Sequence of oxygen binding by hemoglobin

(hemoglobin subunits/spin-labeling/electron paramagnetic resonance/heme-heme interactions)

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ABSTRACT A nitroxide spin-label probe was attached directly to a propionic acid group of heme in either the α or the β chain of hemoglobin. The electron paramagnetic resonance (EPR) spectrum of the spin label is altered by the spin-state change of the heme iron to which the spin label is attached. These hybrid hemoglobins showed normal optical and functional properties, indicating that the attachment of the spin label did not perturb the function of hemoglobin. Upon deoxygenation of α -heme-spin-labeled hemoglobin, EPR signals changed proportionally with oxygen saturation (determined by measuring absorption spectra). This result indicates that there is no binding preference between the α and β chains of hemoglobin. However, the cross plot for the fraction of the EPR changes vs. the fraction of oxygen saturation deviated significantly from the diagonal straight line in response to the addition of 2,3-diphosphoglycerate and inositol hexaphosphate. The deviation indicated that the EPR change precedes the optical change at low oxygen tension. This result implies that, in the presence of organic phosphate, oxygen binds preferentially to the α subunit of deoxyhemoglobin. This conclusion was supported by the result obtained with β -heme-spin-labeled hemoglobin: the direction of the deviation for β -heme-spin-labeled hemoglobin in the presence of diphosphoglycerate and inositol hexaphosphate was opposite to that obtained for α -heme-spin-labeled hemoglobin. However, the curve deviated even in the absence of organic phosphate. This deviation for β -heme-spin-labeled hemoglobin can be explained by the intersubunit interaction of hemoglobin. From these results, it was concluded that, in the absence of organic phosphate, oxygen combines with the α and β chains with equal probability whereas, in the presence of organic phosphate, oxygen binds preferentially to the α chains of hemoglobin.

Although the structural, spectral, and functional nonequivalence of isolated α and β chains of hemoglobin is well known, the affinity of these individual chains for oxygen in native tetrameric hemoglobin is not well understood. Published data concerning the sequence of oxygen binding by the four hemoglobin subunits are conflicting.

Perutz (1) speculated that oxygen will first combine with the α subunit because there is ample room in the heme pocket of α chain in both the oxy and deoxy forms. Gray and Gibson (2) reported that the β chain binds carbon monoxide at a faster rate than does the α chain. We have concluded from the analysis of oxygen equilibrium curves of hemoglobin that the oxygen affinities of unlike chains are relatively similar in stripped hemoglobin but differ greatly in the presence of 2,3-diphosphoglycerate (2,3-DPG) and inositol hexaphosphate (InsP₆) (3). However, we could not determine which of the two chains has a higher affinity for oxygen. On the basis of NMR studies of normal hemoglobin (Hb A), Johnson and Ho (4) reported that the α subunit binds oxygen more strongly than does the β subunit in the presence of organic phosphates. In contrast, by similar

NMR studies, Huang and Redfield (5) did not detect a clear binding preference for oxygen by the α subunit relative to the β subunit. These discrepancies may be attributed to the lack of a technique by which the changes specific to each chain can be measured accurately without affecting the oxygen binding properties of hemoglobin. The heme-spin-label method may be an advantageous method by which to study the sequence of oxygen binding by hemoglobin. The direct attachment of a spin label to the propionic group of heme at either the α or the β chain does not affect the oxygen binding properties of hemoglobin (6), although the spin label can sense changes in paramagnetism during the oxy-deoxy transition of the specific heme to which it is attached. This paper describes studies on the sequence of oxygen binding to hemoglobin by using α - or β -heme-spin-labeled hemoglobin ($\alpha_2^{SL}\beta_2$ or $\alpha_2\beta_2^{SL}$).

