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Interaction of Human Adult Methemoglobin in Low-Spin State with Inositol Hexaphosphate. A Proton Magnetic Resonance Study

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SUMMARY The hyperfine-shifted proton nuclear magnetic resonance (NMR) spectra of the low-spin complexes of human adult methemoglobin were found to be much altered by the addition of inositol hexaphosphate (IHP). The stoichiometry and pH-dependence of IHP binding, and the spin equilibrium of azide methemoglobin are parallel to those of high-spin human methemoglobin and of carp methemoglobin, both of which are proposed to be switched from the R to T states with IHP. The present NMR results show that IHP affects the structure of human methemoglobin regardless of the spin state of the heme iron, suggesting that there is no correspondence between quaternary structure and the spin state of ferric heme iron.

Interaction of hemoglobin with allosteric effectors has been the subject of continuing interest. Perutz et al. (1) reported that inositol hexaphosphate (IHP) induces significant changes in the spectroscopic and magnetic properties of human methemoglobin in the high-spin state. They emphasized that high-spin complexes of methemoglobin can be swiched to the T structure with IHP while low-spin ones cannot, implying that there is a quaternary structural transitior of methemoglobin linked to the spin state of the heme iron (1,2). Scholler and Hoffman (3) also reported that no changes in the EPR g-values and in the resonance Raman frequencies of human cyanide and azide methemoglobins were observed upon addition of IHP. Thus the interaction of low-spin human methemoglobin with IHP has not been intensively studied.

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In this communication we show that IHP induces large changes of the hyperfine-shifted proton nuclear magnetic resonance (NMR) spectra of human adult methemoglobin in low-spin state. The NMR results on human low-spin methemoglobin are compared with the already reported results on human high-spin methemoglobin and on carp methemoglobin.

MATERIALS AND METHODS Human adult methemoglobin was prepared from whole blood as reported previously (4). Organic phsohate was removed according to the method of Berman et al. (5). Methemoglobin was prepared by adding a two-fold excess of potassium ferricyanide to oxyhemoglobin in 10 mM bistris buffer, pH 6.0. The methemoglobin solution was applied to a cellulose column (Whatman, CM52) equilibrated with the same buffer. The cellulose column with adsorbed methemoglobin was washed with the same buffer to remove residual ferri- and ferro-cyanides. The methemoglobin solution was eluted with 0.1 M bistris buffer, pD 7.0, in D_2O. Then a five- to ten-fold molar excess of azide and imidazole was added to the methemoglobin solution.

Inositol hexaphosphate (IHP) was purchased from Sigma (type V). Appropriate amounts of a 50 mM solution of IHP, titrated to pD 6.3 with DCl, was added to the methemoglobin complexes. None of the denatured materials was found in the samples throughout the experiment. The pD value noted was the direct reading of a pH meter (Radiometer, model 28) with a micro combination electrode (Ingold).

Proton NMR spectra were obtained on a Varian HR-220 spectrometer with a Nicolet TT-100 accessary in a pulse Fourier transform mode at 220 MHz. The spectra consisting of 10,000 to 60,000 transients were collected over a 40 kHz spectral width with 4 K data points and a 30 μsec 75° pulse. Chemical shifts in parts per million (ppm) were referenced from internal 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) with positive sign indicating lower field shifts.

Results

Figure 1A shows the titration of human azide methemoglobin with IHP as followed by proton NMR. Upon addition of increasing amounts of IHP, the intensities of azide methemoglobin resonances around 27, 22 and 14 ppm gradually decreased with concomitant appearance of the characteristic resonances at 31.1, 24.9, 16.5 and about 21 ppm. This implies that the IHP-induced structural change of azide methemoglobin occurs slowly on the NMR time scale. These resonances observed at pD 6.4 reversibly disappeared with increasing pD and the spectrum above pD 7.8 was almost the same as that without IHP.

