & Gibson, 1977).

For evaluation of the extent of α–β nonequivalence which must be taken into account to describe the cooperative ligation of hemoglobin, a probe must be selected which allows a direct observation of α and β chains of an intact tetrameric Hb molecule at different stages of ligation. The most promising one is proton nuclear magnetic resonance (NMR) spectroscopy over the spectral region from 6 to 20 ppm downfield from HDO. This region contains the hyperfine shifted proton resonances of deoxyhemoglobin and is flat for ligated Hb A. At least two resonances, one at 18 ppm assigned to the β heme and the other at 12 ppm assigned to the α heme (Davis et al., 1971; Lindstrom et al., 1972) are well enough resolved to be used for a quantitative determination of the oxygenation process. Ho and co-workers (Ho & Lindstrom, 1972; Lindstrom & Ho, 1972; Johnson & Ho, 1974) measured the area ratios of the α peak (at 12 ppm) and β peak (at 18 ppm) as a function of CO and O_2 saturations. They found an essentially constant ratio when Hb A combines with CO in the presence and absence of organic phosphates. This area ratio still remains constant upon oxygenation of Hb A in the absence of organic phosphate but is found to decrease at partial oxygen saturations in the presence of P_2-glycerate and even more so in the presence of Ins-P_6 (Johnson & Ho, 1974).

Recently, Huang & Redfield (1976) have applied a long-pulse NMR technique (based on a "214" pulse sequence) to investigate the binding of O_2 to the α and β chains of Hb A. They observed a nearly linear relationship between the intensity of the β-heme resonance at 18 ppm and O_2 saturation of Hb A in the presence and absence of organic phosphates. On this basis, they questioned if there is a higher O_2 affinity of the α chains of Hb A as reported by Ho and co-workers. They further observed a significant line broadening of both the α-heme resonance at 12 ppm and the β-heme resonance at 18 ppm (especially the former resonance) on increasing temperature and O_2 saturation. The observed broadening is greatest in the presence of Ins-P_6. They interpreted the line broadening as a kinetic effect and emphasized that there is a kinetic difference between the α and β chains on oxygenation. It should be mentioned that the greater broadening of the α-heme resonance is in disagreement with the interpretation that the β-chain kinetics are more rapid than those of the α chain as reported by Gibson and co-workers (Gibson, 1973; Salhany et al., 1975; Sawicki & Gibson, 1977). However, there are two major agreements between ^1H NMR results of Ho and co-workers and those of Huang & Redfield (1976). First, there is no preferential O_2 binding to α and β chains of Hb A in 0.1 M Bistris buffer at pD $\sim$7. Second, both sets of results exclude the possibility that β chains have a higher O_2 affinity as compared to the α chains. On the other hand, the data of Lindstrom & Ho (1972) and of Johnson & Ho (1974) show that the α chains have a higher O_2 affinity than the β chains in the presence of organic phosphates, especially in the presence of Ins-P_6. Due to the base-line uncertainty as well as the limited accuracy of all previous studies, no quantitative conclusions could be made on the binding of O_2 to the α and β chains of Hb A.

In the present work, we have made major improvements in obtaining a better base line in our ^1H NMR spectra by means of NMR correlation spectroscopy. We have obtained a satisfactory base line over the spectral region from 6 to 30 ppm from HDO and have also made improvements in the analysis of our data. Our results may be summarized as follows. First, we found no significant line broadening in both α-heme and β-heme resonances upon oxygenation (at low O_2 saturations). Second, we found that, upon oxygenation and in the presence of P_2-glycerate or Ins-P_6, the area of the α-heme resonance at 12 ppm decreases more than that of the β-heme resonance at 18 ppm, in agreement with the earlier results reported by this laboratory (Ho & Lindstrom, 1972; Lindstrom & Ho, 1972; Johnson & Ho, 1974). Third, by comparing the areas of α- and β-heme resonances with the fractional population of partially ligated Hb tetramers [Hb, HbO_2, $Hb(O_2)_2$, $Hb(O_2)_3$, and $Hb(O_2)_4$], we have found that, in the presence of organic phosphate, most of the singly oxygenated Hb tetramers, HbO_2, undergo structural changes of both α chains but not the β chains. Fourth, the behavior of the hyperfine shifted proton resonances of Hb A upon oxygenation is not linear. The α-heme resonance at 12 ppm and the β-heme resonance at 18 ppm both have a smaller area than expected from the available deoxy chains of Hb based on the degree of oxygenation. Fifth, our results clearly show that two-state allosteric models are not adequate to describe the cooperative oxygenation of hemoglobin. Sixth, from the measurements of the values of Hill coefficient and partial pressure of O_2 at 50% oxygenation (P_{50}) as a function of pH in both D_2O and H_2O media, we have concluded that D_2O does not alter the mechanism of oxygenation of hemoglobin under our experimental conditions.

Materials and Methods

Preparation of Samples. Human adult hemoglobin (Hb A) was prepared from fresh blood samples obtained from the local blood bank according to Drabkin (1946) and stripped of organic phosphates according to the procedure of Berman et al. (1971). All procedures for exchange of samples with D_2O (obtained from Bio-Rad Laboratories), removal of CO, and deoxygenation have been described by Lindstrom & Ho (1972). Stock solutions of [bis(2-hydroxyethyl)imino]tris(hydroxyl)methane (Bistris; obtained from Aldrich), 2,3-diphosphoglycerate (obtained from Calbiochem), and inositol hexaphosphate (obtained from Sigma Chemical Co.) were prepared in D_2O as described previously (Wiechelman et al., 1974). All other chemicals were obtained from commercial companies as the reagent grade and were used without further purification.

Partial oxygen saturation for NMR studies was obtained by mixing oxy- and deoxyhemoglobin samples in a specially designed NMR sample system as shown in Figure 1. This setup consists of a standard 5-mm precision NMR sample tube (Wilmad No. 527-PP; i.d. 5.0 mm) and a precision coaxial inner tube (Wilmad No. WGS-4BL). The bottom of the coaxial inner tube is cut open and attached to a nylon insert with a narrow channel as shown in Figure 1A. A small glass ball is dropped to the bottom of the 5-mm NMR sample tube, and a certain amount of deoxy-Hb A solution is added to the sample tube under nitrogen atmosphere (Figure 1B). The

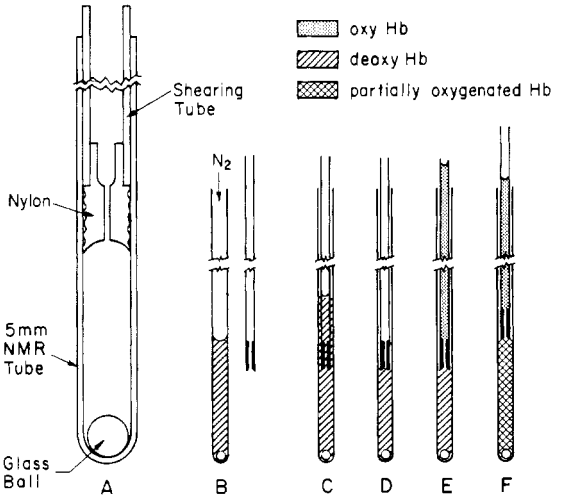

FIGURE 1: NMR sample system for preparation of partially oxygenated hemoglobin samples. For details, see the text.

