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An Investigation of the Distal Histidyl H-bonds in Oxyhemoglobin: Effects of Temperature, pH, and Inositol Hexaphosphate[†]

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Abstract

On the basis of X-ray crystal structures and electron paramagnetic resonance (EPR) measurements, it has been inferred that the O₂ binding to hemoglobin is stabilized by the hydrogen bonds between the oxygen ligands and the distal histidines. Our previous study by multinuclear nuclear magnetic resonance (NMR) spectroscopy has provided the first direct evidence of such H-bonds in human normal adult oxyhemoglobin (HbO₂ A) in solution. Here, the NMR spectra of uniformly ¹⁵N-labeled recombinant human Hb A (rHb A) and five mutant rHbs in the oxy form have been studied under various experimental conditions of pH and temperature, and also in the presence of an organic phosphate, inositol hexaphosphate (IHP). We have found significant effects of pH and temperature on the strength of the H-bond markers, i.e., the cross peaks for the side-chains of the two distal histidyl residues, α58His and β63His, which form H-bonds with the O₂ ligands. At lower pH and/or higher temperature, the side-chains of the distal histidines appear to be more mobile and the exchange with water molecules in the distal heme pockets is faster. These changes in the stability of the H-bonds with pH and temperature are consistent with the changes in the O₂ affinity of Hb as a function of pH and temperature and are clearly illustrated by our NMR experiments. Our NMR results have also confirmed that this H-bond in the β-chain is weaker than that in the α-chain and is more sensitive to changes in pH and temperature. IHP has only a minor effect on these H-bond markers compared to the effects of pH and temperature. These H-bonds are sensitive to mutations in the distal heme pockets, but not affected directly by the mutations in the quaternary interfaces, i.e., α₁β₁ and/or α₁β₂ subunit interface. These findings provide new insights regarding the roles of temperature, hydrogen ion, and organic phosphate in modulating the structure and function of hemoglobin in solution.

Hemoglobin (Hb) is a tetrameric, heme-containing protein consisting of two α- and two β-chains and is the carrier of oxygen from lungs to tissues in vertebrates. Oxygen binding to Hb is reversible and cooperative (1). The ratio of the equilibrium-binding constants for CO and O₂ is approximately 20,000 for free heme, and is lowered to about 250 in the case of Hb A (2). Thus, the relative affinity for O₂ versus CO is greatly increased in Hb, due to having its hemes embedded in the heme pockets of the protein molecule and interacting with their environment. It is currently accepted that O₂ bound to Hb is stabilized by the hydrogen bonds between oxygen ligands and the distal histidyl residues. Early X-ray crystal structural studies at moderate resolution of 2.1 Å (3) suggested the existence of a H-bond in the α-

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SUPPORTING INFORMATION

Figure 1S. Histidine side-chain region of 600-MHz (¹H, ¹⁵N) HSQC spectra of fully ¹⁵N-labeled normal and mutant rHbs in the oxy form in H₂O under various experimental conditions. This material is available free of charge via the Internet at <http://pubs.acs.org>.

heme pocket between the bound oxygen and the distal histidine E7 (α His58), and possibly a very weak one in the β -heme pocket to the E7 (β His63) histidine. High-resolution (1.25 Å) X-ray crystal structures (4) gave further support to this idea. The existence of such an H-bond has been inferred from the crystal structures (4) to explain the stronger binding of O₂ to Hb and Mb, relative to the free heme. Although reasonable, this suggestion has remained controversial due to a lack of direct experimental evidence, especially for Hb in solution, under physiological conditions (5–8). Our previous NMR study (9) has provided the first direct evidence for the presence of the distal histidyl H-bonds in HbO₂ A in solution. Here, we investigate the stability of these H-bonds as affected by pH, temperature, and an organic phosphate, inositol hexaphosphate (IHP). We have also examined the change to these H-bonds caused by mutations at the subunit interfaces ($\alpha_1\beta_1$ and $\alpha_1\beta_2$) and at the distal heme pockets. Uniformly ¹⁵N-labeled recombinant human normal adult hemoglobin A (rHb A) and five recombinant mutant rHbs were prepared and the NMR spectra were recorded on Bruker AVANCE DRX-600 MHz spectrometers. The changes in the stability of the H-bonds with pH and temperature are consistent with the changes in the affinity of Hb for O₂ and are clearly shown by our NMR experiments. We have found that the H-bond markers, namely the cross peaks for α 58His (¹H ϵ_2 , ¹⁵N ϵ_2) and β 63His (¹H ϵ_2 , ¹⁵N ϵ_2) are significantly affected by pH and temperature. At lower pH and/or higher temperature, the side-chains of the distal histidines appear to be more mobile and the proton exchange with water molecules in the distal heme pocket is faster, causing these markers to lose intensity and even to disappear. Our present findings have confirmed that the H-bond in the β -chain is weaker than that in the α -chain as suggested by the crystallographic studies (3–4). The allosteric effector, inositol hexaphosphate (IHP), binds to Hb and decreases its affinity for O₂. However, the H-bond markers, the cross-peaks for α 58His (¹H ϵ_2 , ¹⁵N ϵ_2) and β 63His (¹H ϵ_2 , ¹⁵N ϵ_2), do not change significantly upon the addition of IHP at pH 8.0 and pH 7.0, although these peaks become weaker, suggesting that the effect of IHP might not be strong enough to break these H-bonds under those conditions.

