Electron Paramagnetic Resonance Studies of Spin-Labeled Hemoglobins and Their Implications to the Nature of Cooperative Oxygen Binding to Hemoglobin*

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Abstract. The spin label technique has been used to study human hemoglobins A, F, Zürich, and Chesapeake as a function of carbon monoxide saturation. The experimental results suggest that the changes in the electron paramagnetic resonance spectra of hemoglobin labeled with N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide depend on the state of ligation of more than one heme group. For those hemoglobins with full or large cooperative ligand binding (such as A, F, and Zürich), there is a lack of isosbestic points in the spectra as a function of CO saturation. However, for those hemoglobins with little or no cooperative ligand binding (such as Chesapeake and methemoglobins), there is a sharp set of isosbestic points. These findings confirm and extend the early work of McConnell and co-workers. The absence of a set of isosbestic points in those hemoglobins with full cooperative ligand binding is consistent with the sequential model of Koshland, Némethy, and Filmer for cooperative oxygen binding to hemoglobin. The present results, with hemoglobin variants having known amino acid substitutions, also focus on the importance of the interactions among the amino acid residues located at α1-β2 or α2-β1 subunit contacts for the functioning of hemoglobin as an oxygen carrier. In addition, the resonance spectra of the spin label are very sensitive to small structural variations around the heme groups in the β- or γ-chains where the labels are attached. The results of the spin label experiment are discussed in relation to recent findings on the mechanism of oxygenation of hemoglobin from the nuclear magnetic resonance studies of this laboratory and the x-ray crystallographic analysis of Perutz and co-workers.

Introduction. Recently, McConnell and his co-workers have developed an elegant technique known as the "spin label" method to study macromolecular conformations.1 The basis of this technique is to introduce a stable nitroxide-free radical into a macromolecule. The electron paramagnetic resonance (epr) spectrum of a spin label attached to a macromolecule in solution or in a single crystal is very sensitive to the correlation time which describes the motion of the spin label.1 Since the rotational diffusion rate of the entire macromolecule is usually too slow to affect the epr spectrum of the attached label, the resonance spectra depend on the motion of the spin label relative to the macromolecule.1 Hence, the resonance spectra of the spin labels reflect changes in local conformation.
McConnell and co-workers\textsuperscript{3-4} have used this technique to investigate the nature of the cooperative oxygen binding to hemoglobin (Hb). Based on the epr spectra of human adult Hb A and horse Hb labeled with N-(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl)iodoacetamide (I) and N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide (II), they have found that (a) at intermediate stages of oxygenation there are some molecules in solution which must have a structure that differs from either the oxy- or deoxyhemoglobin structure in the vicinity of the labeled cysteine $\beta$-93; and (b) the spin label detects conformational changes which arise from changes in the state of ligation of the $\alpha$- as well as the $\beta$-hemoglobin. They pointed out that the spin labels I and II are attached to $\beta$-93 SH group of cysteine (helix position F9) and this amino acid is next to proximal histidine $\beta$-92(F8) whose imidazole ring is directly bonded to the iron atom of the heme group. They also observed that the cooperative oxygen binding to hemoglobin is not markedly affected by the spin labels (the Hill coefficient, $n = 2.3$ instead of $n = 2.9$ for unlabeled Hb), but the oxygen affinity of the spin-labeled Hb is increased by a factor of approximately 10.