coaxial tube is then inserted into the 5-mm NMR outer sample tube until it reaches a mark about 3 cm from the bottom (Figure 1C). The deoxy-Hb solution in the upper part is sucked away by a long, thin disposable pipet (Figure 1D), and the inner tube is now filled with oxy-Hb solution (Figure 1E). Raising the inner tube allows a given amount of oxy-Hb to flow into the lower chamber (Figure 1F). The small glass ball at the bottom is used to mix deoxy- and oxy-Hb solutions inside the NMR sample tube. No significant diffusion of oxy-Hb from the upper chamber to the lower one was detected even after 24 h. The volumetric reproducibility, which was checked by weighing, is consistent with a saturation accuracy better than 5%. The Hb preparations sometimes show a slow oxygen consumption, apparently due to bacterial contamination. This problem can be eliminated by filtering the Hb solution through a 0.45-μm Millipore filter (No. HAWPO 1300). This filtration process produces no observable changes in either optical or ^{1}H NMR spectra. Correction for dissolved oxygen is computed by using

$$Y_{vol} = \frac{V_{oxy}}{V_{oxy} + V_{deoxy}} \quad (1)$$

where V_{oxy} and V_{deoxy} are the volumes of oxy- and deoxy-Hb A solutions, respectively. Y_{vol} is the O_2 saturation based on a volumetric determination as described above. The amount of oxygen in V_{oxy} is given as

$$\text{amount of } O_2 \text{ in } V_{oxy} = V_{oxy}(Y_{oxy}C + \alpha P_{oxy}) \quad (2)$$

This amount of oxygen must be equal to that after mixing deoxy- and oxy-Hb A solutions:

$$\text{amount of } O_2 \text{ after mixing} = (V_{oxy} + V_{deoxy})[Y(P_{O_2})C + \alpha P_{O_2}] \quad (3)$$

where Y_{oxy} is the O_2 saturation of oxy-Hb, P_{oxy} is the partial pressure of O_2 of oxy Hb, C is the hemoglobin concentration expressed in milliliters of O_2 per milliliter of solution, α is the oxygen solubility factor [α = 0.0275 mL of O_2/(mL of Hb atm) at 27 °C] (Tucker, 1967; Severinghaus et al., 1971), $Y(P_{O_2})$ is the O_2 saturation after mixing of oxy- and deoxy-Hb solutions, and P_{O_2} is the partial pressure of O_2 after mixing of oxy- and deoxy-Hb solutions. Substituting in eq 1–3, we obtain

$$Y_{vol} = \frac{Y(P_{O_2})C + \alpha P_{O_2}}{Y_{oxy}C + \alpha P_{oxy}} \quad (4)$$

From eq 4, a table relating Y_{vol} to $Y(P_{O_2})$ can be constructed. From this table, one can interpolate the actual oxygen saturation, $Y(P_{O_2})$, at a given value of Y_{vol} used in each experiment.

Measurement of Oxygen Dissociation Curves of Hemoglobin. Two methods have been used to measure the oxygen dissociation curves of Hb A as a function of pH in H_2O and D_2O media. First, O_2 dissociation curves were measured by using an Aminco Hem-O-Scan apparatus. The partial pressure of oxygen (P_{O_2}) was monitored by an oxygen electrode and the fractional O_2 saturation was monitored by dual-wavelength spectroscopy at 390 and 480 nm. The gas mixtures (obtained from Lif-O-Gen) used were 25% O_2–75% N_2 for oxygenation and 100% N_2 for deoxygenation. For samples in D_2O media, D_2O instead of H_2O was used in the humidifying chamber of the instrument to minimize exposure of the samples to atmospheric moisture. The values for P_{50} were obtained directly from the plots of fractional O_2 saturation vs. P_{O_2}, and the Hill coefficients were calculated from the slopes of the plots [log $Y/(1 - Y)$ vs. log P_{O_2}]. The concentration of Hb A used in these measurements was 4%.

The second method was based on an apparatus designed by Rossi-Bernardi et al. (1975), in which the O_2 dissociation curves were obtained by mixing oxy- and deoxy-Hb A samples to reach various O_2 saturations. Partial O_2 saturations (Y_{i+1}) can be calculated from the equation

$$Y_{i+1} = \frac{1}{V_cC_{i+1}}[C_iY_i(V_c - V_i) + \alpha P_{O_2(i)}(V_c - V_i) + V_iC_{oxy}Y_{oxy} + V_i\alpha P_{oxy} - V_c\alpha P_{O_2(i+1)}] \quad (5)$$

where V_c is the volume of the measuring chamber in the apparatus, V_i is the volume of oxy-Hb added at the ith step (to proceed to the $i + 1$ step), C_i is the concentration of deoxy-Hb at the ith step, C_{oxy} is the concentration of oxy-Hb, $P_{O_2(i)}$ is the partial pressure of O_2 at the ith step, and Y_i is the O_2 saturation of Hb at the ith step. The concentration of Hb A used in these measurements was 1%. The starting O_2 saturations of Y_1 and Y_{oxy} were measured as O_2 released by ferricyanide (Tucker, 1967) or Van Kampen reagent (Van Kampen & Zijlstra, 1961).

The hemoglobin concentration was determined by spectrophotometry on diluted oxy-Hb samples using the extinction coefficients provided by Van Assendelft (1970). No more than 2% of the total hemoglobin was detected as methemoglobin.

pH and pD Measurements. Hydrogen ion concentrations were determined on a Beckman Model 3100 pH meter with a Beckman combination electrode (No. 39505). Deuterium ion concentrations were estimated by adding 0.4 pH unit to the pH meter readings for the D_2O solutions (Glasoe & Long, 1960).

NMR Measurements. ^{1}H NMR spectra were obtained on the MPC-HF 250 MHz superconducting spectrometer by using the NMR correlation spectroscopy technique of Dadok & Sprecher (1974). The ambient temperature of the probe was 27 °C. The rectangular spectral window of the fast-sweep mode eliminated many of the problems associated with both the residual water proton signal and the contiguous aromatic proton resonances which are very large compared with the hyperfine shifted proton resonances. A systematic check of the experimental reproducibility has been carried out over a wide range of spectrometer settings. The tests on ~12% deoxy-Hb A samples with and without Ins-P_6 at neutral pD and 27 °C have been carried out by using sweep rates between 12 000 and 300 Hz/s and a spectral window mainly from −7400 to −1400 Hz downfield from the residual water proton signal (this window cuts out the aromatic proton resonance

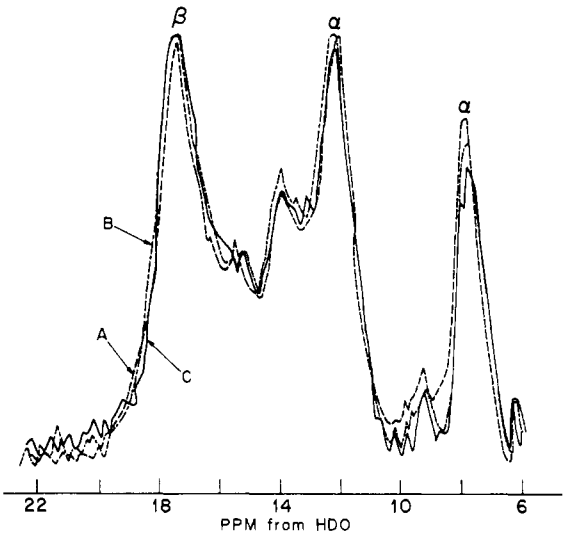

FIGURE 2: Effect of oxygenation on the line width of hyperfine shifted proton resonances of ~12% Hb A in 0.1 M Bistris at pD 7 and 27 °C. (A) (---) fully deoxy sample; (B) (-·-) 23.5% oxygenation; (C) (—) 31.6% oxygenation. The intensities of spectra B and C have been prescaled to give the same intensity as spectrum A.

