An Infrared Study of NO Bonding to Heme B and Hemoglobin A. Evidence for Inositol Hexaphosphate Induced Cleavage of Proximal Histidine to Iron Bonds†

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ABSTRACT: Five- and six-coordinate nitrosyl hemes have been prepared and their infrared, electron paramagnetic resonance (EPR), and visible-Soret spectra compared with the corresponding spectra for nitrosyl hemoglobin A (HbA-NO) determined both in the presence and the absence of inositol hexaphosphate (IHP). The five- and six-coordinate NO complexes prepared from either dipyridine or pyridine carbonyl protoheme dimethyl ester had N-O stretch bands (ν_{NO}) near 1675 and 1625 cm^{-1}, respectively. These frequencies are sensitive to change in solvent (ν_{NO} decreased as the dipole moment of the solvent increased) and, with six-coordinate species, to changes in trans ligand. However, these solvent and trans ligand effects were small compared with the difference (ca. 50 cm^{-1}) between five- and six-coordinate species. The nature of the trans ligand affected the relative proportions of the two coordination complexes present in solution; 1-methylimidazole (MI) forms the six-coordinate NO complex more effectively than does pyridine. Similar infrared experiments were more difficult with HbA-NO because both water and amide bonds appear in the frequency region where ν_{NO} is expected. However, by careful matching of CaF_2 cells, it was possible to employ difference spectroscopy in D_2O solutions (i.e., HbA-NO vs. HbA-CO) to show that $^{15}N^{16}O$ bound to HbA absorbs at 1587 cm^{-1} corresponding to a six-coordinate nitrosyl heme; $^{14}N^{16}O$ was less precisely located at 1615 cm^{-1}. These frequencies are fully consistent with bent-end-on (Fe—N⟍O) bonding with appreciable double bond (covalent) character to the Fe-N bond wherein Fe^{II} serves as π donor and NO as σ donor with net donation of electron density from Fe^{II} to NO. This bonding thus resembles that in HbA-CO and HbA-O_2 as shown by ν_{CO} at 1951 cm^{-1} and $\nu_{O_2}^{*}$ at 1106 cm^{-1}. In contrast, ferric horseradish peroxidase nitrosyl (i.e., Fe^{III}-NO) exhibits $\nu_{^{15}N^{16}O}$ at 1865 cm^{-1} consistent with net donation of election density from NO to Fe^{III}. In the presence of IHP, the NO stretch for HbA-NO appears as two bands of about equal intensity, one at the frequency found with IHP absent and the other at about 50 cm^{-1} higher frequency (1668 cm^{-1} for $^{14}N^{16}O$ and 1635 cm^{-1} for $^{15}N^{16}O$). The IHP induced shifts strikingly parallel changes in $\nu_{^{14}N^{16}O}$ from MI-Fe^{II}-NO in MI (1618 cm^{-1}) to five-coordinate Fe^{II}-NO in 1,2-dichloroethane (1668 cm^{-1}). Changes in EPR, Soret, and visible spectra also closely parallel differences found between six- and five-coordinate heme nitrosyls. Thus, these data give evidence that the binding of IHP to protein results in cleavage of proximal histidine to iron bonds in two of four subunits. The manner in which this effect is transmitted from IHP binding site to heme site remains unclear in detail. A straightforward and seemingly sufficient explanation for the results seen with IHP is a "pulling away" of proximal histidines from heme iron. Nevertheless, present evidence does not permit an evaluation of the relative importance of several possible IHP induced alterations of amino acid residue interactions with the porphyrin ring and the NO ligand.

It has recently become clear that infrared spectroscopy provides a uniquely useful means for direct observation of certain ligands bound to proteins and thereby to gain important insight into the chemistry of ligand binding under physiological conditions. These spectra permit examination of ligand binding in intact tissue to establish the nature of different binding sites and to quantify the ligand at each site (Maxwell et al., 1974a; Volpe et al., 1975). The first ligand studied was CO bound to hemoglobin A (HbA[1]) within as well as isolated from the erythrocyte (Alben and Caughey, 1968). These studies were then extended to include carbonyl complexes of abnormal hemoglobins (Caughey et al., 1969), myoglobins (McCoy and Caughey, 1971; Maxwell et al., 1974b), hemoglobins and myoglobins reconstituted with abnormal hemes (McCoy and Caughey, 1971; O'Toole, 1972), acid-denatured hemoglobins (O'Toole, 1972), cytochrome *c* oxidase (Caughey et al., 1970; Volpe et al., 1975), and heart and other tissue (Maxwell et al., 1974a). With methemoglobins and metmyoglobins, azide and cyanide are among several useful infrared active anionic ligands (McCoy and Caughey, 1970; Alben and Fager, 1972; O'Keeffe, 1974). The observable parameters of greatest interest are the frequency of the band maximum, the bandwidth at half-peak height, and the integrated intensity of the band. These parameters have been shown to vary significantly with changes in protein structure and to reflect differences in porphyrin structure, in the ligand trans to observable ligand, and in the environment (medium) about the ligand.

The ligands first studied (CO, CN^-, N_3^-) had vibrations which appear in a region of the infrared spectrum between 1900 and 2200 cm^{-1}. This region is relatively easy to study due to the "window" of relatively high transmittance for aqueous protein solutions. Recently a more difficult region has been examined to discover the O-O stretch frequency

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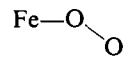
[1] Abbreviations used are: HbA, hemoglobin A; IHP, inositol hexaphosphate; MI, 1-methylimidazole; py, pyridine.

Table I: Infrared Spectral Data for CO[a] and NO[b] Bound to Iron(II) Hemes and Hemoglobins.

Solvent	Dipole Moment[c]	ν_{XO}, cm^{-1} ($\Delta\nu_{1/2}$, cm^{-1})			
		py–Fe^{II}CO[d]	MI–Fe^{II}CO[e]	L–Fe^{II}NO	Fe^{II}NO
CCl_4	0	1986 (19)	1980 (18)		1684 (20)
$CHCl_3$	1.1	1976 (30)	1969 (29)		1676 (18)
$ClCH_2$–CH_2Cl	1.7	1966 (26)	1959 (26)		1669 (28)
Pyridine	2.3	1964 (22)		1633 (20)[f]	
1-Methylimidazole	3.7		1954 (24)	1618 (24)[g]	
HbA[h]			1951 (8)	1615 (10)	
HbA + IHP[h]			1951 (8)	1615 (10)	1668 (~10)

[a] $^{12}C^{16}O$. [b] $^{14}N^{16}O$. [c] McClellan (1963). [d] py, pyridine. [e] MI, 1-methylimidazole. [f] Assumed to be pyridine–Fe^{II}–NO complex. [g] Assumed to be 1-methylimidazole–Fe^{II}–NO complex. [h] See text for specific conditions; with the proteins, histidine rather than MI is present as trans ligand.

for O_2 bound in erythrocytes, isolated hemoglobins, myoglobin, and cobalt hemoglobin A, each of which gave ν_{O_2} at about 1105 cm^{-1} thereby establishing unequivocally[2] bent-end-on bonding, i.e.



within the protein (Barlow et al., 1973; Maxwell et al., 1974b; Maxwell and Caughey, 1974; Caughey, et al., 1975).

