Spin-Labeled Hemoglobin Derivatives in Solution, Polycrystalline Suspensions, and Single Crystals*

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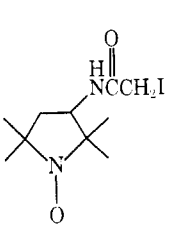
ABSTRACT: The paramagnetic resonance spectra of the carbon monoxy, met, met azide, and met fluoride derivatives of horse hemoglobin spin labeled at the β93 cysteines have been observed in solution, polycrystalline suspensions, and single crystals. The β93 cysteines were alkylated with either *N*-(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl)iodoacetamide (I) or *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide (II). At room temperature, the resonance spectra of all four derivatives labeled with either I or II show evidence of an equilibrium between two isomeric states of the spin label relative to the protein. The strong dependence of the resonance spectra upon buffer concentration and the ligand at the sixth coordination position of the iron atoms is mainly due to a displacement of this equilibrium. This dependence suggests small differences in the structures of the above derivatives that are evidently linked to the spin state of the iron atoms. However, in single crystals the principal hyperfine axis of each label has essentially the same set of orientations in all four derivatives, indicating that the protein conformation in the vicinity of the β93 cysteines must be very similar in all four. Differences in both the solution and crystal spectra decrease as the buffer concentration increases. Changes in the resonance spectra concomitant with crystallization can be detected but, again, the associated changes in protein structure are small.

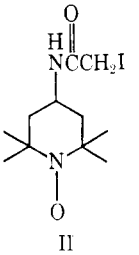
The present paper gives the results of a comparative study of the paramagnetic resonance of several derivatives of spin-labeled horse hemoglobin in solution, in polycrystalline suspensions, and in single crystals. This work was undertaken for the purpose of studying two quite distinct problems. First, a comparison of the resonance spectra of hemoglobin in solution and in single crystals offers the possibility of probing conformational differences in these two states, since such spin-label spectra depend upon the local protein conformation in the vicinity of the label (Hamilton and McConnell, 1968). We were particularly stimulated to make this study for carbonmonoxy and met horse hemoglobin since the resonance spectra of these two derivatives spin labeled at the cysteines β93 with iodoacetamide labels were distinctly different from one another (Ogawa and McConnell, 1967; McConnell and Hamilton, 1968), whereas Perutz and Mathews (1966) found in single crystal X-ray studies that these two derivatives must have very similar secondary and tertiary structures, and identical quaternary structures. A preliminary study of the paramagnetic resonance of spin-labeled carbonmonoxy and met horse hemoglobin has already been published (McConnell and Hamilton, 1968). This earlier work made it clear that the protein structures in the vicinity of β93 must be very nearly identical in the two derivatives, but left open the question as to whether or not the resonance spectra of the two derivatives were distinguishable in single crystals under identical solvent conditions. The second problem is concerned with the interpretation of the paramagnetic resonance spectra of carbonmonoxy or oxyhemoglobin in solution. The low-field hyperfine component in the paramagnetic resonance spectrum of either of these two derivatives in solution is split into two signals of comparable intensity (Ogawa and McConnell, 1967). Since no splitting of this type has ever been seen for labels dissolved in solutions of varying viscosity, the most obvious interpretation of these two signals is that they are due to isomeric states of the label relative to the protein (McConnell and Hamilton, 1968). This interpretation is fully confirmed in the present work and has been used elsewhere in relating label resonance spectra to conformational changes associated with the "heme-heme" interaction and protein conformational changes linked to the spin state of the iron atoms (McConnell *et al.*, 1968; Ogawa *et al.*, 1968; H. M. McConnell, and A. Horwitz, 1968).

Materials and Methods

Two spin labels were used in the present work, *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).



