Title

RESONANCE RAMAN SPECTRA OF METHEMOGLOBIN DERIVATIVES SELECTIVE ENHANCEMENT OF AXIAL LIGAND VIBRATIONS AND LACK OF AN EFFECT OF INOSITOL HEXAPHOSPHATE

Author

Asher, Sanford A.

Publication Date

1977-11-01

Submitted to Biochemistry

LBL-7219
Preprint

RESONANCE RAMAN SPECTRA OF METHEMOGLOBIN DERIVATIVES SELECTIVE ENHANCEMENT OF AXIAL LIGAND VIBRATIONS AND LACK OF AN EFFECT OF INOSITOL HEXAPHOSPHATE

Sanford A. Asher, Larry E. Vickery,
Todd M. Schuster, and Kenneth Sauer

November 1977

Prepared for the U. S. Department of Energy
under Contract W-7405-ENG-48

DISCLAIMER

Resonance Raman Spectra of Methemoglobin Derivatives
Selective Enhancement of Axial Ligand Vibrations
and
Lack of an Effect of Inositol Hexaphosphate †

Sanford A. Asher*‡, Larry E. Vickery§, Todd M. Schuster and Kenneth Sauer

Running Title: Resonance Raman of Methemoglobin

Footnotes

†From the Department of Chemistry and Laboratory of Chemical Biodynamics, University of California, Berkeley, California 94720 (S.A.A., L.E.V. and K.S.) and the Biological Sciences Group, University of Connecticut, Storrs, Connecticut, 06268 (T.M.S.). This work was supported in part by the U.S. Energy Research and Development Administration and in part by a National Science Foundation Grant, PCM 76-05074 to K.S., a National Science Foundation Grant, PCM-76-20041 to T.M.S. and a National Institute of Health Grant, HL - 17494 to T.M.S. The dye laser was purchased with funds provided by an institutional grant from the National Science Foundation to the Chemistry Department of the University of California, Berkeley.

‡Present Address: Gordon McKay Laboratory, Division of Applied Sciences, Harvard University, Cambridge, Massachusetts 02138.

§Present Address: Department of Physiology, College of Medicine, University of California, Irvine, California, 92717.

[1]Abbreviations used are: EDTA, ethylenediaminetetraacetic acid; HEPES, N-2-hydroxyethylpizerazine - N-2-ethanesulfonic acid; IHP, inositol hexaphosphate; MCD, magnetic circular dichroism; MetHb, methemoglobin; MetMb, metmyoglobin; MnETP, manganese (III) etioporphyrin I.

Abstract

Resonance Raman spectra have been obtained for the OH^-, N_3^- and F^- derivatives of methemoglobin by excitation in the 550-650 nm region. A selective enhancement with excitation in the charge transfer bands is observed for peaks at 413 cm^{-1}, 497 cm^{-1} and a doublet at 471 and 443 cm^{-1} in the N_3^-, OH^- and F^- complexes, respectively. These peaks are assigned to Fe-axial ligand stretches on the basis of: 1. A 20 cm^{-1} shift of the 497 cm^{-1} peak of the hydroxide complex to lower energy on isotopic substitution of $^{18}OH^-$ for $^{16}OH^-$; 2. The proximity of the 413 cm^{-1} Raman peak to the 421 cm^{-1} IR peak previously assigned to the Fe-N_3^- stretch in a model heme azide complex (Ogoshi, H., Watenabe, E., Yoshida, Z., Kincaid, J., and Nakamoto, K., (1973), J.Am.Chem.Soc. 95, 2845). 3. The selective appearance of the 471 and 443 cm^{-1} peaks in the Raman spectra of the F^- complex. The doublet observed at 471 and 443 cm^{-1} in the F^- derivative may reflect a heterogeneity in the heme cavity due to hydrogen bonding of H_2O to the F^- ligand in both the α and β subunits, as has been previously suggested based on X-ray diffraction results (Deatherage, J.F., Loe, R.S., and Moffat, K., (1976), J. Mol. Biol. 104, 723). It is suggested that the frequency of the Fe-F^- vibration reflects the out-of-plane distortion of the Fe from the heme plane. The lack of a shift in the frequency of the Fe-F^- stretch suggests that a less than 0.02 Å displacement of the Fe occurs upon the addition of inositol hexaphosphate, which is thought to alter the allosteric equilibrium between the R and T-forms of methemoglobin. Excitation profile measurements suggest that the charge transfer band in methemoglobin OH^- like that in methemoglobin N_3^- is z-polarized, while in methemoglobin F^- the charge transfer transition is mixed with a $\Pi \rightarrow \Pi^*$ transition.

Resonance Raman spectroscopy can serve as a structural probe for biological molecules such as hemoglobin and myoglobin (Spiro, 1975 and references therein; Yamamoto, et al, 1973; Kitagawa et al, 1975; Ozaki, et al, 1976). Upon excitation within the absorption bands of the heme chromophore a selective enhancement occurs in the intensity of the Raman peaks resulting from heme vibrations (Spiro, 1975). In previous reports the dominant Raman bands which have been observed appear to result from in-plane porphyrin macrocyclic modes and occur at energies between 600-1700 cm^{-1}. This is because excitation occurred within $\Pi \rightarrow \Pi^*$ electronic transitions of the porphyrin macrocycle such as in the α, β and Soret bands. The energies of some of these Raman peaks have been shown to be sensitive to the oxidation state, spin-state and/or planarity of the metal with respect to the porphyrin plane (Spiro, 1975; Spaulding et al, 1975). However, vibrational modes of the iron in heme complexes, such as Fe-axial ligand modes, are rarely observed (Brunner, 1974; Spiro and Burke, 1976) because the Π orbitals involved in the α, β and Soret bands are poorly conjugated with the metal orbitals (Asher and Sauer, 1976). Unfortunately, these vibrational modes are precisely the ones that contain the greatest information on ligand binding in heme proteins. In addition, these modes would be expected to be sensitive to constraints imposed by the protein such as the proposed tension on the heme iron by the proximal histidine during the transition between the R and T allosteric forms (Perutz, 1972).

Recently, Asher and Sauer (1976) demonstrated the specific enhancement of vibrational modes involving the metal when excitation occurred within the charge transfer bands of Mn (III) etioporphyrin and suggested that a similar enhancement of vibrational modes which involve the metal should occur upon excitation into charge transfer bands of heme. The heme in methemoglobin, like Mn (III) porphyrins, has electronic transitions between 600 and 640 nm which have been assigned to charge transfer bands

(see Smith and Williams, 1970). Excitation within these bands should enhance vibrational modes such as axial ligand stretches. We have utilized a tunable dye laser which can excite within these charge transfer bands and report here a study of ligation properties of methemoglobin (Asher, et al., 1977; Asher, 1976).

Experimental Procedure

Human hemoglobin A_0 was purified by the method of Williams and Tsay (1973). Methemoglobin was prepared by oxidation of hemoglobin with excess potassium ferricyanide, followed by extensive dialysis against 0.1 M HEPES[1], containing 1mM EDTA, pH 7.0. Azide and fluoride complexes were formed by the addition of buffered solutions of the sodium salts directly to the capillary tubes to be used for Raman excitation.

The absorption spectra of diluted samples were measured on a Cary 118 recording spectrophotometer to confirm complete ligation. In addition, absorption spectra were measured for some of the Raman illuminated samples to determine if sample degradation had occurred; these were measured with thin films of the material spread on a microscope slide and held in place by a cover slip. No degradation was detected.

The $^{18}OH^-$ and $^{16}OH^-$ derivatives of MetHb used for the Raman spectra in Fig. 7 were prepared by freeze drying a buffered solution of MetHb:H_2O at about -60°C. The freeze dried material was redissolved in $H_2{}^{16}O$ or $H_2{}^{18}O$ (95% enriched in ^{18}O, Bio Rad Lab., Richmond, CA). Each of the resulting solutions was slowly titrated to the alkaline pH value at 0°C with small aliquots of a 30% solution of Na ^{16}OH in $H_2{}^{16}O$ with rapid stirring. Heme concentrations for the resonance Raman measurements were typically ca 0.6 mM. Spectra were measured with and without 0.2 M Na_2SO_4 as an internal standard. No changes were observed in the Raman spectra on the addition of Na_2SO_4. The $SO_4^=$ line at 983 cm^{-1} is labeled in the spectra.