Another ligand for which infrared spectra have proven highly diagnostic of bond type in coordination chemistry is NO (Enemark and Feltham, 1974). NO complexes of hemoproteins are also well known (Antonini and Brunori, 1971) but the NO stretch frequencies have not been reported due in part to the fact they are usually found in regions that are difficult to study in aqueous protein solutions. Hemoglobin nitrosyls have been studied by visible-Soret and electron paramagnetic resonance (EPR) spectra with particular recent interest in effects of inositol hexaphosphate (IHP) and other organic phosphates on these spectra (Perutz et al., 1976; Rein et al., 1972; Trittelvitz et al., 1972). In this paper we report infrared bands for NO bound to hemoglobins with and without IHP present. We also consider EPR and visible-Soret as well as infrared spectra of heme nitrosyls in explanation of the nature of NO bonding in stripped and IHP-treated hemoglobin A nitrosyls.

Materials and Methods

Hemin chloride (twice crystallized) from Koch Laboratories was converted to the crystalline FeOFe hemin ester, μ-oxo-bis(protoporphyrin IX dimethyl ester) iron(III) of high purity by the procedure developed in our laboratory (O'Keeffe et al., 1975). The dipyridine heme ester, dipyridine (protoporphyrin IX dimethyl ester) iron(II), was obtained as an analytically pure solid from μ-oxo-bis(hemin) ester according to the procedure of Alben et al. (1968) with slight modification (Barlow, 1973). CP grade CO and technical grade $^{14}N^{16}O$ were obtained from Matheson Gas Products and Scientific Gas Products, Inc., respectively. $^{15}N^{16}O$ (99% ^{15}N) was obtained from Mallinckrodt Nuclear. All other chemicals were of reagent grade.

Preparation of Carbonyl Hemes. Pyridine carbonyl and 1-methylimidazole carbonyl complexes of (protoporphyrin IX dimethyl ester) iron(II) were obtained from μ-oxo-bis(hemin) ester as analytically pure solids via methods to be described in detail elsewhere.[3] The hemin was dissolved in CS_2 with one part in 30 (by volume) pyridine or 1-methylimidazole and reduced under CO with a solution of hydroquinone in methanol. The pyridine carbonyl gave a visible spectrum in $CHCl_3$ with band maxima at 566 nm (α) and 538 nm (β), A_β/A_α = 1.06; for the 1-methylimidazole carbonyl in 1,2-dichloroethane values were 570 nm (α) and 540 nm (β), A_β/A_α = 1.06. Infrared spectra in $CHCl_3$ exhibited ν_{CO} at 1975 and 1968 cm^{-1} for the pyridine carbonyl and 1-methylimidazole carbonyl, respectively; in each case, the carbonyl ligand band height was about 1.2 times that for the ester carbonyl band.

Preparation of Nitrosyl Hemes. Solid five-coordinate nitrosyl heme ester was prepared from dipyridine heme dimethyl ester. The dipyridine heme was heated at 80°C under vacuum until all the liganded pyridine, which was detected quantitatively, had been removed. Upon exposure of the solid to NO, uptake of 1.0 mol of NO/mol of Fe was observed consistent with formation of a nitrosyl heme with one NO ligand and no other ligand; 39.4 mg (52.5 μmol) of dipyridine heme yielded 8.4 mg of pyridine (calculated amount, 8.3 mg) and then took up 53.0 μmol of NO. The infrared spectrum of the product in $CHCl_3$ exhibited ν_{NO} at 1676 cm^{-1} with the NO band intensity essentially equal to the intensity for the ester carbonyl band and a N isotope sensitive band at 480 cm^{-1} assigned to ν_{FeN}. The electronic spectrum in 1,2-dichloroethane exhibited λ_{max}, nm (AmM) values of 566 (11.7), 542 (11.6), and 418 (88).

The five-coordinate nitrosyl heme was also prepared in solution by treatment of solutions of the pyridine carbonyl with NO gas. In the infrared spectrum, an NO stretch band with a frequency that was consistent with the absence of trans ligand was found. The ν_{NO} value was also sensitive to the solvent employed (Table I).

[2] Our assignment of the bands near 1105 cm^{-1} to the ν_{O_2} of bound dioxygen has been questioned by Collman and coworkers (Collman et al., 1974, 1975) on the basis that a nonprotein, nonnatural oxygenated heme which they proposed as a myoglobin model gave rise to a band at 1385 cm^{-1}. However, the 1385-cm^{-1} band has proven to be artifactual (Collman, 1975) but no other band attributable to oxygen could be identified in this compound. The inability of these workers to find a band due to O_2 near 1100 cm^{-1} (Collman et al., 1974) greatly diminishes the usefulness of their "model" for comparison with oxyhemoglobin or oxymyoglobin. That attempts to equate the reversible oxygenation of a heme system with an accurate model for hemoglobins or myoglobins should be made with caution is clearly shown by the findings of Fuchsman et al. (1974) who observed reversible heme oxygen adducts with ν_{O_2} values near 1600 cm^{-1}. Thus oxygen binding must be quite different from that in HbO_2 or MbO_2 where ν_{O_2} is near 1100 cm^{-1}.

[3] W. J. Wallace, J. C. Maxwell, and W. S. Caughey, manuscript in preparation.

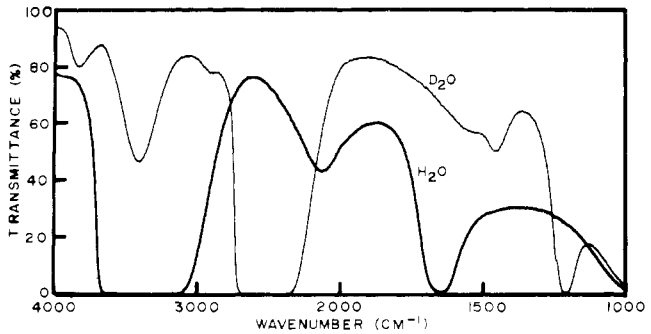


FIGURE 1: Infrared spectra of H_2O vs. air (heavy trace) and D_2O vs. air (lighter trace) recorded in percent transmission mode in a CaF_2 cell with a path length of 0.027 mm. Although both H_2O and D_2O have regions of strong absorption, these regions do not overlap appreciably thereby allowing the entire frequency range from 4000 to 1000 cm^{-1} to be observed by an appropriate selection of the two solvents. Since both H_2O and the CaF_2 window material have strong absorption below 1000 cm^{-1}, this frequency represents the practical low frequency limit for use with aqueous solutions in CaF_2 cells. Use of other window materials allows the range with D_2O to be extended to about 700 cm^{-1}.