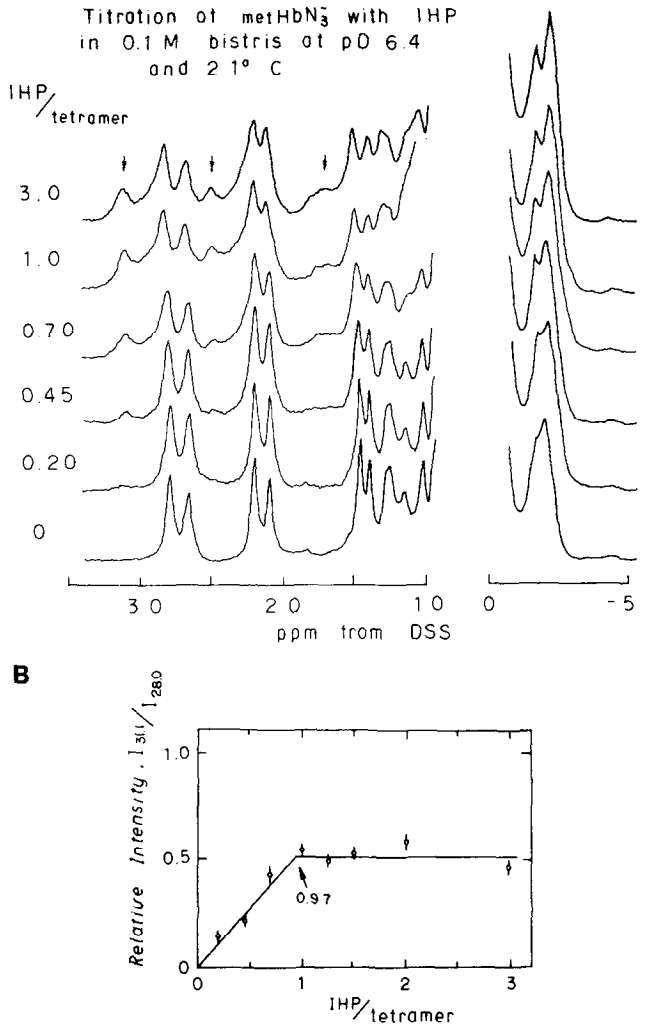

Fig. 1 (A) Titration of human azide methemoglobin with IHP in 0.1 M bistris at pD 6.4 and 21° C. The spectrum at pD 6.4 with IHP is the same as that at pD 5.0. Heme concentration was about 4 mM. (B) Plot of the ratio of the intensities of the resonances at 31.1 ppm and 28.0 ppm against the IHP concentration.

A plot of the intensity of the resonance at 31.1 ppm relative to that at 28.0 ppm against IHP concentration shows that one mole of IHP binds stoichiometrically to one mole of methemoglobin tetramer (Fig. 1B). The hyperfine shifts of the IHP-induced resonances exhibit downfield bias as compared with those of IHP-free azide methemoglobin (bottom spectrum in Fig. 1A). The more downfield shifts are indicative of the larger high spin fraction of azide

