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DEMONSTRATION OF INOSITOL HEXAPHOSPHATE INDUCED CHANGES IN STRUCTURE AT LIGAND BINDING SITES IN CARP HEMOGLOBIN CARBONYL

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Summary. Infrared and ^{13}C NMR spectra for carbonyl ligands bound to hemoglobin from carp, Cyprinus carpio, at pH 6.0 and 25°C undergo marked shifts when inositol hexaphosphate (IHP) is added to the solution. The relative intensities of the two C-O stretch bands at 1951 and 1968 cm^{-1} due to two ligand site conformers are altered by IHP in the same manner as found upon lowering the pH in the absence of IHP. Both the lowering of pH and the binding of IHP enhance the 1968 cm^{-1} band conformer, the conformer normally of much lower stability. ^{13}C NMR spectra indicate the CO sites for only one type of subunit, probably the β-subunit, are altered by IHP. These findings demonstrate directly that the observed IHP-induced shift from high affinity (R) to a low affinity (T) form is accompanied by a significant change in ligand binding site structure at two of the four subunits.

Introduction. Carp hemoglobin provides a convenient model for the study of conformational changes in hemoglobins because it can be switched from a high-affinity (R) state to a low-affinity (T) state while fully saturated with ligand by the addition of IHP at low pH values (1,2). That a two-state model can be used to approximate ligand-binding properties of carp Hb is primarily supported by the observed changes in ligand affinity and Hill coefficient as a function of organic phosphate concentration and pH (1-3). The observation that a ligand such as CO remains bound to all hemes in carp Hb throughout the transition from high to low ligand affinity provides a unique opportunity for the direct detection of a change in conformation by infrared spectroscopy. IR methods permit the direct and quantitative measurement of rapidly interconverting conformers in hemoglobins and myoglobins by use of the C-O stretch band for bound CO (4,5). In addition, differences in α- and β-subunit environments for bound CO

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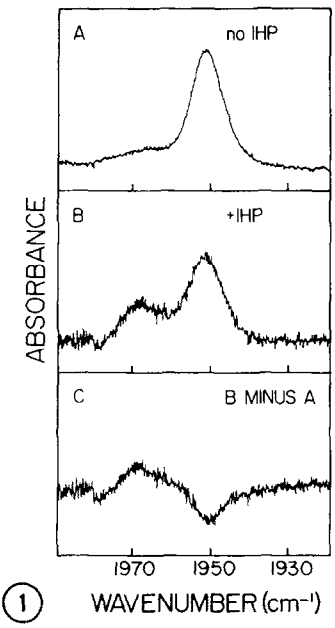
in Hbs can be detected in ^{13}C NMR spectra for bound ^{13}CO (4,6-8). Here we report IR and NMR evidence for IHP-induced changes in the structure of carp HbCO at the ligand binding sites of two of the four subunits which serve to explain, at least in part, the concomitant changes in ligand binding behavior.

Materials and Methods. Carp HbCO, carp HbA CO, and carp HbB CO were purified by the method of Tan et al. (9) as modified (10). For infrared experiments, 0.1 M Bis-Tris buffers with 0.1 M chloride were used. To prepare samples containing IHP, IHP crystals were dissolved in buffer and the pH adjusted with aqueous acetic acid (1 M) prior to addition to the HbCO solution. Both Bis-Tris and IHP were obtained from Sigma Chemical Co. Infrared spectra were recorded on a Perkin-Elmer Model 580B spectrometer in Mode 2A at a resolution of 2.9 cm^{-1}. The cells used had CaF_2 windows and a pathlength of 0.05 mm and were kept at 26°C Ten scans were collected for each sample and averaged to improve the signal-to-noise ratio. The reference cell contained air. The baseline (water vs air) was subtracted from the collected spectrum and the averaged spectrum deconvoluted as previously described (5).