MATERIALS AND METHODS

Sample Preparation

Uniformly ¹⁵N-labeled rHb A and five mutant rHbs, rHb (α V96W), rHb (α V96W/ β N108K), rHb (β D99N), rHb (α Y42D/ β D99N), and rHb (α L29W), were expressed in *Escherichia coli* JM109 from the plasmid pHE2, pHE202, pHE249, pHE208, pHE222 and pHE285, respectively (10–14). rHbO₂ A samples for NMR experiments were prepared in phosphate-buffered solution (100 mM Na₂HPO₄/NaH₂PO₄, 10% D₂O) at pH 6.5, 7.0, and 8.0, respectively, using standard procedures in our laboratory (15). The final protein concentration was 0.7 to 1 mM (tetramer). The samples with IHP were prepared by adding IHP stock solution to the rHb samples to a final concentration of 5 mM. An important consideration in our NMR experiments was sample stability, especially at lower pH and higher temperatures, because the diamagnetic oxy-Hb can readily oxidize to the high-spin ferric, paramagnetic aquomet-Hb, where no heme-pocket resonances can be observed. The freshly prepared oxy-form rHb samples can usually stay in oxy-form without met-Hb being detected for several hours, which gives us time to finish the NMR experiments at one experimental condition. Then, the samples are re-purified according to the standard procedures in our laboratory and prepared carefully for the next experimental condition.

NMR Spectroscopy

NMR spectra were recorded on Bruker AVANCE DRX-600 MHz spectrometers. As the resonances of distal histidine side-chains are essentially under the water resonance (9), the water suppression quality is of paramount importance and no water-directed suppression technique (pre-saturation, excitation sculpting, selective excitation or flip-back) can be

employed, as they would also suppress the resonances of interest. Echo-antiecho gradient-selected HSQC experiments (hsqcetf3gp from the Bruker standard library) (16) had to be used so that only the signals from the protons attached to ^{15}N nuclei are refocused and detected, while the magnetization belonging to protons attached to any other nuclei (^{14}N , ^{12}C , ^{13}C , ^{16}O , etc) remains scrambled and therefore gives no signal. No other water suppression technique was employed and no post-acquisition solvent-filtering software processing was used. A first set of experiments were carried out using a 5-mm TXI z-gradient CryoProbe. Unfortunately, the poor water suppression due to the radiation damping effects and other instrumental causes rendered the higher sensitivity of this probe useless in our case. The rest of the experiments were then carried out at 600 MHz, but with a conventional 5-mm TXI xyz-gradients probe, using magic-angle gradients, which gave satisfactory results. Typical spectral widths were 20 ppm (^1H) and 200 ppm (^{15}N) with carriers at 4.7 ppm and 175 ppm, 2048 and 256 time-domain points, respectively. The refocusing delay in the INEPT module was 5.5 ms in order to select the signals from the ^1H directly attached to ^{15}N . With 16 or 32 scans per increment, 2D spectra were acquired typically in 2.5- or 5-hr blocks. At lower temperatures, the INEPT delay was shortened to compensate for the signal loss due to the increased line widths.

RESULTS

Effect of pH

In our earlier paper (9), the HMQC spectra for HbO₂ A provided the first direct evidence for the existence of the distal histidyl H-bonds in HbO₂ A, at 29 °C and pH 8.0. That condition was chosen to make use of the increased stability of the Hb molecule against auto-oxidation and also because the oxygen affinity is higher at this pH than at lower pH values. In our present study, the NMR experiments were carried out at three different pH values, in the absence and presence of IHP, respectively. First, HSQC and HMQC experiments were carried out at pH 8.0 and 29 °C in 0.1 M phosphate in the absence of IHP as control. Two cross-peaks, which correspond to the H-bonds between the oxygen ligand and the side-chains of $\alpha 58$ and $\beta 63$ distal histidines in HbO₂ A, are shown clearly in the HSQC (Fig. 1B) and in the HMQC spectra (result not shown). Then, HbO₂ A samples were prepared at pH 7.0 and 6.5 and the HSQC experiments were carried out at 29 °C. At pH 7.0 (Fig. 1E), the cross-peak for $\alpha 58\text{His}$ ($^1\text{H}_{\epsilon 2}$, $^{15}\text{N}_{\epsilon 2}$) is visible, indicating that the H-bond still exists at this pH. However, the cross-peak for $\beta 63\text{His}$ ($^1\text{H}_{\epsilon 2}$, $^{15}\text{N}_{\epsilon 2}$) disappears at pH 7.0. At pH 6.5 and 29 °C, the cross-peaks from $\alpha 58$ and $\beta 63$ distal histidines cannot be observed (Fig. 1G), suggesting that the H-bonds are broken at lower pH. To further illustrate the changes in the peak intensity of $\beta 63\text{His}$ under various experimental conditions, horizontal 1D slices along the ^1H axis through the cross peak of $\beta 63\text{His}$ are taken from the 2D spectra of Figure 1 and shown in Figure 2. These 1D slices also demonstrate that using the technique described in the section on Materials and Methods, we are able to detect the cross peaks of the H-bonded distal histidine side-chains in oxyhemoglobin when they exist, regardless of their positions with respect to the resonance of water. The first column (A, D and F) of Figures 1 and 2 shows the experimental data collected at 7 °C and pH 8.0, 7.0 and 6.5, respectively. At this low temperature, the peak of $\beta 63\text{His}$ is well resolved from the water signal and it is strong at pH 8.0. The peak becomes weaker at pH 7.0 and then disappears at pH 6.5. This peak also shifts upfield slightly, away from the water, as pH is lowered. The residual water signal is practically midway between the $\alpha 58\text{His}$ and the $\beta 63\text{His}$ peaks, so if there was an attenuation of these signals by the “suppression of the water signal”, the $\alpha 58\text{His}$ peak should have been diminished, too. That is not the case. We also collected data at 11 and 20 °C for each pH condition. When the temperature goes up, the water signal is shifting upfield (toward the right-hand side of the spectra) and is getting closer to the peak of $\beta 63\text{His}$. At 11 °C and pH 8.0, the peak of $\beta 63\text{His}$ is clearly observed, but is very weak at pH 7.0 and not seen at pH

