Nuclear Magnetic Resonance and Spin-Label Studies of Hemoglobin Kempsey†

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ABSTRACT: Nuclear magnetic resonance and spin-label studies of Hb Kempsey indicate that the substitution of asparagine for aspartate at G1(99)β produces widespread structural changes. The structural differences as well as concomitant changes in the functional properties indicate that the $\alpha_1\beta_2$ subunit interface has a profound influence on the heme groups as they bind ligands. Comparison of the hyperfine shifted nuclear magnetic resonance (nmr) spectra of deoxyHb A and deoxyHb Kempsey shows that the structure of both α and β hemes is altered. Similar comparison of the ring-current shifted proton resonances of HbCO A and HbCO Kempsey indicates that the substitution at β99 shifts the E11(67)β valine away from the heme group. The nmr spectra of Hb Kempsey as a function of CO concentration show that the β hemes have a higher ligand affinity. The addition of inositol hexaphosphate changes the structure of both the α and β hemes in deoxyHb Kempsey and causes the α and β hemes to bind ligands with equal affinity. The spin-label results show that inositol hexaphosphate alters the mutated $\alpha_1\beta_2$ subunit interface in Hb Kempsey which allows the α chains to influence the structure of the β chains during ligand uptake similar to that observed in Hb A.

Hemoglobin Kempsey is a mutant human hemoglobin which produces erythrocytosis in individuals heterozygous for Hb A[1] and Hb Kempsey (Reed *et al.*, 1968). Hb Kempsey differs from Hb A by a single amino acid substitution in the $\alpha_1\beta_2$ subunit interface where the aspartic acid at β99 is replaced by an asparagine residue (Reed *et al.*, 1968). Normally in deoxyHb A, the carboxy group of the aspartate at G1(99)β forms a hydrogen bond with the phenolic hydroxyl group of the tyrosine at C7(42)α and this hydrogen bond is broken in the transformation to the liganded form of Hb A (Perutz and Ten Eyck, 1971). In deoxyHb Kempsey, this important intersubunit hydrogen bond cannot be formed and the deoxy quaternary structure is less stable (Morimoto *et al.*, 1971). In fact, the crystals of deoxyHb Kempsey have been found to have oxy-like quaternary structure (Perutz *et al.*, 1974). This quaternary structural change in Hb Kempsey is thought to be the cause of the increased oxygen affinity and reduced cooperativity. In 0.05 M bis-tris + 0.1 M NaCl at pH 7.2 and 20°, the partial pressure of oxygen necessary to saturate 50% of the hemes (p_{50}) in Hb Kempsey is only 0.25 Torr whereas the p_{50} of Hb A under similar conditions is 2.5 Torr.[2] The addition of 1 mM inositol hexaphosphate to Hb Kempsey causes the p_{50} to rise to 1.1 Torr.[2] The Hill coefficient for the oxygenation of Hb Kempsey is 1.1 to 1.2 indicating that the hemes bind oxygen rather independently (Reed *et al.*, 1968).[2] However, in the presence of 1 mM Ins-P_6, the Hill coefficient becomes 1.6 as the cooperative interactions between ligand binding sites become apparent.[2] The Bohr effect of Hb Kempsey (in the absence of organic phosphate) is less than half that of Hb A.[2] In light of these interesting properties, we thought it appropriate to investigate the structure-function relationships of Hb Kempsey using the techniques of nmr and spin labeling.

Nmr and spin-labeling techniques have been shown to be useful in the study of hemoglobin structure and function (Davis *et al.*, 1969, 1971; Lindstrom *et al.*, 1972a,b; Lindstrom and Ho, 1972, 1973; Ho and Lindstrom, 1972; Ho *et al.*, 1970, 1973; Baldassare *et al.*, 1970; Ogawa *et al.*, 1968; Ogata and McConnell, 1971, 1972a,b; Ogata *et al.*, 1972; Ogawa and Shulman, 1971, 1972; Cassoly *et al.*, 1971). Nmr spectra of the hyperfine (or contact) shifted proton resonances have shown that the α and β hemes in deoxyHb A are not structurally equivalent and under certain circumstances, nonequivalent in their ligand affinities (Davis *et al.*, 1971; Lindstrom *et al.*, 1971, 1972a; Lindstrom and Ho, 1972; Ho and Lindstrom, 1972). In some cases, these hyperfine shifted resonances are sensitive to changes in the quaternary structure of the hemoglobin molecule (Davis *et al.*, 1971; Ho *et al.*, 1973; Perutz *et al.*, 1974). They arise from the interactions between the unpaired electrons of the paramagnetic iron atom and the porphyrin protons as well as those amino acid protons positioned sufficiently close to the iron atom (Kurland *et al.*, 1971). These resonances would vanish when ligands (such as O_2 or CO) bind to the hemes and the hemes become diamagnetic. The ring-current shifted proton resonances have shown to be very sensitive to changes in the tertiary structure around the ligand binding sites in carbonmonoxyhemoglobin (Lindstrom *et al.*, 1972b; Lindstrom and Ho, 1973). Electron paramagnetic