I



II

The preparation of these labels has been described previously (Ogawa and McConnell, 1967; McConnell and Hamilton, 1968). During the course of the present work, the following simpler synthesis of II was developed: 15.6 g of 2,2,6,6-tetra-

* From the Stauffer Laboratory for Physical Chemistry, Stanford, California 94305. *Received January 24, 1969.* This research was supported by the Office of Naval Research, Contract Number 225(88). This research has also benefited from facilities made available by the Advanced Research Projects Agency through the Center for Materials Research at Stanford University.

methyl-4-aminopiperidine (Aldrich Chemical Co.) was acetylated with an equivalent amount of chloroacetyl chloride. The reaction mixture was extracted with dilute hydrochloric acid and the extract was neutralized with aqueous sodium hydroxide. The white solid which precipitated was collected on a filter and suspended in *ca.* 600 ml of water; 3 g of sodium tungstate, 3 g of disodium EDTA, and 60 ml of 30% hydrogen peroxide were added to the suspension and the resulting mixture was stirred until all solid material dissolved. The solution was allowed to stand for 2 hr, and then the resulting orange solution was saturated with sodium chloride and extracted with ether. The ether phase was washed with water to remove excess peroxide. The dark orange crystals obtained after drying over magnesium sulfate and removal of the ether *in vacuo* were dissolved in anhydrous acetone and a slight excess of sodium iodide dissolved in acetone was added. After standing overnight, the mixture was filtered and the acetone was removed *in vacuo*. The dark oil remaining was dissolved in 200 ml of hot toluene and after 3 days the product (II) crystallized as dark orange needles which melted at 114–117°.

Anal. Calcd for $C_{11}H_{20}IN_2O_2$: C, 39.0; H, 5.9; I, 37.4. Found: C, 39.3; H, 6.2; I, 37.5.

The procedure for preparing hemoglobin was adapted from those of Perutz (1968) and Benesch and Benesch (1962). Erythrocytes were separated from the plasma, washed, lysed, and shaken with toluene. The hemoglobin layer was separated from the toluene cell wall fraction, saturated sodium chloride was added to bring the total salt concentration to 2%, and the solution was centrifuged for 1 hr at 17,000 rpm. The supernatant was desalted by passage through a G-25 Sephadex column previously equilibrated against a 0.1 M phosphate buffer, pH 7.8. Hemoglobin was labeled as described previously (Ogawa and McConnell, 1967), except that the labeling reaction with II was carried out for 2–3 days at 5°. Under the labeling conditions used, I and II react with groups other than the β93 cysteines to the extent of *ca.* 5%, as judged by resonance spectra obtained when the β93 cysteines are preblocked with *p*-mercuribenzoate (Ogawa and McConnell, 1967; S. Ogawa, A. Horwitz, and H. M. McConnell, 1968, unpublished data, and S. Ogawa, 1967). Unreacted spin label was removed by passing the solution through a G-25 Sephadex column equilibrated against a 0.01 M phosphate buffer, pH 7.0.

Crystals of labeled hemoglobin were grown at room temperature by the method of Perutz (1968) using either crystallizing solution "A" (2 volumes of 4 M $(NH_4)_2SO_4$ to 1 volume of 2 M $(NH_4)_2HPO_4$) or "B" (2 volumes of 4 M $(NH_4)_2SO_4$ to 1 volume of a phosphate buffer consisting of 0.95 volume of 2 M $(NH_4)_2HPO_4$ and 0.05 volume of 2 M $NH_4H_2PO_4$). Solution B has a pH of about 7.3 (measured with a glass electrode) and an anion molarity of 3.3. Crystals were grown from solutions prepared by mixing 2.0 ml of 2% hemoglobin with between 2 and 3 ml of one of the crystallizing solutions. Monoclinic single crystals with linear dimensions of 0.1–2.0 mm were used (Reichert and Brown, 1909). The monoclinic structure with space group C2 has two molecules per unit cell. These two molecules have identical orientations; hence the resonance spectra of labels attached to each are identical. Single crystals were mounted in plastic holders. The holders were positioned accurately in the center of the microwave resonance cavity. The mount was covered with a tight-fitting cap whose inside edge was coated with silicone grease in order to prevent evaporation of mother liquor. Crystal axes were identified by inspection under a polarizing microscope. Magnetic resonance spectra were obtained with a Varian X-band spectrometer.