6.5. At 20 °C and pH 8.0, the signals from water and $\beta 63\text{His}$ are very close. But, we are still able to recognize the signal of $\beta 63\text{His}$ since it is broad and the water signal is sharp. When the temperature is raised to 29 and 37 °C, the water signal already shifts to the right side of the signal from $\beta 63\text{His}$ (Figures 2 B and C). At pH 8.0, the peak of $\beta 63\text{His}$ is observed at 29 °C and getting weaker at 37 °C. In conclusion, the peak of $\beta 63\text{His}$ is observed at pH 8 and the peak intensities are getting weaker when the temperature changes from 7 to 37 °C. However, at pH 7.0, the peak of $\beta 63\text{His}$ can only be observed at 7 and 11 °C (Figure 2). The β -chain peak is visible when it exists, and not visible when it does not. Our results clearly show a trend, namely, the peak is weaker in the β -chain distal heme pocket and the distal histidine peaks in both α - and β -chains are weaker when the affinity of Hb for oxygen is lower.

Effect of Temperature

It is known that the temperature affects the oxygen binding to Hb (17). An increase in temperature decreases its affinity and thus enhances the release of oxygen. For example, at pH 6.5, the measurements show P_{50} values of 4.3 mmHg at 7 °C and 21.5 mmHg at 29 °C (Fig. 3). The effect of temperature on the stability of the H-bond marker has been examined in the range from 7 to 37 °C and at different pH values. At pH 8.0 and 29 °C, the cross-peaks for both $\alpha 58\text{His}$ ($^1\text{H}\epsilon_2$, $^{15}\text{N}\epsilon_2$) and $\beta 63\text{His}$ ($^1\text{H}\epsilon_2$, $^{15}\text{N}\epsilon_2$) are clearly visible, but that for $\beta 63\text{His}$ is weaker (Figures 1B and 2B). When the temperature is increased to 37 °C, the peak of $\beta 63\text{His}$ ($^1\text{H}\epsilon_2$, $^{15}\text{N}\epsilon_2$) becomes very weak, but still observable (Figures 1C and 2C), and when the temperature is decreased to 7 °C, this peak becomes much stronger (Figures 1A and 2A). At pH 7.0, the cross-peak for $\beta 63\text{His}$ ($^1\text{H}\epsilon_2$, $^{15}\text{N}\epsilon_2$) is missing at 29 °C (Figure 1E and 2E), but is clearly seen at 7 °C (Figures 1D and 2D), while the cross-peak for $\alpha 58\text{His}$ ($^1\text{H}\epsilon_2$, $^{15}\text{N}\epsilon_2$) is visible at all temperatures shown and its intensity decreases at higher temperatures. At the lowest pH, pH 6.5, the cross-peak for $\alpha 58\text{His}$ ($^1\text{H}\epsilon_2$, $^{15}\text{N}\epsilon_2$), is missing at 29 °C (Figures 1G and 2G), but appears at 7 °C (Figures 1F and 2F). However, at pH 6.5, the cross-peak for $\beta 63\text{His}$ ($^1\text{H}\epsilon_2$, $^{15}\text{N}\epsilon_2$) is undetectable even at 7 °C (Figures 1F and 2F).

Figure 4 shows the non-exchangeable ring-current-shifted proton resonances from -1 ppm to -3 ppm from DSS. These resonances provide valuable information about the environment in the heme pockets. On the basis of our previous studies, the resonances at -1.75 and -1.82 ppm in the spectra of HbCO A at 29 °C (results not shown) have been assigned to the γ_2 -CH₃ group of the $\alpha 62\text{Val}$ and $\beta 67\text{Val}$ (E11) respectively (18–19). In rHbO₂ A at 29 °C, the ring-current-shifted resonances from $\alpha 62\text{Val}$ and $\beta 67\text{Val}$ occur around -2.4 ppm, are overlapped at pH 8.0 and become clearly resolved at pH 6.5. However, at 7 °C, these two peaks shift only slightly from pH 8.0 to pH 6.5 (Figures 4A and B). This is consistent with the changes in the intensity of the distal H-bond markers under different pH and temperature conditions. For example, the H-bond markers for the α -heme pockets change significantly at 29 °C (Figures 1B, 1E, and 1G) and change only slightly at 7 °C (Figures 1A, 1D, and 1F). As shown in Figure 3, the Bohr effect is also dramatically affected by temperature. At 29 °C, the P_{50} value increases about seven fold between pH 6.0 (21.5 mmHg) and pH 8.4 (3.1 mmHg), but changes much less at 7 °C between pH 6.5 (4.3 mmHg) and pH 8.1 (1.5 mmHg). Thus, the changes in the chemical shifts of the E11 valyl residues and the intensity of the H-bonds markers are consistent with the effect of pH and temperature on the oxygen-binding affinity.

Effect of IHP

A number of studies have been reported regarding the functional changes induced by organic phosphates, e.g., 2,3-bisphosphoglycerate (2,3-BPG) and IHP, in Hb A and mutant Hbs (12–13,20). On the basis of the X-ray crystallographic studies, 2,3-BPG and IHP are believed to bind to the central cavity, between the two β -chains of the Hb molecule in the

deoxy form and thus stabilize the deoxy quaternary and lower the oxygen affinity (21–24). On the other hand, molecular dynamics simulations suggest that allosteric effectors, such as 2,3-BPG, bezafibrate (BZF) and IHP, affect only the ligation-linked tertiary structural changes rather than the homotropic ligation-linked T→R quaternary structural transition (25–27).

