Proc. Nat. Acad. Sci. USA
Vol. 69, No. 7, pp. 1707–1710, July 1972

Functional Nonequivalence of α and β Hemes in Human Adult Hemoglobin

(NMR/preferential ligand binding/organic phosphates/models of cooperative ligand binding)

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Communicated by Harden M. McConnell, April 21, 1972

ABSTRACT **Nuclear magnetic resonance studies of the contact-shifted spectra of heme protons in deoxyhemoglobin A from human adults show conclusively that oxygen binds to the α hemes in preference to the β hemes. The preferential binding is produced in 10% hemoglobin solution at neutral pH by either a 15-fold molar excess of 2,3-diphosphoglycerate or a 5-fold molar excess of inositol hexaphosphate. Preferential binding is not observable in the absence of the organic phosphates. The results indicate that the oxygenation of hemoglobin may be described by a sequential model, or by a concerted model that allows the α hemes to bind ligand first.**

Our previous nuclear magnetic resonance (NMR) studies have indicated that the α and β hemes in a deoxyhemoglobin A tetramer are not equivalent (1–3). This conclusion was based on our identification of separate contact-shifted NMR resonances due to the α and β hemes in Hb A. In the NMR spectrum of deoxy Hb A, contact-shifted resonances appear at −17.9, −12.1, and −7.4 ppm downfield from the HDO signal. Studies of modified human hemoglobins (1, 2), especially Hb M Milwaukee-1 [E11(67)β Val → Glu]†, have enabled us to assign the contact-shifted line at −17.9 ppm to the β-chain hemes and the other two resonances at −12.1 and −7.4 ppm to the α-chain hemes.

The contact-shifted proton NMR signals result from hyperfine interactions between the unpaired electrons of a paramagnetic iron atom and of porphyrin protons, as well as amino-acid protons positioned sufficiently near the iron atom (4). These hyperfine interactions vanish when ligands bind to the hemes and the hemes become diamagnetic. Since we are able to distinguish proton resonances of the deoxy α hemes from those of the deoxy β hemes in Hb A, we should be able to observe whether ligands bind to one type of heme in preference to the other. This effect has been demonstrated by us for the preferential binding of *n*-butyl isocyanide to the β-chain hemes in Hb A (3), the preferential binding of CO to the α-chain hemes in Hb Chesapeake [FG4(92)α Arg → Leu] (2), and the preferential binding of CO to the β-chain hemes in Hb Yakima [G1(99)β Asp → His] (T. R. Lindstrom, R. T. Jones, and C. Ho, unpublished results). This preferential binding observed by the NMR method is consistent with the results of other methods of analysis, notably with the kinetics of *n*-butyl isocyanide binding to Hb A (5, 6), and with spin-label studies of Hb Chesapeake (7) and Hb Kempsey [G1(99)β Asp → Asn] (8).

Perutz's atomic model of deoxyhemoglobin indicates that ligands should first bind to the α hemes before the β hemes, because the valine methyl groups of the E11(67)β block the ligand binding site of the β hemes (9). According to Perutz, the E11(67)β valine moves away from the ligand binding site only after a series of structural transitions have occurred that are triggered by the ligand binding to the α hemes (9). Perutz further describes how 2,3-diphosphoglycerate(P_2 glycerate) and hydrogen ion inhibit the structural transition from the deoxy to the oxy quaternary structure (9). Based on indirect kinetic and spectroscopic evidence, Gray and Gibson (10) have proposed that CO combines first with the β chains before the α chains. As yet, there is no direct experimental evidence showing that oxygen binds to one type of heme in Hb A in preference to another. This is a preliminary report on the preferential binding of oxygen to the α-chain hemes in the presence of P_2 glycerate or inositol hexaphosphate(IP_6), as monitored by selective decrease of the contact-shifted resonances of the α chains.

