NUCLEAR MAGNETIC RESONANCE STUDIES OF HEMOGLOBINS. VIII. EVIDENCE FOR PREFERENTIAL LIGAND BINDING TO β CHAINS WITHIN DEOXYHEMOGLOBINS.*

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Summary

When deoxyhemoglobin is titrated with n-butyl isocyanide in the presence of inositol hexaphosphate at pD 7.1, the intensity of the heme proton resonance positioned at ∿-18 ppm downfield from HDO decreases more rapidly than that at ∿-12 ppm. The lower field line was previously designated as a β heme methyl resonance and the other line an α heme methyl resonance. It has also been reported that in the presence of inositol hexaphosphate n-butyl isocyanide is bound preferentially to the β chains within native human hemoglobin. The results presented here support both these previous conclusions.

As indicated in prior reports (1,2), the proton nuclear magnetic resonance (NMR) spectrum of human deoxyhemoglobin has hyperfine shifted resonances at approximately -18, -12, and -7 ppm downfield from HDO. These contact shifted resonances offer extraordinary potential for the study of hemoglobin structure because they allow direct observation of the properties of the heme environment. Evidence has already been

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presented to show that the α and the β hemes in deoxyhemoglobin have non-identical hyperfine shifted heme proton spectra indicating a dissimilarity in their heme environments (1,2). The α and β components of the heme proton spectrum have been tentatively assigned through the use of modified and mutant hemoglobins (1,2). The resonance at ∿-18 ppm is believed to be due to a β heme methyl group and the resonance at ∿-12 ppm an α heme methyl group.

Two of the authors have reported that the time course of the reaction of n-butyl isocyanide (BIC) with deoxyhemoglobin is strikingly biphasic and that the amplitudes of the fast and slow components are dependent on the wavelength of observation, the fast component having an isosbestic point at ∿420 nm and the slow component an isosbestic point at ∿423 nm (3,4). They also found that in the presence of organic phosphates the kinetic but not the spectral differences between the two components, presumably the α and β chains, are enhanced and that marked deviations from isosbesty exist in equilibrium titration experiments in which the Soret absorption spectrum is monitored. In fact, when hemoglobin is titrated with BIC in the presence of inositol hexaphosphate (IHP), binding occurs exclusively to the kinetically fast component over a range of ligand concentrations which gives rise to total saturation values of 0 to about 20%. Thus the presence of IHP not only amplifies the functional differences between the chains but also allows the visualization of equilibrium populations of partially saturated intermediates. Through several indirect experiments this kinetically fast spectral component which possesses a higher affinity for BIC in the presence of IHP has been identified as β chains (4), but a more direct spectral identification was desired. The application of the NMR technique to this IHP-BIC-hemoglobin system is a logical extension of these previous studies.

Experimental

Deuterated human hemoglobin was prepared in the CO form as described previously (1,2). Following D_2O exchange, appropriate amounts of 1 M deuterated 2,2-bis(hydroxymethyl)-2,2',2"-nitrilotriethanol (bis-tris) (Aldrich Chemical Co.) at pD 7.1 and 0.1 M deuterated IHP (Sigma Chemical Co.) at pD 7.2 were added so that the hemoglobin solution was 10 mM heme, 100 mM bis-tris and 4 mM IHP. To obtain deoxyhemoglobin, the solution was flushed with oxygen in a rotary evaporator to replace the carbon monoxide with oxygen and subsequently flushed with prepurified nitrogen to remove the oxygen. A 18.4 mM stock solution of BIC was prepared by dissolving 0.04 ml of BIC (Aldrich Chemical Co.) in 20 ml of deoxygenated D_2O. Five NMR samples were prepared by flushing 5 mm NMR tubes with nitrogen, sealing the tubes with serum stoppers (NMR Specialties, Inc.), introducing 0 to 0.3 ml of BIC stock solution, and diluting the solutions to a total volume of 0.3 ml with deoxygenated D_2O. Deoxyhemoglobin was transferred into these tubes in 0.3 ml aliquots with a 0.5 ml syringe taking precautions to avoid contamination with atmospheric oxygen. The NMR tubes were then permanently sealed with a coating of quick setting epoxy cement (Hardman, Inc.).