EXPERIMENTAL PROCEDURE

Heme-Spin-Labeled Hybrid Hemoglobin. The oxy form of heme-spin-labeled hemoglobin in which all four hemoglobin subunits contain mono-spin-labeled protoheme was prepared by a method described elsewhere (6, 7). The α and β chains containing spin-labeled heme were isolated from the heme-spin-labeled hemoglobin by the method of Bucci and Fronticelli (8). *p*-Chloromercuribenzoate was removed from the subunits according to DeRenzo *et al.* (9). The α - and β -heme-spin-labeled hemoglobin subunits were then recombined with non-spin-labeled partner subunits prepared by the same technique from native hemoglobin. This hemoglobin was purified by chromatography on CM-cellulose to remove excess hemoglobin subunits and small amounts of denatured product (6).

Measurements. Electron paramagnetic resonance (EPR) spectra of spin-labeled hemoglobins were measured in a quartz flat cell (Varian) by using a Varian E-9 EPR spectrometer at ambient temperature. The EPR spectra were analyzed with a Nicolet Instrument computer, model 1074. The oxygen saturation of the sample in the quartz flat cell was measured optically with a Perkin-Elmer Coleman 126 spectrophotometer. The oxygen equilibrium curves of hemoglobins were determined with an Imai-type automatic apparatus (6, 10).

Reagents. Protohemin and other reagents were purchased from Sigma. The spin-label, 2,2,5,5-tetramethyl-3-aminopyrrolidine-1-oxyl, was purchased from Eastman.

Abbreviations: 2,3-DPG, 2,3-diphosphoglycerate; InsP₆, inositol hexaphosphate; EPR, electron paramagnetic resonance; $\alpha_2^{SL}\beta_2$, α -heme-spin-labeled hemoglobin; $\alpha_2\beta_2^{SL}$, β -heme-spin-labeled hemoglobin.

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RESULTS

Optical and Oxygen Binding Properties of α - and β -Spin-Labeled Hemoglobins. To determine if the physical properties as well as the oxygen-binding properties of reconstituted hemoglobins were perturbed by the attachment of a spin label at the propionic acid group of the heme, we measured the absorption spectra and oxygen-binding properties of α -, β -, and α , β -heme-spin-labeled hemoglobins. The absorption spectra of the oxy, deoxy, and carboxy forms of these hemoglobins were identical to those of corresponding native hemoglobins. All hemoglobin samples used for these experiments contained no detectable amounts of methemoglobin or hemichrome. The oxygen-binding properties of these hemoglobins were normal and within the range of experimental error (Table 1). These results confirmed results of previous studies (6, 11) showing that chemical modifications at the propionic acid group of heme do not alter either the optical or oxygen-binding properties of hemoglobin. This may be explained by the orientation of the heme in the heme pocket. X-ray crystallographic studies of hemoglobin show that the propionic acid groups are extruded outside the pocket to the polar medium (12).

EPR Spectrum of α - and β -Spin-Labeled Hemoglobins. EPR spectra of the oxy and deoxy forms of two types of hybrid hemoglobins are compared in Fig. 1. The line shapes (particularly the line shapes of lower magnetic fields) for α - and β -spin-labeled hemoglobins showed distinct differences, an indication that there is a difference between protein conformations in the α and β chains in the vicinity of the spin label.

Effect of Deoxygenation on EPR Spectra of Hybrid Hemoglobin. As shown previously (3, 6), the spin label attached directly to heme is affected by the conformational changes in the vicinity of the spin label and by magnetic dipolar interaction due to the spin-state change of the iron in the heme to which the spin label is attached. The conformational changes give rise to changes in the EPR line shape, whereas the magnetic effect results in a decrease in the EPR signal amplitude (3, 6, 13). If an oxygen is removed from a spin-labeled heme, the amplitude of the EPR signal of that spin label decreases because the spin state of the heme changes from diamagnetic oxyheme to paramagnetic high-spin deoxyheme. Because the spin label is attached only to one kind of hemoglobin subunit, we can assume that the change in the amplitude of the EPR signal during the oxy-deoxy transition is mainly due to a change of the ligand state of those spin-labeled subunits. On the other hand, the change in visible spectrum is due to changes in both spin-labeled and non-spin-labeled subunits. Therefore, a cross plot of the change in amplitude of the EPR signal vs. the change in visible absorbance at 577 nm during the oxy-deoxy transition of hemoglobin will allow us to determine the sequence of oxygen binding by hemoglobin.