For these studies no attempt was made to isolate the nitrosyl hemes with trans ligand as solids. Rather the six-coordinate species were observed in solutions in which either the solid five-coordinate NO compound had been added to solvent containing nitrogenous base or the heme carbonyl had been exposed to NO gas in a solution with excess nitrogenous base present. The presence of the six-coordinate compound with nitrogenous base trans to NO (as well as some five-coordinate species) was detected by infrared, uv-visible, and EPR spectroscopy as discussed below. In each case, the equilibrium between five- and six-coordinated species shifted in favor of six-coordination as the concentration of base was increased. In pure 1-methylimidazole, where only the six-coordinate species was detected in the infrared spectrum, the uv-visible spectrum exhibited λ_{max} nm(AmM) values of 575 (11.8), 545 (12.9), and 418 (142).

Preparation of Oxyhemoglobin Solutions. Oxyhemoglobin A was prepared in water solution from human blood by the method of Geraci et al. (1969). To achieve exchange of D_2O for H_2O, the hemoglobin solution was passed down a G-25 Sephadex column equilibrated with 0.01 M phosphate in D_2O (pD 7.0). Alternatively, erythrocytes were washed repeatedly with D_2O saline (0.9 g of NaCl/100 ml of D_2O) to achieve exchange of H by D within the cell, followed by lysis in pure D_2O and by centrifugation to remove the ghosts. The resulting supernatant used directly in the CO and NO experiments described herein gave results undistinguishable from those obtained from hemoglobin subjected to D for H exchange on a Sephadex column.

The hemoglobin solutions used for the experiments on effects of inositol hexaphosphate (IHP) were simultaneously exchanged with D_2O and stripped, at least partially, of organic phosphate (Berman et al., 1971) by passage down a G-25 Sephadex column equilibrated with 0.005 M Tris buffer in D_2O (pD 8.0). The resulting solution was concentrated under O_2 in an Amicon ultrafiltration cell to a concentration approximately 10 mM in heme. To prepare a solution 10 mM in IHP and 5 mM in heme, the concentrated solution was treated with an equal volume of a solution with 0.2 M bis-tris buffer and 0.02 M IHP in D_2O (pD 6.0).

Infrared Spectral Studies. Spectra were recorded on a Perkin-Elmer Model 180 infrared spectrophotometer operated in the absorbance mode with a resolution of 1.5–2.7 cm^{-1}. The CaF_2 windows in the infrared cells transmit radiation well over a sufficiently broad range to permit the recording of spectra in the Soret, visible, and infrared regions with the same cell. Path lengths were approximately 0.025 mm for hemoglobin solutions and 0.1 mm for solutions of hemes. The absolute path length is not critical but, in any given experiment, it is essential that the path lengths of sample and reference cells be so nearly identical that the bands due to the protein cancel to leave a nearly flat baseline. Such matching is achieved by overlapping the interference fringe patterns of the empty cells. An infrared spectrum of each empty cell vs. air is recorded from 4000 to 1200 cm^{-1}. Such a spectrum contains an interference fringe pattern from which the distance between the cell windows can be accurately computed. However, unless band intensities are to be determined, the actual path lengths need not be established. Rather, it is most important that the distances in the sample and reference cells be the same. This will be the case when the wave patterns for the two cells are superimposable. This can be achieved by choosing spacers with nearly identical thickness and by careful adjustment of the pressures applied on the spacers. In this way, path lengths can be closely enough matched to permit satisfactory difference spectra of protein solutions in the N–O stretch region to be recorded so long as the protein concentrations were also closely matched in the sample and reference cells. This close matching of the protein solutions was conveniently achieved with HbCO solution in the reference cell and the same protein solution in the HbNO form where CO has simply been replaced by NO in the sample cell. Typically, ca. 0.5 ml of HbCO solution is treated with 1.5 ml of NO gas for 5 min in a syringe under agitation, the exposure to NO gas is repeated, and the sample cell is filled with HbNO solution directly from the syringe. In this procedure O_2 can be readily excluded from the HbNO solutions.

Infrared spectra of the five-coordinate heme nitrosyls were obtained from ca. 10 mM solutions of the solid in various solvents or from 2 mg of heme NO in 200 mg of KBr. The solutions used to observe the six-coordinate nitrosyls were prepared as described above.

Ultraviolet and Visible Spectral Studies. Spectra for the Soret and visible regions were recorded with the same cells used for the infrared observations on a Cary Model 17 spectrophotometer using 0–1.0 absorbance range for the Soret region and the 0–0.2 absorbance range for the visible region.

EPR Spectral Studies. Solutions of nitrosyl hemes and hemoglobins were injected into EPR tubes that contained CO to exclude O_2. Spectra were recorded at −160°C with a JEOL Me-X spectrometer operating at 9.28 GHz.

Results and Discussion

Detection of the N–O Stretch Band in the Infrared Spectrum of HbA-NO. Since heme nitrosyls were found to exhibit ν_{NO} between 1575 and 1700 cm^{-1}, it seemed reasonable to expect the nitrosylhemoglobins to absorb in this frequency range also. However, unlike the hemes, the hemoglobins must be examined in aqueous solution and this frequency range presented several obstacles. In the first place, H_2O is unsuitable as a medium in that too little energy is transmitted between 1700 and 1575 cm^{-1}; fortunately D_2O does have a "window" in this region (Figure 1). Thus, while either H_2O or D_2O may be used as solvent for the C–O region near 1950 cm^{-1} or the O–O region near 1100 cm^{-1},

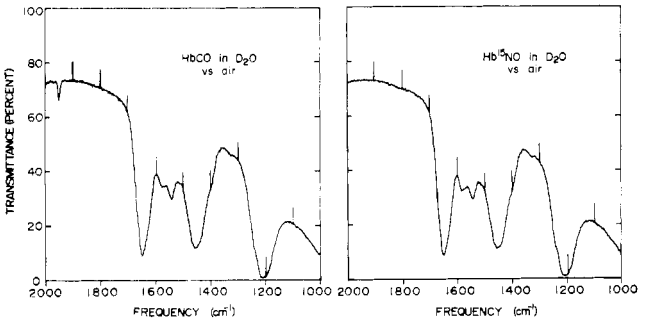


FIGURE 2: Infrared spectra recorded as percent transmittance vs. air for hemoglobin solutions in D_2O 4.9 m*M* in heme: carbonyl species on the left; nitrosyl ($^{15}N^{16}O$) species on the right. Cells had CaF_2 windows and path length of 0.26 mm. Wavenumber markers appear at 100-cm^{-1} intervals on each spectrum.