The main purpose of this communication is an attempt to answer the question: Do the changes observed in the epr spectra of the spin-labeled Hb in going from deoxy- to oxy-form relate to the cooperative oxygen binding to hemoglobin? Our basic approach to this problem is to choose those human hemoglobin variants whose structure-function relationship can help us settle the above problem. We have chosen the following three human genetic variants of Hb: fetal, Zürich, and Chesapeake. The Hill coefficient which is a measure of cooperative oxygen binding is the same for fetal and adult Hb (i.e., $n = 2.9$).\textsuperscript{2} Hemoglobins A and F have the same normal but different reverse Bohr effects, the reverse Bohr effect in Hb F being only about half that of Hb A.\textsuperscript{6,7} Hence, the results of Hb F will be a good check on those of Hb A. Hb Zürich is an abnormal hemoglobin in which the distal histidine residue at $\beta$-63 position has been replaced by an arginine residue.\textsuperscript{8,9} According to Winterhalter et al.\textsuperscript{,10} Hb Zürich has 2.5-4 times higher oxygen affinity than Hb A, a decreased value of the Hill coefficient ($n = 1.8$), and a normal Bohr effect. Nagel, Gibson, and Charache\textsuperscript{11} reported that Hb Chesapeake is characterized by a higher oxygen affinity (six times higher than Hb A), a significantly decreased or absent cooperative oxygen binding (Hill coefficient, $n = 1.3$), and a normal Bohr effect. There is no difference in the reactivity of the sulfhydryl group at $\beta$-93 toward iodoacetamide when Hb Chesapeake is going from oxy- to deoxy-form.\textsuperscript{11} The position of amino acid substitution in Hb Chesapeake is $\alpha$-92(FG4), i.e., arginine is replaced by leucine\textsuperscript{12} which is located at $\alpha_1-\beta_2$ subunit contacts.\textsuperscript{12,14} All known human Hb variants which have amino acid substitutions at $\alpha_1-\beta_2$ contacts exhibit diminished subunit interactions.\textsuperscript{13} The recent proton nuclear magnetic resonance (nmr) results of Ho et al.\textsuperscript{,14} on Hb A and Hb Chesapeake suggest that there are differences in the aromatic proton resonances between these two hemoglobins which are related to the altered interactions among the amino acid residues located at $\alpha_1-\beta_2$ contacts. Furthermore, Perutz and Greer\textsuperscript{16} have recently made a comparison of the x-ray diffraction patterns of Hb A and Hb Chesapeake in both oxy- and deoxy-forms at a resolution of 5 Å. They found that there is only a small difference in the electron density maps between Hb A and Hb Chesapeake.
in the deoxy-form. According to them, this small difference can be explained by the substitution of a bulky arginine by leucine in Hb Chesapeake. They found that crystals of HbO₂ Chesapeake were isomorphous with those of HbO₂ A. From an analysis of the difference Fouriers between oxyhemoglobins A and Chesapeake, they found that there are considerable differences between these two proteins. For these reasons, Hb Chesapeake is an excellent genetic variant for checking whether the conformational changes detected by spin label II are involved in the cooperative binding of oxygen.

Materials. In some experiments, Hb A was prepared from fresh human blood samples obtained from the local blood bank by the usual procedure and the red cells were lysed either by toluene or distilled water. In other studies, Hb A was isolated from heterozygotes of Hb Chesapeake and of Hb Zürich. Red cells were lysed with distilled water and CCl₄ and Hb A and Hb Chesapeake as well as Hb A and Hb Zürich were separated by chromatography on DEAE-Sephadex by the respective procedures of Huisman and Dozy. Hb F was eluted from fresh cord blood samples obtained from the Magee Women's Hospital on CM-Sephadex by the procedure of Zade-Oppeg. All Hb solutions used in this study were converted to the CO-form before column chromatography. The purity of the Hb samples was tested by electrophoresis on the Gelman cellulose acetate strips. In the case of Hb F, it is also assayed by the standard alkaline denaturation procedure.

The spin label used in this study was N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide (II). The procedure for introducing the spin label to hemoglobin was the same as that described by Ogawa et al. In our studies, we have used HbCO instead of HbO₂. This is particularly advantageous because Hb Zürich and Hb Chesapeake are very sensitive to autooxidation. Deoxyhemoglobin was prepared from HbCO by first passing oxygen to remove CO and then passing oxygen free nitrogen or argon to remove oxygen. As judged by visible spectrum, deoxyhemoglobin samples used in our study were better than 95% in the deoxy-form and contained less than 2% methemoglobin. Hb samples for the spin labeled epr studies were prepared in 0.05 M phosphate at pH ~7. pH readings were obtained directly from a Radiometer pH meter model 26 in conjunction with a Beckman model 39030 frit junction combination electrode. All other chemicals used were of the best commercial grades and were obtained from major suppliers. They were used without further purification.

Method. A Bruker B-ER418s electron paramagnetic resonance spectrometer operating at the x-band frequency (about 9.5 kHz) was used to obtain the epr spectra. The ambient temperature inside the microwave cavity was 23°C.