The Raman spectra were measured on an instrument constructed in the Chemistry Department of the University of California, Berkeley. The laser used to excite the Raman spectra is a CMX-4 Xenon flashlamp-pumped dye laser (Chromatix Corp., Mountain View, CA). The laser produces 1 μsec pulses at a repetition rate between 5 and 30 Hz. The output of the laser was focussed onto 1.5 mm I.D. melting point capillaries which contained 10-50 μl volumes of the samples. The scattered light was collected by an ellipsoidal mirror and imaged into a Spex 1400 monochromator equipped with an EMI 9558QB photomultiplier. A polarization scrambler was placed in front of the entrance slit of the monochromator to avoid intensity artifacts resulting from the polarization

bias of the monochromator gratings. A quartz Wollaston prism was used to analyze the polarization of the scattered light.

The output of the Raman scattered light was detected and normalized to the intensity of the incident laser light by a dual channel box car integrator. Prior to the sample, part of the incident laser beam was split off and was monitored by a second photomultiplier. The output of each electrometer was integrated with a 2 sec time constant, and the integrated output of the sample photomultiplier was divided by that of the reference photomultiplier. Further details of the spectrometer are given elsewhere (Asher, 1976).

The spectrometer was calibrated with an Eppley Laboratories' standard incandescent intensity lamp. The excitation profiles were calculated from peak height measurements of the Raman spectra and then normalized to the spectrometer efficiency curve and to the internal standard $SO_4^=$ line at 983 cm^{-1}. The Raman spectra themselves have not been normalized to the spectrometer efficiency profile, however.

Results and Discussion

Methemoglobin-Azide

Figures 1 and 2 show the resonance Raman spectra of the azide complex of MetHb excited at 5590.8 and 6383.2 Å, respectively. As the absorption spectrum of MetHb N_3^- in Fig. 3 shows, excitation at 5590.8 Å occurs between two absorption bands which have been assigned to the α and β bands of metalloporphyrins (Smith and Williams, 1970). Excitation at 6383.2 Å occurs within a weak absorption band at ca 6400 Å, which has been assigned to a charge transfer transition (Smith and Williams, 1970; Eaton and Hochstrasser, 1968).

The Raman spectrum shown in Fig. 1 is similar to previously reported spectra of the azide complex of MetHb excited at 5682 Å between the α and β bands, (Strekas and Spiro, 1972) and at 4416 Å in the Soret band (Yamamoto et al, 1973), and to the azide complex of metmyoglobin, excited at 4880 Å, between the

β and Soret band, (Kitagawa, et al, 1976). The dominant features in the resonance Raman spectrum excited at 5590.8 Å, between the α and β bands (Fig. 1) appear at energies greater than 600 cm^{-1}. The bands occurring at 1640, 1586, 1308, 1132, and 755 cm^{-1} are due to porphyrin macrocyclic vibrational modes (Spiro, 1975). A comparison between the Raman spectrum of an aqueous solution of NaN_3, and a solution of MetHb N_3^- with lower concentrations of N_3^- (not shown) indicates that the feature at 1344 cm^{-1} in Figs. 1 and 2 is due to uncomplexed azide because the 1344 cm^{-1} peak does not appear at lower concentrations of NaN_3^- (0.04 M), while the spectrum of the MetHb solution is still characteristic of the MetHb N_3^- complex.

The resonance Raman spectrum of MetHb N_3^- excited at 6383.2 Å in the charge transfer band (Fig. 2) shows a new Raman peak appearing at 413 cm^{-1}. A depolarization ratio measurement of the 413 cm^{-1} peak indicates that the peak is polarized, as is expected for an axial ligand stretch (Asher and Sauer, 1976), while an examination of the excitation profiles in Fig. 3 shows that the 413 cm^{-1} peak is in resonance with only the charge transfer band at 6400 Å. All of the other Raman peaks which appear upon 6383.2 Å excitation are more intense upon excitation at shorter wavelength and appear to be in resonance with the α and β bands.

The 413 cm^{-1} peak enhanced by excitation in the charge transfer band of MetHb N_3^- is close in energy to a vibration at 421 cm^{-1} observed in the IR spectrum of Fe^{3+} octaethylporphin-N_3^-, which was assigned to the Fe-azide stretching vibration (Ogoshi, et al., 1973). The unique enhancement of the 413 cm^{-1} peak by the charge transfer band of MetHb N_3^-, the close correspondence between the energies of the 413 cm^{-1} peak and the Fe-N_3^- stretch observed in Fe(III) octaethylporphin-N_3^-, the fact that the peak is polarized and the selective appearance of the 413 cm^{-1} peak in the resonance Raman spectrum of MetHb N_3^- in contrast to its absence in MetHb OH^- and MetHb F^- (vide infra), all, suggest that the 413 cm^{-1} peak results from a vibration of the azide nitrogen against the heme iron.

In contrast to the Raman spectra obtained from MnETP excited in the charge transfer band (Asher and Sauer, 1976; Shelnutt et al, 1976; Gaughan et al, 1975)

the porphyrin macrocyclic vibrational modes do not show an excitation profile maximum within the charge transfer band of MetHb N_3^-. In MetHb N_3^- the peaks resulting from porphyrin macrocyclic modes appear to derive their intensity from the α and β bands. Even the pyrrole-nitrogen-Fe vibrations are not visibly enhanced, suggesting that the electronic transition which is responsible for the absorption band at 6400 Å in MetHb N_3^- is different from the electronic transition which is responsible for the charge transfer band in MnETP. This conclusion is supported by polarization studies of the absorption spectra of single crystals of MetHb N_3^- (Kabat, 1967), and MetMb N_3^- (Eaton and Hochstrasser, 1968), which indicate that a z-polarized electronic transition is responsible for the absorption band at 6400 Å, and the MCD spectrum of MetMb N_3^- which shows a negative extremum due to the 6400 Å absorption band (Vickery, et al., 1976). In contrast, the MCD spectrum of the charge transfer band of MnETP exhibits a Faraday A term, indicating a degenerate, x, y-polarized electronic transition (Boucher, 1972; Asher, 1976). Eaton and Hochstrasser assigned the ∿ 6400 Å absorption band of MetMb N_3^- to either a porphyrin $a_{2u}(\pi) \rightarrow d_{z2}$ or an azide (π) → iron (d) charge transfer band. It is less likely that the band results from a d → d transition, because its molar absorptivity ($\varepsilon \sim 10^3$) is an order of magnitude higher than the molar absorptivity expected for d → d transitions (Smith and Williams, 1970).

The Raman data do not distinguish the $a_{2u}(\pi) \rightarrow d_{z2}$ from the azide (π) → iron (d) charge transfer transition. In-plane porphyrin and pyrrole-nitrogen - Fe^{3+} vibrations may not couple well to a z-polarized electronic transition. However, enhancement of out-of-plane vibrations involving the Fe^{3+} pyrrole linkages would be expected. Unfortunately, the magnitude of this enhancement is difficult to predict. No new features which might be due to out-of-plane vibrations of the heme are observed in the Raman spectrum of MetHb N_3^-. The vibration of the Fe-N_3^- linkage is expected to be the major vibration enhanced in an a_{1u} or $a_{2u} \rightarrow d_{z2}$ transition since the azide is bound by the $d_2 2$ orbital of the iron (Kitagawa, et al, 1976).

A charge transfer transition from the azide Π orbitals to the d orbitals of the iron would be expected to enhance the Raman peak due to the vibration of the Fe-N_3^- linkage. However, internal vibrations of the N_3^- might also be enhanced, since an analogous enhancement of pyridine vibrations occurs upon excitation in a Fe^{2+} → pyridine charge transfer band at 4765 Å in pyridine complexes of Fe^{2+} mesoporphyrin IX (Spiro and Burke, 1976). Raman spectra of MetHb N_3^- with lower concentrations of N_3^- (0.04 M) show spectra similar to Fig. 2. The only difference is the disappearance of 1344 cm^{-1} peak, due to free N_3^-. There is no obvious enhancement of internal azide vibrations upon excitation in the charge transfer band at 6400 Å.