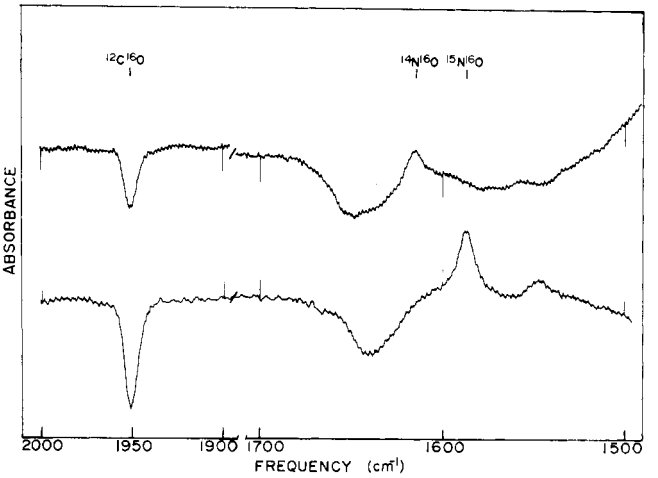
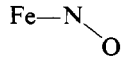


FIGURE 3: Infrared difference spectra recorded in the absorbance mode. The upper trace shows the spectrum for hemoglobin solutions 2.7 mM in heme in 0.01 M sodium phosphate in D_2O buffer (pD 7.0) with the $^{14}N^{16}O$ species in the sample cell and the $^{12}C^{16}O$ species in the reference cell. The lower trace shows a similar spectrum for $^{15}N^{16}O$ vs. $^{12}C^{16}O$ species and was obtained from the same cells used to obtain the spectra of Figure 2. Conditions were the same for both isotopes except for a difference in protein concentration. The negative band at 1951 mm^{-1} is due to bound CO and the positive bands at 1615 and 1587 cm^{-1} result from bound $^{14}N^{16}O$ and $^{15}N^{16}O$, respectively. The broad negative band at ca. 1650 cm^{-1} is primarily due to a slight mismatch in cell path lengths. The HbCO solution was always placed in the longer path length cell so that any absorptions due to protein would appear as negative bands.

only D_2O is satisfactory for the N–O region. A further complication in the N–O region is the high intensity of protein amide bands which are the main contributors to the bands shown at 1650, 1540, and 1450 cm^{-1} in Figure 2. The 1540- and 1450-cm^{-1} bands are isotope sensitive and as peptide bond hydrogens are replaced by deuterium, a shift in absorption from 1540 to 1450 cm^{-1} occurs. The amide bands absorb strongly and consequently limit the amount of protein that can be introduced in the light path and still provide sufficient energy through the sample to allow the spectrum to be recorded. Furthermore, the much greater intensity of the amide bands compared with the N–O stretch band makes the matching of cell path lengths and protein concentrations particularly critical when it is desired to detect the N–O band in the presence of these other stronger bands. Figures 2 and 3 illustrate the nature of the problem. In Figure 2, transmission spectra taken with only air in the reference beam show strong absorption in the amide region which makes the relatively weak band due to Hb bound $^{15}N^{16}O$ at 1587 cm^{-1} very difficult to detect (trace on the right). Under similar conditions, the CO band for Hb-CO is easily seen at 1951 cm^{-1} (trace on the left). Nevertheless, when the Hb-$^{15}N^{16}O$ and the Hb-CO are placed in the sample and reference beams, respectively, the $^{15}N^{16}O$ band is readily apparent in the difference spectrum (Figure 3, lower trace) recorded in the absorbance mode. The band for $^{15}N^{16}O$ experiences less interference from the amide I band than does the $^{14}N^{16}O$ band. Satisfactory difference spectra can only be obtained by this procedure if the path lengths of reference and sample cells are nearly the same and the protein concentrations are also essentially identical. Similar precautions are required for the observations of O–O bands near 1100 cm^{-1} a frequency where, with CaF_2 windows, variations in window thickness can also be a problem.

The identification of bands at 1615 and 1587 cm^{-1} as due to $^{14}N^{16}O$ and $^{15}N^{16}O$ bound to human hemoglobin A in the absence of IHP is confirmed by the magnitude of isotopic shift which is nearly that calculated from simple reduced mass considerations for a diatomic molecule. The narrow band width ($\Delta\nu_{1/2}$, 10 cm^{-1}) is only slightly greater than was found for CO and O_2, as expected if the environment for each ligand is the same. The band intensity was about 75% of that found for the CO of HbCO. In the absence of IHP or another allosteric effector, each of the four NO ligands of the hemoglobin tetramer appear at the same frequency near the value found for the six-coordinate 1-methylimidazole protoheme nitrosyl in 1-methylimidazole solution (Table I). The fact that the frequencies for each of the four NO ligands are the same, or nearly so, indicates the close similarity of NO to heme bonding at each heme site as noted earlier for CO (Alben and Caughey, 1968). This frequency is fully consistent with bent-end-on bonding, i.e.,



(Enemark and Feltham, 1974) with iron(II) serving as π donor and the N of NO as σ donor with an overall shift of electron density from iron to NO upon bonding. This interpretation is not consistent with conclusions drawn from EPR studies of certain hemoproteins to the effect that the electron-density shift was in the opposite direction, namely from NO to iron(II) to give a partially positive NO ligand (Yonetani et al., 1972). However, since EPR data give evidence of *spin* density but do not indicate the *charge* distribution, the EPR data need not be considered inconsistent with the conclusions drawn from infrared data. It is of interest here that iron(III) horseradish peroxidase binds NO to form a diamagnetic nitrosyl complex having no EPR spectrum presumably due to spin-pairing (Yonetani et al., 1972). In preliminary infrared experiments for such a complex, we found ν_{NO} at 1865 cm^{-1} for $^{15}N^{16}O$, a frequency 22 cm^{-1} higher than the free gas value. Such a high frequency is consistent with net electron donation from NO to iron(III) to give a positively charged NO ligand. Thus, a shift of ca. 300 cm^{-1} occurs on going from the iron(II) of hemoglobin to the iron(III) of the peroxidase.