To obtain ^{13}C NMR spectra a solution of 3.4 mM carp HbA CO at pH 6.0 in 0.1 M Bis-Tris/HCl with 30% D_2O was equilibrated with ^{13}C-enriched CO (90.7%, Prochem) at 4°C by rotating the solution exposed to ^{13}CO under light from an incandescent bulb (100 watt) for 1 hour. To prepare a solution containing IHP, IHP was added to give a final concentration 0.5 mM in excess of the heme concentration. The replacement of ^{12}CO by ^{13}CO was confirmed by the shifts of the C-O stretch band in the IR spectrum from 1951 cm^{-1} to 1906 cm^{-1} (11). The NMR spectrum of HbCO solution (1 ml) in a 5 mm diameter tube was recorded at 25° on a JEOL FX-100 Fourier transform instrument operating at 25.1 MHz. The resonance at 186.5 ppm from sodium 2,2-dimethyl-2-silapentane sulfonate (DSS) for free (i.e., unbound) ^{13}CO was used as internal reference. No DSS was added to the HbCO solutions.

Results and Discussion. The infrared spectra of Fig. 1 for pH 6.0 show the marked effect of the addition of excess IHP on the C-O stretch band. With solutions at pH 6.5, but otherwise the same, the effect of the addition of IHP on these spectra are much less but observable. Purified A and B carp HbCO fractions behaved similarly to the unseparated mixture at pH 6.0. Fig. 1 demonstrates a shift in C-O band intensity from the 1951 cm^{-1} band into the 1968 cm^{-1} band upon binding of IHP. Similar shifts in intensity have been noted for human HbA CO upon lowering the pH from neutrality (4). The effects of changes in pH on C-O stretch bands for carp HbCO are compared with those for human HbA CO in Fig. 2. At all pH values the carp Hb has greater relative intensity in the 1968 cm^{-1} band. Carp Hb also exhibits an intensification of the 1968 cm^{-1} band at higher pH values upon acidification from pH 7 than does the human Hb. In general, the carp Hb is less stable toward acidification. From the data of Fig. 2, it can be estimated that the effect of IHP at pH 6.0 shown is comparable to that expected if the pH were lowered by about 1 pH unit in the absence of IHP.



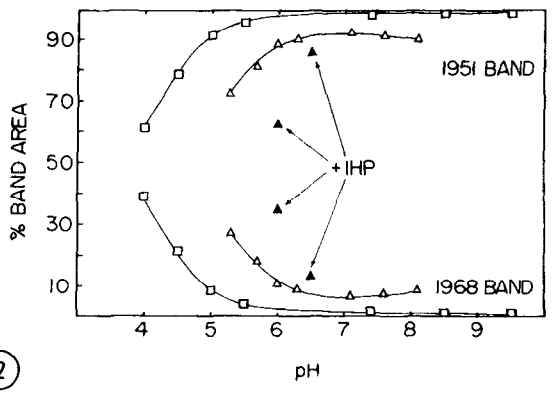


Fig. 1. Infrared spectra in the C-O stretch region for carp hemoglobin carbonyl at pH 6.0 and 25°C with and without inositol hexaphosphate present. Buffer: 0.1 M Bis-Tris with 0.1 M chloride. A. HbCO, 15 mM in heme; IHP, none. B. HbCO 2.0 mM in heme; IHP, 2.5 mM. C. Difference spectrum obtained from subtraction of spectrum A from spectrum B after normalization of absorbance values to reflect the difference in HbCO concentrations. The addition of IHP is shown to enhance absorption near 1968 cm^{-1} and to reduce absorption near 1951 cm^{-1}.

Fig. 2. Effects of changes in pH on the relative contributions of 1951 cm^{-1} and 1968 cm^{-1} band areas to the C-O stretch region of infrared spectra for human hemoglobin A and carp hemoglobin carbonyls. (□) Data for human HbA CO from Choc and Caughey (4). (△) Data for carp HbCO 2 to 4 mM in heme in 0.1 M Bis-Tris buffer, 0.1 M Cl^- at 25°C with no IHP present. (▲) Carp HbCO, 2 mM as in (△) with 2.5 mM IHP for solutions at pH 6.0 and HbCO 3.0 mM and IHP 3.6 mM at pH 6.5.

