Comparative Study of Oxygen and Carbon Monoxide Binding by Hemoglobin*  

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A spin label is attached covalently to a propionic acid group of the heme in either the α or β subunits of hemoglobin. Hemoglobins, so chemically modified, show functional properties similar to those of native hemoglobins. Since electron paramagnetic resonance is sensitive to the ligation state of hemoglobin, electron paramagnetic resonance changes have been used to "follow" the sequence of ligand binding to the α and β subunits of hemoglobin. Studies on the binding of carbon monoxide with heme spin-labeled hybrid hemoglobins show that carbon monoxide binds preferably to the β chains of hemoglobin. A similar study is also carried out on the process of oxygen binding with heme spin-labeled hemoglobins. The results show that: 1) oxygen binding to hemoglobin is a random process in the absence of an allosteric effector; 2) 2,3-diphosphoglycerate causes preferential binding of oxygen to the α subunits of deoxyhemoglobin; and 3) a similar but more striking effect is observed in the presence of inositol hexaphosphate. These results, coupled with the oxygen equilibrium curves of these modified hemoglobins, are used to generate the parameters of the Monod-Wyman-Changeux model of oxygen binding of hemoglobin. It is concluded from the comparison of parameters that an allosteric effector stabilizes the T state of hemoglobin and decreases the oxygen affinity of the β subunits in the T state. The difference in the binding sequence of oxygen and carbon monoxide by hemoglobin suggests that the hemoglobin affinities for oxygen and carbon monoxide are controlled by different factors.  

A marked advancement in hemoglobin research was the discovery of significant differences in the ligand binding properties of the α and β subunits of the hemoglobin molecule. Gibson and his co-workers (1, 2) first reported the difference in the kinetics of the reaction between the methionine form of the hemoglobin subunits and various ligands (1-4). Nonequivalence of the α and β chains was also observed in both binding and dissociation of ligands with hemoglobin (5-9). For example, in oxygen pulse experiments, in which a solution of oxygen was mixed with a solution containing hemoglobin and dithionite, it was found that oxygen was bound to and then rapidly dissociated from β chains (9). Nonequivalence of the ligand binding property of α and β chains was also demonstrated in equilibrium experiments, which may be more relevant to understanding the mechanism of the cooperativity of hemoglobin binding with ligands. Ogata and McConnell (10, 11) suggested differences in oxygen binding affinity on the basis of spin label studies. By means of high resolution proton nuclear magnetic resonance (NMR), Johnson and Ho (12) showed that oxygen exhibited a slight preferential binding to the α-hemes in the absence of organic phosphates, marked preferential binding to the α-hemes in the presence of 2,3-diphosphoglycerate (DPG), and almost exclusive binding to the β-hemes in the presence of inositol hexaphosphate (IHP). They did not, however, measure the fractional saturation of hemoglobin with oxygen, and the method used for estimation may subject the values to some degree of uncertainty (13). Moreover, their assignment of α and β NMR peaks, whose changes in intensities in the ligation process were used to monitor ligand binding to the α and β chains, was questioned by others (14). More recently, Huang and Redfield (15) repeated the same experiment over a wider range of conditions. In addition, they measured the percentage of oxygen saturation spectrophotometrically in the same NMR tube. In their study, they did not find evidence for any large difference in oxygen affinity between the α and β subunits in the presence or absence of organic phosphates. From 31P NMR studies on hemoglobin labeled with trifluoracetyl groups at β93 cysteine, Huestis and Raftery (16) observed that, on the average, the chain ligation exceeds chain ligation by approximately 10% throughout most of the range. However, it was suggested that the 31P-trifluoracetyl group was more likely to be a probe of the transition of the quaternary conformers (R ↔ T) instead of that of the ligation of the β subunits (17).  

Using hybrid-heme hemoglobins containing proto- and mesohemes, Makino and Sugita (18) measured the oxygen equilibrium curves of the α and β chains separately at the isosbestic points of the partner chains. They concluded that the β chains have a slightly higher affinity for the first oxygen molecule than do the α chains.  

