Proc. Natl. Acad. Sci. USA
Vol. 76, No. 2, pp. 549–552, February 1979
Chemistry

Absence of heme-localized strain in T state hemoglobin: Insensitivity of heme-imidazole resonance Raman frequencies to quaternary structure

(protein dynamics/cooperativity/strain energy/heme vibrations)

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Communicated by John J. Hopfield, November 17, 1978

ABSTRACT **Substitution of pentadeuterated 2-methylimidazole in (2-methylimidazole)-Fe(II)-protoporphyrin IX, a model complex for deoxyHb, shifts three bands in the low-frequency resonance Raman spectrum 380 → 373 cm^{-1}, 348 → 345 cm^{-1}, and 220 → 218 cm^{-1}. The first of these is assigned primarily to Fe-imidazole stretching, and the other two are assigned to porphyrin deformation modes with substantial Fe-pyrrole stretching contributions. The three bands are observed in deoxyHb and Mb. The Fe-pyrrole modes are at essentially the same frequencies in the two proteins, but the Fe-imidazole mode is 6 cm^{-1} lower in deoxyHb than Mb, implying a slight alteration in the heme-imidazole linkage. No change greater than 2 cm^{-1} is observed when Hb Kempsey is switched from the R to the T state. This observation places an upper limit on the energy stored in the Fe-imidazole bond of T state deoxyHb, which is estimated to be <0.2 kcal/mol (<836.8 J/mol).**

The iron-imidazole bond is a key chemical link in heme proteins, in most of which at least one of the heme axial ligands is an imidazole side chain. For hemoglobin (Hb) and myoglobin (Mb), the iron-imidazole bond is the only point of covalent attachment of the heme prosthetic group. Along with the noncovalent contacts, it supports the structural changes (1) that are known to accompany the binding of O_2 to the five-coordinate iron atom in deoxyHb (2) and Mb (3). Moreover, the forces that induce the quaternary structural change that accompanies O_2 binding to deoxyHb must be transmitted at least in part through the iron-imidazole linkage (4).

Accordingly, it would be valuable to have available a probe of the iron-imidazole bond strength. The vibrational frequency associated with stretching of this bond should provide a suitable probe, because bond strengths and force constants are directly correlated. Resonance Raman spectroscopy is capable of monitoring vibrational modes associated with the heme group (5). The most intense resonance Raman bands arise from porphyrin ring vibrations (6). Resonance enhancement is much weaker for the vibrational modes associated with the iron atom. Moreover, these occur in the low-frequency region, <600 cm^{-1}, which also contains porphyrin deformation modes of comparable intensity. Despite these difficulties, several iron axial ligand stretching modes have been found and confirmed by isotopic frequency shifts. The bonds so identified include Fe—O_2 in O_2Hb (7) and the O_2 adduct of Collman's "picket fence" porphyrin (8); Fe—OH and Fe—N_3 (and plausibly Fe—F, although not confirmed isotopically) in MetHb derivatives (9); Fe—F in Fe(III)-octaethylporphyrin fluoride (10); Fe—O—Fe in the μ-oxo dimer of Fe(III)-tetraphenylporphine (11); and Fe-pyridine (also internal pyridine modes) in bispyridine-Fe(II)-mesoporphyrin IX dimethyl ester (12). Stretching modes have also been assigned, but not confirmed isotopically, for Fe—X (X = F, Cl, or Br) in Fe(III)-octaethylporphyrin halides and for Fe-imidazole in bisimidazole adducts (13).

Herein we report the location of the Fe-imidazole stretching mode, near 380 cm^{-1}, in deoxyHb and Mb, with the aid of the model complex, (2-methylimidazole)-Fe(II)-protoporphyrin IX [(2-MeIm)Fe(II)PP], for which a 2-MeIm/pentadeuterated 2-MeIm (2-MeIm-d_5) isotope substitution study has been carried out. The 6-cm^{-1} decrease between Mb and Hb indicates a slight change in the nature of the heme-imidazole linkage. Two low-frequency porphyrin modes, ≈350 and ≈220 cm^{-1}, mix with the Fe-imidazole mode via substantial contributions from Fe-pyrrole stretching. They are shifted only slightly, if at all, 2 and 0 cm^{-1} between Mb and Hb. Hb Kempsey exhibits the same low-frequency spectrum as Hb A. No frequency shifts (<2 cm^{-1}) are observed upon inositol hexaphosphate addition. This observation implies that no significant strain is developed at the heme-imidazole linkage of the deoxy T state in Hb Kempsey.

