Heme-Spin Label Studies of Hemoglobin

I. PREPARATION AND PROPERTIES OF HEME-SPIN-LABELED FERRIHEMOGLOBIN*

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SUMMARY

In order to investigate the protein conformation in the vicinity of the heme and the spin states of the heme-iron in dissolved hemoglobin, a nitroxide spin label was covalently attached to one of the propionic acid groups of the porphyrin ring. The optical spectra of the heme-spin labeled ferrihemoglobins were identical to those of native hemoglobins indicating that the spin labeling did not affect significantly the electronic structure of the prosthetic group. The EPR spectra of the nitroxide moiety in the fluoride, cyanide, and azide derivatives of the heme-spin-labeled hemoglobin and of the corresponding acid methemoglobin in solution were not identical, suggesting that the protein conformation in the vicinity of the label is different in each of these hemoglobin derivatives.

From the strength of this interaction, the distance between the heme-iron and the nitroxide radical was estimated as 11.8 Å. Since the spin label attached to heme is sensitive to changes in the magnetic moment of the iron, the heme-spin-labeled ferrihemoglobin can also be used for studying the thermal equilibrium between high and low spin electronic states of the heme-iron. By comparing the nitroxide resonance amplitudes of the hydroxide form with those of the high spin acid met form and the low spin cyanide form of hemoglobin, the ratio of high to low spin components of the hydroxide was calculated as 53:47 at 0°. This ratio was increased at higher temperatures due to the shift of the equilibrium composition in favor of the high spin form.

Optical and EPR spectra of free spin-labeled protohemoglobin were also investigated. EPR spectra of protohemoglobin were sensitive to dimer formation of hematin in aqueous alkaline solution.

Two empirical indices are presented for convenient numerical expression of the mobility of a nitroxide label from its EPR line shape.

X-Ray crystallographic studies by Perutz and associates (1, 2) have provided three-dimensional structures of human and horse hemoglobins and have made possible discussions of the relations between hemoglobin structure and function at the molecular level. Structural studies of dissolved hemoglobin are the next urgent requirement for determination of the physiologically important regulation of hemoglobin function, especially the molecular mechanism of the effect of pH, organic phosphates, and CO2 on oxygen binding by hemoglobin. The intermediate structure between oxy and deoxy forms of hemoglobin is of potential value in understanding the mechanism of heme-heme interaction as well as the sequence of oxygen binding to hemoglobin tetramers.

For this purpose, the spin label technique developed by McConnell et al. (3) has been used in studies of hemoglobin in solution by labeling the heme group directly with nitroxide-free radicals. Preliminary experiments with this heme-spin label method showed that conformational changes in the vicinity of the heme and the spin state of the heme-iron are reflected in the nitroxide EPR spectra (4-7).

The present paper describes the preparation and properties of heme-spin-labeled ferrihemoglobin in which all four subunits contain mono-spin-labeled protoheme.

METHODS AND MATERIALS

Hemoglobin and Apohemoglobin—Crystalline hemoglobin was obtained from human blood by the method of Drabkin (8). The hemoglobin crystals were dissolved in cold-distilled water at a final concentration of 3 mM and bubbled with carbon monoxide gas to convert oxyhemoglobin to the carbonmonoxym form. The pH of the solution was then adjusted to 2.5 by the addition of 1 N HCl while stirring gently with a Vortex mixer. The solution was immediately mixed with 2 volumes of cold 2-butanol containing a few drops of 1 N HCl and shaken by inverting the test tube 2 to 3 times. The mixture was allowed to crystallize at 4°C over a period of 2 to 3 days.

The crystal was then quickly washed with cold water and transferred to a glass cell containing 1.0 M NaCl. The cell was placed in a cold room at 4°C. To dissolve the crystals, the solution was allowed to warm to 6°C and then brought to 6°C by adding 1 N HCl drop by drop to the solution.

The solution was then allowed to equilibrate at 6°C for a period of 2 to 3 days. The solution was then brought to 6°C by adding 1 N HCl drop by drop to the solution.

