# Effects of Ligands and Organic Phosphates on Functional Properties of Human Adult Hemoglobin†

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ABSTRACT: The equilibrium binding of oxygen and carbon monoxide to human adult hemoglobin is compared through nuclear magnetic resonance studies of contact shifted resonances of the heme protons in neutral pD solutions. It is found that CO exhibits random binding between the $\alpha$ and $\beta$ hemes in the absence of phosphate. In the presence of 2,3-diphosphoglycerate, CO exhibits a possible, but very slight preference for the $\alpha$ chains, while in the presence of inositol hexaphosphate a small, but definite preference for the $\alpha$ chains is exhibited. In contrast, $O_2$ exhibits a slight preferential binding to the $\alpha$ hemes in the absence of phosphate, marked preferential binding to the $\alpha$ hemes in the presence of 2,3-diphosphoglycerate, and almost exclusive binding to the $\alpha$ hemes in the presence of inositol hexaphosphate. These, as well as other results from this laboratory, suggest that the hemoglobin–ligand reaction is ligand, phosphate, and pH specific.

In recent years proton nuclear magnetic resonance (nmr) studies have shown that the $\alpha$ and $\beta$ chains in the human adult hemoglobin (Hb A)[1] are nonequivalent (Davis *et al.*, 1969, 1971; Lindstrom *et al.*, 1972; Lindstrom and Ho, 1972; Ogawa and Shulman, 1972). This conclusion was based on the identification of specific contact shifted proton resonances due to groups on or near the $\alpha$ and $\beta$ hemes. In deoxy Hb A the $\beta$ heme resonance occurs at approximately $-18$ ppm downfield from HDO, while the $\alpha$ heme resonances appear at approximately $-12$ and $-8$ ppm (Davis *et al.*, 1971; Lindstrom *et al.*, 1972). Upon ligand binding, the hemoglobin molecule becomes diamagnetic and these resonances are shifted upfield to their usual positions.

Although it has been shown that the $\alpha$ and $\beta$ chains are nonequivalent, little attention has thus far been given to the possibility that the Hb–ligand reaction may also be ligand-specific. For example, it is usually assumed that, with the exception of a scaling factor, the reactions of Hb A with oxygen and carbon monoxide are equivalent (Antonini and Brunori, 1971). And in fact, the evidence for ligand-specific binding is somewhat contradictory. Lindstrom and Ho (1972) have shown in a preliminary $^1$H nmr study of the contact-shifted resonances that $O_2$ binds preferentially to the $\alpha$ chains in the presence of a large excess of 2,3-diphosphoglycerate (DPG) or inositol hexaphosphate (IHP). From $^{19}$F nmr studies on Hb A labeled with trifluoroacetonyl groups at $\beta$93 cysteines, Huestis and Raftery (1972a) have also suggested that in the presence of DPG, $O_2$ binds initially to the $\alpha$ chains and that binding to the $\beta$ chains lags $\sim$10% behind overall ligand binding throughout the binding curve. Using kinetic measurements, Gibson (1973) has suggested that both the association and dissociation rates of $O_2$ with the $\beta$ chains are much faster than with the $\alpha$ chains, but that the equilibrium constants are probably such that the $\alpha$ chains have a higher $O_2$ affinity than the $\beta$ chains at low fractional saturations. Indirect agreement with the general conclusion that the $\alpha$ chains have a higher $O_2$ affinity than the $\beta$ chains has also been obtained in a study of chain oxidation rates where it was found that decreasing the $O_2$ pressure increased the $\beta$ chain oxidation rate much more than the $\alpha$ chain rate, indicating that the $\beta$ chains have a lower $O_2$ affinity (Mansouri and Winterhalter, 1973).

For CO the situation is somewhat less clear. In preliminary nmr studies using the contact-shifted proton resonances, Davis *et al.* (1971) have found no evidence for preferential binding of CO to either of the chains of Hb A in 0.1 M phosphate buffer (pD 7). Likewise, Lindstrom and Ho (1972) found no evidence for preferential binding of CO to either of the chains in the presence of a 1–2 molar excess of DPG or IHP at pD 7. Using spin-labeled triphosphates, Ogata and McConnell (1972) have shown that in the presence of these triphosphates the $\alpha$ chains exhibit a very slight preference for CO ($\lesssim$2%) at low fractional saturation. On the other hand, Huestis and Raftery (1972b, 1973) have concluded both from $^{19}$F nmr measurements of their fluorine-labeled Hb A and from $^{31}$P nmr studies of the DPG bound to Hb A that CO follows essentially the same course as $O_2$ in binding to Hb A: namely, that it is initially bound by the $\alpha$ chains and that the binding by the $\beta$ chains lags behind the overall ligand binding by $\sim$10%. Kinetic studies by Gray and Gibson (1971a,b) suggest that in the absence of phosphate the binding rates of the different chains with CO are indistinguishable, but that upon addition of inorganic phosphate, DPG, or IHP, the $\beta$ chains exhibit a faster binding rate than the $\alpha$ chains. The data on dissociation rates, however, were insufficient to give any information on the relative affinities of the $\alpha$ and $\beta$ chains under equilibrium conditions.

The evidence for preferential binding by *n*-butyl isocyanide (BIC) is also confusing. From direct nmr measurements of the contact-shifted proton resonances, Lindstrom *et al.* (1971) have shown that in the presence of IHP, BIC exhibits preferential binding to the $\beta$ chains. This conclusion has been extended in a series of kinetic measurements by Olson and Gibson (1971, 1972, 1973a,b) and Cole and Gibson (1973) who have shown that the association rates of the $\beta$ chains for BIC are greater than those of the $\alpha$ chains both in the absence and in the presence of DPG or IHP. They also have shown that in the absence of organic phosphates the equilibrium affinities of the chains are approximately equal. However, in the presence of DPG or

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[1] Abbreviations used are: Hb, hemoglobin; DPG, 2,3-diphosphoglycerate; IHP, inositol hexaphosphate, BIC, *n*-butyl isocyanide; Bis-Tris, 2,2-bis(hydroxyethyl)-2,2′,2″-nitrilotrimethanol.