In our study, the oxygen-binding affinity for rHb A is measured in the presence of IHP under different conditions of pH (pH 8.0, 7.0 and 6.5) and temperature (29, 11 and 7 °C). As shown in Figure 3, at lower pH and/or higher temperature, IHP has a greater effect on the oxygen binding. When the oxygen-binding affinity is highest at high pH (pH 8.0), the P_{50} values do not change significantly upon the addition of IHP. The effect of IHP on P_{50} becomes obvious when the pH is lower than 7.5 and when the temperature is higher. At pH 7.0 and 29 °C, the P_{50} values for Hb A with and without IHP are 45.7 and 15.0 mm Hg, whereas at 11 °C the values are 21.3 and 5.0 mm Hg, respectively (Fig. 3). In order to investigate if the H-bond marker would be affected by the addition of IHP, HSQC spectra were collected under each of the corresponding experimental conditions. It is found that upon the addition of IHP, the H-bond marker for the α -heme pocket, the cross-peak of $\alpha 58\text{His}$ ($^1\text{H}\epsilon_2$, $^{15}\text{N}\epsilon_2$), does not change significantly and that for the β -heme pocket becomes weaker at pH 8.0 and 7 °C and disappears under the other conditions (Fig. 5). Looking at the ring-current shifted resonances shown in Figures 4A and 4B, the changes in the chemical shifts of $\alpha 62\text{Val}$ and $\beta 67\text{Val}$ due to the temperature and pH are also quite similar in the presence and absence of IHP. IHP slightly perturbs the position of the E11 valyl residue in the β -heme pocket (Fig. 4B), consistent with the previous findings (18–19).

Mutations in the $\alpha_1\beta_1$ and $\alpha_1\beta_2$ Interfaces

It is well-known that mutations in the $\alpha_1\beta_2$ subunit interfaces of Hb A can significantly change the oxygen affinity (1,28). For example, Hb Kempsey (βD99N) is a naturally occurring mutant of Hb A with high oxygen affinity and very low cooperativity (29). The functional defect for Hb Kempsey is believed to be due to the substitution of Asn for Asp at $\beta 99$, which prevents the formation of an important inter-subunit H-bond between $\alpha 42\text{Tyr}$ and $\beta 99\text{Asp}$ in the T-state. This structural change destabilizes the T-state of Hb Kempsey, thus causing an increase in the oxygen-binding affinity. In contrast, rHb (αV96W) was designed as a low-oxygen affinity mutant by introducing a new water-mediated H-bond between the indole nitrogen atom of $\alpha 96\text{Trp}$ and $\beta 101\text{Glu}$ across the T-state interface (12,30). Thus, it would be interesting to know whether the H-bond markers in the distal heme pockets would be affected by the mutation in the subunit interfaces of Hb A. Four rHbs with mutations in the $\alpha_1\beta_2$ and/or $\alpha_1\beta_1$ interfaces of Hb A were selected to examine this correlation. The NMR results show that the H-bond marker for the α -heme pocket in a low-affinity mutant, rHb (αV96W) (Figures 6A and B), and a high-affinity mutant, rHb Kempsey (βD99N) (Figures 6C and D), exhibit a pattern similar to that observed for rHb A, i.e., showing a stronger signal at high pH and/or low temperature and a weaker signal at low pH and/or high temperature. The H-bond marker for the β -heme pocket, the cross-peak of $\beta 63\text{His}$ ($^1\text{H}\epsilon_2$, $^{15}\text{N}\epsilon_2$), also exhibits similar behavior as in rHb A, except that the cross-peak does not appear at pH 7.0 and 7 °C.

Further examination of the H-bond markers has been carried out on a compensatory mutant for Hb Kempsey, rHb ($\alpha\text{Y42D}/\beta\text{D99N}$), which was designed to restore its functional properties by providing an alternate H-bond to replace the one that is missing in Hb Kempsey (11). The H-bond cross-peaks for this mutant rHb exhibit a very similar pattern compared to that for rHb A at all experimental conditions (pH and temperature) (Figures 6E and F).

Another mutant tested in this study, rHb (α V96W/ β N108K), has the greatest tendency to switch to the T-type structure, even when it is still in the ligated state (13). The mutation at β 108 (G10) Asn $\rightarrow$ Lys is located in the $\alpha_1\beta_1$ subunit interface in the central cavity of the Hb molecule (1). The combination of mutations in the $\alpha_1\beta_1$ and $\alpha_1\beta_2$ interfaces produced a mutant with good cooperativity and the lowest oxygen affinity among the hemoglobins designed in our laboratory. There are two cross-peaks, at 12.9 and 12.1 ppm proton chemical shift, which have been assigned to the side-chains Ne^2H group of α 122His and α 103His, H-bonded to β 35Tyr and β 131Gln, respectively, in the $\alpha_1\beta_1$ interface (15,31–32). At pH 8.0, these cross-peaks are very weak at 7 °C and missing at 29 °C (Fig. 7), suggesting that the $\alpha_1\beta_1$ interface is disturbed by the mutation at β 108 as observed from the previous study for other $\alpha_1\beta_1$ interface mutants (13,32). However, the H-bond markers from both α 58His and β 63His are clearly visible at 7 °C and still can be observed at 29 °C (Fig. 7), similar to those seen for rHb A. This result clearly suggests that although the quaternary structure is affected by the mutation in the subunit interfaces, the conformation of the heme pocket does not appear to change significantly and shows that the affinity for oxygen is controlled by multiple mechanisms involving quaternary and tertiary structural effects as well as the dynamics of this complicated molecule (33).