MATERIALS AND METHODS

HbCO A was prepared (11) from whole blood obtained from a local blood bank; distilled water was used to lyse the cells. The hemoglobin was made phosphate-free by gel filtration with a Sephadex G-25 column (2.5 × 50 cm); 0.05 M Tris·HCl–0.1 M NaCl, pH 7.45, was used as a buffer (12). Deuterium oxide was exchanged for H_2O by repeated concentration by ultrafiltration through an Amicon UM-20E membrane and dilution with D_2O (Merck, Sharpe & Dohme). Stock solutions of either 0.15 M P_2 glycerate or 0.09 M IP_6 and 1.0 M Bis–Tris (Calbiochem) were added, so the solutions used for measurement were 9% Hb A, 32 mM P_2 glycerate, 0.1 M Bis–Tris·HCl at pD 6.8, and 11% Hb A, 10 mM IP_6, 0.1 M Bis–Tris·HCl at pD 7.1. The pD of the solutions was determined by addition of 0.4 pH units (13) to the value obtained with a Radiometer model 26 pH Meter equipped with a Beckman 39030 electrode. The carbon monoxide was replaced by oxygen by flushing the solution in a rotory evaporator in an ice–water bath under a Sylvania 150-W flood lamp. Oxygen was removed by flushing the solution with purified nitrogen. Partially oxygenated samples were obtained by transfer of aliquots from the rotory evaporator into NMR tubes at intervals before complete deoxygenation was reached. Because of the concentration of hemoglobin, we did not determine the percent of oxygen saturation of the solutions in

Abbreviations: P_2 glycerate, 2,3-diphosphoglycerate; IP_6, inositol hexaphosphate.

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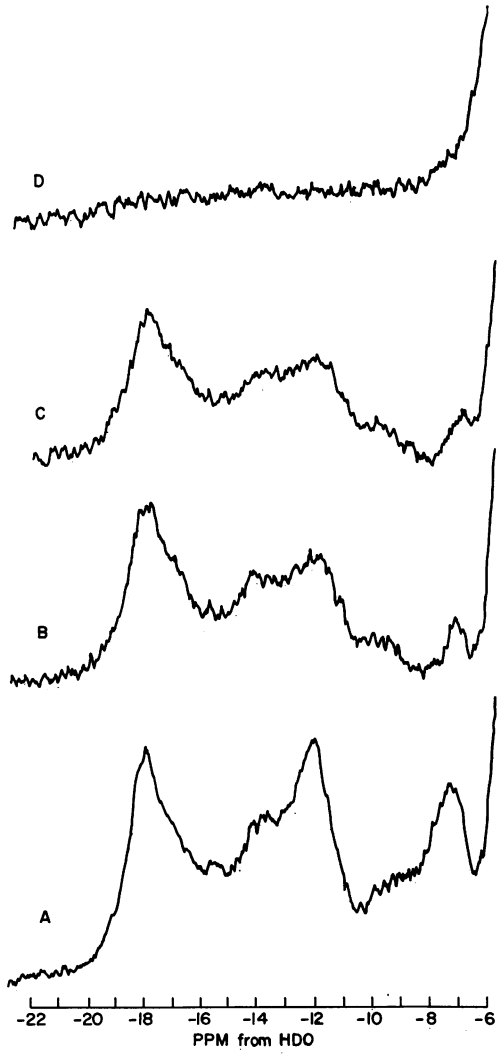

FIG. 1. 250-MHz proton NMR spectra of 9% Hb A in 32 mM P_2 glycerate, 0.1 M Bis-Tris·HCl at pD 6.8 and at 31°: *A*, deoxy Hb; *B* and *C*, partial oxygenated Hb (10–40% saturation); and *D*, fully oxygenated Hb.

the NMR tubes. A thin film of hemoglobin solution adhering to the inside walls of the NMR tubes was used to obtain the absorption spectrum between 500–700 nm with a Cary 14 spectrophotometer. The absorption spectrum indicated that the samples were between 10 and 40% oxygenated, and that samples obtained in the latter stages of the deoxygenation process contained less oxygen.

NMR spectra were obtained on an MPC-HF 250-MHz superconducting spectrometer (14) and a Bruker HFX 90-MHz spectrometer. 5-mm NMR tubes were used with the 250-MHz instrument and 10-mm NMR tubes were used for measurements at 90 MHz. Probe temperatures of the instruments were 31° for the 250 MHz and 28° for the 90 MHz. Chemical shifts are referenced with respect to the HDO signal, and all resonances reported are downfield from HDO and are accurate to ±0.1 ppm. The HDO signal is 4.72 ppm downfield from the proton resonance of 2,2-dimethyl-2-silapentane-5-sulfonate(DSS) at 31° and 4.80 ppm with DSS at 28°. The signal-to-noise ratios were improved by signal averaging with a Northern Scientific model NS-544 digital memory oscilloscope when the MPC-HF 250-MHz spectrometer was used and with a Fabri-Tek model 1074 computer when the Bruker HFX 90-MHz NMR spectrometer was used.