EXPERIMENTAL

Hb A solutions were prepared by well-established procedures (14) from samples of whole blood obtained from a local hospital. The myoglobin used was Sigma type I. Buffered solutions of the oxygenated species were reduced with a small amount of dithionite, and spectra were recorded immediately. Mutant Hb Kempsey was separated from Hb A according to the procedures of Bunn *et al.* (15).

Hemin chloride (Sigma type III) was used as received. 2-MeIm (Aldrich) was recrystallized from benzene and sublimed. Deuterium exchange was carried out with 4% (wt/vol) solutions of 2-MeIm in 99% ^2H_2O at 250°C in an autoclave for 6 hr. The solution was evaporated to dryness at reduced pressure, and the resulting residue was recrystallized from benzene and sublimed. The extent of deuteration (>97%) was determined by NMR spectroscopy. Hemin chloride was dissolved (≈1 mg/ml) in H_2O; the solution was adjusted with NaOH to pH 10.5 and contained 5 mg of 2-MeIm per ml. The solution was filtered and equilibrated with argon, and a small amount of solid sodium dithionite was added.

Resonance Raman spectra were obtained by flowing the solution through a 1-mm capillary tube with Tygon or Viton A tubing and a peristaltic pump (16). The data were collected digitally at 0.2-cm^{-1} increments. Frequencies were determined from the derivative (17) spectrum after using a 25-point quadratic smooth.

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Abbreviations: PP, protoporphyrin IX; 2-MeIm, 2-methylimidazole; -d_5, pentadeuterated.

RESULTS AND DISCUSSION

Model Compound and Assignments. Five-coordinate Fe(II) hemes can be prepared by reducing Fe(III) hemes in the presence of excess 2-MeIm (18). The bulky methyl group adjacent to the imidazole nitrogen atoms provides steric hindrance to the formation of planar bisligated complexes, which is observed for imidazole itself. The crystal structure of (2-MeIm)-Fe(II) tetraphenylporphine (19) shows the iron atom to be 0.55 Å out of the mean porphyrin plane, toward the axial ligand, which is the same as the corresponding distance determined for deoxyMb (3). 2-MeIm complexes of Fe(II) porphyrins of the physiological variety, with pyrrole rather than methine substituents [the methine phenyl groups of tetraphenylporphine change the vibrational pattern markedly (11)], give porphyrin vibrational frequencies that are essentially the same as those of deoxyHb and Mb (12).

Fig. 1 shows the low-frequency resonance Raman spectrum of an aqueous solution of (2-MeIm)Fe(II)PP, and of the complex prepared with the five hydrogen atoms on the 2-MeIm carbon atoms [C(2)CH_3, C(3)H, and C(4)H] replaced with deuterium. Three bands, all of them polarized and therefore associated with totally symmetric vibrations, are seen to shift: 380 → 373 cm^{-1}, 348 → 345 cm^{-1}, and 220 → 218 cm^{-1}. The 380 cm^{-1} mode experiences the largest shift, 7.0 cm^{-1}, and is therefore assigned to Fe-imidazole stretching.

The 348 and 220 cm^{-1} bands are also observed in other metalloporphyrins (13, 20), and are assigned to totally symmetric porphyrin deformation modes, to which Fe-pyrrole bond stretching is expected to contribute. When the iron atom is out of the porphyrin plane, the Fe-imidazole and Fe-pyrrole internal coordinates are not orthogonal and are necessarily coupled. The 2- to 3-cm^{-1} shifts observed for 2-MeIm-d_5 imply substantial coupling.

There are other bands in the region that do not shift. Presumably these arise from other porphyrin modes that are not significantly coupled to the Fe-imidazole stretch.