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to stand at 0° for a few minutes until the organic phase was clearly
separated from the colorless lower globin phase. The butanone
phase which contained free hemin was siphoned off. The colored
butanone phase remaining on the top of the aqueous globin solu-
tion was completely removed by repetitive addition and removal
of cold 2-butanol. Each new solution of 2-butanol was care-
fully added so that no mixing occurred with the aqueous globin
solution. The remaining aqueous phase was treated again with 2
volumes of butanone as described above. The globin solution was
dialyzed first against several changes of cold-distilled water
and then against 10 mM potassium phosphate buffer, pH 7.0. The
slight precipitates formed during the dialysis were removed either
by centrifugation or by filtration through Whatman No. 5 filter
paper. The globin solution can be stored for a few days at 0°.

Preparation of Mono- and Di-spin-labeled Protohemin—A nitro-
xide compound, 2,2,5,5-tetramethyl-3-aminopyrrolidine-1-oxyl
was prepared by decarboxylation of 2,2,5,5-tetramethyl-3-car-
boxamide-1-oxyl according to the method of Morrisett (12). The
starting material was kindly supplied by Dr. H. R. Drott or was
purchased from Eastman Kodak Co. Di-spin-labeled protohemin
in which two spin labels are covalently bound to positions 6 and
7 of the porphyrin ring, was prepared from protohemin (Sigma)
and 2,2,5,5-tetramethyl-3-aminopyrrolidine-1-oxyl as described
elsewhere (4).

Mono-spin-labeled protohemin, spin-labeled at only one of the
two propionic acid groups at positions 6 and 7 of the porphyrin
ring, was prepared by the partial reaction of protohemin with
the nitroxyl-free radicals, followed by purification by column
chromatography on silicic acid (Bio-Gel SA). Conditions for the
chromatography were the same as that used for the preparation
of protohemin monomethyl ester (13).

The pyridine hemochromogen spectra of the di- and mono-spin-
labeled protohemins are identical with that of protohemin. A
millimolar extinction coefficient of 34.4 nm⁻¹ cm⁻¹ at 550 nm was
used to determine the concentration of these spin-labeled hemins.

Identification of Spin-Labeled Protohemin—The pyridine hemo-
chromogen spectra of the di- and mono-spin-labeled hemins have
the same absorption maxima as that of protohemin, indicating
that positions 2 and 4 of the porphyrin ring were intact. Optical
spectra of mono- and di-spin-labeled protohem in dimethyl-
sulfoxide were also identical with those of protohemin (cf. Fig. 2).
Di-spin labeled protohemin was soluble only in organic solvents
such as ethyl acetate and chloroform, and completely insoluble
in aqueous alkali. Mono-spin-labeled protohemin has a solu-
bility intermediate between that of di spin labeled protohemin
and free hemin. By optical and EPR measurements, the ratio
of hemin to nitroxyl-free radical was calculated as approximately
1:2 in di-spin-labeled hemin and 1:1 in mono-spin-labeled hemin.

| Table I |

Thin layer chromatography of spin-labeled protohemin

<table>
<thead>
<tr>
<th>Hemin</th>
<th>Lutidine-water (20:1)</th>
<th>Hexane-chloroform-methanol (1:1:0.3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protohemin</td>
<td>0.05</td>
<td>0.1</td>
</tr>
<tr>
<td>Mono-spin-labeled protohemin</td>
<td>0.45</td>
<td>0.33</td>
</tr>
<tr>
<td>Di-spin-labeled protohemin</td>
<td>0.95</td>
<td>0.56</td>
</tr>
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</table>

Thin layer chromatography on glass fiber impregnated with silicic
acid (ITLC-SA, Gelman Instrument Co.) showed that the di-spin-
labeled hemin moved as a single spot with RF values of 1.0 and
0.52 in the solvent systems, lutidine-water (1:2), and hexane-
chloroform-methanol (1:1:0.3). Free protohemin had RF values
between 0 and 0.1 in these two solvent systems. The mono-spin-
labeled protohemin had intermediate RF values under the same
conditions. These results are summarized in Table I. The in-
fraed spectrum of the di-spin-labeled protohemin in chloroform
shows carbonyl stretching vibrations of amide groups at 1655 and
1526 cm⁻¹. The mono-spin-labeled protohemin exhibited the above-mentioned amide bands together with the free carboxylic
acid group having an absorption at 1705 cm⁻¹.