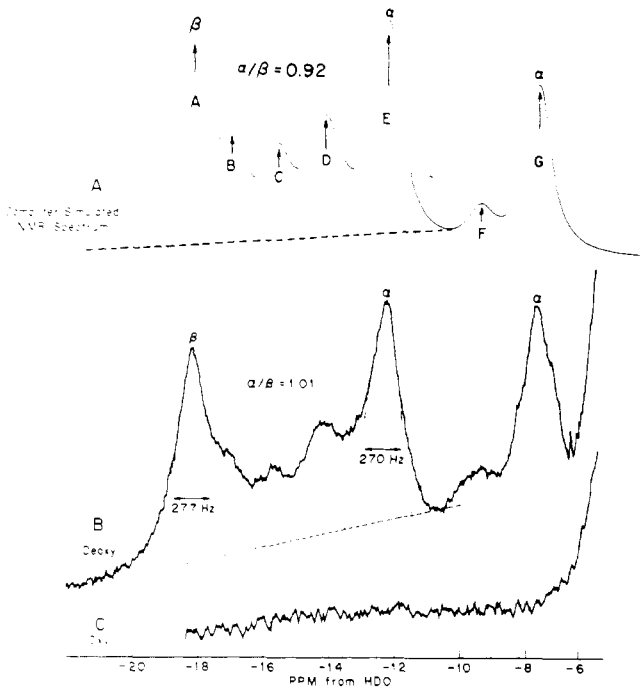


FIGURE 1: The 250-MHz spectrum of 16% Hb A in 30 mM DPG, 0.1 M Bis-Tris·HCl at pD 7.0 and 26°. Spectrum A is a computer simulation of a 100% deoxy sample (for spectral assignments, refer to Table III), spectrum B is as observed for a 100% deoxy sample, and spectrum C is as observed for a fully oxy sample. Dotted lines in A and B illustrate the methods for drawing the base line and determining the area ratios of the two resonances. Full widths at half-height of the peaks at −18 and −12 ppm are also shown in B.

IHP, and at low ligand saturation, the equilibrium affinity of the $\beta$ chains is 20–40 times that of the $\alpha$ chains, with the affinities becoming approximately equal at high ligand saturation. Huestis and Raftery (1972c), however, have again suggested from their $^{19}F$ nmr measurements of fluorine-labeled Hb A that in the presence of DPG the $\alpha$ chains are preferentially bound by BIC, but that in the absence of phosphate the chains exhibit random binding.

This communication is an investigation of some of the differences between CO and $O_2$ binding to Hb A, and of the effects of organic phosphates on the ligand binding properties of Hb A using $^1H$ nmr measurements of the contact-shifted heme resonances.

## Experimental Section

*Materials.* Human adult hemoglobin was prepared according to Drabkin (1946) and stripped of all DPG according to the procedure of Berman *et al.* (1971). All procedures for exchange of samples with $D_2O$, removal of CO, deoxygenation, and anaerobic filling of the nmr tubes have been described by Lindstrom and Ho (1972). After $D_2O$ exchange, the final $H_2O$ concentration was approximately 6%. Solutions used in these measurements were 11–16% Hb A in 0.08–0.1 M Bis-Tris·HCl (Aldrich) and pD 7.0 ± 0.1. For observing the effects of organic phosphates 11–17 mM IHP (obtained as phytic acid from Sigma) or 32–35 mM DPG (obtained as the pentacyclohexylammonium salt from Calbiochem and then exchanged against AG 50W-X8, Bio-Rad cation exchange resin) was included in the appropriate samples. The pD of solutions was determined by adding 0.4 pH unit (Glasoe and Long, 1960) to the value obtained from a Radiometer Model 26 pH meter equipped with a Beckman 39030 combination electrode. In 5-mm nmr samples tubes, samples which were partially ligand saturated were obtained by mixing appropriate aliquots of deoxy Hb A and CO or $O_2$ saturated Hb A. In 10-mm tubes partially saturated samples were obtained by using a Hamilton

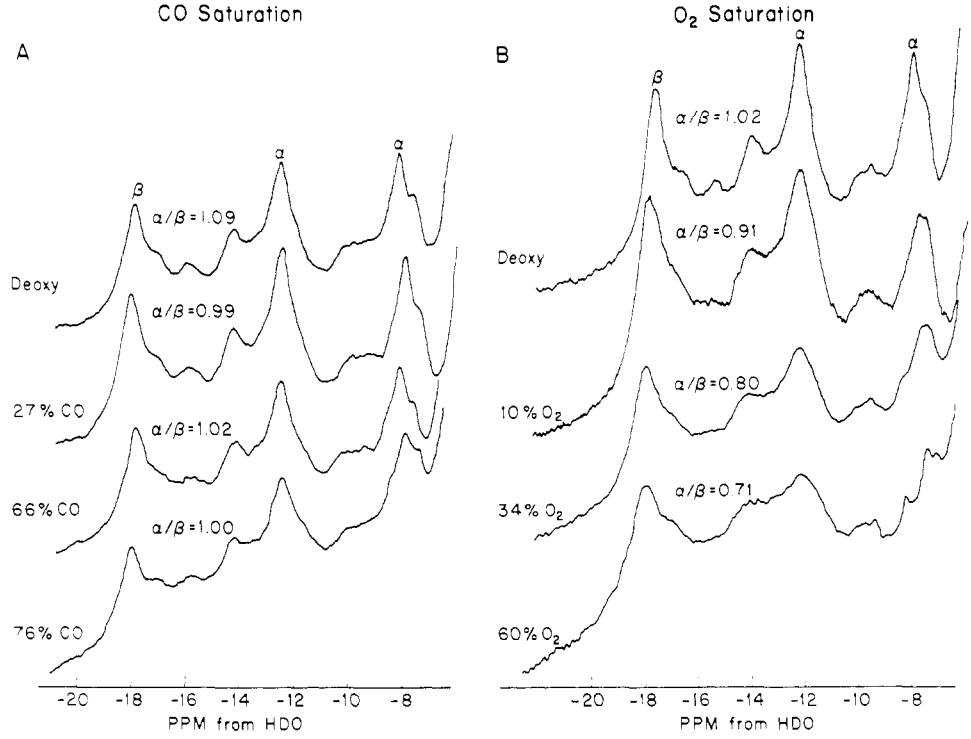


FIGURE 2: The 250-MHz proton nmr spectra of 11% Hb A in 33 mM DPG, 0.09 M Bis-Tris·HCl at pD 7.0 and 27°. The series on the left represents increasing saturation of CO, while that on the right represents increasing saturation of $O_2$. Due to slight variations in scale expansion during computer printout of the spectra, the chemical shift scale shown above is exact for the bottom spectra only. To an accuracy of ~0.2 ppm no chemical shift variations were observed during any saturation series.