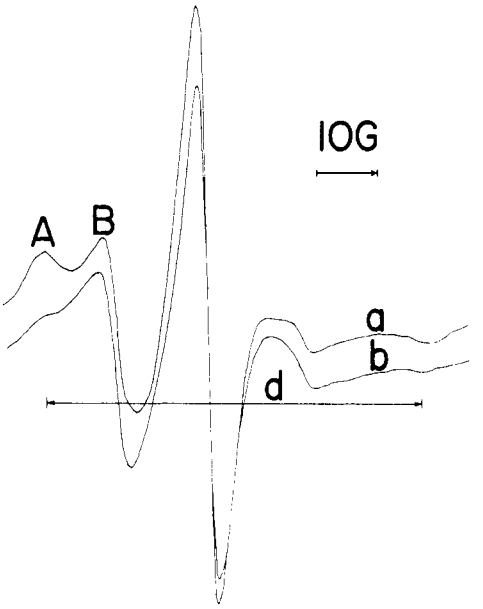


FIGURE 1: Solution (0.01 M anion) paramagnetic resonance spectra of hemoglobin labeled with I: (a) HbCO and (b) metHb. Buffer concentrations were ordinarily controlled by the dilution of solution B (see text).

The analysis of paramagnetic resonance spectra of spin labels having fixed orientations in single crystals in terms of the principal axes and other parameters of a spin Hamiltonian has been discussed extensively elsewhere (McConnell and Hamilton, 1968; Deal, 1969). For each label, there is one unique axis which gives the largest N^{14} hyperfine splitting (about 62 gauss for five-membered and 65 gauss for six-membered ring nitroxide radicals). This axis is approximately in the direction of the axis of the 2 pπorbital on the ring nitrogen. In the present work, the direction of only this axis was determined; it was located by systematic searches in which the applied field was rotated relative to the crystal axes. The unique axis direction, designated $\pm\pi$, is the direction of maximum splitting. (The sign of π is not defined.)

Polycrystalline hemoglobin was obtained either from sonicated crystals (monoclinic form) or from small crystals grown during attempts to grow large crystals. Crystals in the latter samples were mostly of the monoclinic form; a few were of the orthorhombic form. Spectra of samples from either source and of crystals grown from either solution A or B were identical under the same solvent conditions. The polycrystalline spectra were isotropic and the intensity due to dissolved hemoglobin was only a few per cent of the total. Buffer concentrations were ordinarily controlled by varying the dilution of solution B.

Results

Before considering the experimental results, it may be helpful to describe two problems that stimulated the study of single crystals of spin-labeled hemoglobin. Figure 1 shows the paramagnetic resonance spectra of labeled HbCO and met Hb. (The spectrum of HbO_2 is essentially identical with that of

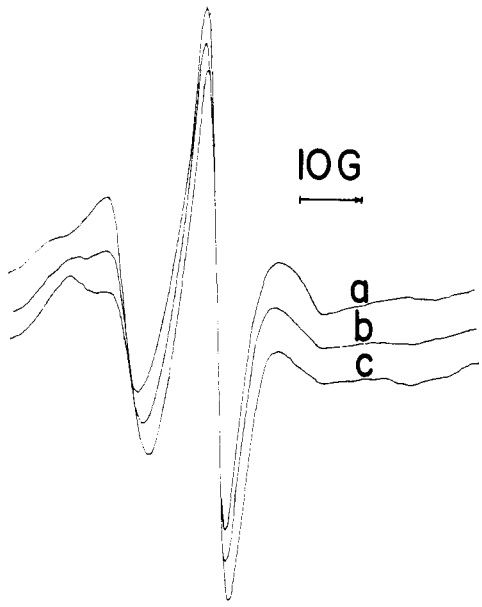


FIGURE 2: Variation with buffer concentration of the solution resonance spectrum of metHb labeled with I: (a) 0.01 M anion, (b) 1.25 M, and (c) 2.5 M.