Further reaction of the mono-spin-labeled protohemin with the
nitroxide produced a heme indistinguishable from the di-spin-
labeled protohemin.

Recombination of Apohemoglobin with Spin-labeled Heme Recom-

bination was carried out by essentially the same technique
as that used for the recombination of apocytochrome c peroxidase
with protoheme alkyl esters (14). A stoichiometric amount of
spin-labeled protohemin (0.5 mM) dissolved in dimethylsulfoxide
was added dropwise to 0.01 mM apohemoglobin in 10 mM potas-
sium phosphate buffer, pH 7.0, at 0°. The solution was stirred
for 5 min, and the mixture was dialyzed against three changes of
cold-distilled water for 6 to 9 hours. The mixture was then dia-
lyzed against 5 mM potassium phosphate buffer, pH 7.0, from the
column. Since the material contains some dena-
tured low spin component (cf. Fig. 5A, Trace VI), the spin labeled
ferrihemoglobin was reduced enzymatically to the oxy form with
ferredoxin, ferredoxin reductase, and TPNH-generating system
(15). A heat treatment for this oxyhemoglobin was carried out
at 40° for 10 min. After centrifugation at 10,000 X g for 10 min,
the supernatant was oxidized with excess potassium ferricyanide,
which was removed immediately with gel filtration on Sephadex
G-25. The ferrihemoglobin eluted shows an absorption spectrum
indistinguishable from that of native ferrihemoglobin in potas-
sium phosphate buffer, pH 6.0 (cf. Fig. 5D, Trace VI).

Definition of Mobility Index—Perhaps the most rigorous defini-
tion of the mobility of a free radical is by the correlation time
for relaxation of its electron spin. However, calculation of the cor-
relation times for radicals in the "slow tumbling region" at
present, requires a considerable amount of computer time (16). Therefore, two empirical equations were used which allow a
convenient expression of the spectral changes by a "mobility
index." Index A is used for homogeneous systems and Index B
is used for complex EPR spectra where two or more subspectra
are observed. The Index A is calculated from two parameters:
the peak to peak line width of the central resonance (P in Fig. 1A)
and the distance from the central point to the outer line peak in
the higher magnetic field (D in Fig. 1A).

Index A = PDP/550

Table II illustrates the relations of the Mobility Index A to vari-
ous EPR spectra of 2,2,5,5-tetramethyl-3-aminopyrrolidine-1-
oxyl dissolved in water-glyceral mixed at various temperatures.
When the label is tumbling very fast, the index shows a value
less than 1, while the index is about 100 when the label is strongly

Fig. 1. Notation of various parameters used for the calculation of mobility index A and B.
The EPR spectra of 2,2,5,5-tetramethyl-3-aminopyrrolidine-1-oxyl were measured under conditions described in the table with a Varian E-4 EPR spectrometer. The mobility Indices A and B were calculated as shown in the text.

<table>
<thead>
<tr>
<th>lability Index P</th>
<th>Mobility Index A</th>
<th>Mobility Index B</th>
<th>( \tilde{r} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.6</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2.7</td>
<td>5</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>15.4</td>
<td>26</td>
<td>15</td>
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<tr>
<td>34.7</td>
<td>33</td>
<td>40</td>
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</tr>
<tr>
<td>77.0</td>
<td>47</td>
<td>70</td>
<td></td>
</tr>
</tbody>
</table>

*The rotational correlation time (\( r, \text{ns} \)) was estimated from the Stokes law (17).*

immobilized. Table II also compares Index A with the rotational correlation time \( r \) which was estimated from the results obtained by Hsia using the Stokes equation (17). It can be seen that the Mobility Index A is approximately proportional to the correlation time although there is no theoretical justification for this relation.

The Mobility Index B is used to compare mobility changes in complex systems which contain more than two discernable spectra. This index is equal to the sum of the products of the relative amplitude \( k_i/\text{H} \) and the cube of the distance \( d_i \) from the point where the central resonance crosses the base-line (Fig. 1B).

\[
\text{Mobility Index B} = \left( \frac{1}{\text{H}} \times 10^6 \right) \sum k_i \cdot d_i^3
\]

The sign of \( k_i \) is negative when the curve is below the baseline. The product is calculated at 1-G intervals, and the results are shown in Table II.