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[1] Abbreviations used are: Hb A, human adult hemoglobin; HbCO, carbonmonoxyhemoglobin; p_{50}, the partial pressure of oxygen necessary to saturate 50% of the hemes in hemoglobin; Tris, tris(hydroxymethyl)-aminomethane; bis-tris, 2,2-bis(hydroxyethyl)-2,2′,2″-nitrilotrimethanol; Ins-P_6, inositol hexaphosphate.

[2] H. F. Bunn, T. B. Bradley, R. C. Wohl, and Q. H. Gibson, unpublished results.

resonance (epr) measurements of hemoglobins labeled with *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide at F9(93)β cysteine SH groups have provided information on the $\alpha_1\beta_2$ subunit interactions during the ligand binding (Ho *et al.*, 1970; Baldassare *et al.*, 1970).

Experimental Section

Materials. Hb Kempsey was isolated and purified by chromatography on both DEAE-Sephadex (Huisman and Dozy, 1965) as previously described (Davis *et al.*, 1971) and on CM-cellulose.[2] Two different sources of Hb Kempsey (one obtained from Dr. Virgil F. Fairbanks through Dr. Richard T. Jones and one from H. F. B.) were used and we found no observable differences in the nmr and epr spectra of these two Hb Kempsey samples. Hb A was isolated in the sample preparation of Hb Kempsey or was prepared by standard methods from fresh whole blood samples obtained from the local blood bank (Davis *et al.*, 1971). The hemoglobin was made phosphate free by gel filtration with a Sephadex G-25 column (2.5 × 50 cm) using as buffer 0.01 M Tris-HCl with 0.1 M NaCl at pH 7.45 (Berman *et al.*, 1971). Deuterium oxide (Merck, Sharp & Dohme of Canada, Ltd.) was exchanged for H_2O by repeated dilution with D_2O and concentration by ultrafiltration through an Amicon UM-20E membrane. Appropriate amounts of 1.0 M deuterated potassium phosphate buffer were added to the hemoglobin solutions after the final concentration so that the hemoglobin solutions were buffered at pD 7.0 with 0.1 M phosphate. Sodium inositol hexaphosphate (Sigma Type V) was dissolved in D_2O and titrated to pH 6.7 with NaOH and then repeatedly lyophilized and redissolved in D_2O. This resulted in a 0.09 M stock solution of Ins-P_6 in D_2O at pD 6.6. When necessary, this stock solution was added to the hemoglobin solutions in varying amounts. The pD of solutions was determined by adding 0.4 pH unit (Glasoe and Long, 1960) to the value read from a Radiometer Model 26 pH meter equipped with a Beckman 39030 combination electrode. To prepare deoxy samples, the CO ligand was replaced by O_2 by flushing the HbCO solution in a rotatory evaporator in an ice–water bath under a Sylvania 150-W flood lamp. Oxygen was then removed by flushing the solution with prepurified nitrogen. Partially saturated samples were prepared by anaerobic mixing of appropriate amounts of deoxy- and carbonmonoxyhemoglobin solutions. The hemoglobin concentration in our nmr studies varied from 6 to 17%.

Hemoglobin samples for the spin-label studies were prepared in the same manner as those used in nmr studies except that no D_2O exchange was needed. The HbCO samples were labeled with *N*-(1-oxy-2,2,6,6-tetramethyl-4-piperidinyl)-iodoacetamide (Synvar Associates) as previously described (Ogawa *et al.*, 1968; Ho *et al.*, 1970; Baldassare *et al.*, 1970). The hemoglobin concentration in our spin-label studies varied from 0.5 to 1%.