Effects of IHP on Hemoglobin Carbonyls and Nitrosyls. In HbA-NO the bands at 1615 cm^{-1} (for HbA-$^{14}N^{16}O$) and at 1587 cm^{-1} (for Hb-$^{15}N^{16}O$) were both reduced to about one-half the original intensity (compared with the HbA-CO ν_{CO} band which remained unchanged) upon addi-

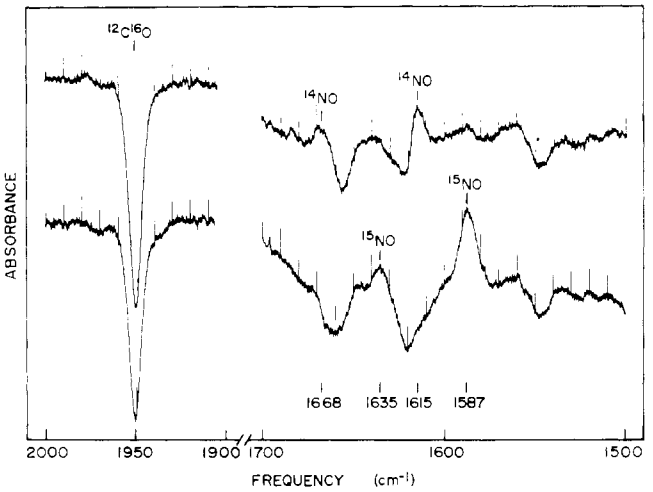


FIGURE 4: Infrared difference spectra recorded in the absorbance mode. The upper trace shows the spectrum for hemoglobin solutions ca. 4.6 mM in heme in 0.1 M bis-tris and 10 mM IHP (pD 6.1) with the $^{14}N^{16}O$ species in the sample cell and the $^{12}C^{16}O$ species in the reference cell. The lower trace shows a similar spectrum for $^{15}N^{16}O$ vs. $^{12}C^{16}O$ species under identical conditions. To observe the effect of IHP binding on band intensities, note that the band height for the $^{15}N^{16}O$ at1587 cm^{-1} is nearly the same for the lower spectrum in this figure and for the lower spectrum in Figure 3. However, the band height of the CO band in this figure is approximately twice that of the CO band in the lower trace of Figure 3. Thus, approximately one-half of Hb bound ^{15}NO no longer has a stretching frequency of 1587 cm^{-1}. Isotope sensitive bands at 1668 and 1635 cm^{-1} for $^{14}N^{16}O$ and $^{15}N^{16}O$, respectively, are visible among the irregularities of the baseline. These baseline irregularities may be due to differences in protein conformation between HbCO and HbNO as a result of IHP binding. It should be noted that these spectra were recorded at a twofold higher ordinate expansion than those shown in Figure 3.

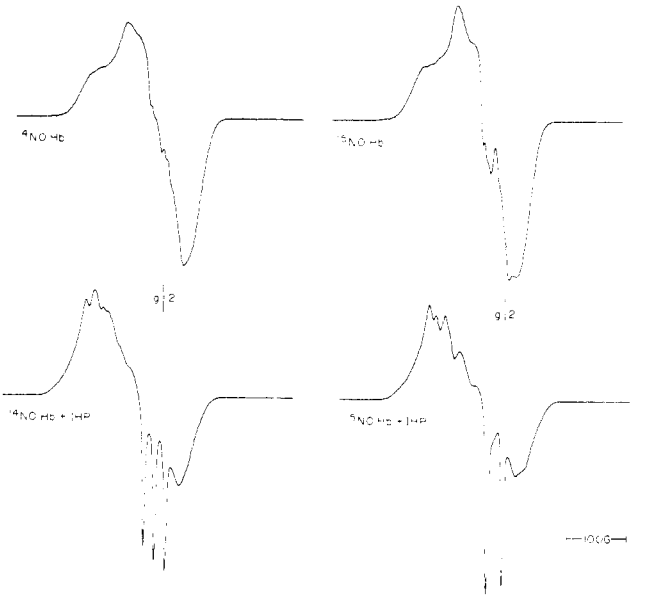


FIGURE 5: X-band EPR spectra for $^{14}N^{16}O$ and $^{15}N^{16}O$ hemoglobin A complexes with and without IHP (2 mol per Hb tetramer) in 0.1 M bis-tris buffer (pH 6 3). Instrument settings were as follows: microwave power, 10 mW; microwave frequency, 9.28 GHz; modulation width, 2 G; temperature, −160°C.

tion of IHP to the respective solutions (Figure 4). In each case, a second band appeared at about 50 cm^{-1} higher frequency. The new bands appeared of lower intensity than the remaining lower frequency bands but the intensities of the new bands were less easily measured due to baseline irregularities.

The effects of IHP on the EPR spectra of our preparations with both nitrogen isotopes were also examined (Figure 5). The results for the $^{14}N^{16}O$ ligand paralleled those observed by Rein et al. (1972) with IHP binding the cause of striking differences. Substitution of ^{15}N for ^{14}N caused not only the expected change from three strong hyperfines to two in the g_z region, but differences are also seen in the g_x region (Figure 5).

Uv-visible spectra were examined both before and after recording of ir spectra. The effects of IHP found were similar to those reported by Perutz et al. (1976) in the accompanying paper. Without IHP, the hemoglobin A nitrosyl exhibited λ_{max} values of 418, 543, and 571 nm. With 10 mM IHP and the hemoglobin nitrosyl 4.7 mM in heme at pD 6.1 in bis-tris buffer, λ_{max} values were 417, 542, and 570 nm with a noticeable drop in the intensity of the Soret band.

As will be discussed below, comparison of these data with those from protein-free heme carbonyls and nitrosyls, the IHP-induced shifts in ν_{NO} band, and in EPR and uv-visible spectra are fully consistent with the formation of an essentially five-coordinated heme nitrosyl in two subunits upon IHP binding with the remaining two subunits unaffected by IHP binding. In contrast, the lack of change of the C–O stretch band upon addition of IHP shows no significant change at heme–CO site as a consequence of IHP binding.

Further discussion of the support for these conclusions and their implications will follow consideration of the properties of protein-free heme carbonyls and nitrosyls.

Heme Carbonyls. Carbonyl hemes are low spin, iron(II) complexes with a great preference, although possibly not an absolute requirement (Rougee and Brault, 1973; Mehne and Wayland, 1975), for six-coordination. The CO ligand serves as an excellent infrared probe because the C–O stretch band is highly responsive to changes in binding site. ν_{CO} varies with change in trans ligand, solvent, and porphyrin structure (Caughey et al., 1973). Wide variations in ν_{CO} are found for protoheme dimethyl ester carbonyls in chloroform with a variety of trans ligands; for example, values of 1969, 1976, and 2008 cm^{-1} are found for 1-methylimidazole, pyridine, and methylisonitrile, respectively (Caughey et al., 1973; Table I). It can be seen from data in Table I that frequency variations nearly as large have been observed with changes in solvent. These solvent effects on ν_{CO} (but not on bandwidth) correlate well with the dipolar nature of the solvent; ν_{CO} decreases as dipole moment increases. The lack of observable change in the C–O stretch band of HbA-CO when IHP was added can thus be considered strong evidence that the presence of IHP under these conditions did not produce significant structural changes at the heme site.