Similar studies comparing the ligand affinities of α and β chains of hemoglobin for CO at equilibrium conditions have been carried out in a few cases. By means of high resolution NMR, Johnson and Ho (12) showed that CO exhibits a possible but very slight preference for the α chains in the absence of organic phosphates and a small but definite preference for the α chains in the presence of HHP. These studies may have ambiguities as a result of the uncertainties mentioned above regarding their oxygen binding experiment. Raftery and Huestis (19) have extended their 31P NMR studies on CO binding to hemoglobin labeled with trifluoracetyl groups at β93 sulphydryl groups. They interpreted their ob-  

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1 The abbreviations used are: HHP, inositol hexaphosphate; DPG, 2,3-diphosphoglycerate; SL, spin label; oxyHb, oxyhemoglobin; COHb, carbonmonoxyhemoglobin; MWC, Monod-Wyman-Changeux; bis-Tris, 2,2-bis(hydroxymethyl)-2,2'-nitroethanol.
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In order to clarify the controversies surrounding the comparative affinities of the α and β chains of hemoglobin for oxygen and carbon monoxide, we addressed ourselves to this problem by using the heme spin label method.

We have shown that a spin label attached to the propionic acid group of the heme of the hemoglobin molecule is a sensitive probe of the ligand state of the heme (20–22). In the deoxygenation process, the amplitude of the EPR signal decreases as the result of changes in the spin state of the heme. The advantage of this labeling method is that it does not perturb the functional properties of hemoglobin.

Recently, we have prepared hybrid hemoglobins with the spin label attached to the propionic groups of either the α- or the β-hemes. By measuring concurrently the changes in EPR and visible spectra of the hybrid hemoglobins as the function of ligand saturation, one may be able to study the ligand binding sequence of hemoglobin. In this study, we used oxygen and carbon monoxide as ligands to demonstrate the feasibility of this new approach. An attempt was made to interpret the experimental results in terms of the currently generally accepted Monod-Wyman-Changeux (MWC) model of cooperative binding of hemoglobin with oxygen.

A preliminary report on the sequence of oxygen binding to the heme spin-labeled hemoglobins has been published elsewhere (23).

MATERIAL AND METHODS

Preparation of α2β2, and α2β2−SL=−1α2SLβ2 and α2β2SL, in which the spin label (2,2,5,5-tetramethyl-3-amino-pyrrolidine-1-oxyl) was attached to one of the propionic acid groups of either the α-heme or β-heme of hemoglobin, were prepared according to the procedure which has been described elsewhere (24). In one experiment (binding of carbon monoxide to α2β2−SL=−1), the spin labels attached to the β-hemes are 2.2.6,6-tetramethyl-4-amino-piperidine-1-oxyl. α2β2SL with these two different spin labels attached gave essentially similar results.

Measurements—Oxygen equilibrium curves of hemoglobin were measured by an Imai-type automatic apparatus (21, 26). Carbon monoxide equilibrium curves of hemoglobin were measured by the method of Anderson and Antonini (27). Partial laser flash photolysis of carboxyhemoglobin was measured by Dr. J. A. McCray of Drexel University with an apparatus described previously (28). Visible and EPR spectra were measured by a Perkin-Elmer double beam spectrophotometer and a Varian E9 EPR spectrometer interfaced with a Nicolet instrument computer (model 1074), respectively.

Carboxyhemoglobin was measured photometrically by the method of Anderson and Antonini (27). It was found that they gave identical results. Further confirmation comes from the result of partial laser of carbon-monoxidehemoglobins. The result indicates that native and spin-labeled hemoglobins are indistinguishable. These results are not unexpected because the propionic acid groups, where a spin label is attached, are extruded toward the outside of the heme pocket. In addition, as we will show later, the oxygen equilibrium curves of heme spin-labeled hemoglobin are normal.