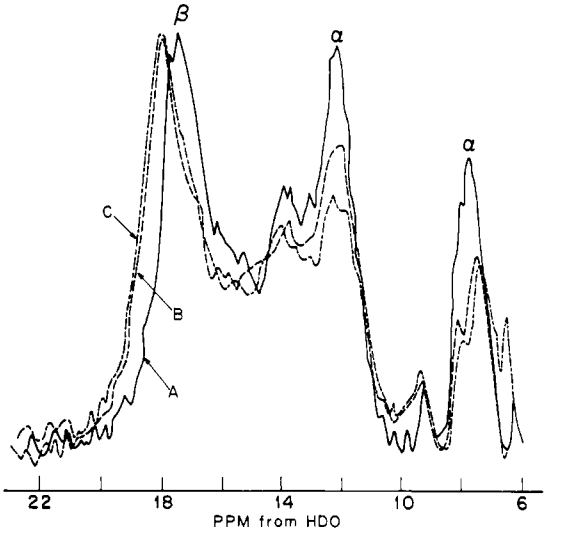

FIGURE 3: Effects of inositol hexaphosphate and oxygenation on the line width of hyperfine shifted proton resonances of ~12% Hb A in 0.1 M Bistris at pD 7 and 27 °C. (A) (—) 31.6% oxygenation (same as spectrum C of Figure 2); (B) (---) 31.6% oxygenation in the presence of 11 mM Ins-P_6; (C) (-·-) 48% oxygenation in the presence of 11 mM Ins-P_6. The intensities of the 18-ppm peaks in spectra B and C have been prescaled to give the same intensity as that of the 18-ppm peak in spectrum A.

region) and sometimes up to −500 Hz (including the aromatic proton resonance region). It has been found that the most critical parameter is the phase; the spectra recorded with a careful phase setting are essentially superimposable. The results obtained in these experiments have provided us with a reasonable setting for the acquisition of hyperfine shifted proton resonances of Hb A at 250 MHz, namely, a spectral window of 6000 Hz between −7400 and −1400 Hz downfield from HDO at a sweep rate of 7500 Hz/s. With these settings, we have found that 1600 scans per spectrum can give a reasonable signal to noise ratio. All the spectra reported in this paper have been run with this set of spectrometer parameters. The proton chemical shifts are expressed as parts per million (ppm) with respect to the residual water proton signal (HDO), which resonates 4.83 ppm downfield from the proton resonance of 2,2-dimethyl-2-silapentane-5-sulfonate at 27 °C. The chemical shift scale is presently defined as positive in the low-field direction with respect to the residual water proton signal.[2]

Results

(*A*) *Effects of Oxygenation on the Hyperfine Shifted Resonances of Hb A at Partial O_2 Saturation.* The experiments shown in Figures 2 and 3 were designed to check whether there is a true decrease in the areas of the α-heme resonance at 12 ppm and the β-heme resonance at 18 ppm upon oxygenation in the presence and absence of Ins-P_6 or if there is a base-line problem (by superimposition of overlapping resonances) or if there is a significant line broadening upon oxygenation. ^1H NMR spectra have been recorded on the same sample of Hb A, first on a fully deoxygenated sample, then on the sample oxygenated to 31.6% in the absence of organic phosphate (Figures 2 and 3), then on the sample with Ins-P_6 added to the same Hb solution, and finally on the sample with the O_2 saturation increased to 48% in the presence of 11 mM Ins-P_6 (Figure 3). The spectra have been prescaled to give the same maximum height of the β peak at 18 ppm. Without organic phosphates, the spectra for completely deoxy-Hb A as well as those at 23.5 and 31.6% O_2 saturations (Figure 2) are essentially identical. The above results clearly show that there is no evidence for any significant line broadening in these spectra and that there is a real but equal decrease in the intensity of both resonances at 18 and 12 ppm upon oxygenation. The line width at half-height can be estimated at about 285 Hz for each peak with an accuracy of ±50 Hz. This puts an upper limit of 3.3 Hz/1% O_2 saturation for the oxygenation-dependent line broadening [a value much less than 8 Hz/1% O_2 saturation reported by Johnson & Ho (1974)]. On the other hand, the actual figure must be well below this maximum because no changes are found for the total area from 10 to 22 ppm and for the height of the peaks in this region. Addition of 11 mM Ins-P_6 at 31.6% O_2 saturation (Figure 3) causes a shift of the β peak by about 0.4 ppm downfield [as first reported by Ho et al. (1973)] and an unquestionable decrease in the intensity of the 12-ppm α peak. This must be a true decrease in the area of the α peak because the total area (from 10 to 22 ppm) has decreased, whereas no substantial change can be observed in the area of the β peak. Furthermore, the right-hand side of the 12-ppm α peak in the spectrum with Ins-P_6 (Figure 3) lies completely inside the α peak of the spectrum taken before the addition of Ins-P_6, ruling out errors that can arise from the area lost in the tail of the resonance. Increasing the O_2 saturation to 48% in the presence of Ins-P_6 further decreases the intensity of the 12-ppm peak (Figure 3). The other peak of the α chain at ~7.9 ppm is also greatly reduced by the addition of Ins-P_6 at partial oxygen saturations. This peak is a superposition of at least two resonances (for details, see Difference Spectra).

(*B*) *Oxygenation of Hb A in the Absence and Presence of Organic Phosphates.* Figure 4 shows a representative series

[2] In conforming with the recommendation for the presentation of NMR data for publication in chemical journals proposed by the International Union of Pure and Applied Chemistry (No. 38, Aug 1974), we have adopted the IUPAC convention, namely, the chemical shift scale is defined as positive in the low-field (or high-frequency) direction. This convention is different from that used by this laboratory. Previously, we had used the negative sign to indicate that the chemical shift of a given resonance is downfield from the resonance of a standard, such as the proton resonance of the residual water (HDO) signal. Hence, this change in the sign of the chemical shift scale should be noted when referring to earlier publications reported by this laboratory.