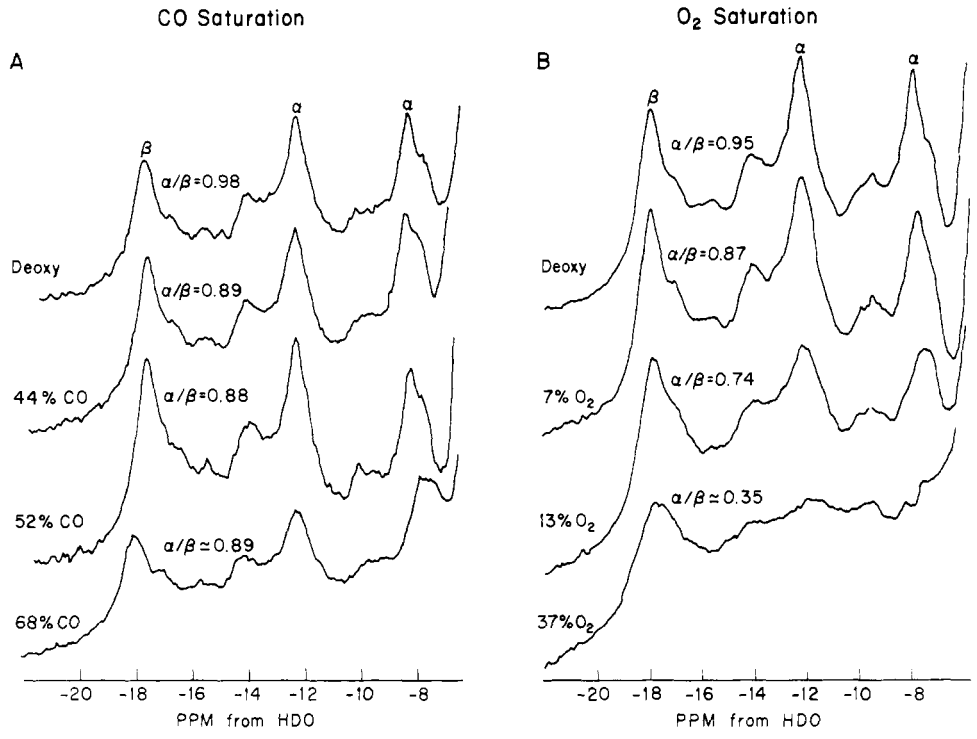


FIGURE 3: The 250-MHz proton nmr spectra of 11% Hb A in 13 mM IHP, 0.09 M Bis-Tris·HCl at pD 7.0 and 27°. The series on the left represents increasing saturation of CO while that on the right represents increasing saturation of $O_2$. Due to slight variations in scale expansion during computer printout of the spectra, the chemical shift scale shown above is exact for the bottom spectrum only. To an accuracy of ~0.2 ppm no chemical shift variations were observed during any saturation series.

microsyringe to inject small amounts of CO or $O_2$ gas through a rubber stopper in the side of the tube. After gas injection of the 10-mm tubes, the optical spectrum was monitored for approximately 30 min until no further change occurred. The sample was then allowed to equilibrate for at least 1 hr before an nmr spectrum was taken. For 5-mm tubes the additional equilibration time was a minimum of 4 hr. The saturation was measured by monitoring the decrease in the deoxy peak at 757 nm with a Cary 14 spectrophotometer. For the 5-mm tubes a holder was constructed to accurately position the tube in the spectrophotometer and the absorbance was determined directly through the nmr sample tube. For the 10-mm tubes a piece of flat glass (~2–3 mm in optical path length) was fused to the top of the tube to act as a cuvet. From measurement repeatability and consistency, the accuracy of the calculated saturations was considered to be approximately 5–10%.

*Methods.* Nmr spectra were obtained on a Bruker HFX 90 MHz spectrometer and the MPC-HF 250 MHz superconducting spectrometer (Dadok *et al.*, 1970); 10-mm tubes were used at 90 MHz while 5-mm tubes were used at 250 MHz. Standard probe temperatures were 28° for the Bruker and 27° for the 250-MHz instrument. For variable-temperature experiments, the Bruker probe was also operated at 12 and 37°. Chemical shifts are referenced with respect to the HDO signal which is 4.56 ppm downfield from the proton resonance of 2,3-dimethyl-2-silapentane-5-sulfonate at 37°, 4.83 ppm downfield at 27°, and 5.23 ppm downfield at 12°. Signal to noise ratios were improved by signal averaging with a Fabri-Tek 1074 computer when using the Bruker spectrometer. The MPC-HF 250-MHz spectrometer was interfaced with a Sigma-5 computer and the nmr correlation spectroscopy technique of Dadok *et al.* (1972) was used for signal enhancement.

For the purpose of measuring the areas of the $\alpha$ and $\beta$ resonances, a base line was drawn between the resonances at −18 and −12 ppm as shown in Figure 1B. To avoid problems of measuring the "tails" of each resonance, lines were drawn along the sides of each resonance, thus giving an essentially "triangular" representation for the area of each resonance. Each of these areas was then measured with a planimeter. The repeatability for area ratio measurements on different spectra of the same sample was found to be approximately ±0.05.

## Results

Representative saturation series for $O_2$ and CO at 27° are shown in the 250-MHz spectra of Figures 2 and 3. These spectra show the three major hyperfine shifted proton resonances, along with a number of previously unreported and unassigned resonances (Davis *et al.*, 1971; Lindstrom *et al.*, 1972; Lindstrom and Ho, 1972). Of these unassigned resonances, it has been previously suggested that the one at ~−14 ppm was due to the presence of methemoglobin A in the sample (Lindstrom and Ho, 1972). However, this peak was unaffected by the introduction of sodium dithionite (which reduces methemoglobin to deoxy Hb) into the hemoglobin solution. Likewise, upon complete CO or $O_2$ saturation, all of the above mentioned resonances disappear leaving a completely flat spectrum in this region (see Figure 1C). Apparently, then, these additional resonances are also contact-shifted resonances due to proton groups on or near the hemes of deoxy Hb A. In particular, a tentative assignment for the resonance at ~−14 ppm may be made by observing that in the $O_2$ saturation series of Figures 2 and 3, the ratio of heights of the resonance at ~−14 ppm to that of the one at −18 ppm appears to be nearly constant, while the relative height of the resonance at −12 ppm appears to be decreasing relative to the height of the one at ~−14 ppm. This suggests that the resonance at ~−14 ppm is due to a proton group on or near the $\beta$ heme. The spectra of deoxy Hb M Milwaukee where the iron atoms in the $\beta$ chains are permanently