It has been shown that the amplitude of the EPR signal decreases upon removal of ligand (O2 or CO) from the spin-labeled hybrid hemoglobin. This observation was ascribed to be the result of the change in the spin state of the heme iron from low spin to high spin accompanied by the removal of ligand from the heme. Therefore, we can use the change in the EPR amplitude of spin-labeled hemoglobin to probe ligand saturation of a particular subunit. For instance, in the process of removal of CO from CO α2β2, the change in EPR amplitude monitors the change in the ligation state of the α chains, while the change in optical spectrum results from the change in the ligation state of both the α and β chains. Therefore, a cross-plot of ΔEPR versus ΔA of α2β2SL in such a process will represent the sequence of carbon monoxide binding by hemoglobin.

RESULTS

Reaction of Carbon Monoxide with Spin-labeled Hybrid Hemoglobins—In order to ascertain that attaching spin labels to the propionic acid groups of the hemes of hemoglobin does not disturb the functional property of binding carbon monoxide, carbon monoxide equilibrium measurements on both native and spin-labeled hemoglobins were performed spectrophotometrically by the method of Anderson and Antonini (27). In Fig. 1, we show the results obtained in this study.
Figs. 2 and 3. Comparison of changes in visible and EPR spectra of heme spin-labeled hemoglobin upon removal of carbon monoxide. (A) Cross-plot of ΔA416 versus ΔEPR of α2β2 in 0.05 M phosphate (pH 7.0) in the process of removing CO from CO α2β2. (B) Cross-plot of αβ2 in 0.05 M phosphate (pH 7.0).

Similar experiments were carried out on hemoglobin in which one of the propionic acid groups of the δ-chain(s) was spin-labeled (αβ2ζζ). As shown in Fig. 2B, the experimental points deviate from the diagonal line with a downwardly concaved curve, the opposite direction from that obtained in similar experiments with αζζζζ. This result also indicates that CO preferentially binds to the δ-chain of hemoglobin.

Functional Properties of α2δβ2 and αβδζζ—Oxygen equilibrium curves of α2δδβ2 and αδζζζζ in 0.1 M phosphate, pH 7.0, are determined at 20°C, in comparison with native hemoglobin (Fig. 3). The P50 values and Hill coefficients are listed in Table I.

It is clear that the oxygen binding properties of these modified hemoglobins are normal, both in the presence and absence of organic phosphates. These results confirm those from the previous studies showing that chemical modifications at the propionic acid groups of heme do not alter the optical or the oxygen binding properties of hemoglobin (21, 29). It is also clear that the protein modification does not affect the interaction between deoxyhemoglobin and DPG. These results are consistent with those of x-ray diffraction studies on hemoglobin which show that the propionic acid groups are extruded outside the heme pocket to the polar medium (30, 31).

Deoxygenation Experiment of Spin-labeled Hybrid Hemoglobins—Fig. 4 shows the changes in visible and EPR spectra of α2δδβ2 in 0.05 M phosphate, pH 7.2, during the deoxygenation process. The fractional changes of optical absorbance at 577 nm and those of the amplitude of the EPR central resonance are cross-plotted in Fig. 5A. Within experimental error, all the experimental points lie along the diagonal line, which indicates that the oxygen binding process is a random process without any preferential binding tendency toward either the α or δ subunits. In order to investigate whether buffer conditions affect the oxygen binding property of the α and δ subunits, αζζζζζζ in different buffer conditions were tried, i.e., concentrations of phosphate ranging from 5 mM to 0.1 M, pH values from 6.5 to 7.8, and with or without 1 mM EDTA. It was found that, under all these conditions, similar results to that of Fig. 5A were observed. It was also found that experiments carried out in bin-Tris buffer gave a result similar to that in phosphate buffer.