Methods. Nmr spectra were obtained on the MPC-HF 250-MHz superconducting spectrometer (Dadok *et al.*, 1970) and on a Bruker HFX 90-MHz spectrometer. Standard 5-mm nmr sample tubes were used. The ambient temperatures of the spectrometers were 31° for the 250 MHz and 28° for the 90 MHz. Proton chemical shifts are referenced with respect to the residual H_2O in the samples. The HDO signal is −4.72 ppm downfield from the proton resonance of 2,2-dimethyl-2-silapentane-5-sulfonate at 31° and −4.80 ppm from dimethylsilapentanesulfonate at 28°. The negative sign of the chemical shifts indicates that the resonance is downfield from that of HDO and the positive sign indicates that the resonance is upfield from the HDO signal. The hyperfine shifted resonances are accurate to ±0.1 ppm and the ring-current shifted resonances are accurate to ±0.05 ppm. The signal to noise ratios were improved by signal averaging with a Northern Scientific Model NS-544 digital memory oscilloscope when using the MPC-HF 250-MHz spectrometer and with a Fabri-Tek Model 1074 computer when using the Bruker HFX 90-MHz spectrometer.

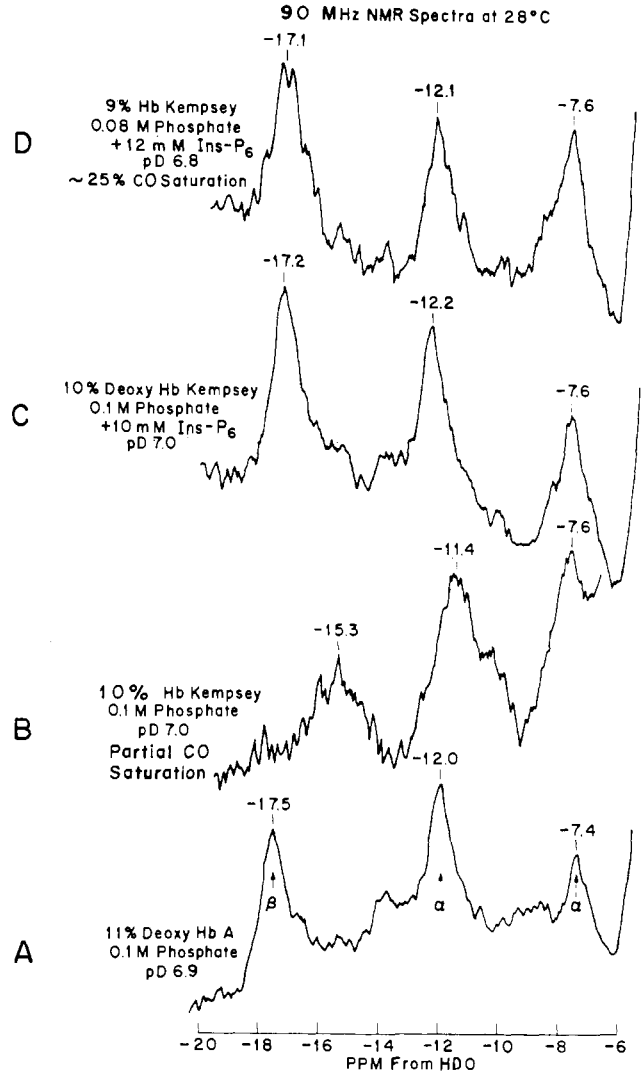

FIGURE 1: 90-MHz nmr spectra at 28°: (A) deoxyHb A in 0.1 M phosphate at pD 6.9; the symbols α and β under the peaks indicate those resonances assigned to α and β hemes, respectively; (B) Hb Kempsey in 0.1 M phosphate at pD 7.0 partially saturated with CO (up to 25% saturation); (C) deoxyHb Kempsey in 0.1 M phosphate + 10 mM inositol hexaphosphate at pD 7.0; and (D) Hb Kempsey in 0.08 M phosphate + 12 mM inositol hexaphosphate at pD 6.8 partially saturated with CO (approximately 25% saturation).

Epr spectra of the spin-labeled hemoglobin samples were obtained on a Bruker B-ER 418s spectrometer and a Varian E-4 spectrometer. Both spectrometers were operated at the X-band frequency (~9.5 GHz). The ambient temperatures inside the microwave cavities of these two epr spectrometers were 22–23°.