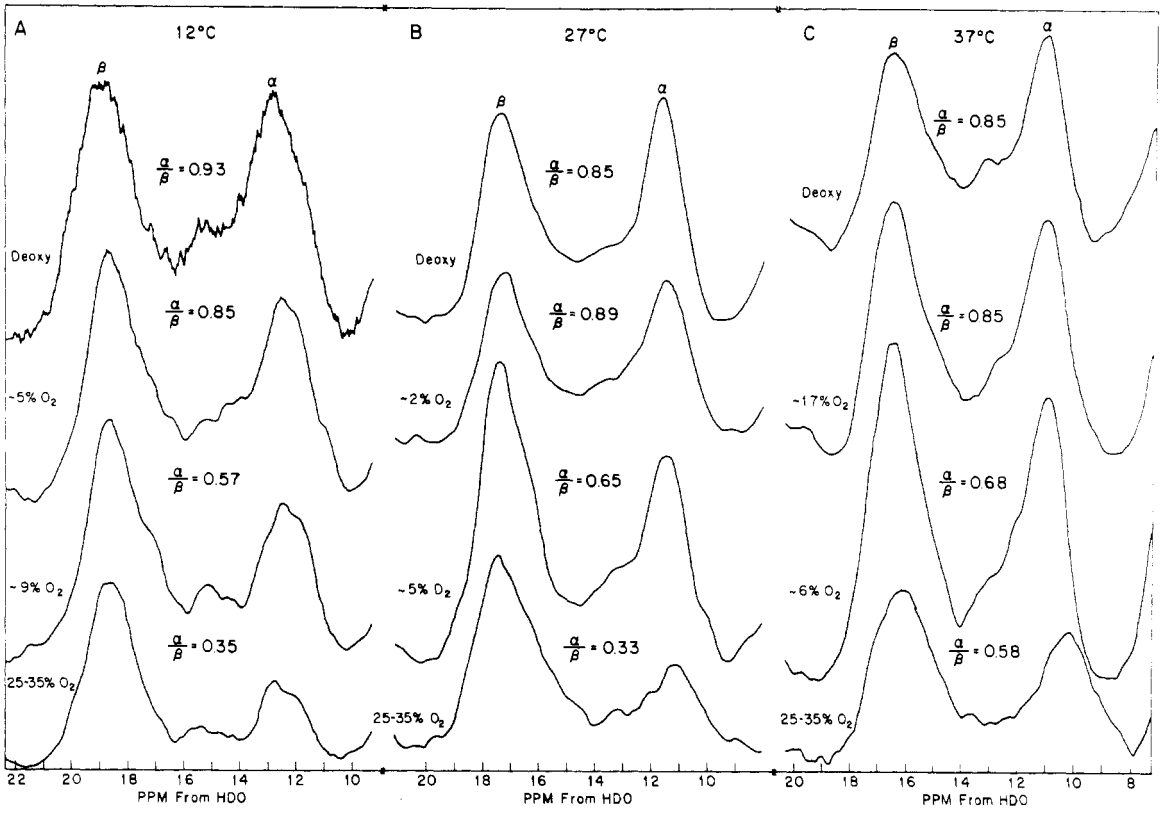


FIGURE 4: The 90-MHz proton nmr spectra of 14% Hb A in 11 mM IHP, 0.09 M Bis-Tris-HCl at pD 7.0. Each of the series represents increasing $O_2$ saturation at the temperature indicated. The $\alpha/\beta$ area ratios of the deoxy spectra at 90 MHz are slightly lower than those at 250 MHz due to the existence of a small resonance at ~−17 ppm. At 90 MHz it is not possible to completely separate this resonance from the $\beta$ resonance at ~−18 ppm and it is thus partially included in the $\beta$ resonance intensity measurement. At 250 MHz they are separable (compare with Figures 1-3) and this second resonance is thus not included in the $\beta$ intensity measurement.

in the ferric form are also consistent with this assignment (L. W. M. Fung and C. Ho, unpublished work). The line widths of the main $\alpha$ and $\beta$ resonances at ~−18 and ~−12 ppm were also found to be field dependent in the deoxy form, increasing from ~200 Hz at 90 MHz to ~270 Hz at 250 MHz.

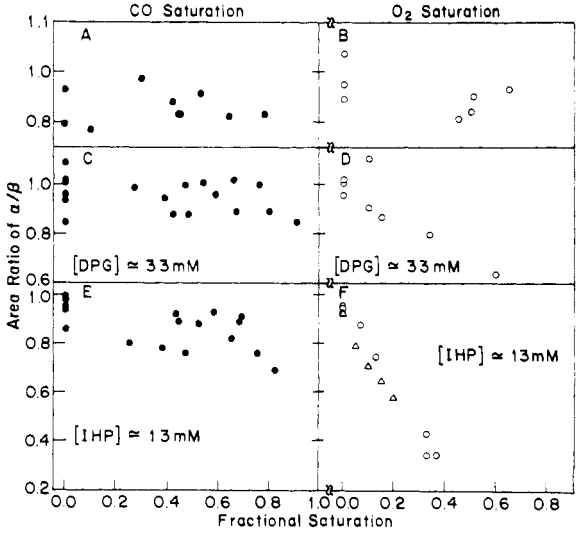


FIGURE 5: Dependence of $\alpha/\beta$ area ratios on ligand saturation. General conditions, 10-14% Hb A in 0.08-0.1 M Bis-Tris-HCl at pD 7.0 ± 0.1 and at 27-28°: (A and B) CO and $O_2$, respectively, in the absence of phosphate; (C and D) CO and $O_2$, respectively, in the presence of 32-35 mM DPG; (E and F) CO and $O_2$, respectively, in the presence of 11-17 mM IHP. The triangle points (△) in F are the computer simulation ratios and are shown for comparison with the experimental points.

Figures 2 and 3 also demonstrate that in the CO saturation series the line widths are essentially unchanged, whereas in the $O_2$ saturation series, the line widths at 250 MHz are significantly broadened. To an accuracy of ~0.2 ppm no changes in the chemical shifts were observed at 250 MHz for either the $O_2$ or CO saturation series. Representative $O_2$ saturation series at various temperatures in the presence of IHP are shown in the 90-MHz spectra of Figure 4. From this figure it can be seen that the line widths at this lower field do not appear to be a function of either temperature or the degree of $O_2$ saturation; the primary change is simply that the $\alpha$ resonance decreases in intensity with respect to the $\beta$ resonance as the $O_2$ saturation increases. A less obvious change at 90 MHz is that the $\alpha$ resonance appears to shift upfield by about 0.5 ppm at high $O_2$ saturation; to an accuracy of ~0.2 ppm the $\beta$ resonance exhibited no shifts upon oxygenation (up to ~60% total saturation).

The ratios of the area of the $\alpha$ peak at ~−12 ppm with respect to the $\beta$ peak at ~−18 ppm were calculated for all of the spectra in these saturation series and are also shown in Figures 2-4. From these ratios, it can be seen that as the CO saturation is increased, the relative areas of the $\alpha$ and $\beta$ peaks remain essentially unchanged in the presence of DPG and show only a slight change in the presence of IHP. For oxygen, however, a significant decrease in the area of the $\alpha$ peak relative to that of the $\beta$ peak is observed upon increasing the $O_2$ saturation. Likewise, it can be seen that the $\alpha/\beta$ area ratio decreases much more rapidly in the presence of IHP (Figures 3 and 4) than in DPG (Figure 2). These results are summarized more concisely in Figure 5. For all of the saturation series in this figure the $\alpha/\beta$ ratio appears to exhibit an approximately linear dependence on saturation. Hence, the data can be fit by least-squares

TABLE I: Preferential Binding of Ligands by $\alpha$ and $\beta$ Chains in Terms of Differences in Saturation between the $\alpha$ and $\beta$ Chains.[a]

| Phosphate Content | CO Saturation (%) | $O_2$ Saturation (%) |
|---|---|---|
| No phosphate | 0.6 ± 2.2[b] (A)[c] | 2.6 ± 3.0 (B) |
| 32–35 mM DPG | 1.8 ± 1.4 (C) | 15.0 ± 4.2 (D) |
| 11–17 mM IHP | 4.2 ± 1.6 (E) | 60.2 ± 4.4 (F) |

[a] All percentage differences are calculated at 50% total saturation except for $O_2$ saturation in the presence of IHP which is at 40% since the data go up to only ~40% saturation. [b] Quoted errors are calculated from the standard deviations of the slopes of the least-squares lines as fit to the data of Figure 5. [c] The letters within parentheses (A–F) refer to Figure 5.