The lack of enhancement of internal azide vibrations may be due to the geometry of heme-azide complex. It is known from X-ray crystallographic studies of MetMb N_3^- that azide binds at an 111° angle to the normal of the heme plane (Stryer, et al, 1964), Fig. 4. Both the symmetric and anti-symmetric vibrations of the N_3^- are at 111° to the z-polarized electronic transition. Thus, these azide vibrations may not couple well with the z-polarized charge transfer transition. It is difficult to predict the enhancement expected for the doubly degenerate azide deformation mode. Another possibility is that the charge transfer transition occurs from a nonbonding orbital of the azide to a d_{z^2} orbital of the iron. This transition, which is z-polarized, would enhance the vibration between the Fe and azide nitrogen, but neither the pyrrole-nitrogen-Fe nor internal azide vibrations would be appreciably enhanced.

Met Hemoglobin Hydroxide

Fig. 5 shows the resonance Raman spectrum of MetHb OH^- at pH = 11.06 excited at 6000.9 Å. Dominant features in the spectrum occur at 497, 755, 1555, 1587, and 1634 cm^{-1}. A number of differences appear between the resonance Raman spectrum

of MetHb OH^- and MetHb N_3^-. The 413 cm^{-1} peak seen for the azide complex is absent in the hydroxide form but a new peak is found at 497 cm^{-1}. An examination of the absorption spectrum and the excitation profiles presented in Fig. 6 indicates that the 497 cm^{-1} peak is in resonance with the shoulder near 6000 Å in the absorption spectrum. The other Raman bands increase dramatically in intensity as the excitation wavelength decreases, indicating that they are resonance enhanced by the 5700 and/or 5400 Å $\pi \rightarrow \pi^*$ absorption bands.

The unique enhancement of the 497 cm^{-1} band by the shoulder at 6000 Å is similar to the selective enhancement shown by the 413 cm^{-1} Raman peak in the charge transfer band of MetHb N_3^- at 6400 Å. To determine whether the 497 cm^{-1} peak results from the Fe-O stretch Raman spectra were obtained for MetHb ^{18}OH. Fig. 7 shows the resonance Raman spectra of MetHb ^{18}OH and MetHb ^{16}OH excited at 6004.7 Å. The 497 cm^{-1} peak which appears in the spectrum of MetHb $^{16}OH^-$ shifts 20 cm^{-1} to lower energy following substitution by $^{18}OH^-$. The increased background in both of the spectra in Fig. 7 over that in Fig. 5 presumably is due to some denaturation of the MetHb during the freeze drying process.

Using a harmonic oscillator model, the energy shift for the 497 cm^{-1} peak is only 2 cm^{-1} less than the 22 cm^{-1} shift predicted if this vibration were due to a pure Fe-O stretch, indicating little mixing with other vibrational modes. The enhancement of the Fe-O stretch at 6000 Å and the lack of observable excitation profile maxima for the other porphyrin macrocyclic modes in this region suggests that the shoulder of the absorption band at ca 6000 Å is a pure charge transfer transition which is not mixed with the $\pi \rightarrow \pi^*$ transitions of the porphyrin macrocycle. The similarity between the excitation profiles in MetHb N_3^- and MetHb OH^- suggests that the electronic transitions are similar charge transfer transitions, and that the shoulder at ca 6000 Å in MetHb OH^- is z-polarized.

Other differences between the resonance Raman spectrum of MetHb OH^- and MetMb N_3^- occur in the spin-state sensitive regions of the Raman spectrum between 1550 and 1640 cm^{-1} (Spiro, 1975; Spiro and Burke, 1976). In contrast to MetHb N_3^-,

which is predominantly low-spin, MetHb OH^- exists in a spin-state equilibrium with comparable concentrations of the high and low-spin forms (George, et al, 1961). Thus, instead of the low-spin peaks at ca 1640 and 1586 cm^{-1} in the Raman spectrum of MetHb N_3^- (Fig. 1) a complex spectrum of overlapping peaks occurs between 1550 - 1640 cm^{-1} for MetHb OH^-.

In contrast to MetHb N_3^- and MetMb N_3^- which show almost identical Raman spectra when excited in the same spectral region (Strekas and Spiro, 1973) and MetHb F^- and MetMb F^- (vide infra) which also show almost identical spectra, Raman spectra of the hydroxide complex of MetHb differ from those of MetMb in the 1550-1640 cm^{-1} region when excited at 4416 Å (Yamamoto et al, 1973), and when excited between 5800-6100 Å (S.Asher, L. Vickery, T. Schuster and K. Sauer, unpublished observations). The differences in the Raman spectra presumably reflect a change in the spin-state equilibrium of MetHb OH^- from that of MetMb OH^- (Yamamoto et al., 1973; Ozaki et al., 1976). Differences between the heme electronic structure of MetHb OH^- and MetMb OH^- are also observed by magnetic susceptibility measurements (George et al, 1961). The concentration of the high-spin form appears to be higher in MetMb OH^- than in MetHb OH^-. In addition, absorption measurements show a decrease in absorption for MetMb OH^- between 5000-5800 Å, and an increase in absorption above 5800 Å with a peak appearing at ca 6000 Å (George, et al, 1961). Raman spectra of MetMb OH^-, excited in the 6000 Å absorption band show a dramatic enhancement of the peak corresponding to the Fe-O stretch, which becomes the dominant feature in the Raman spectrum, (S.Asher, L. Vickery, T. Schuster and K. Sauer, unpublished observations), suggesting an increased charge transfer contribution to the absorption spectrum at ca 6000 Å in MetMb OH^- over that of MetHb OH^-. Additionally, the FeO stretch appears to be shifted to slightly lower frequency, indicating a weaker Fe-O bond in MetMb OH^- than in MetHb OH^-.

Methemoglobin fluoride

In contrast to MetHb N_3^- which is predominantly low-spin and MetHb OH^- which is in a thermal spin-state equilibrium, MetHb F^- is almost purely high spin (Beetlestone and George, 1964). Fig. 8 shows the resonance Raman spectrum of MetHb F^- excited at 6175.1 Å. The dominant features in the Raman spectrum occur at 1610, 1550, 1217, 760, 471 and 443 cm^{-1}. The higher frequency region of the Raman spectrum shown in Fig. 8 (> 700 cm^{-1}) is qualitatively similar to previously reported spectra of MetHb F^- excited at 6328 Å and between 4579 and 5145 Å (Strekas, et al., 1973). In contrast to the Raman spectra of MetHb N_3^- and MetHb OH^-, which show peaks at 413 and 497 cm^{-1}, respectively, intense low frequency peaks in MetHb F^- appear at 443 and 471 cm^{-1}. An examination of the excitation profiles and the absorption spectrum of MetHb F^- shown in Fig. 9 reveals a number of excitation profile maxima. The two peaks at 443 and 471 cm^{-1}, which are polarized, appear to be in resonance with the absorption peak at $\sim$ 6000 Å. However, it should be noted that the 443 cm^{-1} peak shows an intensity maximum at a somewhat higher wavelength than does the 471 cm^{-1} peak; and at excitation wavelengths lower than 6080 Å, the 471 cm^{-1} peak is more intense than the 443 cm^{-1} peak. The appearance of the 443 and 471 cm^{-1} doublet does not arise from subunit differences in MetHb F^- because a very similar Raman spectrum is observed when MetMb F^- is excited in this spectral region (S. Asher, L. Vickery, T. Schuster and K. Sauer, unpublished observations).

In view of the enhancement of Fe-axial ligand vibrations by the charge transfer bands of MetHb N_3^- and MetHb OH^-, the fact that the peaks are polarized, as is expected for axial ligand vibrations (Asher and Sauer, 1976), and the fact that intense vibrations between 400-500 cm^{-1} have not been observed for any metalloporphyrins other than MnETP F^- (495 cm^{-1}, Asher and Sauer, 1976), MetHb N_3^- and MetHb OH^-, it is tempting to assign the 443 and/or 471 cm^{-1} peaks to the Fe-F stretch. However, Ogoshi et al, (1973), in their IR studies of metalloporphyrins, assigned an Fe-F^-

stretch in ferric octaethylporphin F^- to a band at 602 cm^{-1}, while Kincaid and Nakamoto (1976), assigned the Fe-F stretch to a 600 cm^{-1} peak in the resonance Raman spectrum of the F^- complex of ferric octaethylporphin and Spiro and Burke (1976) observed the selective appearance of a 580 cm^{-1} peak in the resonance Raman spectrum of the fluoride complex of ferric mesoporphyrin IX dimethyl ester.