Results

In Figure 1, we show the 90-MHz proton nmr spectra of deoxyHb A and deoxyHb Kempsey in the region of the spectrum where the low-field hyperfine shifted resonances

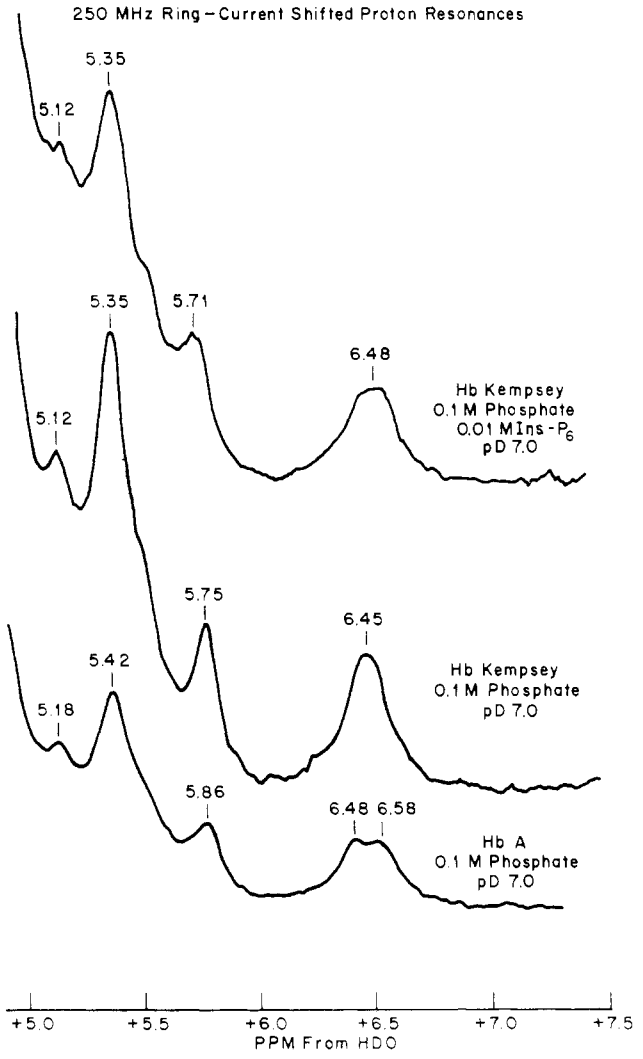

FIGURE 2: 250-MHz ring-current shifted proton resonance of HbCO Kempsey and HbCO A at 31°.

occur. As is well documented, deoxyHb A displays three prominent low-field hyperfine shifted resonances, a β-chain resonance at ~ -17.5 ppm and two α-chain resonances at ~ -12.0 and ~ -7.4 ppm (Davis *et al.*, 1971; Lindstrom *et al.*, 1972a). And as we have previously reported (Davis *et al.*, 1971), three resonance lines in the deoxyHb Kempsey spectrum are at -15.3, -11.4, and -7.6 ppm from HDO. In view of the high ligand affinity of Hb Kempsey, it is difficult to remove all the ligand from a 10% Hb Kempsey sample under our experimental conditions and we estimate that the sample of Hb Kempsey giving the spectra in Figure 1B is up to 25% saturated with CO. The area under the peak at -15.3 ppm is about 50% of that under the peak at -11.4 ppm. We have previously assigned the resonance at -15.3 ppm as due to a β heme and that at -11.4 ppm to an α heme in Hb Kempsey (Davis *et al.*, 1971). The preferential decrease of the β-heme resonance is direct evidence that the β hemes have a higher affinity for CO as compared to that of the α hemes in deoxyHb Kempsey. When Ins-P_6 is added to deoxyHb Kempsey (Figure 1C), the spectrum reverts to one similar to deoxyHb A. The heme proton resonances now occur at -17.2, -12.2, and -7.6 ppm. If a small amount of CO is present, it becomes distributed randomly to the α and β hemes (within the sensitivity of our measurement) when Ins-P_6 is present (Figure 1D).

Figure 2 shows the 250-MHz ring-current shifted proton resonances of HbCO A, HbCO Kempsey, and HbCO Kempsey plus added Ins-P_6 at pD 7.0. In the spectrum of HbCO A, the resonances at $+6.58$ and $+5.86$ ppm from HDO have been assigned respectively to the γ_1 and γ_2 methyl groups of βE11 valine and the resonance at $+6.48$ ppm has been assigned to the γ_1 methyl group of the αE11 valine (Lindstrom *et al.*, 1972b). The ring-current shifted proton resonances of HbCO A and HbCO Kempsey differ in the shifts of βE11 valine methyl groups. This suggests that the tertiary structure of the β-heme pocket in HbCO Kempsey has been altered as a result of the amino acid substitution at G1(99)β which is located at the $\alpha_1\beta_2$ subunit interface, near the proximal histidine residue. The addition of 10 mM Ins-P_6 to HbCO Kempsey in 0.1 M phosphate at pD 7.0 appears to broaden the line at $\sim +6.5$ ppm suggesting that the γ_1 methyl groups of E11 valine in the α and β chains become nonequivalent as in the case of HbCO A.