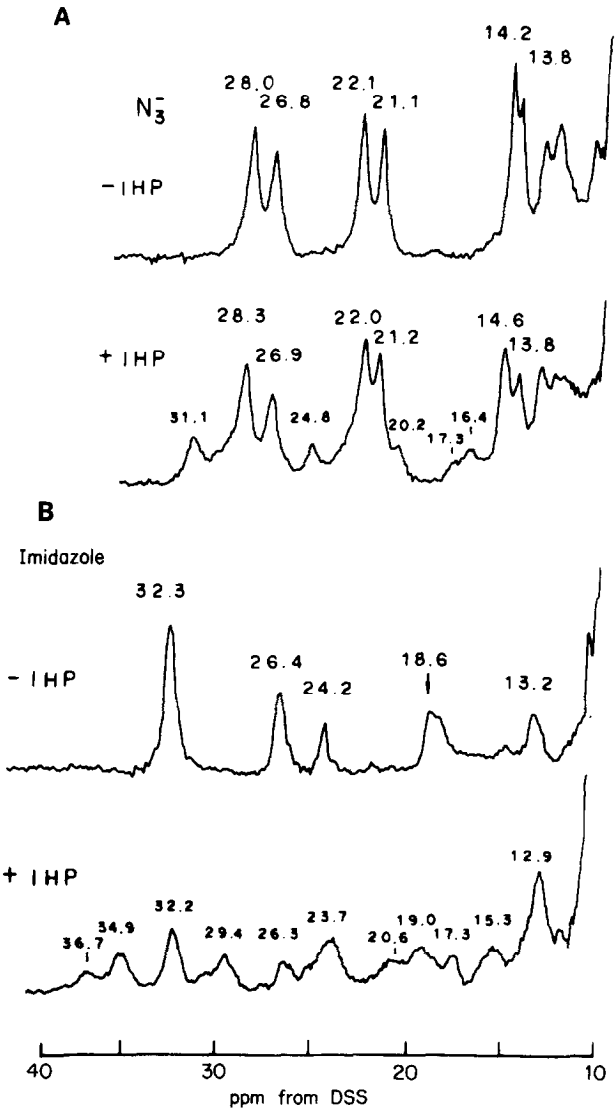

Fig. 2 Proton NMR spectra of human adult methemoglobin in the low-spin state, with and without IHP, in 0.1 M bistris at pD 6.5 and 21° C. (A) azide methemoglobin; (B) imidazole methemolgobin. The IHP/tetramer ratio is about 5.

methemoglobin with IHP than that without IHP, because the NMR formulation of paramagnetic shift suggests that the magnitude of the hyperfine shifts increases, to a first approximation, with increasing high-spin fraction of the heme iron (6). The present NMR result is consistent with the result by Perutz et al. (1), who showed that upon addition of IHP to human azide methemoglobin the

magnitude of high-spin visible absorption bands increases while that of low-spin bands decreases, suggesting an increase in the high-spin fraction of the heme iron.

For imidazole methemoglobin, which is pure low-spin, IHP induced a more pronounced alteration of the hyperfine-shifted proton spectrum as shown in Fig. 2B. Upon addition of IHP to imidazole methemoglobin, new resonances at 36.7, 34.9, 29.4, 23.7, 20.6, 19.0, 17.3, 15.3 and 12.9 ppm appeared. These imidazole methemoglobin resonances in the presence of IHP also exhibited the same pD dependence as those of azide methemoglobin with IHP and were observable only below pD 7.

In order to determine the magnitic properties of the IHP-bound species, the spin equilibrium of azide methemoglobin with IHP was examined. Figure 3 shows the temperature dependence of the hyperfine-shifted resonances of azide methemoglobin with IHP and the