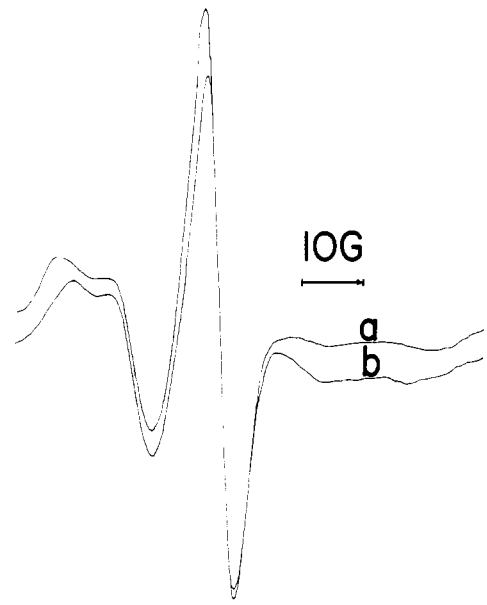


FIGURE 3: High buffer concentration (2.5 M anion) solution resonance spectra of hemoglobin labeled with I: (a) HbCO and (b) metHb.

HbCO.) The spectra of the two derivatives are clearly different, suggesting differences in protein conformation in the vicinity of the label. The first problem is to relate this result to the conclusion of Perutz and Mathews (1966) that, in single crystals, these two derivatives must have very similar secondary and tertiary structures and identical quaternary structures. The second problem is the interpretation of the paramagnetic resonance spectra of spin-labeled hemoglobin in solution. The low-field hyperfine component in Figure 1 is split into two signals, designated A and B. Since no splitting of this type has ever been seen for labels dissolved in solutions of varying viscosity, the most obvious interpretation of these two signals (noting that labeling occurs essentially only at the β93 sulfhydryls) is that they are due to isomeric states of the label relative to the protein and that these states are in equilibrium with a lifetime for interconversion that is long compared with the reciprocal of the frequency separation of signals A and B (10^{-7}–10^{-8} sec). The only troublesome point with this interpretation is that the approximate theoretical calculation of Itzkowitz (1966, 1967) suggests that a radical with a suitable spin Hamiltonian, correlation time and/or anisotropic motion might give rise to two well-defined peaks in the low-field component of the hyperfine spectrum. The results presented here provide a solution to both of these problems, and this solution is important in using the spin-label technique for studying the "heme–heme" interaction (McConnell *et al.*, 1968).

All of our experiments were carried out with both labels I and II, and in all cases the observed spectra are qualitatively similar, and their interpretation leads to similar conclusions. In the interest of brevity we present only a few representative spectra using label I.

Solution Spectra. As seen in Figure 1 the paramagnetic resonance spectra of labeled HbCO and metHb in solution show evidence of two distinct low-field signals, designated A and B. The resonance spectra of the other low-spin derivatives (oxy, met azide, and met cyanide) are essentially the same as that of the carbonmonoxy derivative, and the spectrum of the other high-spin derivative (met fluoride) is very similar to that of the met derivative below pH 7. It will be assumed now, and demonstrated later in single crystal studies, that these two signals are due to isomeric states of the label relative to the protein. The resonance spectra of labels in state A have the large "hyperfine separation" (d in Figure 1; approximately 62 gauss for five-membered spin labels) characteristic of labels referred to as "strongly immobilized." The spectra of labels in state B correspond to more "weakly immobilized" states. The spectra of the high-spin derivatives appear to have a larger proportion of component B than do those of the low-spin derivatives. In addition, especially for label I, the hyperfine separation of component A is less in the high-spin derivatives; thus state A is not precisely the same in all the derivatives. This difference in the hyperfine separations means either that the motion of labels in state A is more severely restricted or that the odd-electron density on the ring nitrogen of the label is greater for the low-spin derivatives; the former is by far the more likely source of the difference.