TABLE II: Characterization of the Deoxy Contact-Shifted Hb A Spectrum at 250 MHz.

| Line[a] | Chemical Shift (ppm) | $\Delta\nu_{1/2}$ (Hz) | Intensity | Assignment[d] |
|---|---|---|---|---|
| A | −18.0 | 270 | 3.0[b] | $\beta$ |
| B | −16.8 | 250 | 1.0 | $\beta$ |
| C | −15.6 | 250 | 1.0 | $\alpha$ |
| D | −14.1 | 320 | 2.0 | $\beta$ |
| E | −12.3 | 250 | 3.0 | $\alpha$ |
| F | −9.4 | 250 | 0.5[c] | $\beta$ |
| G | −7.9 | 250 | 2.0 | $\alpha$ |

[a] See Figures 1A and 6 for a graphic representation of these lines. [b] L. W. M. Fung and C. Ho (unpublished work) have recently determined that this resonance consists of six protons per Hb tetramer or three protons per $\beta$ chain using *cis*-4-*tert*-butylcyclohexanol in the presence of the shift reagent Eu(dpm)$_3$ as a reference. [c] This line appears to be due to partially exchangeable protons and thus the intensity would not be expected to be an integral number of protons. [d] Lines A, E, and G have previously been assigned from studies of mutant hemoglobins (see, *e.g.*, Lindstrom *et al.*, 1972) while line D has been tentatively assigned as discussed above. The assignments for lines B, C, and F were made from observations on deoxy Hb M Milwaukee where the $\beta$ chains are permanently in the met form (L. W. M. Fung and C. Ho, unpublished work) and by observing whether they tended to disappear faster than, or at the same rate as the $\beta$ resonance at −18 ppm when Hb is oxygenated in the presence of IHP, but due to the poor signal to noise ratio for these smaller resonances, the assignments should be regarded as tentative.

lines, with the slope of the lines giving the degree of preferential binding. Some of the scatter shown in the data of Figure 5 is due to the fact that both 90- and 250-MHz data are combined within the figure. At 90 MHz the two lines at ~−18 and ~−17 ppm are not entirely resolved, thus giving a slightly lower intercept at zero saturation. The slope of the curve, however, does not appear to be affected by this factor (and theoretically should not be).

Assuming the $\alpha/\beta$ area ratio is linearly dependent on saturation, it can be put in the form

$$X = 1 - A(1 - d) \quad (1)$$

where $X$ is the $\alpha/\beta$ area ratio and $A$ is the slope of the plot $\alpha/\beta$ *vs.* fractional saturation (refer to Figure 5). The degree of preferential binding may be quantitatively estimated by noting that the observed resonances are due to the $\alpha$ and $\beta$ deoxy components of the Hb solution; hence the total deoxy content is simply the average of the $\alpha$ and $\beta$ intensities

$$(d_\alpha + d_\beta)/2 = d \quad (2)$$

where $d_\alpha$ and $d_\beta$ are the respective resonance intensities at ~−12 and ~−18 ppm from HDO, and $d$ is the fractional deoxy content. The fractional deoxy $\alpha$ content is then simply

$$d_\alpha = 2dX/(1 + X) \quad (3)$$

The difference in saturation between the $\alpha$ and $\beta$ chains is then

$$\Delta = d_\beta - d_\alpha = 2d(1 - X)/(1 + X) \quad (4)$$

And using the least-squares line (eq 1) for the $\alpha/\beta$ area ratio

$$\Delta = 2dA(1 - d)/[2 - A(1 - d)] \quad (5)$$

For moderate slopes, which are observed in all of the saturation series except $O_2$ in the presence of IHP (Figure 5F), the maximum difference in saturation between chains (*i.e.*, the maximum preferential binding) occurs at approximately 50% total saturation.

Numerical values for the preferential binding are given in Table I, where the entries represent the differences in saturation between the $\alpha$ and $\beta$ chains. The errors quoted are calculated from the standard deviations of the slopes of the least-squares lines. (These quoted errors are a measure of the statistical scatter in the data, with an arbitrary assumption of a linear fit. This is not a measure of the "goodness of the fit" for the linear hypothesis.) The data in this table indicate that CO exhibits random binding in the absence of organic phosphate, a possible but very slight preference for the $\alpha$ chains in the presence of DPG, and a definite but still small preference for the $\alpha$ chains in the presence of IHP. $O_2$, on the other hand, exhibits a possible slight preference for the $\alpha$ chains even in the absence of organic phosphate, a strong preference for the $\alpha$ chains in the presence of DPG, and an even more extreme preference for the $\alpha$ chains in the presence of IHP.

The procedure used for measuring the $\alpha/\beta$ intensity ratios as described in the previous section was used due to the fact that the contact-shifted resonances lie on the "shoulder" of a large number of diamagnetic resonances (in particular, the proton signals due to the aromatic amino acids in the Hb molecule along with some of the "tail" of the residual water in the sample), thus giving a slanted base line and preventing the numerical fitting of the spectrum to a sum of lines. However, since the procedure used here might give rise to systematic errors, particularly where line broadening is involved, a series of computer simulations of the spectra were attempted. First, the deoxy spectrum was synthesized by parameterizing the observed spectrum as fully as possible in terms of line positions, widths, and relative intensities (assuming Lorentzian line shapes).[2]

[2] The specific parameters used were the observed positions of maximum intensity for each of the lines, the full line widths at half-height for lines A, C, E, and G, the upfield half-width at half-height for line B, the downfield half-width at half-height for lines D and F, and the intensities of lines B, C, D, and F as measured relative to the sum of the intensities of lines A and E. The base line for measurement of intensities and line widths is as shown in Figure 1.