RESULTS

Fig. 1 shows the 250 MHz spectra, in the region from −22 to −6 ppm, of deoxyhemoglobin A in 32 mM P_2 glycerate at various stages of oxygenation. The amount of oxygen increases from bottom to top. The two intermediately saturated solutions are both less than 40% saturated. The spectrum of the deoxy sample shows the lines at −17.9 −12.1, and −7.4 ppm that have been reported (1–3)†. The resonance at −13.9 ppm is due to acid methemoglobin A that has formed during the preparation and NMR measurements. As larger amounts of oxygen are added, the peaks at −12.1 and −7.4 ppm decrease in preference to the peak at −17.9 ppm.

A similar result at 250 MHz has been obtained for solutions containing 10 mM IP_6 instead of P_2 glycerate. The peaks at −12.1 and −7.4 ppm decrease in preference to the peak at −17.9 ppm. Since it is apparent that appreciable methemoglobin forms during the measurements, we wanted to make sure that the preferential diminution of the lines at −12.1 and −7.4 ppm was not caused by the effect of oxidation. Samples were prepared in 10-mm NMR tubes for use in the 90-MHz spectrometer. The 90-MHz spectrometer is more accessible to our laboratory, and with the large sample tubes, the spectra could be obtained within minutes after removal from the deoxygenation apparatus. Fig. 2 shows the 90-MHz spectra with 11% Hb A and 10 mM IP_6 in 0.1 M Bis-Tris buffer at pD 7.1. In the partially oxygenated sample, the peak due to the methemoglobin is virtually absent. In the partially saturated sample, the area of the line at −12.1 ppm is 70% as intense as the line at −17.9 ppm.

DISCUSSION

There is little doubt that the results we have presented here show for the first time that oxygen molecules bind to the α-chain hemes in preference to the β-chain hemes in Hb A. The preferential oxygen binding to the α chains in Hb A is observable only in the presence of high concentrations of organic phosphates. We have shown (2) that within the sensitivity of our NMR measurements, there is no preferential binding of carbon monoxide to Hb A in 0.1 M phosphate at pD 7. We have found that even in the presence of a one to two molar excess of P_2 glycerate or IP_6, carbon monoxide binds to Hb A in a random order. The large excess of organic phosphate needed to produce preferential binding suggests that the preferential binding is due to effects of phosphate binding to partially liganded forms of the tetramer. Organic phosphates are known to bind to analogues of partially saturated tetramers, the mixed valence hybrids $\alpha_2^{+CN}\beta_2$ and $\alpha_2\beta_2^{+CN}$ (15–17), but with reduced affinity (15). P_2 glycerate binds weakly to oxyhemoglobin (18, 19), but the binding is increased as the pH falls below 7 (19). IP_6 binds to both oxy- and deoxyhemoglobin (10, 20, 21). The high concentration of organic phosphate used in this study insures that the par-

† Lindstrom, T. R., Ho, C. & Pisciotta, A. V. (1972) "Nuclear magnetic resonance studies of hemoglobin M Milwaukee-1 and their implications to heme-heme interactions," manuscript submitted to *Nature*.

tially liganded intermediates bind phosphate, and are presumably stabilized in low affinity forms.