Heme Nitrosyls. The five-coordinate nitrosyl protoheme dimethyl ester is obtained cleanly in the solid state from the dipyridine heme which upon heating under vacuum loses the pyridine (py) ligands (eq 1) (Fuchsman et al., 1974). Exposure of the resulting "bare iron" species to NO gas results in the binding of one NO per iron (eq 2) and, since no other ligand is available to the iron, this must represent a five-coordinate heme nitrosyl. The ν_{Fe-N} band at 480 cm^{-1} confirms NO binding to Fe. (In contrast, similar exposure of the "bare iron" species to CO did not result in CO uptake.[3]) The solid five-coordinate nitrosyl heme, in KBr, exhibits an ν_{NO} at 1660 cm^{-1}. In solutions, the ν_{NO} values varied from 1669 to 1684 cm^{-1} (Table I).

$$py\text{-}Fe^{II}\text{-}py \xrightarrow{\Delta} Fe^{II} + 2py \quad (1)$$

$$Fe^{II} + NO \rightarrow Fe^{II}\text{-}NO \quad (2)$$

Six-coordinate nitrosyls can be prepared in solution from either the five-coordinate nitrosyl or six-coordinate carbonyl. Placing Fe^{II}-NO in a solvent that contains a potential nitrogenous ligand such as pyridine or 1-methylimidazole (MI) will result in an infrared band due to the six-coordinate species; the relative intensities of the two NO bands depend upon the concentration and nature of the trans ligand. An important solution property of nitrosyl hemes is that there exists a facile equilibrium between five- and six-coordinate species illustrated in eq 3 where L is a potential trans ligand.

$$L + Fe^{II}\text{-}NO \rightleftharpoons L\text{-}Fe^{II}NO \quad (3)$$

When the pyridine protoheme carbonyl is dissolved in NO saturated chloroform, the CO is readily displaced by NO with most of the bound NO seen at 1676 cm^{-1}, the frequency noted when the five-coordinate heme is dissolved in chloroform. A second band at 1640 cm^{-1} assumed to be due to six-coordinate pyridine nitrosyl is weak with only about 5–10% of the intensity of the 1676-cm^{-1} band. Thus, eq 4 is the main reaction and eq 5 a minor process here. However, in pure pyridine the band for five-coordinate nitrosyl heme is not clearly seen. Only the 1633-cm^{-1} band is present as is consistent with eq 5 being much more important than eq 4 in this solvent. When MI-Fe^{II}-CO is dissolved in NO saturated chloroform, the band at 1676 cm^{-1} is about three times as intense as the 1630-cm^{-1} band. Since with py-Fe^{II}-CO the five-coordinate band was about 12 times more intense than the six-coordinate band, MI appears more effective than pyridine as a ligand trans to NO. When solid five-coordinate Fe^{II}-NO is dissolved in pure MI, only the six-coordinate species (ν_{NO} at 1618 cm^{-1}) is observed. The correspondence between ν_{NO} in the five-coordinate solid nitrosyl and the species in solution suggests that even in the presence of excess NO only a mononitrosyl is formed.

$$py\text{-}Fe^{II}\text{-}CO + NO \rightarrow Fe^{II}\text{-}NO + CO + py \quad (4)$$

$$py\text{-}Fe^{II}\text{-}CO + NO \rightarrow py\text{-}Fe^{II}\text{-}NO + CO \quad (5)$$

As seen in Table I, solvent and trans ligand effects on ν_{NO} are of similar magnitude to those found with ν_{CO} for heme carbonyls. With suitably chosen solvents, heme carbonyls and nitrosyls represent close models of the hemoglobin environment. Thus, MI-Fe^{II}-CO in pure MI exhibits a ν_{CO} at 1954 cm^{-1} near that of HbA-CO (1951 cm^{-1}) whereas for MI-Fe^{II}-CO in 1,2-dichloroethane, ν_{CO} is 1959 cm^{-1} compared with 1959 cm^{-1} found for Chironimus hemoglobin where the distal histidine is replaced with isoleucine (O'Toole, 1972) and 1958 cm^{-1} for the Hb Zurich β subunit with the distal histidine replaced with arginine (Caughey et al., 1969). Similarly, MI-Fe^{II}-NO in pure MI has ν_{NO} at 1618 cm^{-1} compared with HbA-NO where ν_{NO} is 1615 cm^{-1}. The five-coordinate Fe^{II}-NO in 1,2-dichloroethane has ν_{NO} at 1669 cm^{-1} and the five-coordinate subunit assumed to be present in HbA-NO with IHP bound was ν_{NO} at 1668 cm^{-1}.

The magnitude of the IHP-induced shift (~50 cm^{-1}) is so great as to provide strong evidence for loss of the trans histidine ligand, a shift of precisely the same magnitude as found on loss of imidazole in protein-free hemes. We are unaware of any medium (solvent) effects nearly that large. Thus, these infrared data support a five-coordinate structure for the species represented by the 1668-cm^{-1} band which appears in HbA-NO upon addition of IHP. Further, the relative intensities of the bands at 1618 and 1668 cm^{-1} provide quantitative evidence that one-half of the hemes (two out of four subunits) undergo change from six to five coordination. Thus, the effect of IHP binding to the two β subunits at the core of the tetramer (Arnone and Perutz, 1974) is to cleave proximal histidine to iron bonds in two of the subunits. However, our experiments do not provide evidence as to which two hemes are affected by IHP binding.

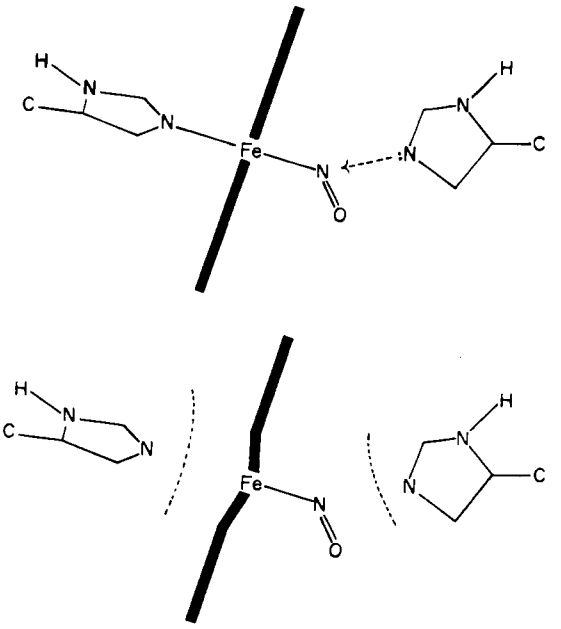


FIGURE 6: Schematic representations of NO binding to hemoglobin. The upper drawing represents the six-coordinate NO heme present at all heme sites in stripped or native HbA NO. The lower drawing represents the five-coordinate heme NO present in half the subunits of HbA as a result of IHP binding where infrared data provide convincing evidence for complete cleavage of the proximal histidine-Fe bond but changes in the distal histidine-NO interaction are less clear.