**Measurements**—The optical and EPR measurements were carried out with a Perkin-Elmer Coleman 124 spectrophotometer and a Varian E-4 spectrometer with variable temperature controls.

**RESULTS**

**Optical Spectra of Spin-labeled Protohemin**—Absorption spectra of di-spin-labeled protohemin in dimethylsulfoxide are shown in Fig. 2. The spin-labeled hemin shows a typical high spin-type spectrum in dimethylsulfoxide in the presence of 2 \( \mu \text{M} \) perchloric acid. The sharp Soret band is indicative of monomeric ferriheme. Dimethylsulfoxide may furnish axial ligands for the heme-iron under these conditions (18). Addition of KCN converts the spectrum to that of the low spin di-cyanide complex. On the other hand, upon addition of aqueous alkali, the hemin is converted to a complex which has relatively low Soret band (Fig. 2). This compound is probably a dimeric form of the hemin (18). All of the optical spectra of di-spin-labeled protohemin are identical with those of protohemin and of mono-spin-labeled protohemin, indicating that the modification of the propionic acid groups at positions 6 and 7 of the porphyrin ring produces no significant effect on the electronic structure of the porphyrin ring, although the modification alters the solubility of the spin-labeled protohemins significantly.

**Effect of Iron Spin State and Dimerization on EPR Spectra of Spin-Labeled Protoheme in Dimethylsulfoxide**—As shown in Fig. 3, both the line shape and the resonance amplitude of the EPR spectra of the mono-spin-labeled protohemin in dimethylsulfoxide are strongly affected by the changes in the spin state of the heme-iron. The low spin cyanide complex (Spectrum III in Fig. 3) shows three sharp lines typical of rapidly tumbling nitroxy1-free radicals, while the high spin complex (Spectrum I in Fig. 3) exhibits relatively weak, broad lines. This broadening in the high spin complex is probably due to the magnetic dipole interaction between spin label and heme-iron (5, 19). The high spin heme-iron has a larger magnetic moment as well as a longer electron spin relaxation time, and both of these factors produce a decrease in the resonance amplitude. By contrast, the low spin iron of the cyanide complex has little effect on the spin label because of the small magnetic moment and the very short relaxation time of the iron.

The effect of temperature on the EPR spectrum of the mono-spin-labeled protohemin is shown in Fig. 4. At higher tempera-
Fig. 4. Effect of temperature on the EPR spectra of low spin cyanide (top) and high spin ferric forms (bottom) of monospinlabeled protocemes (ML-PH). The experimental conditions are the same as in Fig. 3.

Fig. 5. Absorption spectra of mono-spin-labeled hemoglobin at various purification steps. A-I, hemoglobin (80 μM) immediately after elution from carboxymethylcellullose column; A-II, I + 0.2 M potassium ferricyanide. The excess ferricyanide was removed by gel filtration with Sephadex G-25. B-III, II + 0.1 M potassium fluoride; B-IV, methemoglobin fluoride prepared from native hemoglobin; C-V, III + ferredoxin reductase system (15); D-VI, V + potassium ferricyanide; D-VII, VI + ferredoxin reductase system.