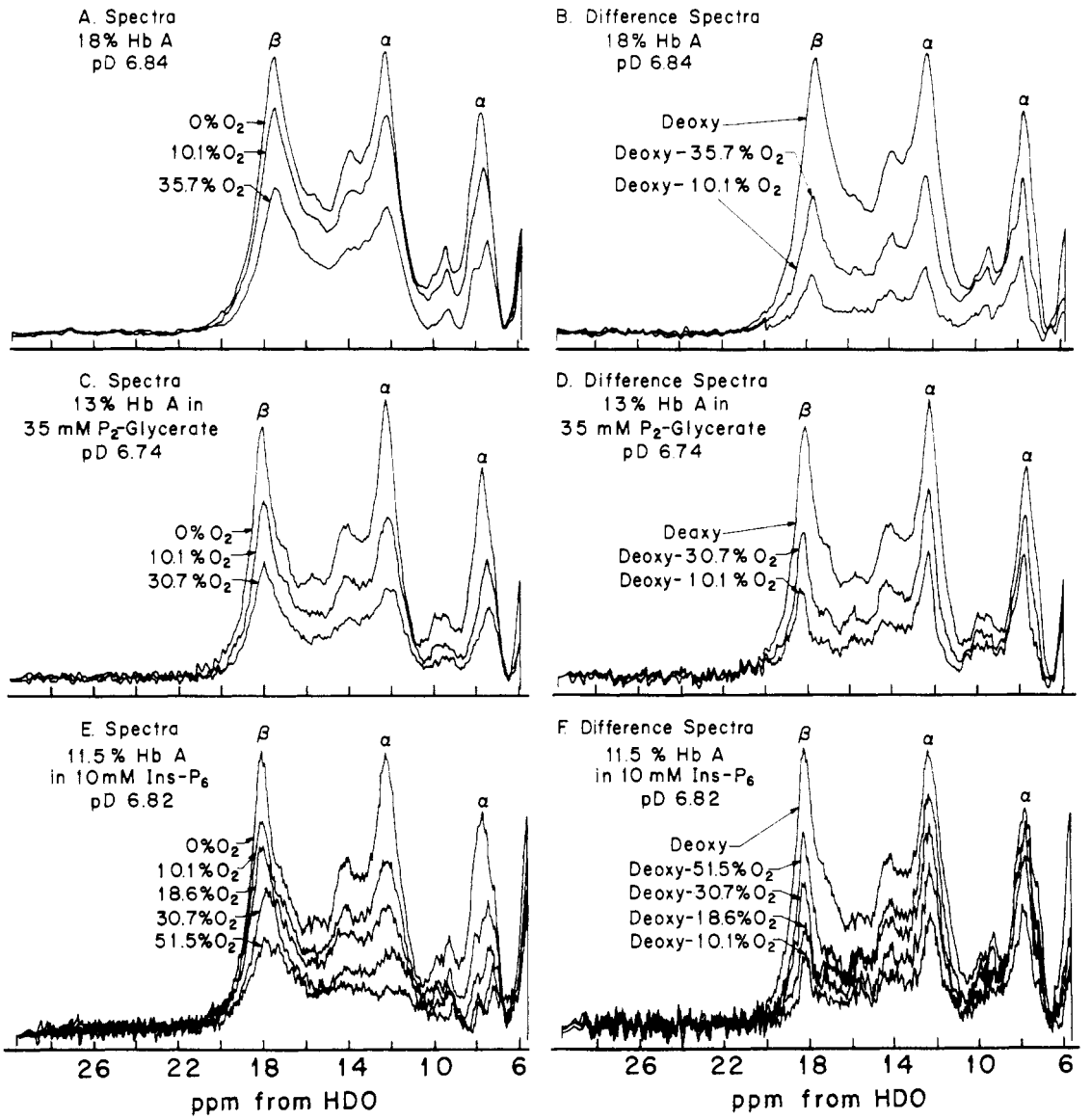

FIGURE 4: The 250-MHz ^1H NMR studies of oxygenation of Hb A in 0.1 M Bistris in D_2O at 27 °C. (A) 18% Hb A at pD 6.84; (C) 13% Hb A in 35 mM P_2-glycerate at pD 6.74; (E) 11.5% Hb A in 10 mM Ins-P_6 at pD 6.82; (B) difference spectra of 18% Hb A at pD 6.84; (D) difference spectra of 13% Hb A in 35 mM P_2-glycerate at pD 6.74; (F) difference spectra of 11.5% Hb A in 10 mM Ins-P_6 at pD 6.82.

of the hyperfine shifted proton resonances of Hb A as a function of oxygenation in D_2O at neutral pD in the absence and presence of organic phosphates. These spectra are in good agreement with the description of Johnson & Ho (1974) for the spectral region from 10 to 20 ppm from HDO. The much improved base line in the present study has cleared up some of the problems associated with the spectral region from 6 to 10 ppm, namely, (1) the ratio of the 9.8-ppm resonance area to the 18-ppm resonance area is 1/3, so that the 9.8-ppm peak corresponds to two protons per tetramer according to the calibration of the 18-ppm β peak as six protons per tetramer reported by Fung et al. (1977), and (2) the resonance at 7.9 ppm shows a shoulder at ~7.6 ppm. The phenomenon of a higher oxygen affinity of the α chains as compared to that of the β chains in the presence of Ins-P_6, first reported by Lindstrom & Ho (1972), can readily be seen by looking at the intensities of the peaks at 18 ppm (β chain) and 12 ppm (α chain) as shown in Figure 4E. The flatness of the base line strongly suggests that there is a real change in the area ratio of these resonances upon oxygenation, not a change in line width associated with the kinetic effect.[3]

The areas of the 18-ppm β peak and the 12-ppm α peak have been measured (Figure 5) from the integrated intensities

[3] The analysis by Shulman et al. (1975) on the ligand binding data of Hb A by Ho and co-workers is incorrect in several aspects. First, the legends for Figure 23 in the review article by Shulman et al. (1975) are wrong. The spectra in this figure, which were taken from Figure 2 of Johnson & Ho (1974), referred to the effect of P_2-glycerate, not Ins-P_6, on the binding of O_2 to α and β chains of Hb A. Second, the factor of 10 in the O_2 affinity difference between α and β chains of Hb A suggested by Shulman et al. (1975) is dependent on the model used to calculate it, and does not necessarily reflect the original data of Johnson & Ho (1974). Third, even if a kinetic line broadening of the magnitude suggested by Shulman et al. (1975) were induced, it would not have reduced the total integrated line intensity. Thus, the empirical line-area measurements of Johnson & Ho (1974) would not have been affected by the moderate (≥100 Hz) line broadening predicted by Shulman et al. (1975). Finally, Lindstrom et al. (1971) did not find any observable asymmetric line broadening in the hyperfine shifted proton resonances of the α and β hemes when Hb A binds *n*-butyl isocyanide. They did observe a decrease in the intensity of the β-heme resonance at 18 ppm as compared to that of the α-heme resonance at 12 ppm (downfield from HDO) when the molar ratio of *n*-butyl isocyanide to heme was 1.9. This is quite contrary to the statement made by Shulman et al. (1975) and the data presented in Table 14 of their paper.