Fig. 2A shows the cross plot of $\alpha_2^{\text{SL}}\beta_2$ in 0.05 M phosphate buffer (pH 7.2) at room temperature. Within experimental error, all points lie on the diagonal line, indicating an equal probability of oxygen binding to either the α or the β subunit.

Table 1. Oxygen binding parameters of normal adult hemoglobin and two heme-spin-labeled hybrid hemoglobins

	P_{50} , mm Hg	n
HbA	8.7	2.70
$\alpha_2^{\text{SL}}\beta_2$	8.5	2.62
$\alpha_2\beta_2^{\text{SL}}$	8.4	2.72

Experiments were carried out in 0.1 M potassium phosphate buffer, pH 7.0, at 20°C.

FIG. 1. EPR spectra of the oxy (—) and deoxy (---) forms of α - and β -heme-spin-labeled hemoglobin (Upper and Lower, respectively).

On the other hand, the addition of DPG (Fig. 2B) or InsP_6 (Fig. 2C) caused a significant deviation from the diagonal line, with the EPR change preceding the optical change at low oxygen pressure. Moreover, InsP_6 had a greater effect than DPG. These results imply that, in the presence of organic phosphate, oxygen binds preferentially to the α subunit of deoxyhemoglobin and that this preferential oxygen binding is stronger in the presence of InsP_6 than with DPG.

Similar experiments were performed using $\alpha_2\beta_2^{\text{SL}}$. As shown in Fig. 2F, in the presence of InsP_6 , the change of EPR amplitude occurred after the change in absorbance at 577 nm. Furthermore, this delay was greatest at high oxygen saturation. When the oxy form of $\alpha_2\beta_2^{\text{SL}}$ was subject to deoxygenation, however, the change of EPR signal preceded the change in absorbance at 577 nm. The result is consistent with the conclusion derived from $\alpha_2^{\text{SL}}\beta_2$.

The results in the absence of InsP_6 (Fig. 2D) are somewhat unexpected. The curve for $\alpha_2\beta_2^{\text{SL}}$ deviated significantly from the diagonal line, whereas that for $\alpha_2^{\text{SL}}\beta_2$ did not. This discrepancy may be the result of heme-heme interaction, a geometrical change in the iron-spin-label distance of the β chains brought about by the binding of oxygen to the α chains.

DISCUSSION

Heme-Spin-Label Method. The spin-label method was first introduced by McConnell and his associates as a sensitive probe technique for studying the structure of proteins and membranes (14, 15). We have succeeded in the covalent attachment of a spin label directly to the propionic acid groups of heme in hemoglobin (6). The advantage of this spin-labeling method is that the optical and oxygen-binding properties of hemoglobin do not change after labeling. Therefore, results obtained from heme-spin-labeled hemoglobin also apply to native hemoglobin. Furthermore, the spin label is sensitive to the heme environment, as demonstrated by its ability to discern the α - β non-

FIG. 2. Fraction of changes in central resonance of EPR spectra (ΔEPR) vs. fraction of optical changes (ΔA). Experiments were carried out with a heme concentration of 1 mM in 0.05 M potassium phosphate buffer, pH 7.2. (A) $\alpha_2^{SL}\beta_2$; (B) $\alpha_2^{SL}\beta_2$ + 2 mM DPG; (C) $\alpha_2^{SL}\beta_2$ + 2 mM $InsP_6$; (D) $\alpha_2\beta_2^{SL}$; (E) $\alpha_2\beta_2^{SL}$ + 2 mM DPG; (F) $\alpha_2\beta_2^{SL}$ + 2 mM $InsP_6$.

equivalence in the oxy and deoxy states (Fig. 1), and is sensitive to changes in the ligand state of heme iron (Fig. 1). It is this latter property that we utilized to study the oxygen-binding sequence of hemoglobin.