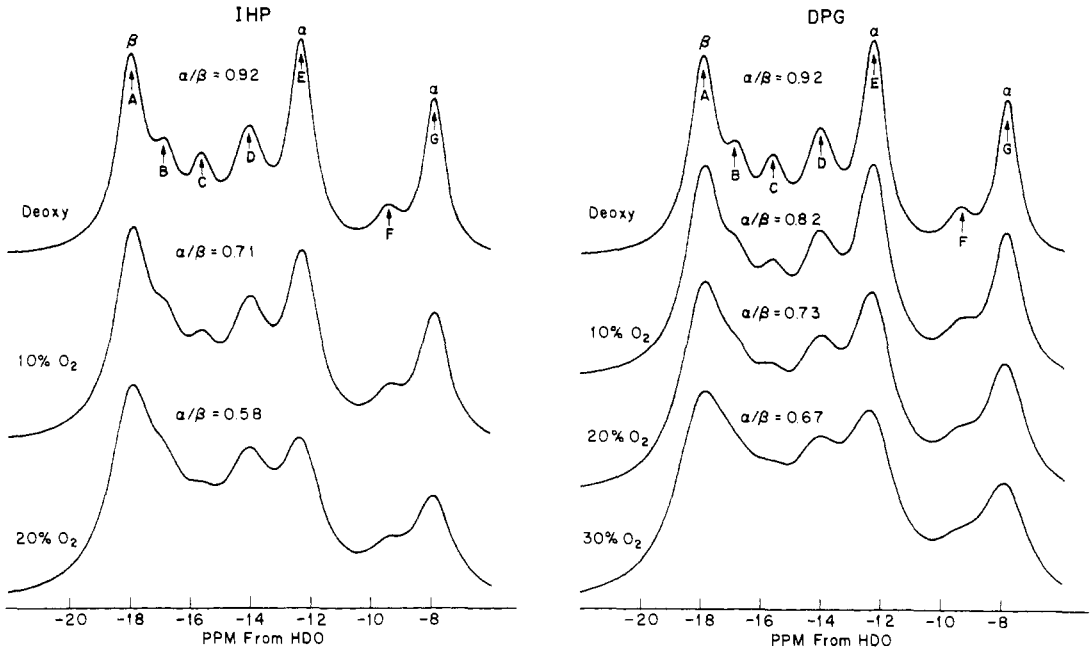


FIGURE 6: Computer simulations of 250-MHz spectra during oxygen saturation in the presence of IHP or DPG. Parameters used in the simulation of the initial spectrum are given in Table II.

Then the parameters of the computed spectrum were adjusted until measurements on the computed spectrum, made in the same fashion as in the experimentally observed spectrum, agreed as closely as possible with the observed spectrum. The final line positions, widths, and intensities are given in Table II. A comparison of the observed and simulated deoxy spectra is shown in Figure 1. After a satisfactory deoxy spectrum was synthesized, simulations of the saturation series were attempted (see Figure 6) by reducing the intensity of the $\alpha$ resonances by the amount predicted from the observed slope of the $\alpha/\beta$ intensity ratio and by increasing the line widths of all resonances by 8 Hz/1% $O_2$ saturation. (This line width increase was the average value observed for the −18 and −12 ppm resonances during $O_2$ saturation in the presence of IHP.) Empirical measurements of the $\alpha/\beta$ intensity ratios and least-squares fits for the simulated spectra were then made in exactly the same fashion as for the experimentally observed spectra. The slopes experimentally observed and those derived from the simulated spectra are compared in Table III.

## Discussion

The results outlined above clearly indicate that CO and $O_2$ differ significantly in their equilibrium reactions with Hb A. CO exhibits little preferential binding even in the presence of high concentrations of DPG or IHP, while $O_2$ exhibits very marked preferential binding under these conditions.

For example, even assuming a maximum error of one standard deviation, the difference in saturation between the $\alpha$ and $\beta$ chains for CO in the presence of IHP is still only ~6% at 50% total saturation (Table I). This indicates that approximately 53% of the $\alpha$ chains and 47% of the $\beta$ chains, respectively, are liganded, differing very little from random binding. For $O_2$ in the presence of IHP, on the other hand, a 60% difference at 40% total saturation corresponds to approximately 70% of the $\alpha$ chains and 10% of the $\beta$ chains being respectively liganded (Table I). This indicates nearly exclusive binding to the $\alpha$ chains at low saturation.

Although the possibility of systematic error in these calculations cannot be entirely ignored, at least two of the results detailed above suggest that any major systematic error is unlikely. First, the consistency between the observed and simulation derived slopes of the $\alpha/\beta$ intensity ratios (see Table III) is quite good. For the IHP series, the agreement is excellent. Although it is acceptable, the agreement for the DPG series is not quite so good, but this may in part be due to the fact that the line width increases used in the simulated saturation series were derived from the IHP series. Second, in deriving the information for preferential binding, data at both 90 and 250 MHz were used. The spectral base lines at these two frequencies were handled in somewhat different fashions and line broadening during $O_2$ saturation appeared at only 250 MHz, yet the data from the two sources gave equivalent results. Thus, the conclusions derived above appear to be on quite firm ground.

From X-ray diffraction studies, Arnone (1972) has shown that the anionic groups of DPG form salt bridges with cationic groups of the $\beta$ chains. This stereochemical binding was stated to be specific for the deoxy structure and to be lost on transition to the oxy form (Perutz, 1970; Arnone, 1972). From these observations it was suggested that DPG stabilized the deoxy conformation at the expense of the oxy structure. Since the organic phosphate is bound between the two $\beta$ chains, it would be expected to have its greatest effect upon ligand binding by the

TABLE III: Comparison of Experimentally Observed $\alpha/\beta$ Intensity Slopes with Those Derived from Computer-Simulated Spectra of $O_2$ Saturation Series.

| Phosphate | Obsd Slope | Simulation Derived Slope |
|---|---|---|
| DPG | $-0.52 \pm 0.13^a$ | $-0.68 \pm 0.08$ |
| IHP | $-1.67 \pm 0.08$ | $-1.63 \pm 0.14$ |

[a] Quoted error is the standard deviation of the slope of the least-squares lines as fit to the data of Figure 5.

$\beta$ hemes. For $O_2$ binding this is quite clearly the case, with IHP having a much stronger effect than DPG (see Figure 5). The difference between the effect of DPG and IHP is not particularly surprising since DPG has been shown to bind strongly only to deoxy Hb (Garby *et al.*, 1969; Riggs, 1971), while IHP binds strongly to both oxy- and deoxyhemoglobin (Gray and Gibson, 1971b; Janig *et al.*, 1971; Tyuma *et al.*, 1971). Similarly, Tyuma *et al.* (1973) have recently shown that the Hill coefficient for oxygenation of Hb A in the presence of IHP is significantly lower than in the presence of DPG and that it varies from 1.0 to 2.4 depending on the degree of oxygenation. Using the Adair model the oxygenation data were interpreted as suggesting that during oxygenation there is a substantially greater population of partially oxygenated intermediates in the presence of IHP than in the presence of DPG (Tyuma *et al.*, 1973). Our nmr results confirm the existence of substantial amounts of partially $O_2$ saturated Hb molecules [*i.e.*, $HbO_2$, $Hb(O_2)_2$, and $Hb(O_2)_3$] in the presence of IHP.