The titration of apohemoglobin with mono-spin-labeled protohemin shows a quantitative binding of 4 moles of heme per mole of apoprotein. The optical spectrum taken immediately after elution of the first column chromatography on carboxymethylcellulose exhibits a spectrum characteristic of a mixture of high and low spin compounds (Fig. 5A, Trace I). The two peaks at 554 and 570 nm are in part due to the oxyhemoglobin which can be oxidized to the met form upon addition of potassium ferricyanide (Fig. 5A, Trace II). Since the spin-labeled hemein used for the recombination experiment is 100% ferric form, the hemooglobin must have become reduced and oxygenated during the preparation. This reduction of the ferric heme-iron has recently been attributed to the spin labels. The visible spectrum of the ferricyanide-treated hemoglobin (Fig. 5A, Trace II) still contains unidentified low spin-type absorptions at around 530 and 570 nm. Although the over-all spectrum resembles that of ferric hemoglobin, this cannot be the explanation because the spectrum is insensitive to changes in temperature. The low spin peaks are therefore attributed to contamination by heme-chrome type denatured protein. Since the ratio of this hemechrome type compound to that of the high spin complex is always approximately 1:1, the formation of the high and low spin compounds may be attributed to the two isomers of 6 or 7 mono-spin-labeled protohemin (see below). As shown in Fig. 5B, Trace III, the low spin component of the ferricyanide-treated hemoglobin does not react with fluoride, while the high spin component forms a typical fluoride complex. If all the heme reacted with fluoride, we would expect Spectrum IV in Fig. 5B. Separation of the denatured component on carboxymethylcellulose column did not occur when the ferric sample was used. As shown elsewhere (15, 20), spin-labeled ferric hemoglobin can be reduced enzymatically to the oxy form without destroying the free radical (Fig. 5C, Trace V). After this reduction, the hemoglobin obviously contains some nonreducible impurity which has a broad absorption between 600 and 700 nm. This impurity can be completely removed by a heat treatment at 40° for 10 min, followed by gel filtration on Sephadex G-25 (Fig. 5D, Trace VII). The oxidized product of this oxyhemoglobin with ferricyanide now shows a spectrum identical with that of native high spin methemoglobin, as shown in Fig.
the resonance amplitude of the acid met form of the spin-labeled relaxation times of the heme-iron (5). It should be noted that hemoglobin is intermediate between the high spin fluoride and cyanide and azide complexes have relatively large resonance amplitudes, while the fluoride complex has a small amplitude.

These spectra belong to those of so-called moderately immobilized globin (Fig. 6).

Absorption Spectra of Spin-labeled Methemoglobin and Its Complexes with Cyanide, Azide, and Fluoride—Sugita and Yoneyama (21) reported that the alkylation of the two propionic acid groups at positions 6 and 7 of the porphyrin ring did not affect the optical and oxygen-binding properties of hemoglobin. As shown in this and the following papers (22), the optical properties of the synthetic hemoglobins containing mono-spin-labeled protohemin and its complexes with various ligands are also identical (within experimental errors) to those of native hemoglobin (Fig. 6).

EPR Spectra of Mono-spin-labeled Hemoglobin and Its Complexes with Various Ligands—In Fig. 7, the EPR spectra of synthetic hemoglobin containing mono-spin-labeled protohemin in 0.1 M potassium phosphate buffer, pH 6.0, are compared to those of the cyanide, azide, and fluoride complexes. Although all these spectra belong to those of so-called moderately immobilized labels, the mobility indices are slightly different from one another indicating that there are differences in the heme environments of the various hemoglobin derivatives (Table III).

Ionization of Spin-labeled Ferric Hemoglobin in Alkaline Solution—The absorption spectra of the spin-labeled ferric hemoglobin at various pH values are shown in Fig. 8. The pK values for the acid-alkali transition of the spin-labeled ferric hemoglobin is 7.6 which is about one pH unit lower than that of native hemoglobin (25). The room temperature absorption spectrum of the alkaline form clearly shows the presence of two components which are thermal mixtures of high and low spin forms as will be described below.

Effect of Temperature on EPR Spectra of Spin-labeled Hemoglobin—The temperature-dependent spectral changes of various complexes of the spin-labeled ferrihemoglobins are shown in Fig. 9. In all complexes examined, the labels are less immobilized at higher temperatures, and there are concomitant increases in the resonance amplitudes. These changes are completely reversible at temperatures between 0 and 30°. The mobility indices and the resonance amplitudes at various temperatures are illustrated in Fig. 10.