The environment of the iron in MetHb F^-, however, is much different from that in fluoride complexes of non-protein bound metalloporphyrins. In 5-coordinate, high-spin complexes of ferric porphyrins the iron lies $\sim$ 0.45 Å out of the plane towards the 5th axial ligand (Hoard, 1975). However, in MetHb F^- the iron lies 0.3 Å out of the plane displaced toward the proximal histidine on the opposite side from which the F^- must bind (Deatherage, et al., 1976a; Perutz, et al.,1974b). Thus, the iron is displaced about 0.75 Å in MetHb F^- compared to ferric porphyrin fluoride. The $\sim$ 120 cm^{-1} decrease in the frequency of the Fe-F^- stretch in MetHb F^- from that in non-protein bound F^- complexes of ferric metalloporphyrins may thus result from non-bonding interactions between the charge cloud of the F^- and the Π orbitals of the pyrrole nitrogens. This is represented diagrammatically in Fig. 10. Assuming an equilibrium bond length of 1.97 Å for Fe-F, i.e., the sum of their ionic radii, little steric interaction would be expected to occur between the Van der Waals radii of the pyrrole nitrogens (1.70 Å, Hoard, 1975) and the ionic radius of the F^- in Fe^{III} porphyrins in which the Fe is out-of-plane towards the F^- atom. In MetHb F^- and MetMb F^-, however, the steric constraints imposed by the nitrogen Π orbitals would be expected to cause an elongation of the bond and a decrease in the frequency of the vibration. If the orbitals were hard spheres the bond would elongate by 30% to $\sim$ 2.6 Å. The Fe-F^- bond appears to be like a stretched spring. The $\sim$ 120 cm^{-1} decrease for the 0.75 Å movement of the Fe atom through the heme plane suggests that this vibration should provide a sensitive measure of any movement of the iron with respect to the porphyrin plane. Assuming a linear decrease in the frequency

of the Fe-F vibration with increasing bond length a 1.7 cm^{-1} shift is expected for a change of 0.01 Å in the out-of-plane distance of the iron atom.

The existence of the two polarized, adjacent peaks at 471 and 443 cm^{-1}, which have similar excitation profiles suggests a correlation between them. The observation by Deatherage et al. (1976a), using X-ray difference Fourier diffraction, that the environment of the F^- ion in MetHb F^- is heterogeneous may account for the presence of two peaks assignable to the Fe-F^- stretch. Deatherage et al. (1976a) observed the presence of a previously unnoticed feature within the heme cavity which was proposed to be a water molecule stabilized by hydrogen bonding to the fluoride ion in the heme cavities of both the α and β subunits. The magnitude of the feature suggested that the water molecule is stabilized in the heme pocket only part of the time, leading to a heterogeneity in the fluoride environment. Hydrogen bonding of water with the fluoride ion should decrease the frequency of the Fe-F^- vibration. Thus, the 471 cm^{-1} peak may correspond to an unperturbed Fe-F^- stretch while the 443 cm^{-1} peak may correspond to the Fe-F^- stretch shifted to lower energy due to hydrogen bonding of water to fluoride.

The excitation profiles of the resonance Raman peaks of MetHb F^-, (Fig. 9) show a more complicated pattern than those of MetHb OH^- and MetHbN_3^-, presumably because of the complexity of the visible absorption spectrum, which consists of at least four overlapping bands (Eaton and Hochstrasser, 1968). In their measurements of the single crystal polarized absorption spectrum of MetMb F^-, Eaton and Hochstrasser observed that in contrast to MetMb N_3^-, which shows a z-polarized transition at ca 6400 Å, the entire visible absorption spectrum of MetMb F^- was x, y polarized. However, they noted that an inequivalency of x and y polarized electronic transitions occurred at ca 6250 Å, indicating a splitting of the degeneracy of the x and y directions. It has been proposed that the complexity of the visible absorption spectrum of MetHb F^- results from the mixing of charge transfer bands with porphyrin $\Pi \rightarrow \Pi^*$ transitions to the extent that none of the absorption

bands in the visible region can be considered as pure transitions (Zerner et al., 1966; Eaton and Hochstrasser, 1968; Smith and Williams, 1970).

In addition to the 443 and 471 cm^{-1} peaks a weak, polarized Raman peak at 347 cm^{-1} appears to be in resonance with the 6000 Å absorption band maximum. The vibrations which show their maximum intensity at 6250 Å occur at 1610 (dp) and 1550 (dp) cm^{-1} (Fig. 9). Since these peaks are depolarized they are either of B_{1g} or B_{2g} symmetry in the D_{4h} point group. Hosever, both the 760 cm^{-1} (dp) and the 1217 cm^{-1} peaks show broad excitation profiles, suggesting that the excitation profiles (Fig. 9) may result from the overlap of two maxima at 6000 and 6250 Å. Weaker, anomalously polarized peaks at 1345 and 1431 cm^{-1} appear upon excitation at ca 6000 and 6250 Å. However, these peaks are not observed with excitation between 6080.8 and 6150.0 Å. The 1217 cm^{-1} peak shows a depolarization ratio, ρ, of 0.62 when excited at 6147.1 Å. However, with excitation at 6328 Å the 1217 cm^{-1} peak is found to be depolarized (Strekas, et al., 1973). For in-plane electronic transitions in the D_{4h} point group, theory predicts that vibrations of A_{1g} symmetry have $\rho = 0.125$, those of A_{2g} symmetry have $\rho = \infty$, and those of B_{1g} or B_{2g} have $\rho = 0.75$ (Pezolet, et al., 1973). A depolarization ratio intermediate between 0.125 and 0.75 suggests an overlap of an A_{1g} vibration with a vibration of B_{1g}, B_{2g} or A_{2g} symmetry. Thus, it appears that only depolarized or inversely polarized peaks show an intensity maximum at 6250 Å.

Since an inequivalency of the x and y directions occurs at 6250 Å, a description of the electronic transitions in the D_{2h} point group is appropriate. In the D_{2h} point group the symmetry of the vibrations which would mix x and y electronic transitions is:

$$\Gamma_x \times \Gamma_y = \Gamma_{B_{2u}} \times \Gamma_{B_{3u}} = \Gamma_{B_{1g}}$$

Thus, vibrations of B_{1g} symmetry are expected to be enhanced at 6250 Å.

However, B_{1g} vibrations in the D_{2h} point group correlate to B_{2g} and A_{2g} vibrations in the D_{4h} point group (Kitagawa, et al, 1975), and depolarized and inversely polarized vibrations are expected to be enhanced at 6250 Å, in agreement with the excitation profile data. None of the polarized vibrations shows an excitation profile maximum at 6250 Å. Instead, the polarized 347, 443 and 471 cm^{-1} peaks show excitation profile maxima at ∿ 6000 Å. The 471 cm^{-1} peak is the most intense feature in the Raman spectra with excitation at 6000 Å.

Eaton and Hochstrasser suggested that the inequivalency of the x and y directions results from the splitting of the d_{xz}, d_{yz} orbitals of the iron. However, the exclusive enhancement at 6250 Å of porphyrin macrocylic modes suggests that the origin of the degeneracy splitting lies mainly in the porphyrin macrocyle and is not a result of an axial ligand-induced inequivalency of the d_{xz} or d_{yz} orbitals. If the inequivalency resulted from the metal orbitals, vibrations of A_{2g} and B_{2g} symmetry about the metal would be enhanced by terms such as $\langle d_{xz} | \frac{\partial H}{\partial Q_a} | d_{yz} \rangle$ in the polarizability expression (Albrecht, 1961; Asher and Sauer, 1976). Thus, the inequivalency in the x and y directions may result from an interaction not through the iron but directly on the porphyrin plane by the heme environment. This could result from a steric influence of the proximal histidine, which might bind to the iron in one particular orientation with respect to the x and y directions of the porphyrin macrocycle. Alternatively, the splitting might result from the interaction of the heme with another species in the heme cavity.