The ^{13}C NMR spectra for carp HbA ^{13}CO at pH 6.0 with IHP present or absent reveal marked effects of IHP binding on one of the two bound ^{13}CO resonances (Fig. 3). In the absence of IHP the resonances for α and β subunits are not as well resolved as is found for human HbA ^{13}CO under these conditions (4) but one resonance is clearly detected as a shoulder at ca. 207.8 ppm on the narrower, higher second resonance at 208.3 ppm (Fig. 3A). With excess IHP present two resonances are well resolved (Fig. 3B); one at 208.3 ppm is unaffected by IHP binding whereas the other resonance is shifted to 207.2 ppm. Human HbA ^{13}CO under these conditions exhibits an α-subunit resonance at 208 ppm and a β-subunit resonance at 207 ppm (4,6-8). Thus the chemical shift of α-^{13}CO for human HbA ^{13}CO is essentially the same as that for the carp Hb resonance that is unaffect-

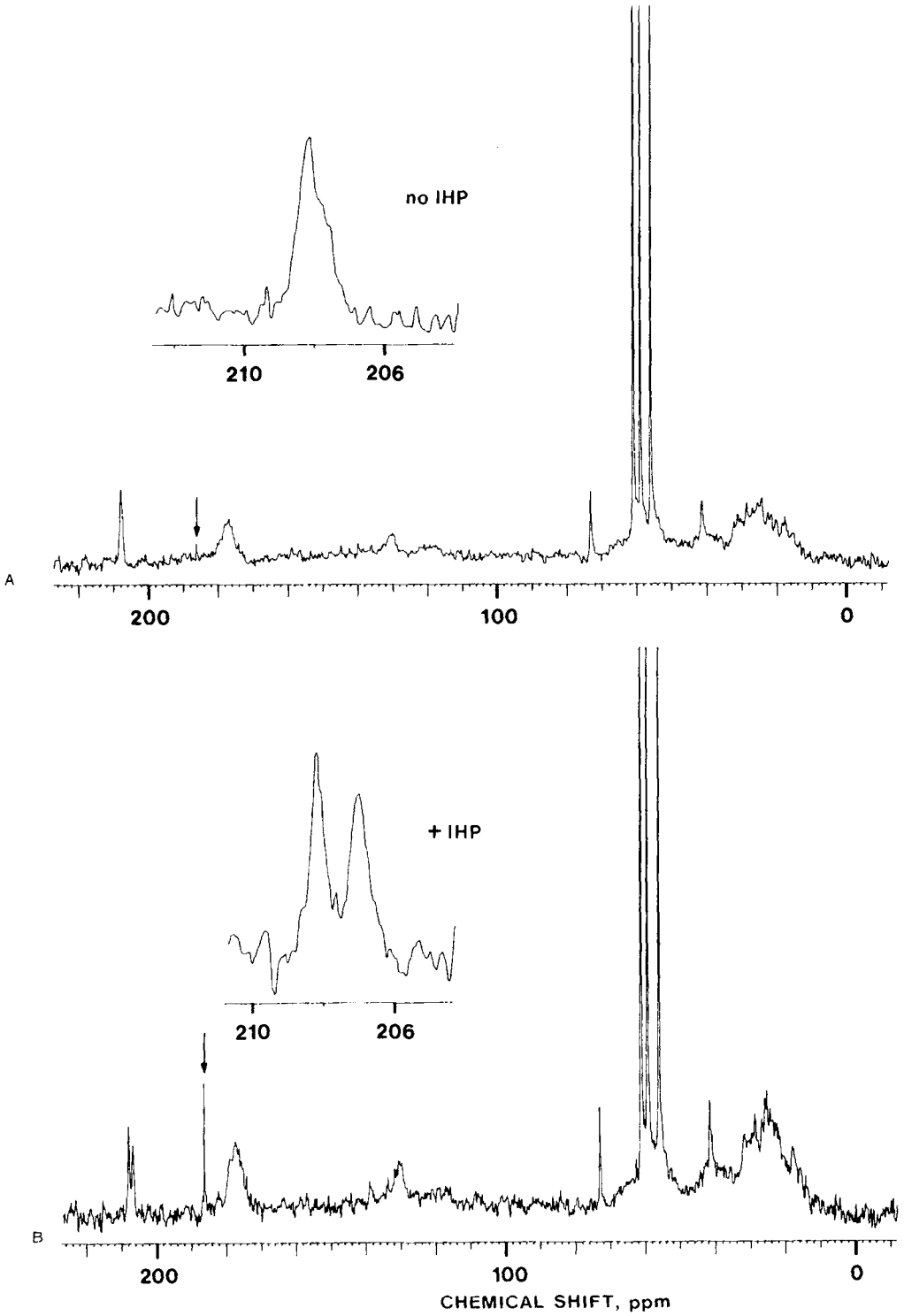


Fig. 3. ^{13}C NMR spectra of carp hemoglobin A carbonyl at pH 6.0 and 25°C with and without inositol hexaphosphate present as obtained after 70,000 pulses. A. HbCO, 3.3 mM in heme; IHP, none. B. HbCO, 3.5 mM in heme; IHP, 4 mM. In each case, the resonances due to liganded ^{13}CO are expanded in insets. The arrows denote the resonance for unliganded "free" CO at 186.5 ppm used as an internal reference.

ed by IHP. If the subunit assignment is the same for the α-subunits of the two hemoglobins, then the NMR spectra provide direct evidence for the ligand binding sites on the α-subunits of carp Hb being unaffected by IHP binding whereas the β-subunit site structures are significantly changed by IHP. In any case, it is clear that the structure of only one type of subunit is IHP-sensitive.