The effect of IHP has also been examined for the low-affinity mutant, rHbO₂ (α V96W/ β N108K), and the high-affinity mutant, rHbO₂ (α Y42D/ β D99N), by carrying out the HSQC experiments in the presence of IHP. Our results show that the H-bond markers in the distal heme pocket of these two mutants do not change significantly upon binding with IHP (Supporting Information Figure 1S). This is consistent with the results observed for rHb A in the oxy form.

Mutation in the Distal Heme Pocket

Our previous studies involving the mutations at the B10 position in Hb A have shown that rHb (α L29W), rHb (β L28F), and rHb (β L28W) exhibit very low oxygen affinity and reduced cooperativity compared to those of Hb A, while rHb (α L29F) exhibits high oxygen affinity (14,33). Proton NMR spectroscopy indicates that these mutations in the B10 helix do not perturb the $\alpha_1\beta_1$ and $\alpha_1\beta_2$ subunit interfaces significantly (an expected result), while the tertiary structures near the heme pockets are affected. rHb (α L29W) was chosen for the present study and the HSQC experiments were performed for this mutant in the oxy form to test if the H-bond would be affected. At pH 8.0, both of the H-bond markers for the α - and β -chains can be observed at 29 °C (Supporting information Figure 1S) with similar peak intensities as those shown for rHbO₂ A. The most significant change detected under this experimental condition is that the cross-peak of α 58His ($^1\text{H}\epsilon_2$, $^{15}\text{N}\epsilon_2$) is shifted upfield by about 0.2 ppm, suggesting that the local environment is changed by the mutation in the distal heme pocket of the α -chain. On the basis of our previous study, the difference in the oxygen-binding affinity between rHb (α L29W) and Hb A becomes larger at lower pH. At pH 7.0 and 29 °C, the P_{50} values for this mutant is about 50 mmHg, which is much higher than that for Hb A (15.1 mmHg) (14,33), indicating a more substantial decrease in the oxygen affinity. Thus, more significant differences in the H-bond markers are expected to be observed at the lower pH. Indeed, at pH 7.0 and 29 °C, the H-bond marker for the α -heme pocket of rHb (α L29W) is much weaker than that in rHb A under the same experimental condition, but can still be observed (Fig. 6H). Meanwhile, the pattern of the H-bond marker for the β -heme pocket does not change significantly compared to that of rHb A. The same experiment was carried out at 20, 11, and 7 °C to confirm that the mutation in the distal heme pocket does make the H-bond marker of the α -chain weaker (Supporting information Figure 1S). Figure 4C shows that the resonance of α 62Val moves downfield and changes due to experimental conditions are easily observed. Thus, the mutation in the α -heme pocket

decreases the binding affinity by changing the geometry of the distal heme pocket, as demonstrated by the H-bond markers and the chemical shift of the valyl residue.

DISCUSSION

Changes in the Heme Pockets due to the Ligation States

The X-ray crystal structures of Hb A in deoxy, oxy, and carbonmonoxy forms determined at 1.25 Å resolution have provided a detailed structural model for hemoglobin (4). As shown in Figure 8, the geometry of the heme pockets changes upon ligand binding, but the major change is in the heme plane. If the structures of HbO₂ A (PDB code 2DN1) and deoxy-Hb A (PDB code 2DN2) are superimposed based on the four nitrogen atoms of the heme, the heme planes fit very well, with the iron atom in the plane in the ligated states, but the iron atom moves down 0.45 Å to a domed heme conformation in the unligated state. In the distal heme pocket, the E11 valyl residues move forward towards the heme in the deoxy-form of Hb A. A more significant movement is observed for β67Val than for α62Val. Meanwhile, the α₁β₁ and α₂β₂ dimers shift slightly and rotate by about 11° with respect to each other. Thus, there are two major structural changes upon ligand binding, i.e., the reorientation of the α₁β₁ and α₂β₂ dimers and the movement of the iron atom with respect to the heme-plane.

The formation of a H-bond between the distal histidine side-chain NH and the oxygen bound to the heme iron is believed to be explanation for the increased relative binding affinity for oxygen versus CO in the heme proteins (myoglobin and hemoglobin). This H-bond stabilizes the NH group against the exchange with the water solvent and thus makes possible to observe the one-bond scalar coupling between ¹H and ¹⁵N (about 95 Hz), manifested as a doublet with the corresponding separation equal to ¹J_{NH} in the 1D spectra or as a cross peak in the 2D HSQC or HMQC spectra. If the histidyl NH proton is able to participate in a solvent proton exchange, the intensity of the cross peak diminishes if the exchange rate is lower than ¹J_{NH} and disappears when the exchange is faster (9). This is why of the 19 histidine residues per αβ dimer that we only observe the side-chain NH cross-peaks for the two histidines in the α₁β₁ interface (α103 and α122) in all ligation forms, and for the distal histidines (α and β) only in the oxy form. We also see the HδN cross peak of the α- and β-proximal histidines in the diamagnetic CO and oxy forms. The H-bond between the distal histidine and the O₂ ligand is a sensitive marker for the changes in the heme-pocket and is directly related to the change in the oxygen affinity. The disappearance or the weakening of the H-bond marker (intensity) means that the ligand is likely to be released from the heme. Our studies have shown that both pH and temperature can change the O₂ binding affinity of Hb A and also affect the H-bond markers directly. On the other hand, the mutations in the subunit interface and/or organic phosphate have a much weaker effect on the H-bond markers, suggesting that these structural perturbations may have major effects on the quaternary structure of Hb A, but only indirectly affect the tertiary structure of the distal heme pocket.