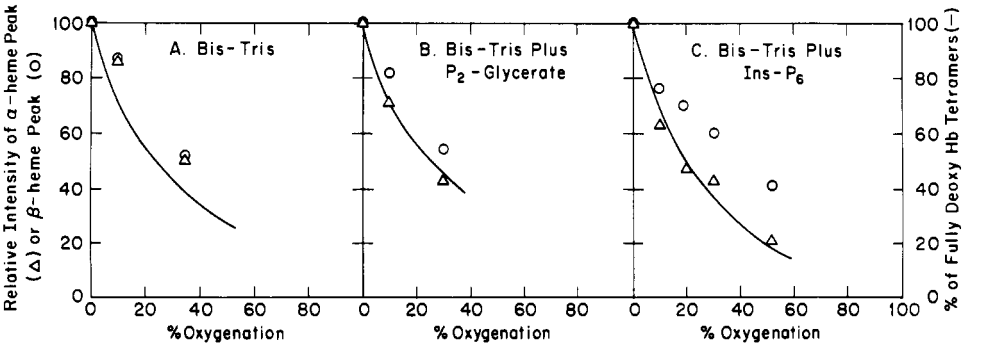

FIGURE 5: A comparison of the relative intensities of the α-heme resonance at 12 ppm (Δ) and the β-heme resonance at 18 ppm (O) with the percent of fully deoxy-Hb tetramers (—) as a function of oxygenation. (A) 0.1 M Bistris; (B) 0.1 M Bistris plus 35 mM P_2-glycerate; (C) 0.1 M Bistris plus 10 mM Ins-P_6. Fraction of fully deoxy-Hb tetramers was calculated from the data of Tyuma et al. (1973). For details, see the text and footnote 5.

of the spectra (by digital integration), taking into account only half of the resonance downfield from the maximum for the 18-ppm peak and upfield for the 12-ppm peak. This procedure avoids most of the problems arising from overlapping resonances at 18 and 12 ppm. This can be checked in the range from 0 to 30% oxygen saturation, where it is possible to fit these portions of the resonances by the Lorentzian line shape. The base line can be determined with good accuracy from the flat downfield region of the spectrum (i.e., ~22 to 23 ppm). The position of the maximum of the resonance can be determined to within ±5 Hz, so the areas of the resonances at 18 and 12 ppm can be estimated with an accuracy of better than 10% (i.e., about twice the vertical size of the experimental points given in Figure 5). The line width at 18 ppm does not increase more than 50 Hz in the range of saturation studied from the initial value of ~250 Hz for the deoxy sample in the presence of Ins-P_6. As pointed out earlier, this figure for the line broadening in the present study is less than that reported by Johnson & Ho (1974). The difference is due to using only the left-hand-half (downfield) portion of the peak in the present treatment to avoid the contribution of the shoulder at 16.8 ppm, which becomes more prominent with increasing O_2 saturation. The line width of the 12-ppm α peak of Hb A varies in the P_2-glycerate series from 270 Hz at 0% oxygenation to 317 Hz at 30% O_2 saturation and varies from 270 Hz to 0% oxygenation to 350 Hz at 30% O_2 saturation for the Ins-P_6 series. At 50% O_2 saturation in the presence of Ins-P_6, the 12-ppm peak overlaps another smaller or broader peak at ~11.5 ppm, so no reliable line width measurement can be obtained. It is also possible that the slightly larger line broadening observed in the presence of Ins-P_6 in comparison with that in the presence of P_2-glycerate is only apparent; instead, it is due to the smaller peaks detectable at higher O_2 saturations.

(*C*) *Difference Spectra.* Deeper insight into the behavior of the hyperfine shifted resonances of Hb A upon oxygenation can be obtained from the difference spectra shown in Figure 4B (without organic phosphate), Figure 4D (in the presence of P_2-glycerate), and Figure 4F (in the presence of Ins-P_6). These spectra are representative of the resonances lost upon oxygenation so they are flat at 0% oxygenation and give the deoxy spectrum at 100% oxygenation. Thus, the intensities of the resonances in the difference spectra give the corresponding oxygenated species. Systematic base-line distortions of NMR spectra which are recorded directly disappear in the difference spectra. The three prominent resonances (~18, ~12, and ~8 ppm) seem to be better resolved and sharper in the difference spectra. There is no observable line broadening at low O_2 saturation range as shown in Figure 6. The two difference spectra at two different O_2 saturations (10.1 and 18.6%) have been prescaled to give the same height for the resonance at ~8 ppm and they are identical. This means that partially overlapping peaks decrease by different amounts upon oxygenation. Namely, the 18-ppm resonance contains two peaks, one centered at 18 ppm and the other under a shoulder at 16.8 ppm (both from β chains). In the difference spectra the 18-ppm peak increases more with oxygenation saturation than the 16.8-ppm signal. This last peak is seen as a small distortion of the 18-ppm resonance even at 50% O_2 saturation in the presence of Ins-P_6 (Figure 4E). This explains the apparent line broadening observed in the directly recorded spectra (Figure 4E) at ~50% O_2 saturation: the 18-ppm peak and the 16.8-ppm peak at this saturation are about the same size and partially overlapping. The same effect is observed in the presence of P_2-glycerate (Figure 4D) and possibly to a smaller extent in the absence of organic phosphates (Figure 4B).

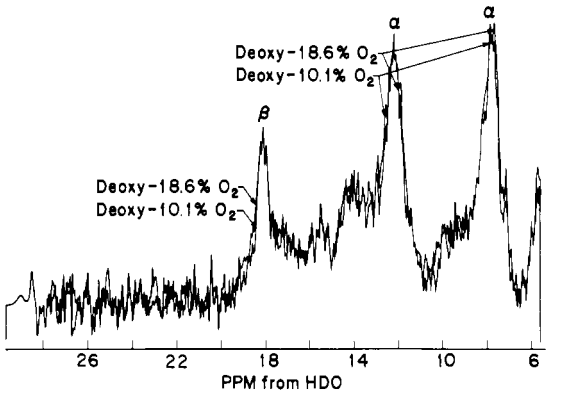

FIGURE 6: Effects of inositol hexaphosphate and oxygenation on the line width of the hyperfine shifted proton resonances of 11.5% Hb A in 0.1 M Bistris plus 10 mM Ins-P_6 at pD 6.82 and 27 °C. Difference spectra of deoxy-Hb A minus 10.1% oxygenation and deoxy-Hb A minus 18.6% oxygenation are shown. The spectra were taken from Figure 4F.

The difference spectra allow a comparison of the α-heme resonances at ~12 and ~8 ppm. This is quite impossible in the directly recorded spectra because the α-heme resonance at 7.9 ppm is composed of at least two strongly overlapping resonances (Figure 4A,C,E). In the difference spectra (Figure 4B,D,F) only one of these resonances is increased in the saturation range studied, and Figure 6 shows the identical behavior of the resonances lost at 12 and 7.9 ppm.