Table I summarizes the Fe-ligand peaks assigned in this report.

Effect of Inositol Hexaphosphate

Inositol hexaphosphate (IHP), which shifts the spin-state equilibrium of MetHb OH^- and MetHb N_3^- to favor the high spin form appears to convert MetHb F^- from the R to the T-form (Perutz et al., 1974b and c). For the hydroxide and azide derivatives this spin change should be reflected in an increase in the time-averaged distance of the iron from the heme plane; a similar relative movement of the iron is expected in the fluoride derivative since Perutz et al, (1974c) propose that in the T-form the iron lies further out of the porphyrin plane than in the R-form. The steric interactions of the sixth ligand with the heme plane suggest that the frequency of the vibration of the sixth ligand to the iron should be a sensitive function of the out-of-plane distance of the metal.

IHP was added to solutions of MetHb X ($X = F^-$, OH^-, N_3^-) and resonance Raman spectra were excited at wavelengths which maximally enhanced the Fe-X vibrations. The addition of IHP to solutions of MetHb X^- had no effect on the entire resonance Raman spectra within the signal-to-noise ratio of the spectra. Frequency shifts of 3 cm^{-1} or changes in peak intensity of 10% should have been readily detected. This lack of an effect of IHP on the porphyrin macrocyclic modes of MetHb F^- was previously noted by Szabo and Barron (1975). The fact that IHP has no effect on the energy of the Fe-O vibration in MetHb OH^-

may simply reflect the fact that IHP does not bind well to MetHb at pH greater than 7, and the lack of a detectable effect on MetHb N_3^- may be due to the fact that the IHP-induced spin-state changes in MetHb N_3^- are quite small (Perutz, et al., 1974c). However, the lack of a shift in the energy of the vibrations which are assigned to Fe-F^- stretching, suggests that little, if any, movement of the iron occurs on the addition of IHP. Based on the discussion given in the preceding section suggesting a 1-2 cm^{-1} shift for a change of 0.01 Å in the iron-ligand bond distance in MetHb F^-, we can conclude that the iron atom moves no more than 0.02 Å. A comparison of the extended x-ray absorption fine structure spectrum (EXAFS) of deoxyHb A with that of DeoxyHb Kempsey also led Eisenberger, et al. to conclude that there was no substantial movement (<0.02 Å) of the iron between the high (R) and the low affinity (T) quarternary forms (Eisenberger, et al, 1976).

It has been suggested that a steric effect of the protein on the heme decreases the accessibility of the heme binding site in the T-form (Perutz, et al., 1976; Deatherage, et al., 1976b). It has also been proposed that a change occurs in the π interactions between the porphyrin macrocycle and the surrounding protein matrix (Maxwell and Caughey, 1976), upon the R to T transition, leading to changes in the electronic structure of the porphyrin,

concomitant changes in the iron d orbitals and changes in the ligand affinities of the heme. The energy of the Fe-axial ligand vibrations reported here may not be a good monitor of these effects. However, it is possible that the excitation profiles may contain some information on steric and π non-bonding interactions between the protein and the heme or both. Polarized single crystal absorption spectra and the excitation profile data both indicate an inequivalency of the x and y directions of MetHb F^- at 6250 Å. If the inequivalency of the x and y directions results from non-bonding interactions between the heme and the protein, changes in the excitation profiles upon the addition of IHP may monitor differences in the interactions between the R and T quarternary forms.

References:

Albrecht, A.C., (1976), J. Chem. Phys. 34, 1476

Asher, S. (1976), PhD. Thesis, Dept. of Chemistry, Univ. of CA, Berkeley, Lawrence Berkeley Laboratories Report LBL-5375

Asher, S. and Sauer, K., (1976), J. Chem. Phys. 64, 4115

Asher, S., Vickery, L.E., Schuster, T.M. and Sauer K., (1977a) Biophys. J. 17, 180a

Beetlestone, J. and George, P., (1964), Biochemistry 3, 707

Boucher, L.J., (1972), Coord. Chem. Rev. 7, 289

Brunner, H., (1974), Naturwiss. 61, 129

Deatherage, J.F., Loe, R.S. and Moffat, K., (1976a), J. Mol. Biol. 104, 723.

Deatherage, J.F., Loe, R.S., Anderson, C.M., and Moffat, K. (1976b) J. Mol. Biol. 104, 687.

Eaton, W.A. and Hochstrasser, R.M., (1968), J. Chem. Phys. 49, 985.

Eisenberger, P., Shulman, R.G., Brown, G.S., and Ogawa S., (1976) Proc. Natl. Acad. Sci. USA 73, 491

Gaughan, R.R., Shriver, D.F., and Boucher, L.J., (1975), Proc. Natl. Acad. Sci. USA 72, 433

George, P., Beetlestone, J., and Griffith, J.S., (1961), Haematin Enzymes, Vol. I., Ed. J. Falk, R. Lemberg and R.K. Morton, Pergamon Press, New York

References: (Continued)

Hoard, J.L., (1975), in Porphyrins and Metalloporphyrins, ed. K.M. Smith Elsevier Scientific Publishing Co., New York

Kabat, K., (1967), Biochemistry 6, 3443

Kincaid, J. and Nakamoto, K., (1976), Spectrosc. Lett. 9, 19

Kitagawa, T., Kyogoku, Y., Iizuka, T., and Saito, M.I., (1976), J. Am. Chem. Soc. 98, 5169

Kitagawa, T., Ogoshi, H., Watanabe, E., and Yoshida, Z-I, (1975), J. Phys. Chem. 79, 2629

Maxwell, J.C. and Caughey, W.S., (1976), Biochemistry 15, 388

Pezolet, M., Nafie, L.A., Peticolas, W.L.,(1973), J. Raman Spectrosc. 1, 455

Ogoshi, H., Watanabe, E., Yoshida, Z., Kincaid, J. and Nakamoto, K., (1973), J. Am. Chem. Soc. 95, 2845

Ozaki, Y., Kitagawa, T., and Kyogoku, (1976), FEBS Lett. 62, 369

Perutz, M.F. (1970), Nature 228, 726

Perutz, M.F. (1972), Nature 237, 495

Perutz, M.F., Ladner, J.E., Simon, S.R., Ho, C. (1974a), Biochemistry 13, 2163

Perutz, M.F., Fersht, A.R., Simon, S.R., and Roberts, G.C.K., (1974b), Ibid. 2174

Perutz, M.F., Heidner, E.J., Ladner, J.E., Beetlestone, J.G., Ho, C., and Slader, E., (1974c),Ibid. 2187

References: (Continued)

Perutz, M.F., Kilmartin, J.V., Nagai, K., Szabo, A., and Simon, S.R., (1976), Biochemistry 15, 378

Shelnutt, I.H., O'Shea, D.C.,Yu, N-T., Cheung, L.D., and Felton, R.H., (1976),J. Chem. Phys. 64, 1156

Smith, D.W. and Williams, R.J.P., (1970), Structure and Bonding 7, 1

Spaulding, L.D., Chang, C.C., Yu, N-T., and Felton, R.H. (1975), J. Chem. Phys. 97, 2517

Spiro, T.G., and Strekas, T.C., (1974), J. Am. Chem. Soc. 96, 338

Spiro, T.G., (1975), Biochem. Biophys. Acta 416, 169

Spiro, T.G., and Burke, J.M., (1976), J. Am. Chem. Soc. 98, 5482

Strekas, T.C. and Spiro, T.G., (1972), Biochem. Biophys. Acta. 263, 830

Strekas, T.C., Packer, A.J., and Spiro, T.G., (1973), J. Raman Spectrosc. 1, 197

Stryer, L., Kendrew, J.C. and Watson, H.C., (1964), J. Mol. Biol. 8, 96

Szabo, A. and Barron, L.D., (1975), J. Am. Chem. Soc. 97, 660

Vickery, L., Nozawa, T., and Sauer, K., (1976), J. Am. Chem. Soc. 98, 343

Williams, Jr., R.C. and Tsay, K-Y., (1973), Anal. Biochemistry 54, 137

Yamamoto, T., Palmer, G., Gill, D., Salmeen, I.T. and Rimai, L. (1973), J. Biol. Chem. 248, 5211

Zerner, M., Gouterman, M., and Kobayashi, H., (1966), Theor. Chim. Acta. 6, 363

TABLE I

Raman vibrations assigned to Fe-X^- stretch in MetHb ($X = N_3^-$, OH^-, F^-)

Derivative	$\Delta\nu$ cm^{-1}	Remarks
MetHb N_3^-	413	
MetHb ^{16}OH	497	
MetHb ^{18}OH	477	
MetHb F^-	471	Fe-F
	443	Fe-F Fluoride anion hydrogen bonded to water

Fig. 1. Resonance Raman spectrum of methemoglobin N_3^- (0.76 mM heme) containing 0.25 M Na_2SO_4 as an internal standard. λ_{ex} = 5590.8 Å. Energy = 10^{-3} joule/pulse. Pulse repetition rate = 30 Hz. Scan speed = 23 Å/min. Slitwidth = 3 Å. Position of Raman lines due to $SO_4^=$ and uncomplexed azide are shown in the figure.