The ν_{NO} value for the five-coordinate heme NO of IHP-HbA-NO (1669 cm^{-1}) is similar to that for the Fe^{II}-NO in 1,2-dichloroethane (1668 cm^{-1}). In contrast the ν_{NO} value of the six-coordinate hemes in HbA-NO (1615 cm^{-1}) is not as close to that of the MI-Fe^{II}-NO in 1,2-dichloroethane (1630 cm^{-1}) as it is for the MI-Fe^{II}-NO in MI (1618 cm^{-1}). (The five-coordinate Fe^{II}-NO in MI, although difficult to detect, due to its low concentration compared with the six-coordinate species, is reasonably expected to exhibit a ν_{NO} near 1663 cm^{-1}; ν_{CO} values are ca. 5 mm^{-1} lower in MI than in 1,2-dichloroethane, Table I.) An attractive explanation for these data is that, not only is the proximal histidine to iron bond broken, but also an interaction between NO ligand and the distal histidine has been interrupted in the conversion from six to five coordination (note Figure 6).

The precise nature of distal histidine-ligand interactions, if any, has not been clearly established. An early crystallographic study of azido and acid metmyoglobins (Stryer et al., 1964) suggested hydrogen bonding interaction between distal histidine and azide (Figure 7). However, McCoy and Caughey (1970) found no change in the azide infrared bands over a range of pH in which protonation and deprotonation of the more remote nitrogen of the distal histidine would likely take place. Clearly, the strength of such hydrogen bonding would be markedly affected by protonation of the other imidazole nitrogen, an affect that should be de-

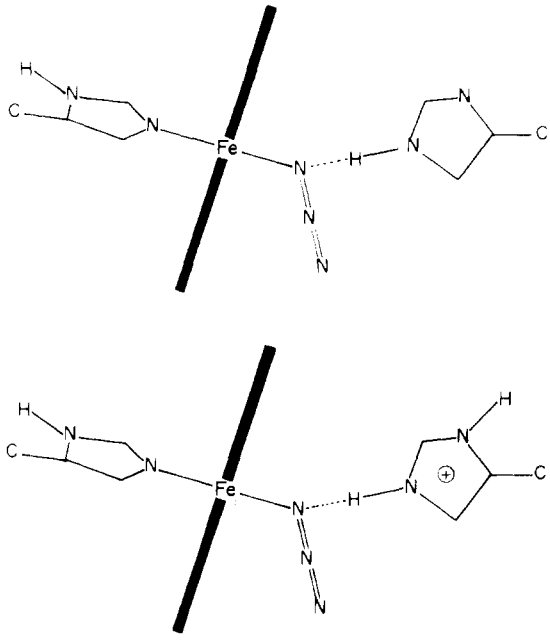


FIGURE 7: Schematic representations of hydrogen bonding between distal histidine and azide. The upper drawing represents hydrogen bonding from a neutral distal histidine in which the more remote nitrogen does not bear a proton. In the lower drawing, the remote nitrogen is protonated to give a positively charged imidazole ring. It is proposed that the strength of any hydrogen bonding to azide, if in fact present, would necessarily differ for the upper and lower cases with the further result that the infrared band due to bound azide should differ for the two cases. The dimensions used approximate those employed by Stryer et al. (1964) and Watson and Nobbs (1968) for metmyoglobins.

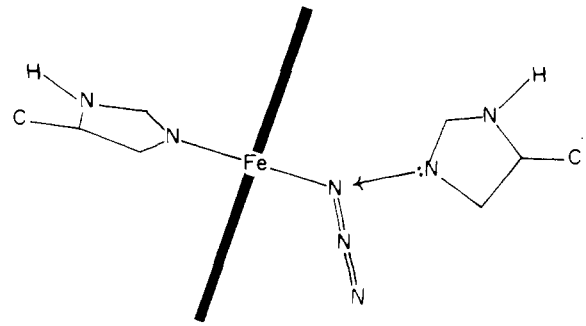


FIGURE 8: Schematic representation of distal histidine–azide interactions of the donor–acceptor type proposed by McCoy and Caughey (1970).

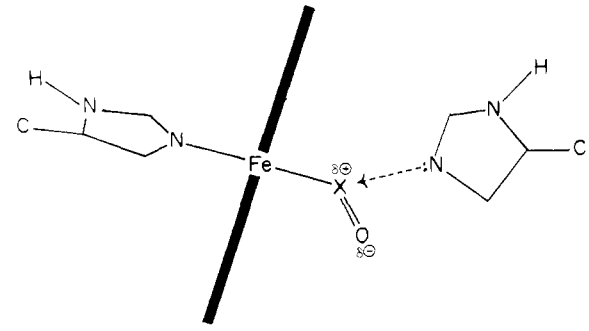


FIGURE 9: Schematic representation of the ligand binding site of hemoglobin where X may be carbon, nitrogen, or oxygen. The partial positive charge on "X" may be stabilized by interaction with the distal histidine wherein a pyridine-like imidazole nitrogen acts as electron donor and "X" as acceptor.