The epr spectra in Figure 3 compare the spin-label response to CO binding to Hb A and Hb Kempsey. The lack of isosbestic points in the Hb A curves demonstrates that the spin label at F9(93)β senses more than two conformations as deoxyHb A is converted to HbCO A. However, as the epr spectra of Hb Kempsey show, the nitroxide label senses only two structures in solution as deoxyHb Kempsey is converted to HbCO Kempsey. When increasing amounts of Ins-P_6 are added to the Hb Kempsey solution (Figure 4), the spin label again senses more than two conformations as evidenced by the deviation from isosbesty in Figure 4B.

Discussion

The nmr contact shifted proton resonances indicate that the structure of deoxyHb Kempsey is significantly different from that of deoxyHb A. As has been noted previously in a variety of hemoglobins (Davis *et al.*, 1971), the heme group in the chain containing the changed amino acid in the $\alpha_1\beta_2$ contacts is perturbed to the greatest extent, but we believe it very important that the α heme group (*i.e.*, the normal chain in this case) is also affected. We have already suggested that this is one way in which structural changes can be transmitted between the α- and β-heme groups (Davis *et al.*, 1971); since the mutation in the interface affects the structure of both heme groups, it is logical to assume that a structural change at one heme can influence the structure at the other heme through one of the residues at the $\alpha_1\beta_2$ subunit interface, such as FG4(92)α, G1(99)β.

Results from a number of investigators have indicated that deoxyHb Kempsey exists in an oxy-like or R-type quaternary structure (Ogata *et al.*, 1972; Perutz *et al.*, 1974). The results of our magnetic resonance studies support the idea that deoxyHb Kempsey is in an R-type quaternary structure. We know from our own studies of chemically modified deoxyhemoglobins known to be stabilized in an R-type quaternary structure that the hyperfine shifted proton resonances are sensitive indicators of R or T type of quaternary structure, especially the β heme resonance found at -17.5 ppm in deoxyHb A (Perutz *et al.*, 1974). This β heme resonance upfield from -16 ppm in all deoxyhemoglobins has an R-like quaternary structure. For deoxyhemoglobins having a T-like quaternary structure, the β-heme resonance is below -16 ppm. The β-heme resonance in deoxyHb Kempsey is at -15.3 ppm indicative of an R-type or oxy-like quaternary structure.

When 10 mM Ins-P_6 is added to deoxyHb Kempsey (Figure 1C), the nmr hyperfine shifted spectrum reverts to one resembling, but not identical with, deoxyHb A. It should be

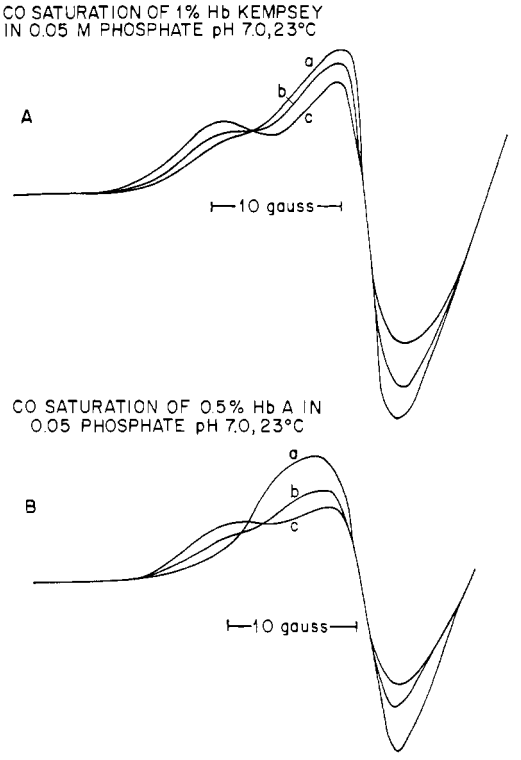

FIGURE 3: Epr spectra of hemoglobins labeled with *N*-(1-oxy-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide at F9(93)β cysteine SH groups as a function of CO saturation: A, Hb Kempsey, and B, Hb A. a represents deoxyHb, b represents intermediate CO saturation, and c represents fully CO form. The spectra were obtained on a Bruker B-ER 418s epr spectrometer operated at the X-band frequency.