Mb, Hb A, and Hb Kempsey. Fig. 2 compares resonance Raman spectra of deoxyHb and Mb in the low-frequency region. Although the relative intensities of the bands are somewhat different from those of (2-MeIm)Fe(II)PP, there is a one-to-one correlation of the frequencies, as shown in Table 1, except for extra, and presently unassigned, protein bands at ≈435, 242(Mb), and 157(Hb) cm^{-1}. The three imidazole-sensitive bands are readily identifiable at 366, 342, and 220 cm^{-1} for deoxyMb and 372, 344, and 220 cm^{-1} for deoxyHb. The frequency shifts between (2-MeIm)Fe(II)PP and the proteins may simply be a consequence of the different ring substitution in 2-MeIm and histidine. The differences between deoxyHb and deoxyMb are significant, however, because the axial ligand is the same. The 6 cm^{-1} lowering of the band assigned to Fe-imidazole stretching implies a structural alteration, which may be linked to tertiary structure differences. It may reflect weakening of the Fe-imidazole bond in Hb, or it might arise, via kinematic effects, from a change in the orientation of the imidazole ring relative to the heme group. The barely discernible lowering of the bands assigned to Fe-pyrrole stretching suggests little change in the Fe-pyrrole bond strength.

Fig. 3 compares resonance Raman spectra of Hb A and Hb Kempsey, a mutant which is known (15) to remain in the R quaternary state upon deoxygenation in the absence of organic phosphate anions. The spectra show no differences comparable

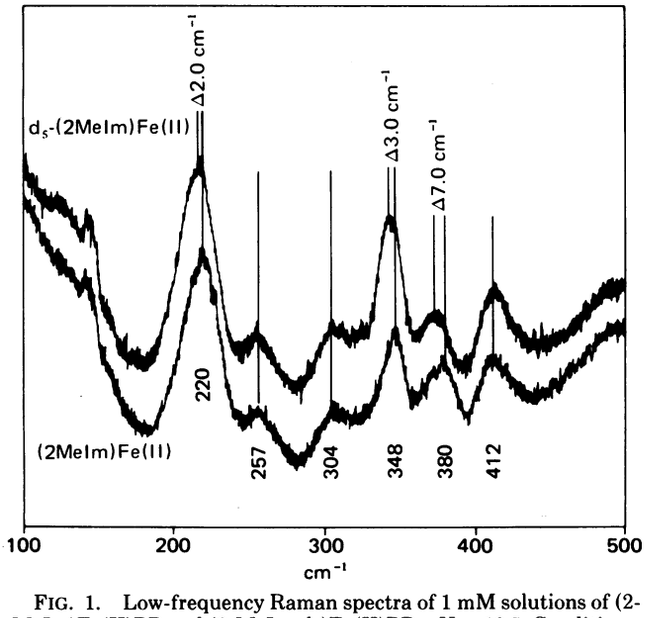

FIG. 1. Low-frequency Raman spectra of 1 mM solutions of (2-MeIm)Fe(II)PP and (2-MeIm-d_5)Fe(II)PP, pH ≅ 10.5. Conditions: 457.9-nm excitation (100 mW), 4 cm^{-1} spectral slit width, 0.2 cm^{-1}/sec scan rate, and digital photon counting. (Smaller slit widths do not improve the band widths.)