Fig. 2. Resonance Raman spectrum of methemoglobin N_3^-. λ_{ex} = 6383.2 Å, conditions as in Fig. 1.

Fig. 3. Absorption spectrum and excitation profiles of methemoglobin N_3^-. The intensities of the Raman lines were corrected for the spectrometer response and then normalized against the $SO_4^=$ line at 983 cm^{-1}.

Fig. 4. a) Geometry of the heme-azide complex of MetHb N_3^- (Stryer, et al., 1964)
b) Vibrational modes of N_3^-

Fig. 5. Resonance Raman spectrum of methemoglobin OH^- (0.76 mM heme).
pH = 11.06. Contains 0.2 M Na_2SO_4 as an internal standard.
λ_{ex} = 6000.9 Å. Energy = 4 X 10^{-3} joule/pulse.
Pulse repetition rate = 30 Hz. Scan speed = 23 Å/min.
Slitwidth = 2.5 Å

Fig. 6. Absorption spectra and excitation profiles of methemoglobin OH^-.
Conditions same as in Fig. 3

Fig. 7. Resonance Raman spectra of methemoglobin $^{16}OH^-$ and methemoglobin $^{18}OH^-$ (ca. 0.9 mM heme) pH = 10.2 λ_{ex} = 6004.7 Å. Energy = 2 X 10^{-3} joule/pulse. Pulse repetition rate = 30 Hz. Scan speed = 16 Å/min. Slitwidth = 4.5 Å.

Fig. 8. Resonance Raman spectrum of methemoglobin F^- (0.76 mM heme) containing 0.25 M Na_2SO_4 as an internal standard. λ_{ex} = 6175.1 Å. Energy = 2 X 10^{-3} joule/pulse. Pulse repetition rate = 30 Hz. Scan speed = 23 Å/min. Slitwidth = 3.7 Å. p, polarized; dp, depolarized; and ip, inversely polarized.

Fig. 9. Absorption spectrum and excitation profiles of methemoglobin F^-. Conditions as in Fig. 3. The points at wavelengths below 5200 Å were obtained from previously reported spectra (Strekas, et al., 1973).

Fig.10. Structure of the heme core

a) non protein bound heme: Fe is $\sim$0.45 Å out-of-plane toward the F^- ligand. The Fe - F^- bond is not strained.

b) Heme in methemoglobin F^-: Fe is 0.3 Å out-of-plane away from the F^- ligand.

The steric interaction between the ionic radius of the F^- and the Van der Waals radii of the pyrrole nitrogens cause an elongation of the Fe-F bond.

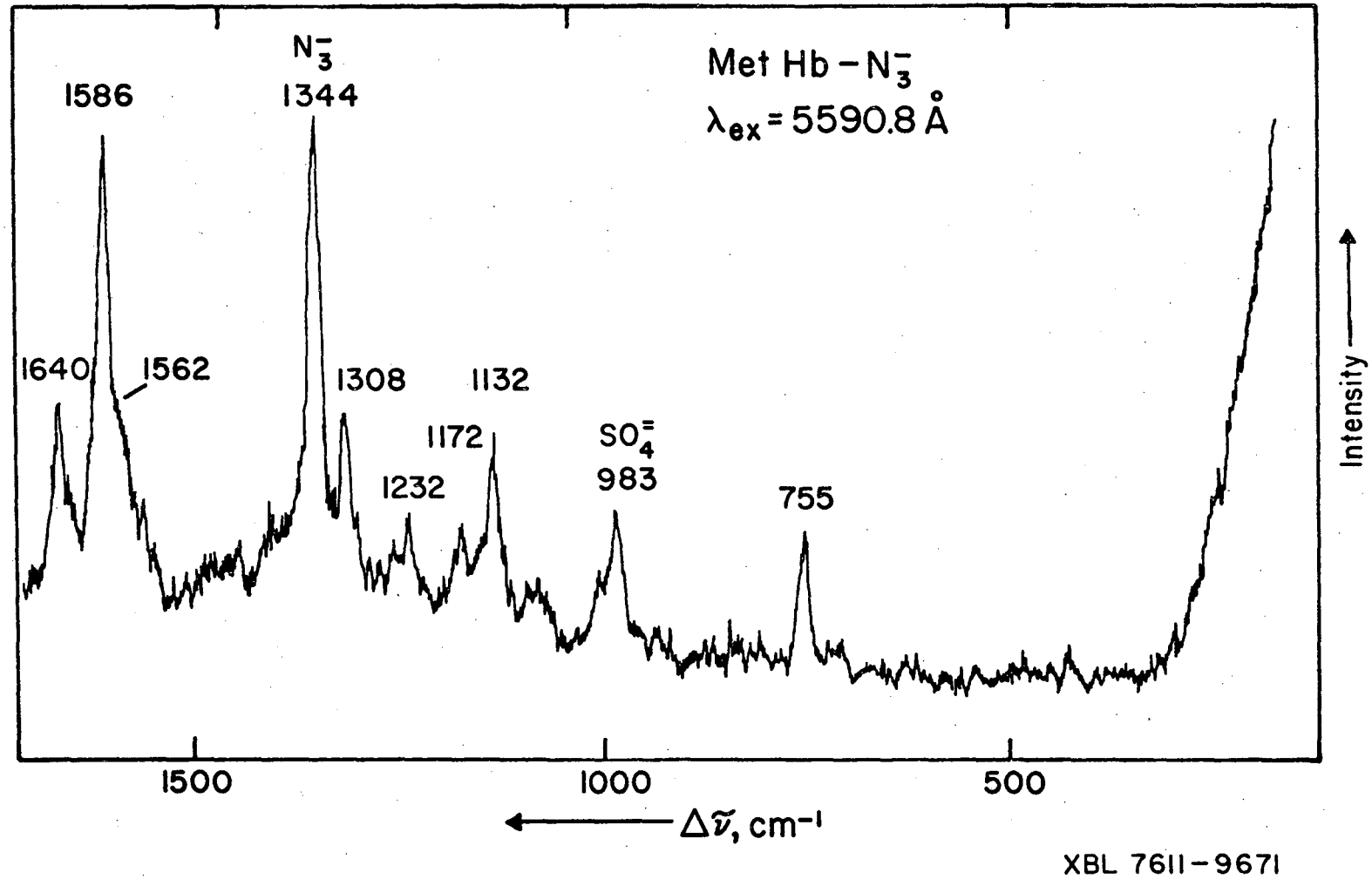

Met Hb – N_3^-
λ_{ex} = 5590.8 Å
N_3^-
1344
1586
1640
1562
1308
1132
1172
1232
$SO_4^=$
983
755
Intensity
1500
1000
500
$\Delta\tilde{\nu}$, cm^{-1}
XBL 7611-9671