It is assumed that, because of the distance, no direct intersubunit iron-spin-label interaction can be detected although indirect effects can occur—i.e., ligand binding to one subunit changes the conformation or the spin state of the partner subunit (see below). Therefore, the measurement of the relative changes of EPR and optical spectra in α - and β -spin-labeled hemoglobins at various oxygenation steps allows us to determine the relative binding of oxygen at α and β chains of hemoglobins.

Interpretation of the Results. As shown in a previous paper (6), deoxygenation of the heme-spin-labeled hemoglobin causes a decrease in the central resonance amplitude of the spin-label signal. This decrease is attributed to the change of the paramagnetism of the heme iron from diamagnetic oxyheme to high-spin deoxyheme. Because the magnetic dipolar interaction between two paramagnetic components decreases relative to the square of the distance (16), the spin-label signal is minimally affected by the spin-state change of the heme in the partner subunit in the same molecule. Thus, from the degree of the magnetic effect on the spin-label signal amplitude, one can estimate the average distance between the heme iron and the spin label (14, 15). The distance calculated for hemoglobin is 12.5 Å, and this value agrees with that calculated by attaching a spin-label molecule to the Perutz hemoglobin model (17).

The same theory can also be applied to the interpretation of the present results. For instance, in the case of β -spin-labeled deoxyhemoglobin, the central resonance amplitude is maximally decreased by the magnetic dipolar interaction of the paramagnetic deoxy β -heme with the spin label. Upon the addition of a small amount of oxygen, the spin-label signal increases only when oxygen binds with the heme to which a spin label is attached. On the other hand, the optical changes are linearly related to the fractional oxygen saturation of hemoglobin regardless of oxygen binding to either the α or β chain. Therefore, if oxygen combines with α chains first, this binding is detected by the optical change but not by the EPR signal. This will result in a deviation of the curve to below the diagonal straight line (Fig. 2D). If this assumption is correct, we should see a deviation in the opposite direction for the same experiment with $\alpha_2^{SL}\beta_2$. As shown in Fig. 2C, in the presence of $InsP_6$ the curve deviated in an upward direction with $\alpha_2^{SL}\beta_2$. These results support the belief that, in the presence of $InsP_6$, the oxygen molecule preferentially combines with the α chain as first speculated by Perutz (1). These results are also consistent with those of Johnson and Ho (4).

Heme-Heme Interaction. The results for the α - and β -spin-labeled hemoglobins in the absence of $InsP_6$ are somewhat conflicting. The EPR changes for $\alpha_2^{SL}\beta_2$ are almost linearly related to the optical changes (Fig. 2A), whereas those for $\alpha_2\beta_2^{SL}$ deviate downward (Fig. 2D). This apparent discrepancy may be explained by the effect of the heme-heme interaction of hemoglobin—i.e., the binding of an oxygen molecule to one subunit affects the conformation or spin state of the other subunits. In other words, the binding of the oxygen to the α subunit indirectly affects the conformation of the deoxy β -subunits, resulting in a change in the dipolar interaction between the deoxy β -heme and the spin label. Two possible changes in the β chains are an increase in the paramagnetism in the β -heme iron or a shortening of the distance between the heme iron and the spin label.

This type of intersubunit interaction has been demonstrated by iron/EPR (18), NMR (19), and spin-label (20) measurements of valency hybrid hemoglobins. The experimental results obtained in the absence of $InsP_6$ can be explained by assuming that oxygen combines with α and β chains almost equally and that the binding of oxygen to the α chain affects the conformation of the β chains. In the presence of $InsP_6$, oxygen combines preferentially with the α chains.

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