H-bond Markers and Changes in the Oxygen-binding Affinity

On the basis of previous studies, there are many factors, such as pH and temperature, that affect the oxygen binding to Hb. The H⁺ ion is a known heterotropic allosteric effector in the oxygenation of Hb. As shown in Figure 3, the oxygen affinity of Hb A strongly depends on pH, and this dependence is known as the Bohr effect (1). This physiologic effect is fundamentally important in the ability of the Hb molecule to deliver O₂ to the tissues, particularly to working muscles, where lactic acid is produced and the pH is thus lower. As suggested by the X-ray crystallographic results and then confirmed by our NMR findings, the binding of O₂ to Hb is stabilized by the H-bonds between oxygen and distal histidines (4,9). Using NMR spectroscopy, we have found that the changes in these H-bonds are

correlated with the changes in the O₂-binding affinity brought upon by pH and/or temperature. At lower pH or higher temperature, the O₂-binding affinity is lower than at high pH and low temperature. Meanwhile, the H-bond markers become weaker, suggesting that under these conditions, the side-chains of the distal histidines experience an increased mobility so that the proton exchange with a water molecule in the distal heme pocket is faster. Thus, the oxygen molecule would be correspondingly less stabilized by the H-bond and become easier to be released, which is consistent with the changes in the O₂ affinity of Hb. This H-bond marker can also show the subtle difference between the α - and β -distal-heme pockets. On the basis of our previous NMR studies, the α -subunit has a higher O₂-binding affinity than the β -subunit in the presence of organic phosphate (34–35). The X-ray crystal structures indicate that the oxygen ligand and distal histidine in the α -heme pocket have a more favorable geometry to form a H-bond than that in the β -heme pocket (4). NMR experiments in our study have confirmed that the H-bond between β 63His and oxygen is weaker and more sensitive to changes in pH and temperature. The weaker H-bond in the β -heme pocket is illustrated by the much smaller intensity of its corresponding cross peak as compared to that of the α -heme pocket (see Figures 1 and 2) under all experimental conditions investigated in this work. It also tends to disappear in most situations when the oxygen affinity is lower.

Effect of IHP on the Structure of Hb A

It is known that allosteric effectors, such as hydrogen ions, chloride ions, and organic phosphates, e.g., 2,3-BPG and IHP, modulate the oxygen affinity of hemoglobin. However, the role of IHP with respect to oxygen binding in hemoglobin is controversial. Previous studies based on the backbone dynamics show a conformational fluctuation of HbCO A upon IHP binding, especially at the inter-dimer interfaces and affecting the α - and β -subunits differently (36). The major conformational changes during the Hb allosteric transition can be attributed to the sliding contacts in the areas including the $\alpha_1\beta_2$ or $\alpha_2\beta_1$ interface, especially in the switch region (α C helix- β FG corner), the joint region (β C helix- α FG corner), and the carboxy-terminals of both α - and β -chains (36). The fluctuation might shift the averaged solution quaternary structure of IHP-bound HbCO A slightly toward the R structure from the mid-point between the R and R2 structures, as suggested by NMR residual dipolar couplings (RDC) experiments for HbCO A and mutant rHbCO (α V96W) in the presence of IHP (37). Yonetani *et al.* (25) have advocated a global allostery model based on molecular dynamics simulation for hemoglobin. Their model proposes that the oxygen-affinities of the T (low-affinity)- and R (high-affinity)-functional states of Hb are changed by heterotropic effector-linked tertiary structural changes without changing the respective T (deoxy)- and R (oxy)-quaternary states. Our previous studies have shown that the side-chain of β 37Trp residue is a sensitive marker for the quaternary structural and dynamic changes in the $\alpha_1\beta_2$ ($\alpha_2\beta_1$) interface caused by IHP (38). In the experiments reported here, the H-bonds between the bound O₂ and the distal histidyl residues in the α - and β -heme pockets are used as markers to investigate if there are any tertiary structural changes upon the addition of IHP. The results show that while IHP affects the oxygen-binding affinity significantly, it has a lesser effect on the H-bond markers. For example, at pH 7.2 and 29 °C in 0.1 M phosphate, the P_{50} value for Hb A is 10.5 and 46.7 mmHg in the absence and presence of IHP, respectively, indicating that the oxygen-binding affinity changes significantly (four-fold) upon the addition of IHP (Figure 3). Under similar experimental conditions, the H-bond marker in the α -heme pocket is only slightly affected (see Figures 1E and 5D). The different effects on the O₂-binding affinity and the H-bond marker can also be observed for a compensatory mutant rHb (α Y42D/ β D99N). At 29 °C and pH 7.2 in 0.1 M phosphate, the oxygen affinity is decreased about 5-fold (P_{50} value of 2.5 mmHg without IHP and 12.9 mmHg with IHP) (11). The H-bond marker in the α -heme pocket can be observed under both conditions (Supporting Information Figure 1S). Thus, the NMR results reported here suggest that the

change in the oxygen-binding affinity is mainly due to the quaternary structural perturbation upon the addition of IHP, although there is a contribution from a perturbation of the dynamics of the β -chain (see below).

Our recent studies of the backbone dynamics and RDC measurements show that in general, IHP affects the α - and β -subunits of Hb A differently (36–37). These differences can also be observed for the H-bond markers. For example, at pH 7.0 in 0.1 M phosphate, the H-bond marker of the α -heme pocket shows up clearly even in the presence of IHP (Figures 5C and D) at all temperatures. But the H-bond marker of the β -heme pocket, which is already weak at pH 7.0 and at 7 °C in the absence of IHP (Figure 1D), disappears upon adding IHP (Figures 5C and D), suggesting that this H-bond is more fragile and is more sensitive to the perturbation by IHP. This result also suggests that the presence of IHP does have an effect on the tertiary structure of Hb A, although this effect may be caused indirectly by its perturbation to the quaternary structure. The recent results are consistent with our earlier studies that IHP exerts its effects on both the tertiary and quaternary structures of Hb A (36–37,39).