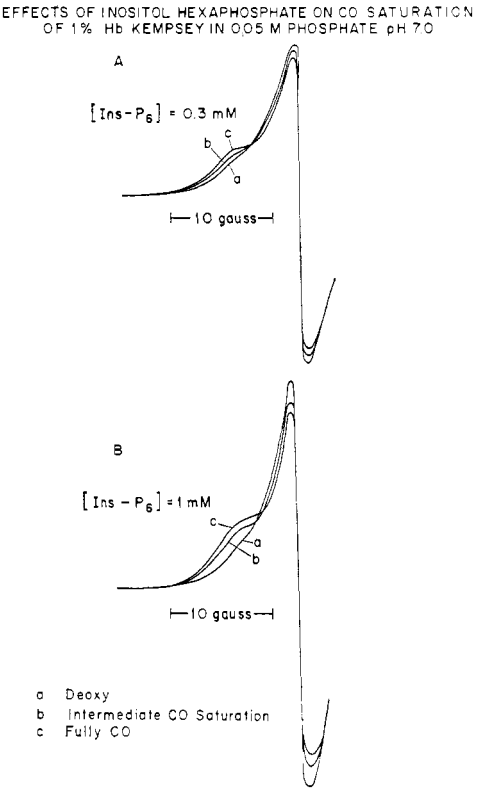

FIGURE 4: Effects of inositol hexaphosphate on the epr spectra of Hb Kempsey labeled with *N*-(1-oxy-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide at F9(93)β cysteine SH groups. A, [Ins-P_6] = 0.3 mM, and B, [Ins-P_6] = 1 mM. The spectra were obtained on a Varian E-4 epr spectrometer operated at the X-band frequency.

mentioned that Ins-P_6 has no significant effect on the heme proton resonances of deoxyHb A in 0.1 M phosphate at pD $\sim$7. Ins-P_6 shifts the quaternary structure of deoxyHb Kempsey from R to T type. This is consistent with the optical difference spectra (Perutz *et al.*, 1974) and also consistent with the higher observed Hill coefficient in the presence of Ins-P_6.[2] The allosteric equilibrium constant, L, for deoxyHb Kempsey is significantly increased by Ins-P_6, but it may still be considerably less than that for deoxyHb A.

The epr spectra of the spin-labeled Hb Kempsey may be interpreted as a similar pattern of structural transitions (Figures 3 and 4). For spin-labeled Hb A, the absence of a set of isosbestic points in the epr spectra as a function of O_2 or CO saturation has been interpreted as showing the existence of more than two conformations predicted by the concerted models of hemoglobin (Ogawa *et al.*, 1968; Ho *et al.*, 1970; Baldassare *et al.*, 1970). These extra conformations are likely produced when only one type of hemes has bound ligands within either the T or R quaternary structure (Perutz, 1970). For example, we can denote these two quaternary structures as $[\alpha_2^r\beta_2^t]^T$ and $[\alpha_2^r\beta_2^t]^R$. Under such circumstances, the $\alpha_1\beta_2$ subunit interface does not dovetail properly, to use Perutz's terminology, and the spin label senses these strained conformations (Perutz, 1970). In Hb Kempsey, the presence of isosbestic points in the spectra at various ligand saturations indicates that the spin label at F9(93)β takes up only two conformations. This behavior is consistent with the presumption that Hb Kempsey exists to a great degree in an R-like quaternary structure in both the deoxy and oxy forms. The spin label senses only the change in tertiary structure of the β chain as it binds carbon monoxide. Other $\alpha_1\beta_2$ mutant hemoglobins (Hb Chesapeake, Hb J Capetown, Hb Yakima) also show isosbesticity with this spin label through the carbon monoxide profile (Ho *et al.*, 1970; Baldassare *et al.*, 1970). However, in contrast to Hb Kempsey, these hemoglobins undergo quaternary structural changes on CO or O_2 binding (Greer, 1971; Nagel *et al.*, 1971; Pulsinelli, 1973). In this respect Hb Kempsey is different from the other $\alpha_1\beta_2$ mutant hemoglobins we have studied (Ho *et al.*, 1970; Baldassare *et al.*, 1970). The details of the paradox will be examined in a subsequent manuscript, but the general conclusion is that the presence of isosbestic points reflects in a first approximation the loss of integrity of the $\alpha_1\beta_2$ interface and a loosening of the dovetail fit that can exert an additional distortion on the β chains (Baldassare *et al.*, 1970).