The areas lost from the 18-ppm β peak and the 12-ppm α peak are equal in the absence of organic phosphate (Figure

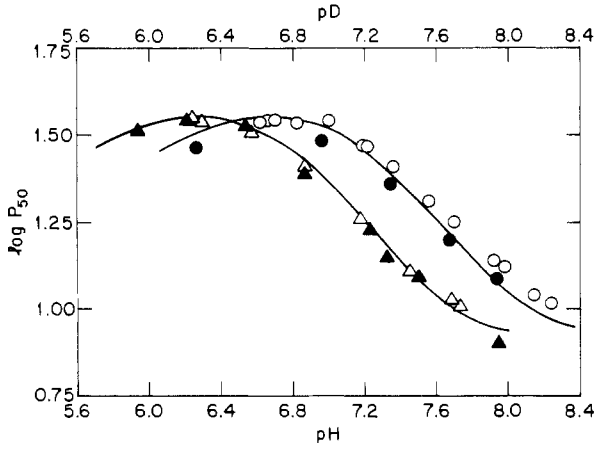

FIGURE 7: Effect of D_2O on the Bohr effect of Hb A in 0.1 M Bistris plus 10 mM 2,3-diphosphoglycerate at 27 °C. Data obtained from Hem-O-Scan by using 4% Hb A: (△) in H_2O and (○) in D_2O. Data obtained from the apparatus designed by Rossi-Bernardi et al. (1975) by using 1% Hb A: (▲) in H_2O and (●) in D_2O.

4B), in agreement with the values reported in Figure 5 measured on the direct spectra of Figure 4A. The 12-ppm α peak loses more intensity in comparisons with the 18-ppm β peak in the presence of organic phosphates (Figure 4D for P_2-glycerate and Figure 4F for Ins-P_6). All the values are in agreement with those of Figure 5 (area measurement from the direct spectra of parts C and E of Figure 4).

(D) Effect of D_2O on the Oxygen Binding to Hb A. Tomita & Riggs (1970) reported that the cooperativity of oxygenation of 0.2% Hb A in 0.1 M Tris at 16.5–35 °C [as measured by the Hill coefficient (n)] was greatly reduced by partial deuteration. They found that the D_2O effect was dependent on the time of exposure: n was found to decrease from 2.8 to 1.9 after Hb A was exposed to D_2O for 20 h and then to return to 2.8 after 240 h at 4 °C. They further found that the alkaline Bohr effect was unchanged by deuteration. Since the present ^1H NMR studies of O_2 binding to Hb A were carried out in D_2O and the Hb A samples were exposed to D_2O for varying amounts of time, we need to assess the effect of deuteration on our results. We have carried out an investigation on the effect of deuteration on the O_2 dissociation curves of ~4.5% Hb A in 0.1 M Bistris plus 10 mM P_2-glycerate at pD 6.73, 7.02, and 7.52 and 0.1 M Bistris at pD 7.02 using an Aminco Hem-O-Scan apparatus at 27 °C. The values of the Hill coefficient were determined from plots of log $Y/(1 - Y)$ vs. log P_{O_2} over the range of the O_2 saturation from 10 to 85%. We have found no significant variations in the Hill coefficient (2.4–2.8) and P_{50} from 0 to 10 days of exposure to D_2O under our experimental conditions. It appears that the time dependence of the Hill coefficient found by Tomita & Riggs (1970) was due to the specific buffer used as first reported by Cupane et al. (1974).

Figure 7 gives plots of log P_{50} as a function of pH and pD respectively in 0.1 M Bistris plus 10 mM P_2-glycerate at 27 °C. These data are a good measure of the Bohr effect of hemoglobin. The concentrations of Hb A used were 1% in measurements using the apparatus designed by Rossi-Bernardi et al. (1975) and 4% in measurements using an Aminco Hem-O-Scan apparatus. It should be noted that these two sets of measurements give essentially the same results.

The data for the pH dependence of the oxygen equilibrium of Hb A in D_2O are found to be displaced by ~0.4 pH unit toward higher pH in comparison with those in H_2O (Figure 7). The theoretical curves in Figure 7 were calculated by using the equation given by Wyman (1948):

$$\log P_{50} = \log K + \log \frac{([H^+] + K_{Hb}')([H^+] + K_{Hb}'')}{([H^+] + K_{HbO_2}')([H^+] + K_{HbO_2}'')} \quad (6)$$

The following values were used to calculate the theoretical curves in a plot of log P_{50} vs. pH in H_2O as shown in Figure 7: $K = 18.3$; $pK_{Hb}' = 5.60$; $pK_{Hb}'' = 7.81$; $pK_{HbO_2}' = 6.40$; $pK_{HbO_2}'' = 6.42$. The corresponding values of pK' and pK'' in D_2O are 0.4 unit larger than those in H_2O. This suggests that the Bohr effect of Hb A in H_2O and D_2O is identical except that the ionization constants of the groups involved are displaced by 0.4 units in going from H_2O to D_2O.

Discussion

The quality of our present ^1H NMR spectra is such that a quantitative investigation of the areas of both the β peak at 18 ppm and the α peak at 12 ppm is feasible over the range of O_2 saturations (0–50%) studied. The present data give strong support to the earlier conclusions reached by this laboratory (Ho & Lindstrom, 1972; Lindstrom & Ho, 1972; Johnson & Ho, 1974) that the addition of organic phosphates decreases the area ratio of the α peak (12 ppm) to the β peak (18 ppm) with increasing O_2 saturation, the effect being larger for Ins-P_6 than for P_2-glycerate. This ratio is equal to 1 for fully deoxy-Hb with and without organic phosphates and remains constant to the limit of the experimental accuracy for Hb A in the absence of organic phosphate at any O_2 saturation studied. These conclusions are also in agreement with the recent spin-label results of Asakura & Lau (1978).

Figure 5 shows that the α- and β-heme resonance intensities lie below the diagonal line, indicating that the sum of deoxy α and β chains giving rise to the 12- and 18-ppm resonances is smaller than that predicted from the total O_2 saturation. No substantial line broadening is detected that can be related to a difference in the kinetic effect[3] of α and β chains even in the presence of Ins-P_6. This means that the structural changes which occur in the partially ligated Hb tetramers [i.e., HbO_2, $Hb(O_2)_2$, and $Hb(O_2)_3$] affect the deoxy chains so as to cause a disappearing of the measured resonances at 18 and 12 ppm.[4] This supports the conclusion that the 12-ppm α resonance and the 18-ppm β resonance are characteristic of the deoxy state or deoxy-like quaternary structure (Perutz et

[4] The mechanism that gives rise to each observed hyperfine shifted proton resonance of deoxy-Hb A is not known at present. In other words, we do not know which hyperfine shifted proton resonances are due to Fermi contact interactions or which to pseudocontact interactions. The Fermi contact shifted resonances result from the proton groups on the porphyrin ring which have direct contact with the iron atom of the heme group and interact with the unpaired electrons, either through chemical bonds or by hyperconjugation. Pseudocontact shifts arise from protons (such as from the amino acid residues in the close vicinity of the heme group) which have no direct contact with the iron atom of the heme group, but have dipolar interactions with the unpaired electrons of the iron atom through space. Pseudocontact shifts can also arise from protons on the heme group. For a recent discussion on the hyperfine shifted resonances of hemoproteins, refer to Ho et al. (1978). The fact that the hyperfine shifted proton resonance pattern of deoxy-Hb A is very different from that of isolated α chain, β chain, and myoglobin, and that these are also different from each other in the deoxy form (Perutz et al., 1974), suggests that these resonances are very sensitive to the detailed electronic structure of the heme group in each protein and/or the detailed conformation or environment of the heme group in each case. The hyperfine shifted resonances of isolated α chain and β chain as well as myoglobin are shifted to higher field as compared to those of deoxy-Hb A. These results provide support for the suggestion that the greater loss of resonance intensities at 12 and 18 ppm, as predicted from the number of deoxy chains available as measured by the degree of O_2 saturation of Hb A, reflects a structural change in the unligated subunits upon ligation of their partners in an intact tetrameric hemoglobin molecule.