The line broadening at 250 MHz and the slight shift in the $\alpha$ resonance position at 90 MHz observed in the $O_2$ saturation series both appear to be due to an exchange between the paramagnetic and diamagnetic heme states (deoxy and oxy forms). This exchange is caused by the alternate association and dissociation of oxygen from the heme. Gibson (1973) has recently shown that the dissociation of oxygen from hemoglobin can be separated into fast and slow components, with the fast component provisionally associated with the $\beta$ chains. At 2°, in 0.05 M phosphate buffer (pH 7.0), the fast and slow dissociation rates are respectively 100/sec and 6/sec. At 20°, the fast rate rose to ~1100/sec; no information was given on the temperature dependence of the slow component (Gibson, 1973). For a comparison with the nmr results, it is noted that the dissociation rate constant is simply $\tau_{oxy}^{-1}$, the inverse lifetime of the oxy state. $\tau_{deoxy}^{-1}$, the inverse lifetime of the deoxy state, is then simply the product of the association rate constant and the $O_2$ concentration. At 50% chain saturation the probability of being in the oxy state is equal to that of being in the deoxy state, thus giving equal oxy and deoxy state lifetimes (*i.e.*, the association and dissociation rates are equal, giving $\tau_{deoxy} = \tau_{oxy}$). For convenience, these equal lifetimes will be refered to simply as $\tau$ in the following discussion.

Assuming the heme resonances in the diamagnetic (ligated) state to be ~2 ppm upfield from HDO, it is apparent that any exchange-induced change in the deoxy resonance position is very small compared to the separation between the two resonance positions. Thus, it can be assumed that the exchange rates are slow compared to the separation between the oxy and deoxy resonance positions (Pople *et al.*, 1959). An estimate of the exchange rate under these conditions may be obtained by noting that the observed line width ($2T_2^{*-1}$) is related to the intrinsic line width ($2T_2^{-1}$) and exchange rate by (Pople *et al.*, 1959)

$$2T_2^{*-1} = 2T_2^{-1} + 2\tau^{-1} \tag{6}$$

or

$$\tau^{-1} = \pi\delta\nu_{1/2} \tag{7}$$

where $\delta\nu_{1/2}$ is the increase in line width beyond the natural line width. At 250 MHz both the $\beta$ (−18 ppm) and $\alpha$ (−12 ppm) heme resonances appear to increase in width by approximately 8 Hz/1% total $O_2$ saturation in the presence of IHP. From the previous analysis on preferential binding it can be determined that in the presence of IHP the $\alpha$ chains are ~50% saturated at ~28% total saturation while the $\beta$ chains are ~50% saturated at ~75% total saturation. This corresponds to ~220 Hz broadening for the $\alpha$ resonance and ~600 Hz broadening for the $\beta$ resonance at 50% chain saturation at 27°. Equation 7 then suggests exchange rates of $\tau_\alpha^{-1} \sim 700$/sec and $\tau_\beta^{-1} \sim 1900$/sec. In the presence of small exchange-induced changes in the resonance position, the exchange rate is related to the shifts by (Pople *et al.*, 1959)

$$\tau^{-1} = \left[\frac{\delta\omega^2 - \delta\omega_e^2}{8}\right]^{1/2} \tag{8}$$

The shift upfield of ~0.5 ppm by the deoxy $\alpha$ resonance (at 90 MHz) along with an equivalent (assumed) shift of the oxy $\alpha$ resonance gives an exchange rate $\tau_\alpha^{-1} \sim 1000$/sec (at 28°). The exchange rate for the $\beta$ chains is in reasonable agreement with Gibson's kinetic measurements of the exchange rates (Gibson, 1973), however, the $\alpha$ rate is several times in excess of the probable rate determined from kinetic measurements. [Assuming that the $\alpha$ chain rate has the same temperature dependence as the $\beta$ chain rate, the $\alpha$ rate of 6/sec at 2° (Gibson, 1973) should increase to ~150/sec at 28°.] Differences between our values and those of Gibson are not entirely unexpected, because our experimental conditions are somewhat different from his. To an accuracy of ~30 Hz no line broadening was observed in the CO saturation series. Gray and Gibson (1971b) have observed a CO dissociation rate of 0.02–0.03/sec at 22°. This rate would give a broadening of only ~0.01 Hz, far less than our experimental error. The lack of any observed line broadening in the $O_2$ saturation series at 90 MHz is not understood, although the quantum mechanical treatment of exchange by Chan and coworkers suggests that in fact less broadening should be expected at lower field (Lee *et al.*, 1972). Although the explanation of spin exchange appears to be at least qualitatively consistent with the observed line broadening and shift in resonance positions, the fact that the deoxy Hb line widths themselves are field dependent may mean that the actual sources of line broadening, etc., are more complex than our analysis would suggest.

Perutz (1970), from his X-ray diffraction studies, has suggested that the stereochemistry of the heme pockets in deoxyhemoglobin may induce preferential ligand binding to the $\alpha$ chains. This is due to observation that in deoxyhemoglobin one of the valine methyl groups of $\beta$67E11 blocks the $\beta$ heme ligand binding site (Pertuz, 1970). Perutz has suggested that this valine moves away from the binding site only after the occurrence of a series of structural transitions which are triggered by the ligand binding to the $\alpha$ chains. The results reported above along with previously published results from this laboratory (Lindstrom *et al.*, 1971; Lindstrom and Ho, 1972) indicate, however, that binding preferences are strongly ligand dependent. BIC, in fact, appears to show exactly opposite behavior from that expected (Lindstrom *et al.*, 1971). Perutz and Ten Eyck (1971) have suggested from model building that this may be due to the bulky butyl group making more short contacts with the interior nonpolar amino acid side chain in the $\alpha$ than in the $\beta$ chains. Oxygen and carbon monoxide, however, are roughly the same size and shape, making it more difficult to explain the differences between their binding properties. On a structural level, the most plausible explanation for this difference appears to be that the detailed stereochemistry of the metal-ligand bonds (such as Fe-CO and Fe-$O_2$) may differ, for example, in the angles between Fe and CO and between Fe and $O_2$. These differences could affect the conformations of the heme pockets near the ligand binding sites in HbCO and $HbO_2$, and thus alter their respective ligand binding properties. Indirect support for this suggestion can be obtained from the observation that the ring-current shifted proton resonances of

the $\gamma_1$ methyls of the $\alpha$- and $\beta$E11 valines in HbCO are approximately 0.7 ppm downfield from those of $HbO_2$ (Lindstrom and Ho, 1973). This indicates that the magnetic environment of Val E11 differs between HbCO A and $HbO_2$ A, which in turn suggests that the stereochemistry of Fe-$O_2$ differs from that of Fe-CO.