When Ins-P_6 is added to the Hb Kempsey solution, the isosbestic points are again absent indicating the presence of more than two conformations (Figure 4). We can suggest two mechanisms by which Ins-P_6 produces this result. When Ins-P_6 binds to the tetramer, it stabilizes the T form. Our nmr results show that Ins-P_6 produces an R to T quaternary structural change to a structure that is similar to deoxyHb A. This change forces an added structural constraint on the tertiary structure of the β chains and produces the additional structures $[\alpha_2^r\beta_2^t]^T$ or $[\alpha_2^t\beta_2^r]^T$. An alternate suggestion is that the presence of more than two conformations is due to the presence of both Ins-P_6 complexed and Ins-P_6 free Hb Kempsey tetramers that themselves may take up R and T quaternary structures. The absence of isosbestic points is then due to incomplete binding of Ins-P_6. We are less inclined to accept this latter explanation because a twofold molar excess of Ins-P_6 does not produce more than two conformations (Figure

4A), that is, the isosbestic points are present in 0.3 mM Ins-P_6, yet the isosbestic points are absent in a sixfold molar excess of Ins-P_6. We should think that if the absence of isosbestic points is due to incomplete Ins-P_6 binding, then the opposite effect would be true. Low concentration of Ins-P_6 would produce a variety of conformations, some with Ins-P_6 bound and some without, and the sixfold molar excess of Ins-P_6 would produce only Ins-P_6-bound structure. This is clearly not the case as shown in Figure 4, and for this reason we believe that Ins-P_6 removes the isosbestic conditions by stabilizing the deoxyHb Kempsey tetramer in a T type of conformation in which the $\alpha_1\beta_2$ subunit interface is responsive to strains and the need for a dovetail fit.

One important feature of the nmr hyperfine shifted proton resonance is that they show directly the occurrence of preferential ligand binding. This feature has already been exploited to show that CO binds preferentially to the α hemes in Hb Chesapeake (Davis *et al.*, 1971), that O_2 binds preferentially to the α hemes of Hb A in the presence of 2,3-diphosphoglycerate or Ins-P_6 (Lindstrom and Ho, 1972), and that *n*-butyl isocyanide binds preferentially to the β hemes of Hb A in the presence of Ins-P_6 (Lindstrom *et al.*, 1971). As is depicted in Figure 1B, carbon monoxide binds to the β hemes of Hb Kempsey in preference to the α hemes. The preferential binding to the β hemes in Hb Kempsey has been predicted by Ogata *et al.* (1972). These predictions were based on the results of the triphosphate spin-label studies which could be explained if the β chains in the deoxyHb Kempsey were assumed to have a ligand affinity 100 times that of the β chains in deoxyHb A (Ogata *et al.*, 1972; Ogata and McConnell, 1972b).

The preferential CO binding to the β chains of Hb Kempsey does not occur when Ins-P_6 is present (Figure 1D). The structure of the inositol hexaphosphate–Hb Kempsey complex is such that both α and β hemes have similar affinities for CO within the accuracy of our nmr measurement.

The ring-current shifted proton resonances are known to be sensitive to changes in the tertiary structure of the heme pocket (Lindstrom *et al.*, 1972a,b; Lindstrom and Ho, 1973) and appear to be related to ligand affinity (Lindstrom and Ho, 1973). In HbCO A the ring-current shifted resonances at +6.58 and +5.86 ppm are the γ_1 and γ_2 methyls of the E11(67)β valine while the resonance at +6.48 ppm is the γ_1 methyl of the E11(62)α valine (Lindstrom *et al.*, 1972a,b). In HbCO Kempsey the βE11 valine resonances appear at +6.45 and +5.75 ppm while the other ring-current shifted resonances are similar to those observed for HbCO A. According to our earlier suggestions (Lindstrom and Ho, 1973), the shift of the βE11 methyl resonances to lower field indicates a movement of the βE11 methyl away from the iron atom and an increase in the ligand affinity of the β-chain hemes within the R quaternary structure. Here again we have a case where a modification at the $\alpha_1\beta_2$ subunit interface influences the structure and function of the heme. According to Perutz (1970), the proper conformation of the βE11 valine is critical for the ability of ligands to enter the heme pocket and the ring-current shifted resonances of HbCO Kempsey provide evidence that structural transitions in the $\alpha_1\beta_2$ subunit interface (on the proximal histidine side of the heme pocket) will alter the conformation of the βE11 valine (a residue located on the distal histidine side of the heme pocket). It is important to remember that the only observable differences between the ring-current shifted resonances of HbCO A and HbCO Kempsey are the βE11 methyl resonances which indicate a rather specific structural change as opposed to the widespread quaternary structural transformation in the ligand-free form of Hb Kempsey.