In the present work no attempt is made to analyze spectra such as those seen in Figure 1 in terms of the relative proportions of the isomeric states A and B. A detailed analysis that could be relied on with confidence might require elaborate computer-type curve fitting, and is certainly not warranted for the present study. However, for a crude order-of-magnitude picture, we may assume (on the basis of unpublished data in this laboratory) that the "extinction coefficient" for signal A is roughly one-half that of signal B. We then estimate from the spectra in Figure 1 that isomeric state A is present to the extent of $\sim$70% for HbCO in spectrum (a) and to the extent of $\sim$55% for metHb in spectrum (b). Changes of this order of magnitude in the relative proportions of states A and B can also be produced by changes of temperature and pH (Ogawa, 1967).

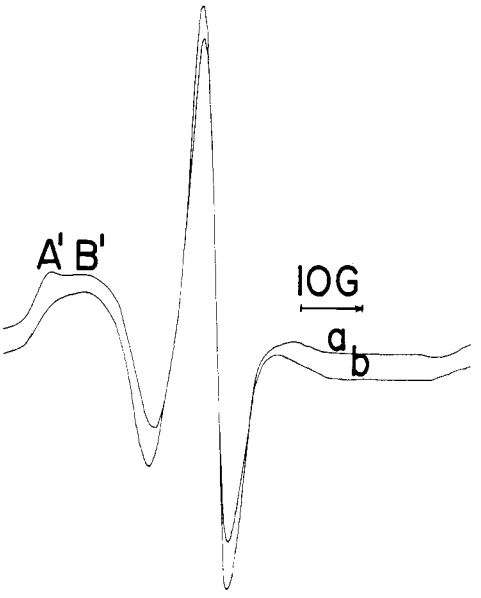


FIGURE 4: Polycrystalline (1.5 M anion) resonance spectra of hemoglobin labeled with I: (a) HbCO and (b) metHb.

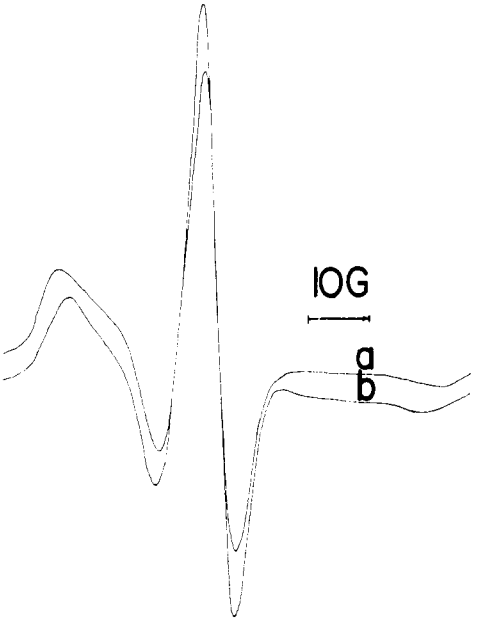


FIGURE 5: High buffer concentration (3.7 M anion) polycrystalline resonance spectra of hemoglobin labeled with I: (a) HbCO and (b) metHb.

The observed variation of the resonance spectra with increasing ionic strength (Figure 2) is consistent with a slight increase in the degree of immobilization of the label states and, mainly, a shift in the equilibria between the populations of states A and B. The spectra of the various derivatives become more similar as the ionic strength is raised, as illustrated in Figure 3. If a perturbation of the protein produces a change only in the relative populations of states A and B (and not, say, in the nature of states A and B themselves), then isosbestic points in the resonance spectra would occur as the strength of the perturbation increases. No search has been made for isosbestic points in the spectra as a function of ionic strength, temperature, or pH. Isosbestic points are seen in the resonance spectra on addition of cyanide to metHb (S. Ogawa, A. Horwitz, and H. M. McConnell, 1968, unpublished data).