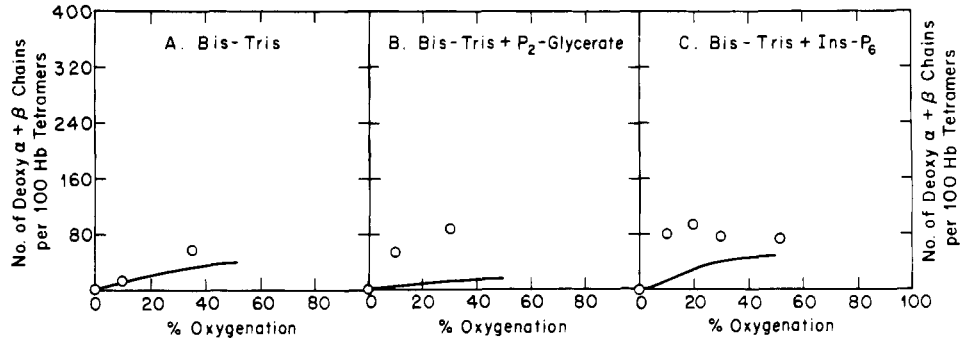

FIGURE 8: A comparison of the number of modified deoxy $\alpha + \beta$ chains (O) and the number of deoxy chains from $Hb(O_2)_2$ plus $Hb(O_2)_3$ in partially oxygenated Hb tetramers (—) as a function of oxygenation of Hb A in D_2O at pD 7 and 27 °C. For details, see the text.

al., 1974; Ho et al., 1975). Other direct evidence that the 18-ppm β peak is characteristic of the deoxy state is shown by the difference spectra. The two peaks, both from the β chains, one at 18 ppm and the other at 16.8 ppm, do change their area ratio upon oxygenation. We have found that, if an oxygen molecule is bound to a β chain, these two resonances would then disappear. However, the experimental results show that if structural changes occur in partially ligated Hb tetramers, which affect a β deoxy chain, the 18-ppm resonance would be disappeared (or shifted) but not the 16.8-ppm peak.

The implications of the present NMR results to the models of the cooperative oxygenation of Hb A can now be considered. In this regard a comparison of the α- and β-peak areas of Hb A with the fraction of fully deoxy tetramers as a function of O_2 saturation is given in Figure 5. The fraction of fully deoxy tetramers was calculated based on the data of Tyuma et al. (1973).[5] In the presence of organic phosphates, the area of the α peak at 12 ppm follows the behavior of the fully deoxy-Hb populations. This means that the presence of even one oxygen molecule in the Hb tetramer is enough to cause a decrease in the area of the 12-ppm α resonance but not the 18-ppm β resonance. The area of the 18-ppm β peak is found to be significantly larger than that of the 12-ppm α peak. The structural changes at partial oxygen saturations in the presence of organic phosphates as seen by NMR spectroscopy cannot be concerted as required by two-state allosteric models, even if extended to take into account the difference in the affinity between α and β chains as proposed by Ogata & McConnell (1971).

Another question which must be taken into consideration is whether or not the changes sensed by the hyperfine shifted resonances are directly related to the cooperative oxygenation of hemoglobin or are energetically important in the oxygenation process. The present data can only allow us to make a qualitative conclusion on this question. We have found that the β peak at 18 ppm is missing in those high-affinity Hb mutants which do not show cooperativity in their binding of O_2, but cooperativity can be restored upon adding Ins-P_6 (Perutz et al., 1974; Ho et al., 1975). Under such conditions, the hyperfine shifted proton resonance pattern is returned to that of normal deoxy-Hb A. The spectrum of deoxy-Hb A is quite insensitive to pH changes, but the spectrum of dearginine-deoxyhemoglobin loses both the 18-ppm β peak and 12-ppm α peak in going from pD 7 to 9 as the mixture of T and R states is shifted toward the R state (Perutz et al., 1974). The β peak of deoxy-Hb A shifts from 17.5 to 18 ppm upon the addition of organic phosphates. On the basis of thermodynamical arguments, Minton & Imai (1974) have proposed that at least a third state must be admitted to describe the behavior of Hb A in the presence of organic phosphate. The spectra of both the isolated deoxy α and β chains of Hb A do not show resonances at 18 or 12 ppm, but by mixture of stoichiometric amounts of α and β chains the ^1H NMR spectrum obtained is that of Hb A (Perutz et al., 1974). Thus, all these results strongly suggest that the hyperfine shifted proton resonances of Hb A are good monitors of the cooperative oxygenation process and the observed spectral changes are energetically significant. For a discussion on the relationship between spectral changes and the energetics of the oxygenation of hemoglobin, refer to Fung et al. (1976).

Figure 8 gives a comparison between the number of deoxy chains belonging to $Hb(O_2)_2$ and $Hb(O_2)_3$ and the total number of modified deoxy chains obtained from NMR measurements. The number of deoxy chains having two and three O_2 bound was calculated from the data of Tyuma et al. (1973). The number of modified deoxy $\alpha + \beta$ chains as determined by NMR spectroscopy was calculated from the equation

$$\text{no. of modified deoxy } \alpha + \beta \text{ chains} = 4(100 - Y) - 200\frac{I_\alpha}{I_{0(\alpha)}} - 200\frac{I_\beta}{I_{0(\beta)}} \quad (7)$$

where Y is the percent O_2 saturation, I_α is the area of the

[5] It is recognized that there is a significant difference in the hemoglobin concentration between the present NMR experiments and the data on O_2 dissociation curves reported by Tyuma et al. (1973) (7.5×10^{-3} vs. 6×10^{-5} M). It is also known that the tetramer–dimer equilibrium at low concentration of Hb ($<10^{-5}$ M) can affect the oxygen dissociation curve as first pointed out by Ackers & Halvorson (1974). However, the present comparison between the fraction of fully deoxy tetramers [calculated from the data of Tyuma et al. (1973)] and the fractions of deoxy α and β chains (derived from our NMR data) is meaningful to a good approximation. First, the shape of the O_2 dissociation curves for Hb A over the concentration range from 10^{-5} to 10^{-4} M is quite similar according to data of Mills et al. (1976). Hence, one can analyze the O_2 dissociation curves of Hb A over the concentration range from 10^{-5} to 10^{-3} by the equation

$$Y = \frac{A_1QP_{O_2} + 2A_2Q^2P_{O_2}^2 + 3A_3Q^3P_{O_2}^3 + 4A_4Q^4P_{O_2}^4}{4(1 + A_1QP_{O_2} + A_2Q^2P_{O_2}^2 + A_3Q^3P_{O_2}^3 + A_4Q^4P_{O_2}^4)}$$

where Y is the fraction of oxygenation, A_1, A_2, A_3, and A_4 are the four Adair constants, P_{O_2} is the partial pressure of O_2, and $Q = 1 + \Delta P_{50}/P_{50}$. P_{50} is the partial pressure of O_2 at 50% oxygenation and ΔP_{50} is the difference in P_{50} between two concentrations of Hb. The fraction of fully deoxy-Hb tetramers, F, can be calculated from the equation

$$F = \frac{1}{1 + A_1QP_{O_2} + A_2Q^2P_{O_2}^2 + A_3Q^3P_{O_2}^3 + A_4Q^4P_{O_2}^4}$$

Thus, the functional dependence of F on Y is independent of Q. On the basis of the data of Mills et al. (1976), the ratio of $\Delta P_{50}/P_{50}$ over the Hb concentration from 3.82×10^{-5} to 3.82×10^{-4} M is no more than 0.04 (or 4%). Hence, within the accuracy of our measurements, the extent of tetramer–dimer dissociation of Hb A under the experimental conditions of Tyuma et al. (1973) would not affect the correlation between our NMR data and those taken from their O_2 dissociation study.