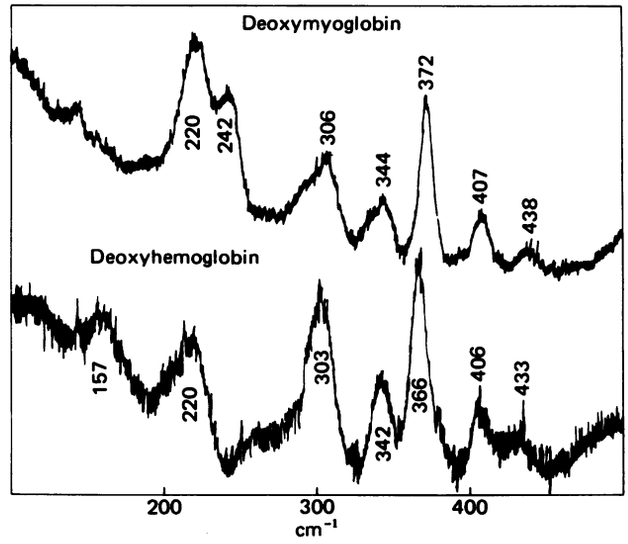

FIG. 2. Low-frequency Raman spectra of deoxyMb and deoxyHb A ≈1 mM in 0.1 M phosphate buffer, pH 7.2. Conditions: as for Fig. 1. The small peak at 143 cm^{-1} is on argon-laser emission line (4609 Å).

Table 1. Low-energy resonance Raman frequencies, cm^{-1}

(2-MeIm) Fe(II)PP	Completely deuterated (2-MeIm) Fe(II)PP	DeoxyMb	DeoxyHb A*	Assignment
		438	433	
380	373	372	366	Fe-Im
348	345	344	342	Fe-P
304	304	306	303	
257	257	†	261	
		242		
220	218	220	220	Fe-P
			157	

Fe-Im, Fe-imidazole; Fe-P, Fe-pyrrole.

* The same frequencies are observed for Hb A and Hb Kempsey with and without inositol hexaphosphate.

† Obscured by the 242-cm^{-1} band.

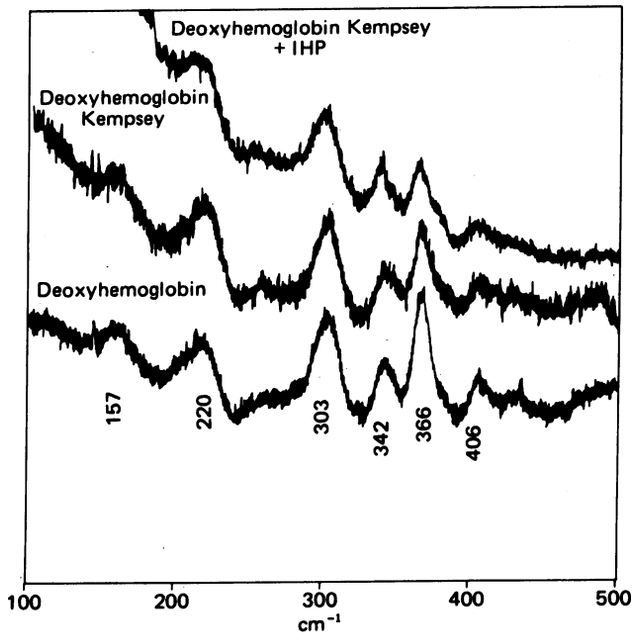

FIG. 3. Low-frequency Raman spectra of deoxyHb Kempsey with and without inositol hexaphosphate (IHP) are compared to deoxyHb A by using the conditions of Fig. 1.

to those observed between Hb and Mb. Also shown is the spectrum of Hb Kempsey after addition of excess inositol hexaphosphate, which is known (15) to switch it to the deoxy T state. For the three bands of interest, frequency shifts as small as 2 cm^{-1} could have been detected, but none are observed. Table 1 summarizes the Raman frequencies observed in this study.

Previous workers have also reported no changes in deoxyHb resonance Raman spectra between R and T states in studies of chemically modified Hb A (21) and of Hb from carp (22). However, this time Fe-imidazole and Fe-pyrrole modes have been identified and monitored. Their insensitivity to the quaternary structure places severe limitations on the amount of strain that can be assumed to be exerted at the heme-imidazole linkage in the deoxy T state.

Estimate of R-T Bond Displacements and Energy Changes. If we model the Fe-imidazole unit with a diatom A-B with masses 56 (Fe) and 82 (2-MeIm), then a stretching frequency of 380 cm^{-1} corresponds to a harmonic force constant k = 2.8 mdyn/Å (1 dyne = 10^{-5} N), a reasonable value for a metal-ligand bond (23). The predicted frequency shift upon increasing the 2-MeIm mass by 5 is 5.3 cm^{-1}, in good agreement with the observed deuterium shift. The model is, of course, greatly oversimplified, leaving out couplings with the Fe-porphyrin system, and also internal motions and changes in orientation of the imidazole ring.