The final solutions contained 6.25 mM heme, 2 mM IHP, 50 mM bis-tris, and from 0 to 9.2 mM BIC. Spectra were obtained on a Bruker HFX NMR spectrometer operating at 90 MHz. Signal to noise ratios were enhanced by 512 repetitive scans using a Fabri-Tex 1074 time averaging computer. Chemical shifts are referenced to HDO and at the probe temperature of the spectrometer (26°), the HDO resonance is -4.82 ppm downfield from sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS.)

Results and Discussion

The proton NMR spectrum of deoxyhemoglobin in the presence of

IHP is, within experimental error (±0.1 ppm), identical to previously published spectra of deoxyhemoglobin in potassium phosphate buffer at pD 7 (1,2) and exhibits three hyperfine shifted resonances positioned at -17.8, -12.1, and -7.4 ppm downfield from HDO (Fig. 1). Normally when oxygen or carbon monoxide is added to deoxyhemoglobin solutions, all three peaks uniformly decrease in intensity as liganded, diamagnetic heme iron is formed (2). However, when BIC is added to deoxyhemoglobin in the presence of IHP, the decrease in intensity is strikingly unequal for the peaks at -17.8 and -12.1 ppm (Fig. 1). At a BIC/heme ratio of 1.2 the peak at -17.8 ppm is clearly less intense than the peak at -12.1 ppm, and when the ratio reaches 1.9, the peak at -17.8 ppm is virtually non-existent while the resonances at -12.1 and -7.4 ppm are still readily visible. Two other spectra of samples with BIC/heme ratios of 0.3 and 0.7 were recorded and gave results consistent with the trends shown in Fig. 1.

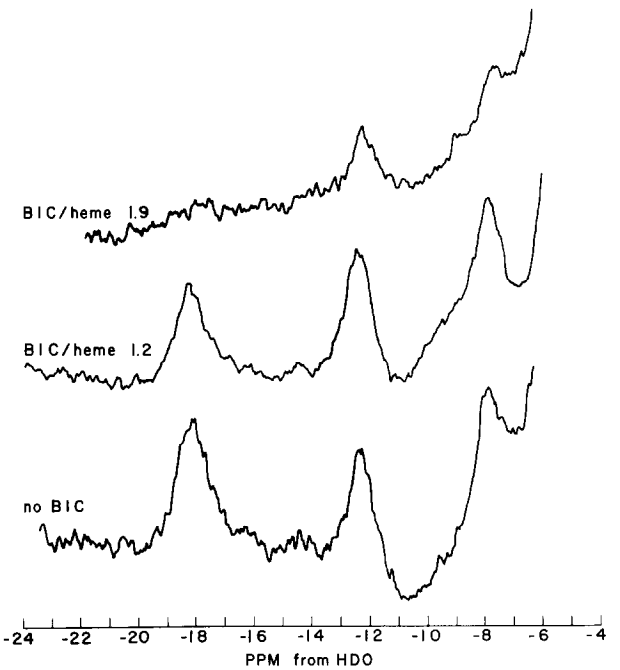

Figure 1. 90 MHz heme proton resonance spectra of 6.25 mM deoxyhemoglobin-BIC mixtures containing 2 mM IHP in bis-tris pD 7.1 at 26°.

Assuming the resonance at -17.8 ppm is a β heme methyl and the resonance at -12.1 ppm is an α heme methyl, the spectra shown in Fig. 1 indicate that BIC binds to the β heme in preference to the α heme in agreement with the previously described kinetic measurements. Any alternative explanation would require that both the assignments of the hyperfine shifted resonances (1,2) and the assignments of the fast and slow kinetic components (3,4) be incorrect, and this appears unlikely.

The results presented here add additional support to the idea that in the presence of organic phosphates the β chains within deoxyhemoglobin possess a higher affinity for ligands than the α chains. This finding is especially timely in view of the recent proposal made by Perutz (5) that due to the stereochemical arrangement of the E11 valine residues in the β chains, oxygen molecules first combine with the α heme groups before those in the β chains. Our results suggest that this is not the case for the bulky ligand n-butyl isocyanide in the presence of inositol hexaphosphate.

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