Consideration of both IR and NMR data leads to the conclusion that the conformer equilibria at subunits of one type are little affected by IHP whereas at the other subunits the conformer equilibria are greatly perturbed in favor of the normally minor 1968 cm^{-1} band conformer. Discrete conformers can be detected by IR spectra but, since the conformers interconvert at rates greater than the NMR time scale, only the resonance that represents the weighted average of all the conformers interconverting at a given site are measured in NMR spectra (4,5). The single, if rather broad, resonance at 207.2 ppm suggests that each of the two perturbed (β?) subunits are affected by IHP to about the same degree. The IR spectra indicate for the perturbed subunits that, although the 1968 cm^{-1} band conformer is greatly stabilized with respect to the 1951 cm^{-1} band conformer due to IHP interactions with the protein, a significant contribution to the binding site structure continues to be made by the 1951 cm^{-1} band conformer; e.g., if α-subunit C-O stretch bands are insensitive to IHP, then the β-subunits under the conditions of Fig. 1B are about 40% in the 1951 cm^{-1} band conformation and 60% in the 1963 cm^{-1} band conformation. The observation of only a single resonance in the ^{13}C NMR spectrum (Fig. 3B) for the perturbed subunits can be explained as due to interconversion among the two conformers at a rate greater than the NMR time scale (4,5). The manner in which these ligand site structural changes detected by IR and NMR spectra relate to IHP-induced effects on ligand binding and other properties of the protein is of great interest.

During the course of these studies, IHP-induced changes in visible spectra and in autooxidation rates were noted. For example, carp HbCO 50 μM in 0.1 M Bis-Tris/HCl at pH 6.0 exhibited a β/α band height ratio of 1.04 without IHP and 1.01 with 0.68 mM IHP present. Also, under these conditions the rate of methemoglobin formation was found to be greater with IHP present than when IHP was ab-

sent if oxygen was allowed access to the solution. Rigorous exclusion of O_2 (e.g., by nitrogen flushing) greatly reduced the rate of metHb formation whether IHP was present or not.

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