Polycrystalline Spectra. As illustrated in Figure 4, the resonance spectra of polycrystalline suspensions of the various derivatives also show evidence for two components, designated A′ and B′. (The resonance spectra of label II show a clearer separation of the peaks A′ and B′ than do the spectra of label I seen in Figure 4.) The hyperfine separation of the first component (A′) is 62 gauss for label I and 66 gauss for label II in the carbonmonoxy and met azide derivatives; as with the solution spectra, it is slightly less in the met and met fluoride derivatives. The line shape for the second label state (B′) is not resolved because of overlapping of the lines, but this component certainly has a reduced hyperfine separation corresponding to some residual motion of the label in the crystallized protein.

Like the solution spectra, the polycrystalline spectra of the met and met fluoride derivatives exhibit a larger amount of the less strongly immobilized component (B′) than do those of the carbonmonoxy and met azide derivatives. As the ionic strength is raised, the polycrystalline spectra change in much the same way as the solution spectra. As illustrated in Figure 5, the spectra of the high- and low-spin derivatives are similar at high ionic strength (~3.7 M anion); however, the differences between the high- and low-spin derivatives never completely disappear in the range of ionic strengths studied.

Single Crystal Spectra. At room temperature, two spin-label conformational states (here designated A″ and B″) with chemically distinct (not symmetry-related) label orientations were previously found in single crystals of HbCO and metHb labeled with II (McConnell and Hamilton, 1968). In the present work, these same label orientations have been found in the met fluoride and met azide derivatives. Most remarkably, the principal axis orientations of label I are the same as those of label II in single crystals of HbCO and metHb.

The label orientations may be specified as follows. A unit vector parallel to the principal axis for the largest hyperfine splitting is designated π. For all labeled derivatives

$$\pm\pi(A'') = \cos(110 \pm 5°)\mathbf{\mu}_a + \cos(55 \pm 5°)\mathbf{\mu}_b + \cos(40 \pm 5°)\mathbf{\mu}_{c^*}$$

$$\pm\pi(B'') = \cos(70 \pm 10°)\mathbf{\mu}_a + \cos(135 \pm 10°)\mathbf{\mu}_b + \cos(50 \pm 10°)\mathbf{\mu}_{c^*}$$

Here $\mathbf{\mu}_a$, and $\mathbf{\mu}_b$ are unit vectors parallel to the monoclinic axes a and b, and $\mathbf{\mu}_{c^*} = \mathbf{\mu}_a \times \mathbf{\mu}_b$. Figure 6 shows the resonance line positions for HbCO labeled with I when the applied field is rotated in the a–c^* plane (the plane perpendicular to the monoclinic twofold axis b). The line positions for metHb are essentially the same as those for HbCO. The maximum hyperfine splitting for label I is 62 ± 1 gauss when the applied field is parallel to $\pm\pi(A'')$ and 57 ± 2 gauss when the field is par-

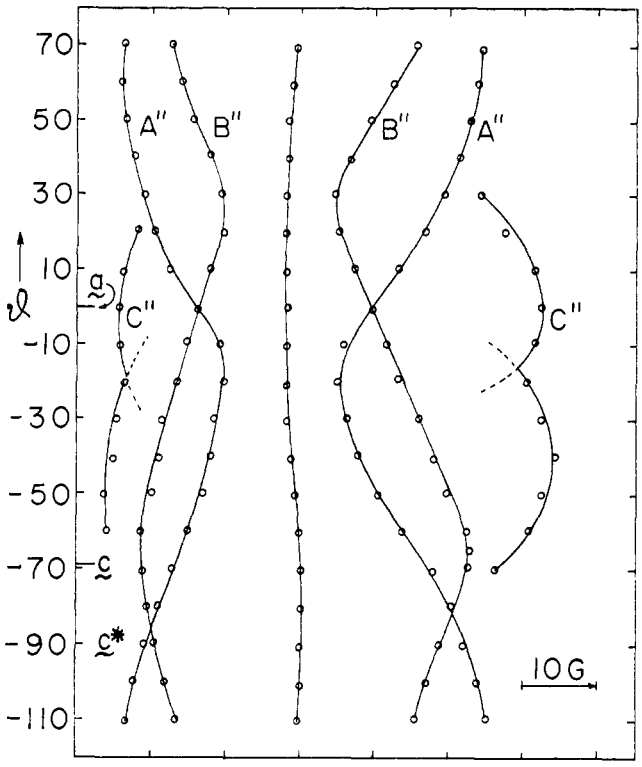


FIGURE 6: Resonance line positions of a single crystal of HbCO labeled with I when the applied field is in the a-c^* plane at an angle of $-\theta$ to a and $69° + \theta$ to c.