H-bond Markers and Structural Changes Induced by Mutations

It is well known that mutations either in the interfaces or in the heme pocket of hemoglobin can significantly change the oxygen-binding affinity. In our studies, when the mutation is made in the α -heme pocket, changes in the H-bond markers and chemical shifts of the valyl residues are observed, suggesting that the affinity changes are related to a geometric change of the α -heme pocket. On the other hand, the mutations at the $\alpha_1\beta_2$ (or $\alpha_2\beta_1$) interface change the O₂-binding affinity by shifting the equilibrium between the R- and the T-states of these rHb mutants toward the T-state (low affinity) geometry or the R-state (high affinity) geometry (11–14). While having more significant effects on the quaternary structure, the mutations at this interface also affect the structure of the heme pockets, which can be suggested by the weaker H-bond markers for the β -chain of both low- and high-oxygen affinity mutants (Figures 6A and C). However, for those mutations in the subunit interfaces, the H-bonds for the α -chain appear to be less affected. Since the high-affinity mutants, such as rHb Kempeszy and its compensatory mutant rHb (α Y42D/ β D99N), prefer to remain in the ligated state, it is understandable that the H-bond markers of these mutants are less affected. For low-affinity mutants, such as rHb (α V96W) and rHb (α V96W/ β N108K), the switching between the R-state and the T-state can occur easily, namely the appearance of the T-state marker is observed at 14 ppm in ¹H NMR spectra while these rHbs are in the ligated state (12–13), especially at lower temperatures. The T-state marker has been previously (40) identified as the intersubunit H-bond between α 42Tyr and β 99Asp in the $\alpha_1\beta_2$ interface in deoxy Hb A and it is very sensitive to the quaternary structural switching between the R- and the T-states. On the other hand, the H-bond markers in the distal heme pockets are not affected significantly (Fig. 7A) at 7 °C. This proves that hemoglobin is in the ligated state and suggests that the tertiary structure of the α -distal heme pocket is quite robust with respect to the perturbations from the subunit interface. At higher temperatures, though, both distal heme pockets become more dynamic and the cross peaks diminish and even disappear (Fig. 7B).

Our present study suggests that the H-bond between ligand O₂ and distal histidines can be used as a sensitive marker for change in the ligation state. For the mutants, it is possible that the two dimers ($\alpha_1\beta_1$ and $\alpha_2\beta_2$) could start to switch the quaternary structure even before the ligation states change. For the low-affinity mutants in the CO form in the presence of IHP and/or lowering the temperature, this could be the reason that the T-state markers in the $\alpha_1\beta_2$ interface can be observed without the ligands leaving the heme.

Dynamic Ensemble of Hemoglobin Structures in Solution

There are at least four R-type ligated Hb A and nine T-type deoxy-Hb A crystallized under different temperature, pH, buffer, and salt conditions (4,6,41–50). Using the graphics program PyMOL (51), the X-ray crystal structure of HbO₂ A (PDB code 2DN1) was superimposed on that of deoxy-Hb A (PDB code 2DN2) and the root-mean-square deviation (RMSD) value was 2.412 Å based on 570 Cα atoms of the tetramer, but only 0.894 Å when based on 285 Cα atoms of the α₁β₁ (or α₂β₂) dimer. This suggests that the major structural changes upon ligand binding are in the α₁β₂ (or α₂β₁) subunit interface, involving several salt bridges and H-bonds. Meanwhile, the α₁β₁ (or α₂β₂) dimer behaves as a rigid body, whose overall structure is essentially insensitive to ligation (52). Further comparisons have been carried out for different R-type or T-type structures of hemoglobin. The RMSD for the four R-type structures is 1.7–1.9 Å based on the tetramer, but is only 0.8–0.9 Å based on the dimer. There is no significant structural difference shown in the heme pocket based on the superimposition of α₁β₁ (or α₂β₂) dimers, suggesting that the major differences between these R-type structures are coming from the differences in the interdimer, i.e. α₁β₂ (or α₂β₁) interface. Meanwhile, for the nine deoxy-form (T-type) structures, the difference between the tetramers is much smaller on the basis of the RMSD values ranging from 0.1 to 0.4 Å, suggesting that the α₁β₂ (or α₂β₁) interfaces exhibit less variance in the unligated form.

It has been suggested that the inter-dimer α₁β₂ (or α₂β₁) subunit interface in ligated Hb A has a weaker interaction than that in the intra-dimer α₁β₁ (or α₂β₂) interface (52–54) and that the interaction in the α₁β₂ (or α₂β₁) subunit becomes stronger in the unligated form of Hb A with the formation of several H-bonds and salt bridges (55). Thus, in the ligated form of Hb A, the α₁β₂ or α₂β₁ interface is more relaxed, more dynamic and is more easily affected by environmental conditions, such as pH, mutations, and organic phosphate. Our previous studies have shown that most of the backbone residues of Hb A are rigid on the time-scale of ns-ps (36,56), which is much faster than the time scale of domain reorientation, that is the basis of hemoglobin's flexibility. The experimentally determined RDC values can be well reproduced by calculations based on either of the X-ray structures for isolated α- or β-chain or that of an isolated αβ dimer. However, a larger χ^2 value is obtained when based on the whole tetramer (39,57). For HbCO A, a better fitting is obtained by a rotation of one αβ dimer with respect to the other (57). Thus, the X-ray crystal structure may only be a snapshot of basically identical α₁β₁ and α₂β₂ dimers, with a particular interdimer interface. Which kind of interface can be observed in the X-ray crystal structure appears to depend on the experimental conditions used for crystallization. Since these interdimer interfaces are “relaxed” in the ligated form of Hb A, the structure of the ligated hemoglobin in solution appears to be an ensemble of two αβ dimers with dynamic α₁β₂ and α₂β₁ subunit interfaces, i.e., a dynamic ensemble of various quaternary structures.