George et al. (26) and Iizuka and Kotani (27) showed that both the acid and alkaline forms of ferrihemoglobin are thermal mixtures of high and low spin electronic states. This property appears to be unaltered after spin labeling of the propionic acid.
groups at positions 6 and 7 of the porphyrin ring. Spin-labeled ferrihemoglobin in alkaline solution clearly exhibits a mixed spectrum of high and low spin compounds (Fig. 8), and the resonance amplitudes of the hemoglobin hydroxide also shows intermediate values between those of the high spin fluoride and low spin cyanide complexes (Fig. 10). If the alkaline form of the spin-labeled ferrihemoglobin is a thermal mixture of high and low spin compounds, then changing the temperature would be expected to influence the equilibrium and a change should therefore be observable in the absorption spectrum and in the magnetic moment. This change in the magnetic moment of the heme-iron should secondarily affect the EPR spectrum of the spin label located close to the heme-iron (5). Such a temperature-dependent magnetic effect is obviously seen in temperature-

![Fig. 8. Effect of pH on the optical absorption spectra of mono-spin-labeled ferrihemoglobin at 20°. The hemoglobin was dissolved in 1 mM potassium phosphate buffer, pH 6.2. The pH was adjusted by adding 1 to 2 µl of concentrated NaOH solution.](image)

![Fig. 9. Effect of temperature on the EPR spectra of mono-spin-labeled ferrihemoglobin in the presence of various ligands. The experimental conditions were the same as in Fig. 10.](image)

![Fig. 10. Effect of temperature on the central resonance amplitude and mobility index A of EPR spectra of mono-spin-labeled ferrihemoglobins. The central resonance amplitudes are normalized to hemoglobin concentrations and the settings of the EPR spectrometer. The dotted line was obtained by assuming that the hydroxide complex is temperature independent.](image)
dependent changes in the resonance amplitudes in Fig. 10. The expected increase of the resonance amplitude of the hydroxide form of heme spin-labeled hemoglobin from increased mobility is suppressed at higher temperatures, indicating that the thermal equilibrium is shifted in favor of the high spin heme at higher temperatures.

**DISCUSSION**

Theoretical treatments for electron paramagnetic resonance line shape of slowly tumbling molecules have been developed using the adiabatic assumption (28, 29) or perturbation assumption (30). More recently, Freed et al. (16), and Gorden and Messenger (31) presented a theory of rotational motion of molecules with the rotational diffusion equation.

In the present paper, attempts were made to find empirical equations which simply express the spectral changes numerically. These equations were obtained on the basis of the fact that the distance between the central peak and outer peak (D and di), and the line width (P) of the central resonance increase as spin labels are more immobilized. It was empirically found that \( P^2D \) in Index A and \( h\cdot d\) in Index B has certain parallelism with the correlation times as shown in Table II. Although the two equations used in this paper have no theoretical basis, relatively small spectral changes are distinguishable numerically by simple calculations. These two indices, however, are not applicable in cases of rapid anisotropic motion of the spin label (cf. Ref. 32).

It has been known that hemin can exist in a dimeric form in aqueous alkaline solutions or in alkaline dimethyl sulfoxide (30, 33-36). Schugar et al. (37) reported that the low magnetic moment of hematin solution is consistent with the idea of antiferromagnetic spin exchange behavior, associated with an Fe-O-Fe bridge. The infrared spectrum of aqueous alkaline heme solution also suggests an oxo-bridged structure (38). The present result obtained by heme-spin label method clearly shows that the spin labels attached to alkaline hemin have no significant magnetic interaction with the heme-iron indicating that the heme-iron is in either a low spin or antiferromagnetic state. The magnetic interaction observed in concentrated solutions may be mainly due to that between spin labels of different molecules.

The mono-spin-labeled protohemin used in the present experiment is assumed to be a 1:1 mixture of the two isomers of 6 or 7 mono-spin-labeled protohemin. Although these isomers cannot be separated, the formation of an equal amount of the high and low spin complexes upon binding with apohemoglobin suggests that the protein may distinguish these two isomers.\(^1\) The conclusion must await the x-ray crystallography of the crystalline hemoglobin. The optical spectra of both ferric and ferrous hemoglobins containing 6 or 7 mono-spin-labeled protoheme are identical with those of natural hemoglobin. The modification of the carboxyl end of the propionic acid chains may be too far removed from the porphyrin ring to affect its electronic properties, although these modifications cause marked changes in the solubility of the heme. A second reason why the modifications at the 6 and 7 carboxyl groups do not affect the optical and oxygen-binding properties of hemoglobin may be the orientation of the heme group in the heme pocket. As reported by Perutz (32), the propionic acid groups in hemoglobin make polar contact with protein in water so that their contributions to the binding energy should be small.

| Table IV |
|------------------|------------------|---------------|
| **Distances between heme-iron and spin label in spin-labeled ferricemoglobin** |
| Ferrihemoglobin   | \( d^* \) | Distance |
| Acid methemoglobin| 0.2  | 0.33  | 11.5 |
| Methemoglobin fluoride | 0.5  | 0.36  | 11.8 |

\( ^* \) Estimated from the EPR spectrum of the iron signal (23).