The results reported above appear to be in good agreement with all of the material reviewed in the introduction with the exception of that reported by Huestis and Raftery (1972a-c, 1973). And, in fact, their results appear to be in some disagreement with much of the other material mentioned in the introduction. Their results for preferential binding of $O_2$ (Huestis and Raftery, 1972a) are in good agreement with our results and those of Lindstrom and Ho (1972) and Gibson (1973), however, this is the only major area of agreement. For CO the direction of preferential binding (*i.e.*, $\alpha$ chains favored over $\beta$ chains) reported by Huestis and Raftery (1973, 1972b) is in qualitative agreement both with our results and those of Ogata and McConnell (1972), however, the magnitude of the preferential binding reported by these authors is approximately four times greater than either our results or those of Ogata and McConnell (1972). This difference is well outside the statistical error calculated above for preferential binding of CO. And for BIC even the direction of preferential binding reported by Huestis and Raftery (1972c) is in disagreement with the results of both Lindstrom *et al.* (1971) and the extensive studies of Olson and Gibson (1971, 1972, 1973a,b) and Cole and Gibson (1973). Likewise an attempt to determine the p*K* of histidine-146$\beta$ through $^{19}F$ nmr studies of fluorine-labeled Hb (Huestis and Raftery 1972d) is in disagreement with a proton nmr study in this laboratory where the C-2 proton resonance of histidine-146$\beta$ was directly monitored as a function of pH (Kilmartin *et al.*, 1973). The reason for all of these discrepancies is not clear since Raftery and coworkers have reported that the functional properties of the fluorine-labeled Hb are essentially identical with those of normal Hb A (Lee *et al.*, 1973). The best method for resolving this question would be a direct comparison of $^1H$ and $^{19}F$ nmr results using the fluorine-labeled Hb.

At first glance, Gibson's conclusion (Gibson, 1973) that $O_2$ exhibits initial binding to the $\beta$ chains appears to contradict Perutz's suggestion (Perutz, 1970) that the stereochemistry of the heme pockets should induce preferential binding to the $\alpha$ chains. Our results, however, suggest that while oxygen may exhibit initial binding to the $\beta$ chains, the relative association and dissociation constants may favor binding to the $\alpha$ chains under equilibrium conditions. In other words, $O_2$ may indeed react with the $\beta$ chains of Hb A before the $\alpha$ chains as suggested by Gibson (1973). However, due to a difference in the stereochemistry of the $\alpha$ and $\beta$ heme pockets, $O_2$ molecules would not bind tightly to the $\beta$ chains until there is a change in the conformation of the $\beta$ heme pocket. Thus, Perutz's suggestion should perhaps be considered as being more relevant to equilibrium conditions than to kinetic studies. Our results also indicate, however, that the process of ligand binding is ligand dependent. More recently, Breen *et al.* (1974) reported that the preferential $O_2$ binding to Hb A is also pH dependent, with $O_2$ binding randomly to the $\alpha$ and $\beta$ hemes in solutions at pH 9.3 in the presence of IHP.

In order to reduce the intense proton signal from $H_2O$ in aqueous medium, $^1H$ nmr studies of proteins are usually carried out in $D_2O$. The effects of partial deuteration on the properties of Hb A have been reported and it has been suggested that the cooperative oxygenation as measured by the Hill coefficient is greatly reduced by partial deuteration with a subsequent return to the normal value upon extensive deuteration (Tomita and Riggs, 1970). Hence, the question arises as to whether or not the results of our nmr studies of hemoglobins in $D_2O$ are equivalent to those carried out in $H_2O$. Recently, we have obtained $^1H$ nmr spectra of Hb A in $H_2O$ and have found that in the presence of DPG or IHP, the $\alpha$ chains have a higher affinity for $O_2$, exactly the same as observed in $D_2O$ (Ho *et al.*, 1973; Breen *et al.*, 1974). Thus, under the conditions of our study, Hb A appears to show equivalent behavior in either $H_2O$ or $D_2O$.

Thus, on the basis of our frequency and temperature-dependent results, we conclude that there is an intrinsic difference between the affinities of the $\alpha$ and $\beta$ chains of Hb A for $O_2$ in the presence of organic phosphates. This observed difference is an equilibrium effect. Likewise these relative affinities are strongly ligand, phosphate, and pH dependent. Hence caution should be used in going from observations of Hb behavior in the presence of a specific ligand, pH, and phosphate to general conclusions regarding hemoglobin function.

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# Asymmetrical Changes in the Tertiary Structure of α-Chymotrypsin with Change in pH†

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ABSTRACT: Changes in the structure of α-chymotrypsin (α-CHT) with change in pH of the surrounding medium have been investigated from pH 1.0 to 10.0 using single crystal X-ray diffraction methods. This pH range is subdivided into seven regions corresponding to distinct and differing structural stability (pH conformers). Transitions between pH conformers occur at pH values close to known p*K* values of different ionizable groups present in proteins. The pH 8.3 conformer shows large asymmetrical changes in tertiary structure in the active site region of α-CHT dimer, where one molecule undergoes a severe reorganization while the other remains essentially unaltered with the pH change. The asymmetrical change is apparently induced near pH 8.0 by the deprotonation of one of the B chain N-terminal amino groups (Ile-16), thus disrupting the active site Ile-16–Asp-194 internal ion pair and causing a major reorganization of other residues in the near vicinity. These residues include the following peptide segments: Ile-16–Asn-18, Leu-143–Tyr-146 (carboxyl terminal, B chain), and Ser-189–Ser-195. The observed difference electron density in the active site regions between the structure of α-CHT at pH 8.3 and 3.6 has been related to changes in tertiary structure on a molecular level. A probable source of the asymmetrical changes is the variability in the tertiary structure previously observed at pH 3.6. The slightly different environments lead to slightly different p*K* values for the two independent Ile-16 residues.

We have already reported indirect evidence for asymmetrical changes in tertiary structure within the dimer interface region of α-chymotrypsin (α-CHT) upon dimerization (Tulinsky *et al.,* 1973a) and similar observations have been made on insulin dimer by Hodgkin and her collaborators (Blundell *et al.,* 1972). We have also reported observing asymmetrical changes in tertiary structure to a lesser extent accompanying a change in pH of the soaking solution of crystals (Vandlen and Tulinsky, 1973) and accompanying the exchange of localized sulfate ions in the crystal structure with selenate ions of a soaking solution (Tulinsky and Wright, 1973). We would now like to report observing fairly spectacular asymmetrical changes in the tertiary structure of the active site region of α-CHT dimer upon further change in pH, where one molecule of the dimer undergoes a severe reorganization within the active site region while the other remains essentially unaltered with the pH change.

## Experimental Section

The pH of the soaking solution above crystals of α-CHT was changed gradually in a series of steps by exchanging an aliquot

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