α-heme resonance at 12 ppm at a given oxygenation, $I_{0(\alpha)}$ is the area of the α-heme resonance at 12 ppm at $Y = 0$, I_β is the area of the β-heme resonance at 18 ppm at a given oxygenation, and $I_{0(\beta)}$ is the area of the β-heme resonance at 18 ppm at $Y = 0$. It is clear that there are a greater number of modified deoxy chains with respect to that of deoxy chains from $Hb(O_2)_2$ plus $Hb(O_2)_3$. So very likely all chains belonging to $Hb(O_2)_2$ and $Hb(O_2)_3$ are modified chains.

The following picture can be considered by looking at the NMR data in the framework of the stereochemical model proposed by Perutz (1970), if one can remove some of the constraints of the two-state allosteric model as recently modified by Perutz (1976, 1978). When an α chain is oxygenated first, this causes the expulsion of the tyrosine HC2(140)α_1 from its pocket and the rupture of the links between arginine HC3-(141)α_1 and the α_2 subunits as first proposed by Perutz (1970). The heme pocket of the α_2 subunits would thus be affected because one of the "salt" bridges broken is with the aspartate H9(126)α_2 that belongs to the H helix. This helix together with the G helix determines the position of the HC3(140)α_2 tyrosine. A pathway for the transmission of information between an α heme and the other α chain is thus established. The α_2 chain is now oxygenated, and the complete rupture of the interchain bridges between the two α chains allows an easier transition toward the R state. This picture does not need to be strictly sequential, depending on how easy it is to jump from the T to the R quaternary state. In the absence of organic phosphates, when the T structure is not stabilized, it is possible to have random ligation of α and β chains. On the other hand, in the presence of P_2-glycerate or Ins-P_6 when there is a strong stabilization of the T structure, the α chains have been found to have a strong preferential oxygen affinity, with some cooperativity in the T state. The present suggestion is also in agreement with the ^1H NMR results on the oxygenation of the α chains of Hb M Milwaukee reported by this laboratory (Fung et al., 1976, 1977). We found that the ferric β chains of Hb M Milwaukee do not undergo concerted changes upon oxygenation of the α chains when organic phosphates are absent but that such changes are hindered by Ins-P_6.

According to our present data, the three states in the absence of organic phosphates are deoxy α chains $\rightarrow$ T state, one oxy α chain $\rightarrow$ modified T state, and two oxy α chains $\rightarrow$ R state or one oxy β chain $\rightarrow$ R state. In the presence of Ins-P_6, the T state is completely locked and its modification cannot be detected at all from the β chains. In this way it is also possible to explain the partial success of the two-state allosteric model, because in the absence of organic phosphates only T and R states play a major role. On the other hand, the effect of the allosteric effectors cannot be explained with a change of the L value [allosteric constant in the two-state concerted model of Monod et al. (1965)] only, but definitely requires a change of the K_T value (oxygen dissociation constant of Hb in the T state). For a recent discussion on the relationship between the binding of O_2 to α and β hemes and the corresponding changes in tertiary and quaternary structure, refer to Viggiano & Ho (1979). By monitoring these structural probes, Viggiano & Ho (1979) have ruled out the applicability of two-state allosteric models to the oxygenation of Hb A (even in the absence of organic phosphate).

Since the present ^1H NMR studies on the binding of O_2 to Hb A were carried out in D_2O, we need to discuss the effect of heavy water on the functional properties of hemoglobin. Until recently, most of the ^1H NMR studies of proteins were carried out in D_2O. The main advantage of substituting H_2O by D_2O in these studies is to suppress the intense proton signal of H_2O absorption in aqueous samples. Samples in D_2O have a wider observable spectral region and resonances near the water proton signal with better resolution and base lines (which allow a more accurate measurement of the signal intensity or the area of the resonance). Our results clearly show that there is no significant difference in the Hill coefficient or the Bohr effect of Hb A in D_2O and H_2O (Figure 7). Thus, the results obtained in D_2O are indeed relevant to those in aqueous media. With recent improvement in NMR instrumentation and techniques, we have been able to carry out ^1H NMR studies of O_2 binding of Hb A in H_2O. The preliminary data indicate that the results obtained in D_2O are essentially the same as those obtained in H_2O (Viggiano & Ho, 1979).

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Nuclear Magnetic Resonance Studies on the Structure of the Tetrapeptide Tuftsin, L-Threonyl-L-lysyl-L-prolyl-L-arginine, and Its Pentapeptide Analogue L-Threonyl-L-lysyl-L-prolyl-L-prolyl-L-arginine†

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ABSTRACT: Nuclear magnetic resonance spectroscopy has been used to investigate the solution conformation of tuftsin, threonyllysylprolylarginine, as well as a pentapeptide inhibitor of tuftsin, threonyllysylprolylprolylarginine. Both proton and carbon-13 studies were performed. In water, neither peptide gives evidence of a preferred conformation. In dimethyl-d_6 sulfoxide, tuftsin appears to prefer a particular conformation, but the inhibitor does not. The conformation of tuftsin is one in which the amide NH proton of arginine is solvent shielded. The conformation does not, however, appear to be such that a normal 4 → 1 β turn exists.

Tuftsin, the phagocytosis stimulating peptide (Najjar & Nishioka, 1970), has been fully characterized chemically (Nishioka et al., 1972, 1973a,b) and biologically (Constantopoulos & Najjar, 1972, 1973a–c; Constantopoulos et al., 1973; Najjar, 1974, 1977). It is an integral part of the CH3 segment of leukokinin, a cytophilic γ-globulin (Fidalgo & Najjar, 1967), residues 289–292 (Edelman et al., 1969), which binds to and stimulates the phagocytic activity of the polymorphonuclear cell and the macrophage (Constantopoulos & Najjar, 1972). This stimulatory effect resides totally in the tuftsin peptide which is active only as the free tetrapeptide (Najjar, 1974). It is released from the carrier molecule by two types of enzymes: one in the spleen and the other on the outer surface of the phagocyte membrane (Najjar, 1974). The splenic enzyme tuftsin endocarboxypeptidase splits the carboxy-terminal arginine from its adjacent glutamyl residue. This enzyme has not been purified or characterized. In the absence of the spleen, the arginyl–glutamyl bond remains intact and the carrier molecule, while still capable of binding to the membrane receptors, is incapable of activating the phagocyte. The

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