Results. Figures 1–4 give the sensitive portion of the epr spectra of hemoglobin A, F, Zürich, and Chesapeake labeled with II as a function of the degree of carbon monoxide saturation. Our results for Hb A are very similar to those reported by Ogawa, McConnell, and Horwitz. In particular, Figure 1 shows a lack of isosbestic points for Hb A as a function of CO saturation which is a direct confirmation of that reported by Ogawa et al. An analysis of the spectral features of Hb A as a function of oxygen saturation was presented by these authors. We have found no isosbestic points for Hb F and Hb Zürich as a function of CO saturation (Figs. 2 and 3). In the case of Hb Chesapeake, there is a set of sharp isosbestic points (Fig. 4). In one experiment, we used 1% Hb Chesapeake in 0.05 M phosphate at pH 7.0 as a function of two intermediate CO saturations and in the second experiment, we used 2% Hb Chesapeake in 0.05 M phosphate at pH 7.0 as a function of only one intermediate CO saturation. We have found a set of sharp isosbestic points in both cases. It is noted that there are spectral
differences among these four hemoglobins (Fig. 5). The meanings of these spectral differences will be discussed in the next section.

Figure 6 gives the spin labeled epr spectra of methemoglobins F and Chesapeake as a function of hydroxide ion concentration. There are sharp sets of isosbestic points for these two hemoglobins in going from pH 6.6-8.8.

**Discussion.** Although McConnell and co-workers have shown that the spin label II senses more than two protein conformations (i.e., there exist intermediate structures during the oxygenation of hemoglobin), it could be argued that the spectral changes detected at cysteine β-93 are not necessarily related to the mechanism of cooperative oxygen binding. We interpret our results as evidence in support of their conclusions that the changes in the epr spectra are involved in the cooperative oxygen binding to hemoglobin. First, the hemoglobins A, F, and Zürich that we looked at with full or large cooperative ligand binding lack sets of isosbestic points. Secondly, there is a sharp set of isosbestic points for Hb Chesapeake labeled with II. The Hill coefficient for Hb Chesape
peake is $n = 1.3$ which suggests that there is very little or no cooperative oxygen binding in this abnormal hemoglobin. Finally, the binding of ligands, e.g., hydroxide, cyanide, and azide ions to methemoglobins, which are noncooperative, give sharp sets of isosbestic points (Fig. 6). McConnell et al. have also observed a sharp set of isosbestic points in the binding of CN$^-$ to methemo-
globin with spin label II. In addition, the absence of a set of isosbestic points indicates that the spin label II senses more than two protein conformations during the oxygenation or the carbomonoxylation process in Hb A, Hb F, and Hb Zürich. Hence, this result is not consistent with the model of Monod, Wyman, and Changeux\textsuperscript{25} for the R \rightleftharpoons T conformational change in the cooperative oxygen binding to hemoglobin but is compatible with the "symmetry-breaking" or the sequential model of Koshland, Némethy, and Filmer\textsuperscript{26} as previously suggested by McConnell and co-workers.\textsuperscript{8}

It is interesting to compare the present results of spin labeled hemoglobins with those of the nmr of Ho and co-workers.\textsuperscript{15} They found that there are differences in the aromatic proton resonances between Hb A and Hb Chesapeake which are most likely due to the altered interactions among the amino acid residues located at \(\alpha_1-\beta_2\) subunit contacts as a result of amino acid substitution at \(\alpha-92\) (FG4) in Hb Chesapeake and that the amino acid residues at FG3 (leucine) and/or FG5 (valine) may be the residues which are observed to undergo conformational change as they transmit information between \(\alpha_1\) and \(\beta_1\)-chains, or vice versa, in the course of oxygenation of hemoglobin. Both nmr and epr results suggest that there are differences in the subunit interactions between Hb A and Hb Chesapeake and these differences could be the cause of some of the abnormal functional properties observed in Hb Chesapeake. Haber and Kosland\textsuperscript{27} recently reported the oxygen saturation curves of the mixed state hemoglobins, [(\(\alpha\)-chains in deoxy)(\(\beta\)-chains in cyanomet)] and [(\(\alpha\)-chains in cyanomet) (\(\beta\)-chains in deoxy)]. They found that the Hill coefficient for the first case is 1.0 and that of the second case is 1.3 (which is the same as that for Hb Chesapeake). They suggested that there are appreciable \(\beta-\beta\) interactions in this second mixed-state Hb and no \(\alpha-\alpha\) interaction in the first mixed-state Hb. The present epr results on the spin labeled Hb Chesapeake suggest that there are essentially no subunit interactions of the type \(\alpha_1-\beta_2\) in this hemoglobin.\textsuperscript{28} It is tempting to speculate that the two abnormal \(\alpha\)-chains in Hb Chesapeake are in some kind of "frozen" conformation similar to that in one of the mixed-state hemoglobins, i.e., [(\(\alpha\)-chains in cyanomet)(\(\beta\)-chains in deoxy)].\textsuperscript{27}