Our results, although definitive as to the sequence of ligand binding, cannot be used to distinguish between the numerous models of cooperative ligand binding that are currently under consideration. Models of cooperative ligand binding generally fall into two categories, those requiring concerted structural transitions (22) and those permitting sequential structural transitions (23, 24). Experimental evidence appears to favor the concerted mechanisms (7–9, 15, 16, 25). According to these concerted models, the effect of P_2 glycerate is to inhibit the quaternary structural transition from the low affinity form to the high affinity form. In order to accommodate our data showing preferential oxygen binding to the α-chain hemes, a concerted model must stipulate that the α-chain hemes have a greater ligand affinity than do the β-chain hemes when the tetramer is in the low affinity quaternary structure. Perutz's model explicitly prescribes this (9). The generalized concerted model described by Ogata and McConnell assumes the α and β hemes are nonequivalent, and their derived dissociation constants indicate that when the tetramer is locked in the low affinity form, the α hemes have a higher ligand affinity than the β hemes (7, 8, 15). Calculations made with the parameters determined by Ogata and McConnell indicate that for 2 mM of hemoglobin and 25 mM of P_2 glycerate at 50% CO saturation, 42% of the α hemes remain unsaturated, while 58% of the β hemes are unsaturated (R. T. Ogata and H. M. McConnell, personal communication). This calculated 2:3 ratio of deoxy hemes agrees with our NMR results, which show that at about 40% saturation with oxygen, the intensity of the deoxy α-heme resonance is 70% that of the deoxy β hemes (Fig. 2). The modified concerted model proposed by Shulman and coworkers (16, 17, 25), while admitting nonequivalence of α and β heme, does not account for the preferential binding to the α-chain hemes. Of course, the sequential model proposed by Koshland and coworkers (23, 24) can easily account for our preferential binding, with the assumption that the P_2 glycerate or IP_6 stabilizes a lower affinity form of the β chains. Our NMR studies are consistent with both the sequential model and those concerted mechanisms that allow the α hemes to bind ligand in preference to the β hemes when the tetramer is stabilized in the low affinity quaternary structure.

We have often speculated that the structure of the deoxy hemes may be influenced by ligand binding at the other hemes in a tetrameric hemoglobin molecule (1, 2)†. We would anticipate that this type of structural change would alter the line position of the contact-shift resonances, since we have observed changes of this nature when amino-acid substitutions are made at the $\alpha_1\beta_2$ subunit interface in several human mutant hemoglobins (1, 2). The spectra in Figs. 1 and 2 do not indicate any chemical shift in the β-heme resonance, but indicate that the line may be broadened slightly. This point is presently being investigated more carefully. We are also in the process of determining the lower limits of P_2 glycerate and IP_6 concentrations needed to produce the preferential binding of oxygen and carbon monoxide to the α chains in Hb A.

In conclusion, our NMR results show that in the presence of organic phosphates at neutral pD, oxygen molecules combine first with the α-chain hemes before those of the β-chain hemes in Hb A. Our present results show that NMR spectroscopy can give valuable and unique information about structure–function relationships in hemoglobin.

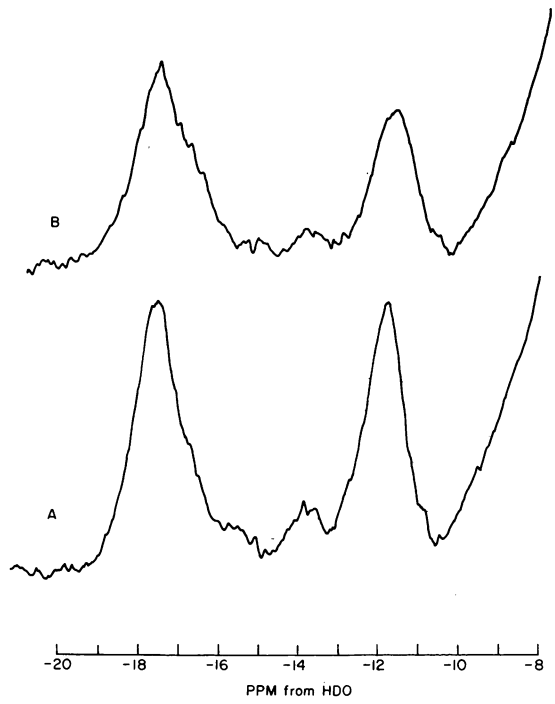

FIG. 2. 90-MHz proton NMR spectra of 11% Hb A in 10 mM IP_6, 0.1 M Bis–Tris·HCl at pD 7.1 and at 28°: *A*, deoxy Hb and *B*, partially oxygenated Hb (about 40% saturation).

This work is supported by research grants from the National Science Foundation (GB-25053), the American Heart Association, Inc., and the National Institutes of Health (RR-00292 and HE-10383).

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