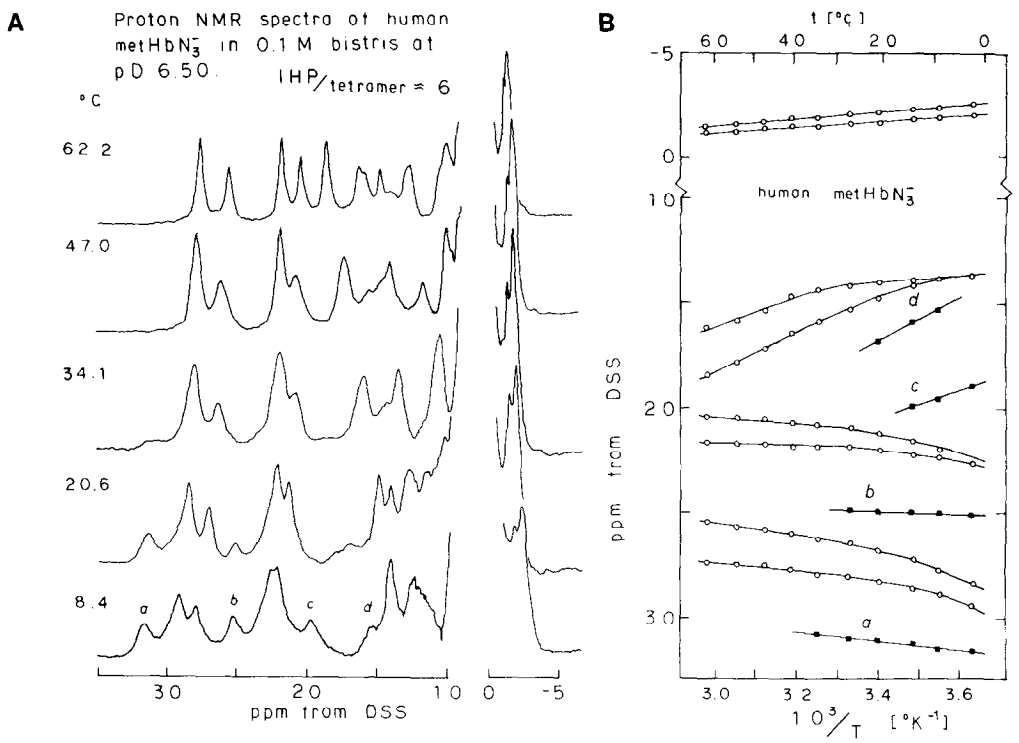

Fig. 3 (A) Temperature dependence of the azide methemoglobin resonances in the presence of IHP; 0.1 M bistris, pD 6.5 (B) Curie plot of the resonances in (A).

corresponding Curie plot. The positive slope and large temperature dependence of the resonances c and d in the plot of Fig. 3B indicates that the IHP-bound species is also in thermal spin equilibrium with larger high-spin fraction than for azide methemoglobin in the absence of IHP (4).

DISCUSSION

The interaction of human adult methemoglobin in the low-spin state with IHP has not been intensively studied. This is probably because the IHP-induced responces in circular dichroism, UV absorption, EPR and resonance Raman spectra (1,2,3) are much smaller than those found in high-spin methemoglobin. The present NMR results, however, show that human adult methemoglobin in the ferric low-spin state binds stoichiometrically with IHP and that this reaction, below pD 7, is accompanied by a change of the heme environmental structure. Perutz et al. (1,2) reported that addition of IHP to human azide methemoglobin produces only a weak difference spectrum and that a change of pH has no effect on the visible absorption spectrum of azide methemoglobin. They also showed that upon addition of IHP to human imidazole methemoglobin, which is pure low-spin, the intensity of low-spin bands of the visible absorption spectrum decreased (2). They interpreted this result as due to the dissociation of imidazole to form acid methemoglobin. However the present NMR results in Figs. 1 and 2 show that new species, whose structures are different from the native ones, are generated by the addition of IHP to azide and imidazole methemoglobins. The chemical shifts of the new resonances are characteristic of the two relevant low-spin complexes and are different from those of acid methemoglobin (1,4). This suggests that the IHP-induced NMR spectral changes are not due to a slight dissociation of the external ligands, as proposed by Perutz et al. (2), but due to a structural change of the low-spin methemoglobins.

It is to be noted that the IHP-induced proton resonances c and d in Fig. 3 exhibit non-Curie type behavior, suggesting that the IHP-bound azide methemoglobin is also in thermal spin equilibrium with an increased high-spin fraction. This result for human azide methemoglobin, in the presence of IHP, is comparable to that for carp azide methemoglobin with IHP, which is proposed to be in a deoxyhemoglobin-like conformation by Perutz et al. (2).

As the above NMR results show, the stoichiometry of IHP binding, pH-dependent profile of the IHP-induced resonances, and the spin equilibrium property of azide methemoglobin with IHP are all parallel to the results on human methemoglobin in the high-spin state as well as those on carp methemoglobin, both of which are proposed to be switched from the R to T state by the addition of IHP (1,2). Thus it is possible to state that the structural changes induced by IHP are not dependent on the spin state of the heme iron in human methemoglobin.

In the NMR spectra discussed above, it is not yet possible to determine the specific structural changes. Several authors suggested that IHP binding induces a localized structural modification of the β subunits of human methemoglobin and that the evidence presented by Perutz et al. (1,2) merely reflect this modification and do not necessarily correspond to the quaternary transition (7,8). In order to clarify this discrepancy, a more detailed investigation is in progress and the results will soon be reported.

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