The results of our magnetic resonance studies of Hb Kempsey may be summarized by reviewing the apparent structural differences between Hb Kempsey and Hb A and their relationship to the altered ligand binding properties. DeoxyHb Kempsey exists predominantly in an R-like quaternary structure which is presumably due to the loss of the hydrogen bond between Asp-G1(99)β and Tyr-C7(42)α which stabilizes the T form of deoxyHb A (Perutz and Ten Eyck, 1971). The R like structure of deoxyHb Kempsey can be shifted to a T like structure by the addition of Ins-P_6 which overcomes the loss of stability of the $\alpha_1\beta_2$ interface. Within the R-like structure, the β chains have a higher affinity for carbon monoxide, but in the T-like structure, the hemes have approximately the same ligand affinity. The substitution of an amide for a carboxylate at the β99 side chain as occurs in Hb Kempsey has a lesser effect on the structure of the liganded form. The heme pockets of HbCO Kempsey differ from those of HbCO A only in the position of the βE11 valine methyls in a manner that should increase the ligand affinity of the β hemes.

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Pressure Denaturation of Metmyoglobin†

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ABSTRACT: When metmyoglobin is subjected to hydrostatic pressure its visible spectrum changes in a manner similar to that observed on denaturation by acid, heat, and urea. This process, which is generally reversible, was studied as a function of temperature and pH at pressures between 1 atm and 6000 kg/cm², temperatures between 5 and 80°, and pH values between 4 and 13. Combinations of pressure, temperature, and pH for the midpoint of the denaturation transition lead to a surface in (P, T, pH) space whose shape has been determined. The most striking feature of this surface is that at any pH from 4 to 13 a range of pressures exists in which the protein undergoes the changes, denatured state → native state → denatured state, when the protein is heated from 0 to 80° at constant pressure. Volume changes for denaturation were obtained by studying the effect of pressure on the equilibrium constant at several values of the pH and temperature. They are of the order of −100 ml/mol at pH <6 and −60 ml/mol at pH 10. Values for the enthalpy and entropy changes of the process under a pressure of 2800 kg/cm² were obtained at temperatures from 5 to 60°. The heat capacity change, ΔC_p, on denaturation appears to be large and relatively insensitive to pressure up to 3800 kg/cm². These results are difficult to explain if the thermodynamics of denaturation is determined by the exposure of buried nonpolar groups to water.

It is widely believed that the native conformation of a protein molecule in aqueous solution is stabilized chiefly by hydrophobic interactions (Kauzmann, 1959; Némethy and Scheraga, 1962; Eisenberg, 1970), that is, the tendency of nonpolar side chains to cluster in the interior of the protein and away from the surrounding water. If hydrophobic interactions are indeed the principal stabilizing forces in proteins and if protein denaturation involves the exposure of a significant number of nonpolar groups to the aqueous environment, then one would expect the thermodynamic changes accompanying the denaturation of proteins to resemble those of a model process in which simple nonpolar molecules are transferred from a nonpolar environment (which should resemble the interior of a protein) to an aqueous one. Certain aspects of the thermodynamics of the denaturation of ribonuclease, chymotrypsinogen, and metmyoglobin at 1 atm do show similarities to this model process (Brandts, 1969). The model is especially successful in accounting for the behavior of the heat capacity, which is much larger for the denatured protein than for the native form. The model is, however, far from successful in accounting for the changes produced by high pressure (Brandts *et al.*, 1970; Kliman, 1969).

The observed three-dimensional structure of metmyoglobin, with its predominantly hydrophobic interior (Kendrew *et al.*, 1961), makes this protein particularly well suited to the study of hydrophobic interactions as a factor in stabilizing the native structure. For this reason it seemed desirable to investigate the effect of pressure on the pH and temperature denaturation of metmyoglobin.

Information is available on the denaturation of metmyoglobin at high and low pH (Theorell and Ehrenberg, 1951; Karodjova and Atanasov, 1964; Acampora and Hermans, 1967), at high temperature (Acampora and Hermans, 1967), and in urea (Khalifah, 1968; Schechter and Epstein, 1968) as well as in the presence of various inorganic ions (Cann, 1964; Hartzell *et al.*, 1967). The denaturation of this protein as a function of temperature and pH has been studied by both optical absorbance and optical rotation measurements. The process was found to be reversible at pH below 5 and above 10 (Acampora and Hermans, 1967). A critical analysis of these data, in conjunction with potentiometric titration data (Breslow

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