Fig. 1

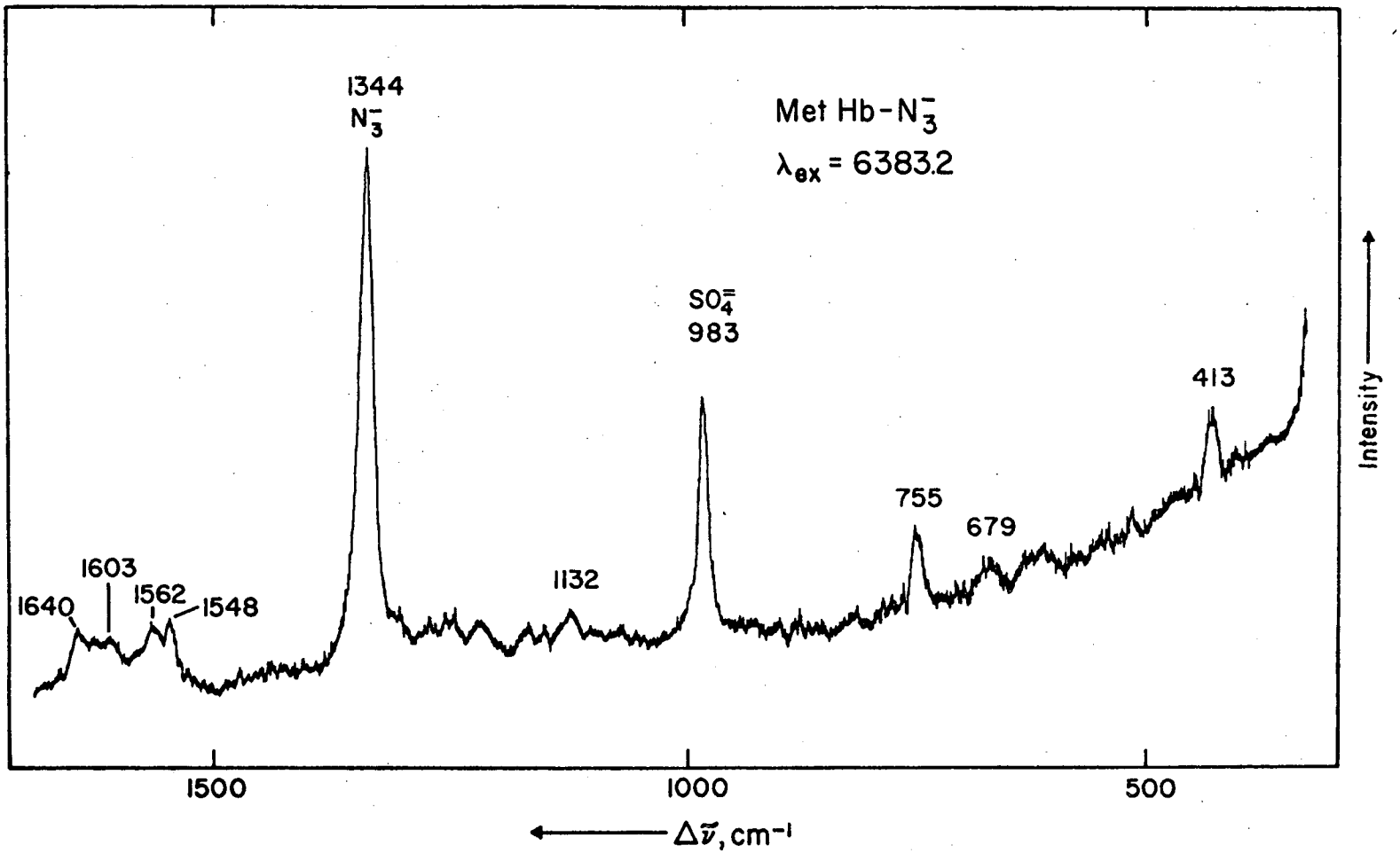
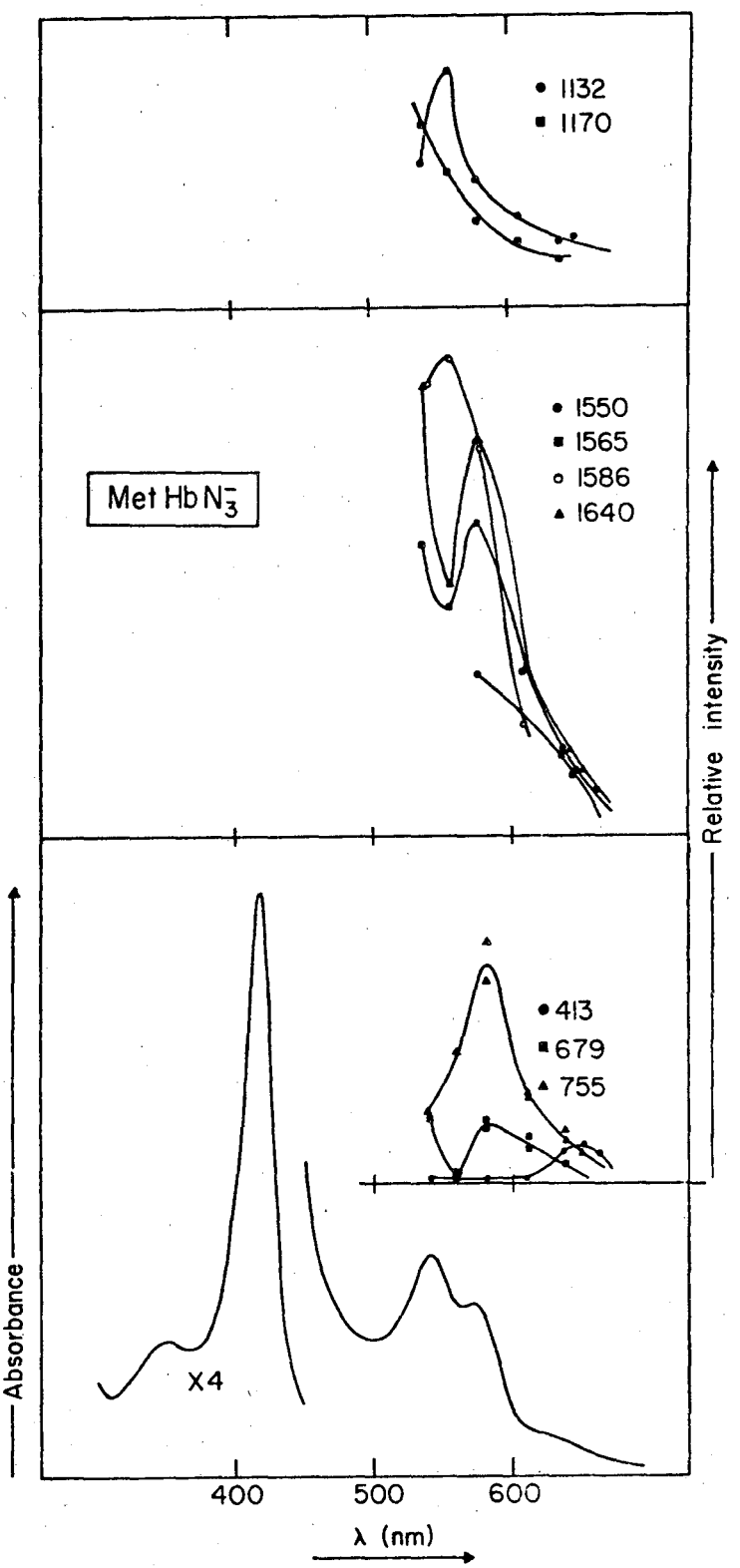
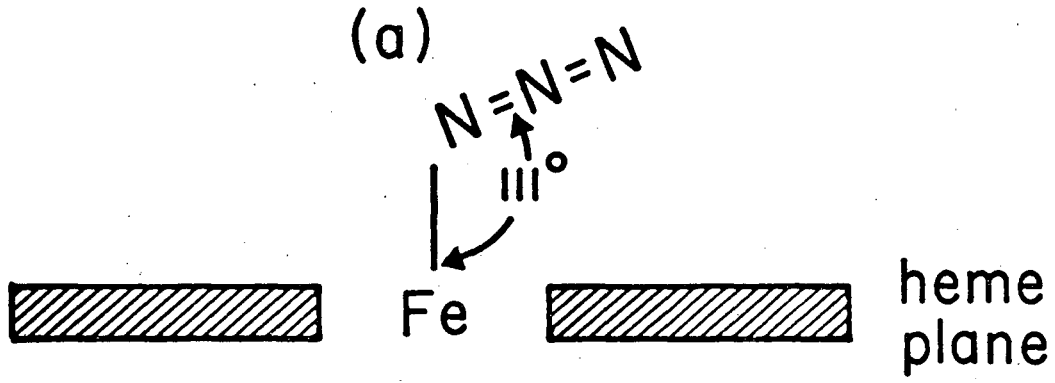