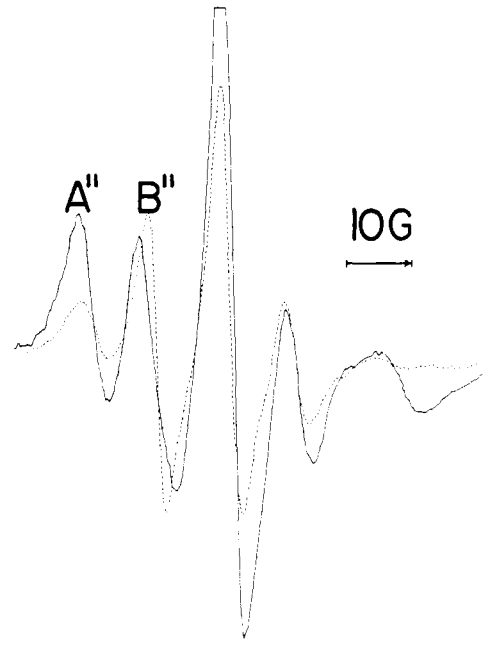


FIGURE 7: Resonance spectra of single crystals of HbCO (———) and metHb (. . . . . .) labeled with I. The applied field is perpendicular to b, makes an angle of $45 \pm 3°$ with a, and an angle of $24 \pm 3°$ with c.

allel to $\pm\pi(B'')$. The fact that the maximum hyperfine splitting for B'' is smaller than that for A'' is undoubtedly due to a greater residual motion of labels in state B''. A similar difference has been noted for label II (McConnell and Hamilton, 1968). The spectra of both labels indicate the presence of other components of relatively low intensity, which are the subject of the next section.

Although the principal axis orientations are essentially the same for all the derivatives, the single crystal spectra of the low- and high-spin derivatives are generally not identical. This is illustrated in Figure 7 where it is seen that the ratios of the signal intensities from states A'' and B'' are quite different in the two derivatives. The differences are even more marked in the case of label II, where the relative intensities of components A'' and B'' are reversed in the two derivatives.

The effect of ionic strength on the spectra was observed by allowing the crystals to dry slowly. (The HbCO crystals were dried in an atmosphere of carbon monoxide.) As the crystals dry, the relative intensities of the components become more nearly equal, and the spectra of all four derivatives eventually become very nearly identical. However, the spectra of the carbonmonoxy and met azide derivatives are at no time the same as those of the freshly mounted met and met fluoride derivatives, and *vice versa*. (The identical relative intensities seen in the spectra of HbCO and metHb reported by McConnell and Hamilton (1968) are evidently due to a differential degree of drying of the two crystals, a possibility which they suggested.) The drying-induced changes in the relative intensities may be reversed by leaving the crystals in a sealed vessel containing mother liquor. As the crystals dry, there appear to be no discontinuities in the relative intensities (until the crystals break up) and no substantial changes in the line width of either component.

Other Label States and Temperature Effects. The single crystal spectra at room temperature indicate the presence of other orientations of the spin labels. The most prominent of these (C'') arises from a label with the principal axis orientation

$$\pm\pi(C'') = \cos(15 \pm 5°)\mathfrak{y}_a + \cos(75 \pm 5°)\mathfrak{y}_b$$

The maximum hyperfine splitting of this component is about 65 gauss for label I and 70 gauss for label II.