\( ^\dagger \) Ratio of the central amplitude of heme-spin-labeled acid met and fluoride methemoglobin to that of cyanide hemoglobin.

\( (1, 2) \), the propionic acid groups in hemoglobin make polar contact with protein in water so that their contributions to the binding energy should be small.

In the present experiment, however, the hemichrome in the spin-labeled hemoglobin was completely removed after reduction of the hemoglobin with a methemoglobin reductase system. It is not clear at present whether the differences in the iron-label distance are due to the conformational difference between the dissolved and crystalline hemoglobin (6), or to the error which might be produced by the

\( ^1 \) Recently, we have succeeded in the chromatographic separation of the two isomers of 2- or 4-monovinylmonomethyldeuterohemin. Although the physical properties of these two isomers are similar to each other, it was found that apohemoglobin can distinguish these two isomers and shows different oxygen-binding properties (48).

\( ^\dagger \) J. S. Leigh, Jr., personal communication.
conformational difference between the cyanide and fluoride complexes.

As to the protein conformation of various hemoglobin derivatives, the present results as well as those obtained by McConnell et al. (24) who spin-labeled at the β-93 cysteine residues of hemoglobin, appear to suggest that the protein conformation in the vicinity of hemoglobin or the β-93 cysteine in various ferric hemoglobin derivatives are not identical.

High and Low Spin Forms of Alkaline Ferric Hemoglobin—The central resonance amplitudes of the spin label signal in the alkaline ferric hemoglobin show values intermediate between those of the completely high spin fluoride complex and the completely low spin cyanide complex (Fig. 10 and Table V). If the hemoglobin hydroxide is a purely high spin complex and if the environment of the label is not altered the resonance amplitude of the alkaline hemoglobin is expected to be similar to that of the acid methemoglobin rather than to that of the fluoride complex (cf. Table V). However, the hydroxide complex contains certain amounts of low spin component, which has both a lower magnetic moment and shorter electron spin relaxation time, the resonance amplitude of the hydroxide is intermediate between those of high spin acid and methemoglobin and low spin cyanide hemoglobin. The values for the relaxation times of the hemoglobin were estimated by measuring the solution EPR spectra of various derivatives of ferric hemoglobin at room temperature (23). If we assume that the electron spin relaxation time for the low spin hydroxide form is as small as that of cyanide complex and that acid methemoglobin is mostly high spin form, then the ratios of the high and low spin compounds at various temperatures can be estimated taking the cyanide and acid met forms as totally low spin and high spin standards, respectively. The fraction of the high spin and low spin forms of the alkaline hemoglobin at different temperatures are calculated and summarized in Table VI. It is noteworthy that the fraction of the high spin and low spin complexes varies with temperature, clearly indicating that the alkaline hemoglobin is an equilibrium mixture of high and low spin states. These results, however, are significantly different from those of George et al. (26) based on magnetic and spectroscopic measurements of alkaline hemoglobin. Although there is no clear explanation for these differences, the relative increase in the fraction of high spin alkaline hemoglobin obtained by the heme spin label method are consistent with that of our room temperature EPR experiment on alkaline hemoglobin (23).

The present results indicate that the spin labeling of the heme group is a useful method for probing the protein conformation in the vicinity of heme and for monitoring changes in the spin state of the heme-iron. An application of the method for ferric hemoglobin will be reported in the following paper (20).

Acknowledgments—The author is indebted to Drs. B. Chance, T. Yonetani, H. R. Drott, J. S. Leigh, G. Reed, and A. Couteel for their valuable discussions. He also thanks Drs. H. Akimoto and R. Hershberg for infrared measurements and helpful discussions.

REFERENCES