However, the presence of organic phosphates, the curve deviated significantly from the diagonal line, especially at low oxygen saturation. Fig. 5B shows the result of deoxygenation of αζζζζζζ in the presence of 2 mM DPG, pH 7.0. It is obvious that the experimental result deviated significantly from the diagonal line at low oxygen saturation. The result indicates that the α subunit has a higher oxygen affinity in the deoxy state. The presence of 2 mM IHP causes a deviation in the same direction and to a greater extent (Fig. 5C). However, it should be noted that in the presence of 2 mM IHP, the oxygen affinity of hemoglobin is so low that about 10% of hemoglobin is in the deoxy form under atmospheric pressure. Therefore, the visible and EPR spectra of spin-labeled, fully oxygenated hemoglobin in the presence of IHP are determined indirectly. The spectra are measured before the addition of IHP and then multiplied by a dilution factor due to the addition. The

Table 1

| P50 and Hill coefficient of native hemoglobin, αζζζζζζ and αδζζζζ, in the absence and presence of 2 mM DPG |
|---|---|---|---|
| | −2 mM DPG | +2 mM DPG |
| | mm Hg | mm Hg | mm Hg |
| Hb A | 8.7 | 2.70 | 15.0 | 3.2 |
| αζζζζζζ | 8.5 | 2.62 | 14.6 | 3.2 |
| αδζζζζζζ | 8.4 | 2.72 | 14.0 | 3.1 |

a 0.1 M phosphate buffer, pH 7.0, 20°C.
b 0.1 M phosphate, 2 mM DPG, pH 7.0, 20°C.
the presence of IHP as shown by visible spectrum. EPR results were the same by both methods.

With the spin label attached to the propionic acid group of the \( \beta \)-hemoglobin (\( \alpha_2 \beta_2^{sl} \)), the cross-plot of \( \Delta \text{EPR} \) versus \( \Delta \text{A} \) in the deoxygenation process gives unexpected results. The experimental points deviate from the diagonal line in the presence or absence of organic phosphates in such a way that a slight removal of oxygen from oxyhemoglobin results in a great change in the amplitude of EPR (Fig. 5 D, E, and F). This experimental result is not unexpected. In the previous deoxygenation experiment on hemoglobin with both the \( \alpha \) and \( \beta \) chains spin-labeled, the cross-plot deviated from the diagonal line (21, 22). It is now clear that this deviation of heme spin-labeled hemoglobin is due to the nonlinear response of the \( \beta \)-heme spin label. The nature of the nonlinearity of the \( \beta \)-heme spin-labeled hemoglobin is unclear and needs to be clarified by further experiments. However, it is reasonable to suggest that the \( \beta \)-heme spin label not only monitors the conformational change of the \( \beta \) chain, but is also sensitive to the change of the quaternary structure of hemoglobin. This suggestion is supported by the experimental result obtained by the gradual addition of cyanide to heme spin-labeled methemoglobin which showed that the cross-plots of changes in EPR and optical spectra are linear for both \( \alpha \) - and \( \beta \)-heme spin-labeled methemoglobin.

In such a process, hemoglobin is always in the oxyhemoglobin-like quaternary structure. If this is correct, subtraction of the best-fitting curve of the experimental points on the deoxygenation experiment of \( \alpha_2 \beta_2^{sl} \) in the absence of organic phosphates (solid line of Fig. 5D) from the curves obtained in the presence of DPG or IHP (solid lines of Fig. 5, E and F) will result in curves which show only local conformational changes. The curves so obtained are indicated by dotted lines in Fig. 5 E and F. These results confirm the conclusion obtained from the deoxygenation experiment on \( \alpha_2 \beta_2^{sl} \) that oxygen binds to the \( \alpha \) chains of hemoglobin with a slight preference in the presence of DPG and with a still higher preference with IHP.

**DISCUSSION**

**Heme Spin-labeling Method to Study Ligand Binding Sequence of Hemoglobin**—Although there have been several studies comparing the affinities of the \( \alpha \) and \( \beta \) chains of hemoglobin for carbon monoxide and oxygen at equilibrium conditions, controversy still remains. To produce meaningful results, the experiment must satisfy several criteria. First, in order to relate the experiment observable to the change must be proportional to the change of the tertiary structure. Preferably, the change should be determined experimentally, preferably by visible spectroscopy.