tectable in infrared spectra for the azide. Therefore, they reasoned that hydrogen bonding between distal histidine and azide was unlikely. The alternative structure in which the iron-bound nitrogen of the azide serves as an acceptor for the electron pair on an unprotonated nitrogen of distal histidine was therefore suggested (Figure 8). The more remote nitrogen of this histidine would thus contain a proton over wide pH ranges. Similar reasoning based on lack of discernable pH effects on infrared spectra of other ligands leads us to suggest similar bonding for O_2, CO, and NO ligands. In these instances, the Fe^{II} bound ligand atom, O, C, or N, respectively, would serve as an acceptor for the basic histidine nitrogen (Figure 9). However, in the aquo complexes, hydrogen bonding between bound water and distal histidine is likely to occur (Caughey, 1966). Specific solvation effects where MI serves as an electron pair donor to CO or NO ligands may well contribute significantly to the rather special influence of solvent MI on ν_{CO} and ν_{NO} in hemes. Donor–acceptor interactions between bound ligand and distal histidine in hemoglobins and myoglobins as shown in Figure 9 could represent an important stabilizing factor in O_2 and NO binding, both ligands that prefer nonlinear bonding to Fe(II). And, with CO, such an interaction would promote bending of the Fe–C–O bonds from the linearity generally preferred in metal carbonyls. The importance of such interactions between distal histidine and bound oxygen is not clear because reversible oxygen binding can readily occur in the absence of a distal histidine as noted in several hemoglobins (e.g., Chironomous, glycera, opposum, Zurich). Nevertheless, it is a striking fact that among the normal hemoglobins from many species of mammals, reptiles, and birds the ν_{CO} values are nearly identical (1950 ± 2 cm^{-1}), a finding highly suggestive that there is something of quite special significance about the structure of the CO (and O_2) binding site.[4] These findings also suggest that in the case reported here, where IHP leads to the cleavage of the proximal histidine to iron(II) bonds there is movement of the iron atom out of the porphyrin plane toward the NO ligand, and a change in Fe–N–O bond angles (Piciulo et al., 1974; Scheidt and Frisse, 1975) which are accompanied by disturbance of the "normal" interactions of the NO with adjacent distal histidine and, possibly, other amino acid residues. The infrared evidence is consistent with a change from an MI-type medium for six-coordinate species to a 1,2-dichloroethane-type medium for the five-coordinate species.

Scheidt and coworkers have prepared and completed x-ray crystallographic structure determinations on five- and six-coordinated iron(II) tetraphenylporphin nitrosyls (Piciulo et al., 1974; Scheidt and Frisse, 1975). The five-coordinate species in KBr exhibited ν_{NO} at 1670 cm^{-1}. However, Wayland and Olson (1974) reported a value of 1700 cm^{-1} for this compound in a Nujol mull and a shift in frequency to 1680 cm^{-1} with piperidine present as a trans ligand. The Scheidt value compares favorably with our value for five-coordinate protohome nitrosyl in KBr (1660 cm^{-1}) when the difference in porphyrin is taken into account. However, the Wayland values are less readily explained in terms of our data with protoheme nitrosyls.

Comparisons of visible–Soret and EPR spectra for heme and hemoglobin nitrosyls provide further evidence for the formation of two five-coordinate and two six-coordinate

[4] J. C. Maxwell, M. C. O'Toole, and W. S. Caughey, unpublished observations.

heme nitrosyls as a result of IHP binding to tetrameric HbA-NO. For example, we have found a 38% reduction of Soret band intensity going from MI-Fe^{II}-NO in MI to five-coordinate Fe^{II}-NO in 1,2-dichloroethane. On the basis of this intensity difference, if one-half of the hemes changes coordination number upon binding IHP, a 19% reduction in Soret band intensity should result. In fact, a very similar reduction does occur, 23% as reported in the accompanying paper by Perutz et al. (1976), a finding we had also made. In general, the differences in relative intensities and λ_{max} values for all the bands in the uv-visible spectra of the hemes compare favorably with the hemoglobin data. Also, evidence from EPR spectra is qualitatively consistent with a six- to five-coordinate transition, but is rather difficult to quantify. The five-coordinate heme nitrosyls have EPR spectra with intense, well-resolved NO nitrogen hyperfine splittings and so do spectra for the IHP-treated Hb solutions (Figure 5). By contrast, the six-coordinate heme nitrosyls give EPR spectra quite similar to stripped or native HbA NO with superhyperfine splitting of ca. 6 G on rather poorly resolved NO nitrogen hyperfine lines. Rather similar EPR data have been reported by Wayland and Olson (1975) and Kon (1975) for $\alpha,\beta,\gamma,\delta$-tetraphenylporphin-iron(II) nitrosyls.

Conclusions

Each of the four hemes in stripped HbA-NO appears in terms of EPR and uv-visible spectra and N-O stretch frequency similar to each other and to the six-coordinate protoheme nitrosyl with 1-methylimidazole as trans ligand and solvent. However, upon binding IHP, these spectra change in a manner that is consistent with two of the four hemes becoming five-coordinate. Thus, the binding of one IHP molecule between two portions of the β subunits at the center of the tetramer (Arnone and Perutz, 1974) alters interactions between protein and heme at two subunits—which two is uncertain—such that the proximal histidine to iron bond is disrupted. These findings provide dramatic evidence that reactions at the protein surface can directly affect the coordination chemsitry at internal heme sites. However, with CO as ligand, IHP did not affect the ν_{CO} values and thus five-coordinate carbonyls could not have been produced. Indeed, this is not unexpected since the protein-free hemes readily form both five- and six-coordinate nitrosyls but usually form only six-coordinate carbonyls.

The IHP effect upon HbA-NO shown here reveals how effects of organic phosphate binding can influence heme coordination and thus affect ligand binding equilibria. Furthermore, the same type of "through protein interactions" that result in the formation of five-coordinate hemes with HbNO and IHP can be expected to stabilize the deoxy forms and thereby decrease the affinity for O_2 without the requirement of a specific effect of IHP on HbO_2 per se. Indeed, present evidence would not suggest that five-coordinate oxygenated hemes are formed or even that IHP binding is extensive for the fully oxygenated HbA. The stabilization of the deoxy form can arise by movement of the proximal histidine and attached iron(II) away from the porphyrin on the side opposite to the side to which the O_2 ligand must bind. The "pulling away" of the proximal histidine would weaken the histidine to iron bonding and thereby render the iron(II) less attractive as a π donor to dioxygen. O_2 binding will also be retarded to the extent that the iron follows after the proximal histidine, thus making the iron(II) less accessible to dioxygen in a steric sense.

The IHP effect observed here may be explained solely in terms of a "pulling away" of proximal histidines in the manner strongly favored by Perutz et al. (1976) in discussions of these Hb reactions in terms of R and T forms. Such an effect is supported by numerous examples of trans effects in metal porphyrin systems (Caughey, 1967, 1973; Caughey et al., 1973; O'Keeffe, 1974) and has been proposed as a mechanistic possibility for heme-heme interactions and related allosteric phenomena (Lumry and Eyring, 1954; Lumry, 1961; Caughey, 1967; 1973). However, other factors which can be highly significant, but are as yet unevaluated, include changes in donor-acceptor (π) interactions between the porphyrin aromatic ring system (and the highly polarizable vinyl groups) and the protein environment, and changes in the environment about the nitrosyl ligand. Thus, both steric and electronic factors influence conformations and reactivity of heme and protein moieties (Caughey, 1967; Caughey, et al., 1969). The importance of the histidine trans effect per se in relation to other factors remains to be established, but the data reported here suggest that nearly all of the organic phosphate effects could arise in this way. Indeed, the trans effect could also be important for many other allosteric processes that control ligand binding.

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