The single crystal spectra change reversibly as the temperature is lowered: the intensities due to A'' and B'' change little relative to each other, but the intensity of C'' decreases and new lines appear whose intensities increase as those of A'' and B'' decrease. A detailed description and discussion of these effects of lower temperatures are given elsewhere together with an extensive compilation of spectra (Deal, 1969). Suffice it to say here that the components which are predominant at low temperature (<0°) have maximum hyperfine splittings larger than those of A'' and B''. The solution and polycrystalline spectra exhibit the same type of effect as the temperature is lowered: there appears to be a shift in the relative populations of the label states and the hyperfine separations increase.

Discussion

At room temperature, the paramagnetic resonance spectra of the carbonmonoxy, met azide, met, and met fluoride derivatives of horse hemoglobin labeled at the $\beta 93$ cysteines with I or II show two distinct components, designated A and B in solution spectra, A' and B' in polycrystalline spectra, and A''

and B'' in single crystal spectra. Qualitatively, the relative intensities of the two components in each case have the same dependence upon the ionic composition of the solvent and upon the ligand at the sixth coordination position of the iron atoms. In all cases, the "A" signals are more strongly immobilized than the corresponding "B" signals. There can be little doubt that A, A', and A'' all represent essentially one state of the label relative to the protein and that B, B', and B'' represent a second. There must be an equilibrium between the weakly and strongly immobilized states, $A \rightleftharpoons B$, $A' \rightleftharpoons B'$, $A'' \rightleftharpoons B''$, that depends in a sensitive way upon the ionic composition of the solvent and upon the ligand bound to the iron atoms. The spectra of the carbonmonoxy and met azide derivatives are essentially identical with one another under all conditions studied. The spectra of the met and met fluoride derivatives are likewise the same. However, the spectra of the former two derivatives are distinctly different from the spectra of the latter two, primarily in the relative intensities of components "A" and "B."

These results show that the local protein conformation in the vicinity of a label at a β93 cysteine depends upon the spin state of the iron atoms. However, the high- and low-spin protein structures must be very similar, since the principal axis orientations for both I and II were found to be very nearly the same for all four derivatives. Evidently the equilibria between the A, B isomeric states are sensitive to *very* small changes in protein conformation.

The present study establishes that the differences in the solution spectra of labeled HbCO and metHb persists in the single crystal spectra. Because the change of protein structure associated with this difference is very small, the present results are not inconsistent with the previously mentioned conclusion of Perutz and Mathews (1966) that the met, oxy, and met azide derivatives have very similar structures.

The present study permits a comparison to be made between the local protein structure in the vicinity of β93 for hemoglobin in solution and in crystals. For a given ionic composition of the solvent the passage from solution to crystals is accompanied by an increase in the degree of immobilization of the labels and very probably also a change in the relative populations of the two conformational states. Here again it can be concluded that there can be no large change in protein structure, since in both cases there is a delicately balanced equilibrium between the two isomeric spin-label states. (On the other hand, such small spectral changes can have biophysical significance. For example, the ionic binding of spin-labeled hemoglobin to erythrocyte membranes at pH 6.15 produces small but definite changes in the label resonance spectra; W. L. Hubbell and H. M. McConnell, 1967, unpublished data.)

The significance of the above conclusions with respect to the problem of the heme–heme interaction has been discussed briefly elsewhere (McConnell *et al.*, 1968) and will be discussed further in a subsequent paper. However, it may be of interest to emphasize here that the paramagnetic resonance of spin-labeled deoxyhemoglobin shows *no* evidence of an $A \rightleftharpoons B$ isomeric equilibrium between two spin-label states, and so is uniquely different from the resonance spectra of all the derivatives (HbCO, HbO_2, metHb, met azide, and met fluoride Hb) discussed in this paper. Thus the local protein conformation that dictates the spin-label resonance spectrum must reflect the quaternary structure of the hemoglobin molecule, since it is known from the work of Perutz and coworkers that this quaternary structure is uniquely different in the case of deoxyhemoglobin (Muirhead *et al.*, 1967; Bolton *et al.*, 1966; Perutz and Mathews, 1966).

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