We believe our approach satisfies the above criteria, at least for the experiment using \( \alpha_2 \beta_2^{sl} \). Fig. 5A shows that in 0.05 M phosphate (pH 7.2) or in 0.05 M bis-Tris (pH 7.0), the change of EPR signal of \( \alpha_2 \beta_2^{sl} \) is linearly proportional to the change of visible spectrum during deoxygenation. This linearity rules out the possibility that the spin label attached to the \( \alpha \)-heme of hemoglobin is a probe for the quaternary structure (32). Therefore, we put more weight on the results from \( \alpha_2 \beta_2^{sl} \) to compare the relative affinities of the \( \alpha \) and \( \beta \) chains of hemoglobin than our results from \( \alpha_2 \beta_2^{sl} \).

Preferential Binding of Carbon Monoxide by Hemoglobin Subunits—The result of the study on binding CO by α-chain β-chain (Fig. 2A) undoubtedly indicates that the β chains of hemoglobin have a higher affinity for CO than do the α chains of hemoglobin. A similar CO binding experiment using αβββ ββαα confirmed the preferential binding to the β chains, i.e., the curve is concave downward instead of upward (Fig. 2B).

It is of interest to compare our result with those from kinetic studies. Geraci et al. (33) showed that the isolated single β chain has about a 4 times higher binding rate constant than does the isolated single α chain (130 × 10^6 and 50 × 10^6 M^-1 s^-1, respectively). It was also shown that their CO dissociation constants are similar (0.021 and 0.012 s^-1, respectively) (34).

The equilibrium constants of the isolated single α and β chains from these kinetic studies are 2.5 × 10^9 and 11 × 10^5 M^-1, respectively, which indicates that the isolated single β chain has about a 4 times higher affinity for CO than does the isolated single α chain. On the basis of kinetic measurements of the binding of carbon monoxide to hemoglobin in 0.1 M phosphate at pH 7 or upon the addition of DPG or IHP, Gray and Gibson (8, 35) concluded that the β chains of hemoglobin have higher binding constants than do the α chains. Recent measurement of the dissociation constants of carbon monoxide from carboxyhemoglobin has been unable to detect aβ nonequivalence (36). Overall, the kinetic studies showed that the β chains of hemoglobin have a higher affinity for CO than do the α chains. This conclusion is in agreement with that obtained by the present heme spin-labeling method.

Preferential Binding of Oxygen by Hemoglobin Subunits—The result obtained by using αβββ ββαα hybrid hemoglobin showed that there was no preferential binding to a particular subunit in the absence of organic phosphates. Changes in pH, ionic strength, and buffers did not change the random binding of oxygen to hemoglobin subunits. This result agrees with that obtained by Huang and Redfield (15) in a similar NMR study. On the other hand, we observed that in the presence of DPG, α subunits exhibit a higher oxygen affinity than the β subunits; moreover, the difference in oxygen affinity is even higher in the presence of IHP. The results are different from those of Huang and Redfield (15), who concluded that no preferential binding of oxygen by subunits was observed in samples containing DPG or IHP. Our results are in qualitative agreement with those reached by Johnson and Ho (12) under this condition. Quantitatively, the two results are significantly different. For instance, in the presence of IHP and at 40% oxygen saturation, Johnson and Ho's result (12) indicates that 70% of the α chains and only 10% of the β chains are liganded; our study indicates 48 and 32%, respectively. In general, their result shows a much higher nonequivalence in oxygen affinity of the subunits than does ours.

The fact that CO and O2 show different binding preferences for the α and β subunits of hemoglobin was, at first, a puzzle to us, considering their similar sizes. However, a recent study comparing the change in ligand affinity for O2 (as well as for CO) of a series of model compounds of hemoglobin suggested that the determining factors responsible for the ligand affinity of hemoglobin for O2 and CO may be different (37). From other studies, it was also demonstrated that the shape of the dissociation curves of hemoglobin for oxygen and carbon monoxide are different (38, 39); the result implies that the binding behavior of O2 and CO to hemoglobin may be different.