CONCLUSION

Ligand binding to hemoglobin is a dynamic and synergistic process involving many tertiary and quaternary structural changes. The cross-peaks of α58His (¹Hε₂, ¹⁵Nε₂) and β63His (¹Hε₂, ¹⁵Nε₂) side chain in Hb A can be used as the H-bond markers to reflect structural and dynamic changes in the distal heme pocket under various experimental conditions in solution. Our results show that the stability of these H-bonds is sensitive to changes in pH and temperature, which are key effectors in regulating the oxygen-binding affinity of Hb and affect the heme-pocket directly. Mutations in the subunit interface can also change the oxygen-binding affinity by shifting the equilibrium between R-type and T-type structures, but may not have a direct effect on the distal heme-pocket. Our results show that two allosteric effectors, H⁺ ion and IHP, exert different effects on the structure and function of the Hb molecule. Using the distal histidine H-bond as a marker, we are able to illustrate how the oxygen-binding affinity of hemoglobin is affected by different effectors in solution and

also provide a better view of the changes in the distal heme pocket upon ligand binding. The stability of the distal H-bonds against perturbation by mutations and allosteric effectors suggests that hemoglobin in the ligated form is a dynamic ensemble consisting of two rigid $\alpha_1\beta_1$ and $\alpha_2\beta_2$ dimers with relaxed but also dynamic $\alpha_1\beta_2$ or $\alpha_2\beta_1$ interface. The structural features of the $\alpha_1\beta_2$ and $\alpha_2\beta_1$ interfaces are dependent on the environmental conditions and can indirectly affect the oxygen-binding affinity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations used

Hb A	human normal adult hemoglobin
rHb	recombinant Hb
HbO₂	oxyhemoglobin
HbCO	carbonmonoxy-hemoglobin
deoxy-Hb	deoxy-hemoglobin
met-Hb	methemoglobin
NMR	nuclear magnetic resonance
IHP	inositol hexaphosphate
HSQC	heteronuclear single-quantum coherence
HMQC	heteronuclear multiple quantum coherence

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Figure 1.

His and Trp side-chain regions of 600-MHz (^1H , ^{15}N) HSQC spectra of fully ^{15}N -labeled HbO₂ A in H₂O at pH 8.0 (upper row); 7.0 (middle row); and 6.5 (lower row) at 7, 29 and 37 °C, respectively. The cross-peaks for the relevant directly bonded ^{15}N - ^1H pairs are identified for each residue.

Figure 2.

Horizontal 1D slices along the ^1H axis through the cross peak of side-chain of $\beta 63\text{His}$ extracted from 600-MHz (^1H , ^{15}N) HSQC spectra of fully ^{15}N -labeled rHbO₂ A in H₂O under various conditions of pH and temperature. The panels are marked with the same letter as the corresponding panels (pH, temperature) from Figure 1.

Figure 3. Oxygen-binding properties of Hb A as a function of pH at 29 °C (red), 11 °C (green), and 7 °C (magenta) in 0.1 M sodium phosphate buffer without IHP (filled triangles) and with 4 times molar concentration of IHP (open circles).

Figure 4.

Ring-current-shifted proton resonances in ^1H NMR spectra (600 MHz) of rHbO₂ A in the absence and presence of IHP (A and B), and mutant rHbO₂ (αL29W) (C) in 95% H₂O, 5% D₂O, 0.1 M sodium phosphate buffer at different pH (8.0, 7.0 and 6.5) and temperatures (29 and 7 °C), respectively.

Figure 5.

His and Trp side-chain regions of 600-MHz (^1H , ^{15}N) HSQC spectra of fully ^{15}N -labeled HbO₂ A in H₂O in the presence of IHP at pH 8.0 (upper row), pH 7.0 (middle row) and pH 6.5 (lower row); and by columns, at 7 °C (left) and 29 °C (right), respectively. The cross-peaks for the relevant directly bonded ^{15}N - ^1H pairs are labeled.

Figure 6.

His and Trp side-chain regions of 600-MHz (^1H , ^{15}N) HSQC spectra of fully ^{15}N -labeled mutant rHb O₂ (αV96W) (A and B); rHb O₂ (βD99N) (C and D); rHb O₂ ($\alpha\text{Y42D}/\beta\text{D99N}$) (E and F); and rHb O₂ (αL29W) (G and H) in 95% H₂O, 5% D₂O, 0.1 M sodium phosphate buffer at pH 7.0, 7 °C (left column) and 29 °C (right column), respectively. The cross-peaks for directly bonded ^{15}N - ^1H of the relevant side-chains are labeled.

Figure 7.

His and Trp side-chain regions of 600-MHz (^1H , ^{15}N) HSQC spectra of fully ^{15}N -labeled mutant rHb O₂ ($\alpha\text{V96W}/\beta\text{N108K}$) in H₂O at pH 8.0, 7 °C (*left*) and 29 °C (*right*), respectively. The cross-peaks for directly bonded ^{15}N - ^1H of the relevant side-chains are labeled.

Figure 8.

Individual heme pockets in the α - and β -subunits of oxy- CO- and deoxy-Hb A with hemes superimposed, colored red for HbO₂ A, blue for HbCO A, and grey for deoxy-Hb A, respectively. The Fe atom in the heme is colored green for HbO₂ A, yellow for HbCO A and blue for deoxy-Hb A, respectively. Illustrations of the Hb A structure produced by using the graphics program PyMOL (51). Coordinates of Hb A in the oxy-, CO and deoxy-forms were obtained from Brookhaven Protein Databank files 2DN1, 2DN3, and 2DN2, respectively.