Nevertheless, the model serves as a useful starting approximation. The calculated force constant corresponds to a calculated bond distance, r = 1.91 Å, using Badger's rule, as modified by Herschbach and Laurie (24):

$$r = d_{ij} + (a_{ij} - d_{ij})k^{-1/3} \quad [1]$$

(For a third-row atom, Fe, bonded to a first-row atom, N, the parameters are d_{ij} = 0.85 Å, with k expressed in mdyn/Å.) This value is somewhat lower than the Fe-imidazole bond distance, 2.16 Å, determined for (2-MeIm)Fe(II) tetraphenylporphine (19). Badger's rule is not expected to yield accurate bond distances, but the errors should cancel in estimating small changes in these distances from frequency shifts. If the Fe-imidazole frequency shifts by 2 cm^{-1}, the upper limit of our experimental error, then the change in the corresponding force constant is 0.03 mdyn/Å. If we assume that Fe-imidazole stretching is quite strongly coupled to other displacement coordinates and that the 380-cm^{-1} mode potential energy has only a 50% contribution from Fe-imidazole stretching, then the allowable force constant change would double to 0.06 mdyn/Å. This translates to an estimated Fe-imidazole bond distance change of less than 0.008 Å, from Badger's rule. A similar estimate can be made for the Fe-pyrrole bonds. The absence of Fe—N displacements greater than 0.01 Å between R and T states of deoxy Hb Kempsey is in agreement with recent extended x-ray absorption fine structure (EXAFS) analyses (25, 26).

The energy change can be estimated on the assumption that the force constant is proportional to the bond energy (i.e., that the curvature at the bottom of the potential well for bond stretching is proportional to its depth). This assumption is borne out empirically where data for comparable bonds are available (23). Drago *et al.* (27) have measured the binding enthalpy of 1-methylimidazole to Co(II)PP in toluene to be 9.6 kcal/mol (40,166 J/mol). This should be a reasonable estimate of the Fe-imidazole bond energy in Mb, considering that Co^{2+} and Fe^{2+} are neighboring divalent ions and that the globin pocket is hydrophobic. A force constant change of <0.06 mdyn/Å, or 2%, then translates to a bond energy change of <0.2 kcal/mol (836.8 J/mol).

An alternative approach is to assume that the Fe-imidazole potential curve is the same for the R and T states of Hb but that the atoms are displaced by a force exerted by the protein. The energy required for elongating the bond from its unconstrained length by a distance x would then be:

$$E = \tfrac{1}{2}kx^2 \quad [2]$$

Assuming that the bond is unconstrained in R state Hb and setting $x_{Hb} = \Delta r$ from the Badger's rule estimate, we obtain E = 0.01 kcal/mol (41.84 J/mol). This is a lower limit estimate, however, because, if the bond in R state Hb is also subject to a protein constraint, then the R–T energy difference is:

$$E_{Hb} - E_{Mb} = \tfrac{1}{2}k(x_T^2 - x_R^2) = \tfrac{1}{2}k(\Delta r^2 + 2x_R\Delta r) \quad [3]$$

Thus, the energy difference rises rapidly with stretching of the R state bond from its hypothetical unconstrained length; if x_R is as much as 0.03 Å, then the energy difference is close to the 0.2-kcal (836.8 J) upper limit obtained above on the assumption of force constant to bond energy proportionality. This estimate, which is somewhat less than that obtained by Perutz (1) [0.3 kcal/mol (1255 J/mol)] from near-infrared spectral shifts, is small compared to the energy of cooperativity [≈3 kcal/heme (≈12,550 J/heme)] (1).

We thank Professor H. F. Bunn for kindly providing a blood sample containing Hb Kempsey, Professor J. J. Hopfield for helpful suggestions, Professor R. S. Drago for communicating results of his work prior to publication, and Dr. M. Leahy for assistance with data acquisition and analysis. This work was supported by National Institutes of Health Grant HL 12526.

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