It is generally assumed that the binding of oxygen and carbon monoxide to hemoglobin is similar, and, therefore, that carboxyhemoglobin can be used as a model for oxyhemoglobin. Our results show that this assumption is not justified. Using 2,2,6,6-tetramethyl-4-aminopiperidine-1-oxo as a probe to monitor the heme environments of the α and β chains of hemoglobin, it can be shown that the heme environments of both α and β chains are different in oxy- and carbonmonoxyhemoglobin.4 This ligand dependence of the heme environment affords a structural basis for the functional difference in hemoglobin binding with oxygen and carbon monoxide.

Interpretation of Experimental Results by the Two-state Model—In order to explain the cooperativity of oxygen binding by hemoglobin, two models have been proposed. The first one, formulated by Koshland et al. (40) who elaborated on a proposal by Pauling (41), is a sequential model defined in terms of the conformations and interactions of the individual chains of the hemoglobin tetramer. The second, introduced by Monod et al. (42), is defined in terms of two quaternary conformers of the whole molecule. Due to its simplicity in mathematical expression and because it is generally satisfactorily fitted to the oxygen equilibrium curves of hemoglobin (43), the latter is widely accepted by scientists involved in hemoglobin research. We shall try to explain our experimental results concerning the comparative oxygen affinity of the α and β subunits of hemoglobin and the oxygen equilibrium curves in terms of the MWC model.

The original MWC model does not take into account the functional nonequivalence of the α and β subunits of hemoglobin. Since its introduction, it has been modified to account for some experimental results (17). We shall adopt Edelstein's formulae (17). If \( K^{'} \) and \( K^{''} \) are defined as the dissociation constants of the respective α and β subunits in the R (relaxed) state, \( K_R^{'} \) and \( K_R^{''} \) are those in the T (tense) state, and \( L \) is the equilibrium constant for the two states (\( L = (T)/(R) \)), the fractional saturation of respective α and β chains, \( Y_a, Y_b \), can be expressed in terms of these parameters plus oxygen partial pressure, \( P \). A plot of \( Y = 0.5 (Y_a + Y_b) \) versus \( P \) will generate a theoretical oxygen equilibrium curve, and the plot of \( Y_a \) versus \( Y_b \) represents binding of oxygen to the α subunits of hemoglobin.

Table II lists parameters of the MWC model which best fit the experimental results in this study. \( P_o \) and \( n \) from the theoretical oxygen equilibrium curve are also listed. The cross-plots of \( Y_a \) versus \( Y_b \) are the solid line of Fig. 5 A, B, and C.

In general, the parameters chosen satisfactorily fit both the oxygen equilibrium curve and the cross-plot of \( Y_a \) versus \( Y_b \). The following conclusions can be derived from parameters of Table II. 1) The addition of DPG or IHP shifts the equilibrium \( R \leftrightarrow T \) toward \( T \), in agreement with previous studies (10, 11, 17, 43). 2) Although the allosteric effectors change the ligand affinity of the α and β subunits in the R state and that of the α subunits in the T state slightly, the predominant effect is a decrease in the oxygen affinity of the β subunits in the T state. The change in oxygen affinity in quaternary conformers as the result of interaction with allosteric effectors is not

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included in the original MWC model of oxygen binding to hemoglobin (42), but it must be included to fit better with the more accurate oxygen equilibrium curve (43). Of course, it is not possible to differentiate the change in the \( \alpha \) and \( \beta \) subunits solely on the basis of the oxygen equilibrium curve. The experimental approach reported in this communication enables us to answer this question. Experiments comparing the affinity of the \( \alpha \) and \( \beta \) subunits of hemoglobin with other ligands are in progress and the results will be reported shortly.

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