Qualitative spectral differences have been observed among Hb A, Hb F, Hb Zürich, and Hb Chesapeake in both CO- and deoxy-forms (Fig. 5). Since spin label II is attached to \(\beta-93\) (F9) or \(\gamma-93\) in the case of Hb F) which is next to the proximal histidine (F8) at \(\beta-92\) or \(\gamma-92\), it is reasonable to assume that the resonance spectra of spin label II can also reflect the heme environment of the \(\beta\)-chain or \(\gamma\)-chain to which it is attached. This conclusion is in agreement with the nmr results of Ho and co-workers on the environment of the heme groups in these four hemoglobins.\textsuperscript{29,30} There are differences in the nmr spectra of the heme group protons between Hb A and Hb F in the cyanomet-form.\textsuperscript{29} They interpreted these differences as caused by altered interactions between amino acid residues at \(\gamma-70\) and \(\gamma-71\) with the corresponding methyl groups in the pyrrole rings IV and I respectively as compared to those at \(\beta-70\) and \(\beta-71\). The amino acid residues at \(\gamma-70\) and \(\gamma-71\) are serine and leucine which replace alanine and phenylalanine at the corresponding positions in the \(\beta\)-chain of human Hb A.\textsuperscript{31} A more significant difference in the nmr spectra of the heme-group protons between MetHbCN A and MetHbCN Zürich has been observed.\textsuperscript{32} This differ-
ence no doubt reflects an altered heme environment in Hb Zürich due to the substitution of the distal histidine (E7) at β-63 by an arginine residue. Similar differences in the proton resonances of the heme group protons have been observed in the azido-methemoglobins A, F, Zürich, and horse. More recently, Ho and co-workers have found differences in the nmr spectra of the heme-group protons in deoxyhemoglobins A, F, Zürich, and Chesapeake. They have been able to assign some of the heme methyl groups to the α- and β-chains by appropriate choice of Hb variants. Even though there are drastic epr spectral differences (i.e., the presence and absence of isosbestic points) between Hb A and Hb Chesapeake, there are minimum differences in the over-all spectral features between these two proteins. It is not difficult to understand the above result if one realizes that the position of the amino acid substitutions in Hb Chesapeake is α-92 which is not located in the immediate vicinity of the heme group. Ogawa et al. have pointed out earlier that most of the spectral changes sensed by spin labels I and II are caused by oxygenation of the β-chain. There are significant spectral differences among hemoglobins A, F, and Zürich. These results are reasonable in view of the amino acid substitutions in the immediate vicinity of the heme groups in the γ-chain of Hb F and in the β-chain of Hb Zürich. It should be mentioned that the spectral differences are more pronounced among these four hemoglobins in the CO-form than in the deoxy-form. These results indicate that epr as well as nmr techniques are extremely sensitive to small structural variations around the heme groups in hemoglobins.

The present experimental results suggest that resonance spectra of the spin label II can detect not only the tertiary structural changes around the heme group in both β- and γ-chains but can also sense the subunit-subunit interactions associated with cooperative oxygen binding to hemoglobin.

In conclusion, we have confirmed the previous observations of McConnell and co-workers that the epr spectra of hemoglobin labeled with N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide can detect intermediate structures in the oxygenation of Hb A as well as several hemoglobin variants, Hb F and Hb Zürich, which exhibit cooperativity and have essentially normal α1-β2 subunit interactions. On the other hand, the epr spectra of Hb Chesapeake, a variant that displays little or no cooperativity and which has an amino acid substitution in the α1-β2 subunit contact region, give no evidence of intermediate structures. This is strong confirmation of the conclusions of McConnell and co-workers that the intermediate structures detected by spin labeling are likely to be associated with cooperativity and that the region of α1-β2 subunit contact is fundamentally important to the cooperative oxygenation process. The latter conclusion is in agreement with Perutz's model of hemoglobin, McConnell and co-workers' earlier work, and Ho and co-workers' recent nmr data. In addition, the epr spectra of hemoglobin labeled with N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide are also very sensitive to changes in the heme environment of the β- and γ-chains where the labels are attached to the SH groups of cysteines located at position 93 in the respective chains.

Our spin labeled epr and nmr studies of hemoglobins have provided some new information on the nature of the cooperative oxygen binding. It should also be mentioned that we would not have been able to propose such detailed interpreta-
tions of the magnetic resonance results without the help of the elegant x-ray crystallographic findings of Perutz and co-workers on the structure of hemoglobin.

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