1344
N_3^-
Met Hb-N_3^-
λ_{ex} = 6383.2
$SO_4^=$
983
413
755
679
1132
1603
1640
1562
1548
Intensity
1500
1000
500
$\Delta\tilde{\nu}$, cm^{-1}
XBL 7611-9670

Fig. 2



XBL 7611-9666

Fig. 3


(a)
N=N=N
111°
Fe
heme
plane

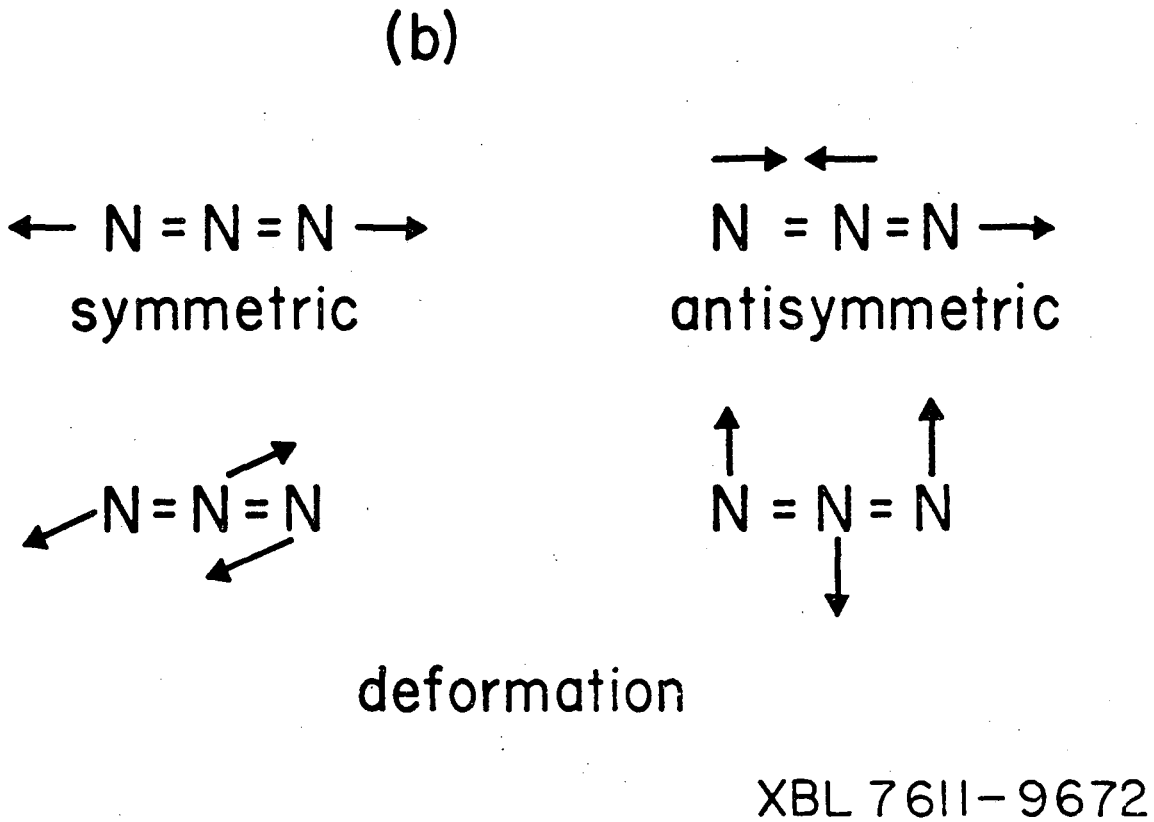

(b)
N = N = N
symmetric
N = N = N
antisymmetric
N = N = N
N = N = N
deformation
XBL 7611-9672

Fig. 4

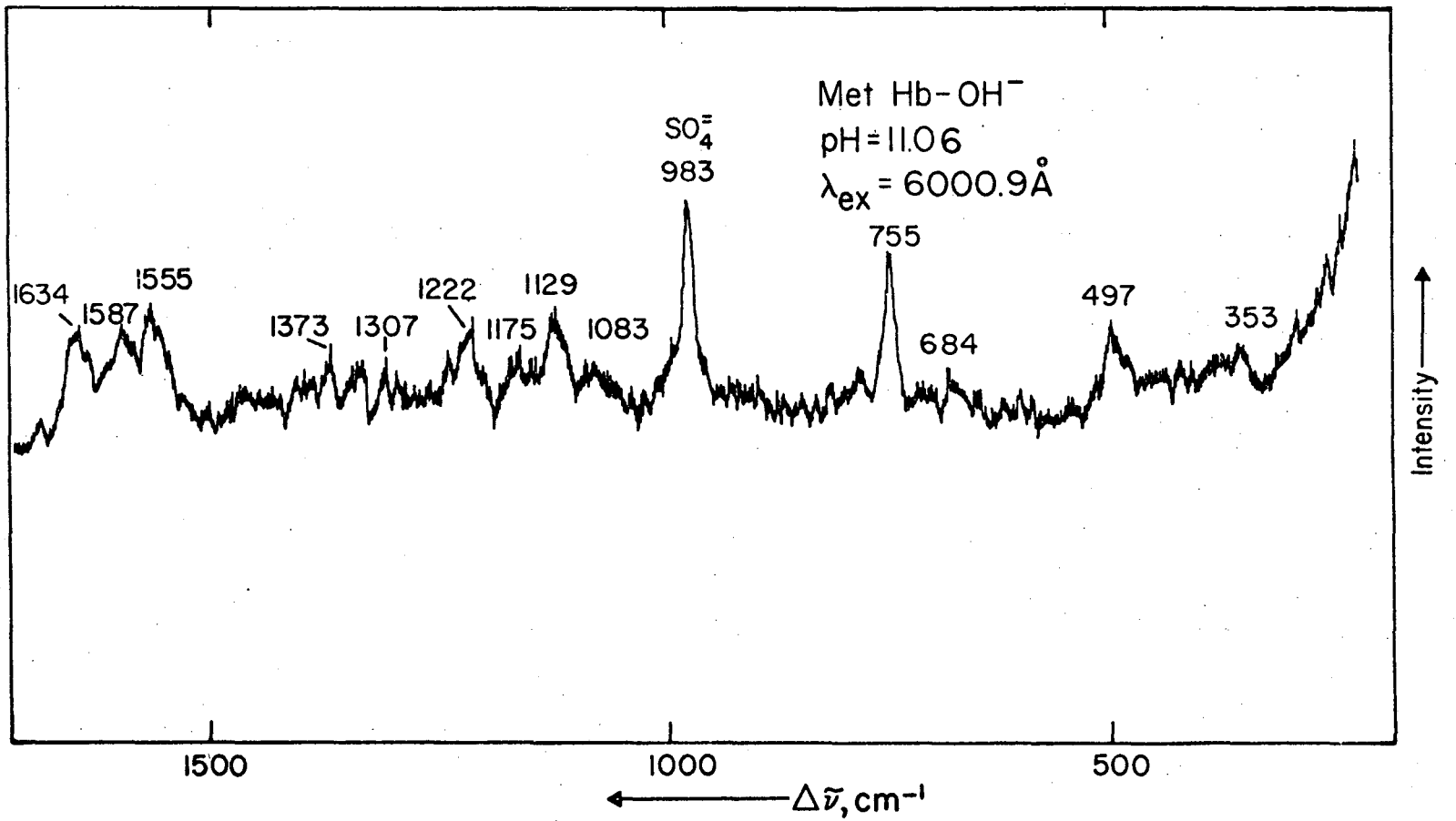

Met Hb-OH⁻
pH = 11.06
λex = 6000.9Å
SO4=
983
755
1634
1587
1555
1373
1307
1222
1175
1129
1083
684
497
353
Intensity
1500
1000
500
Δν̃, cm⁻¹
XBL 7611-9669

Fig. 5

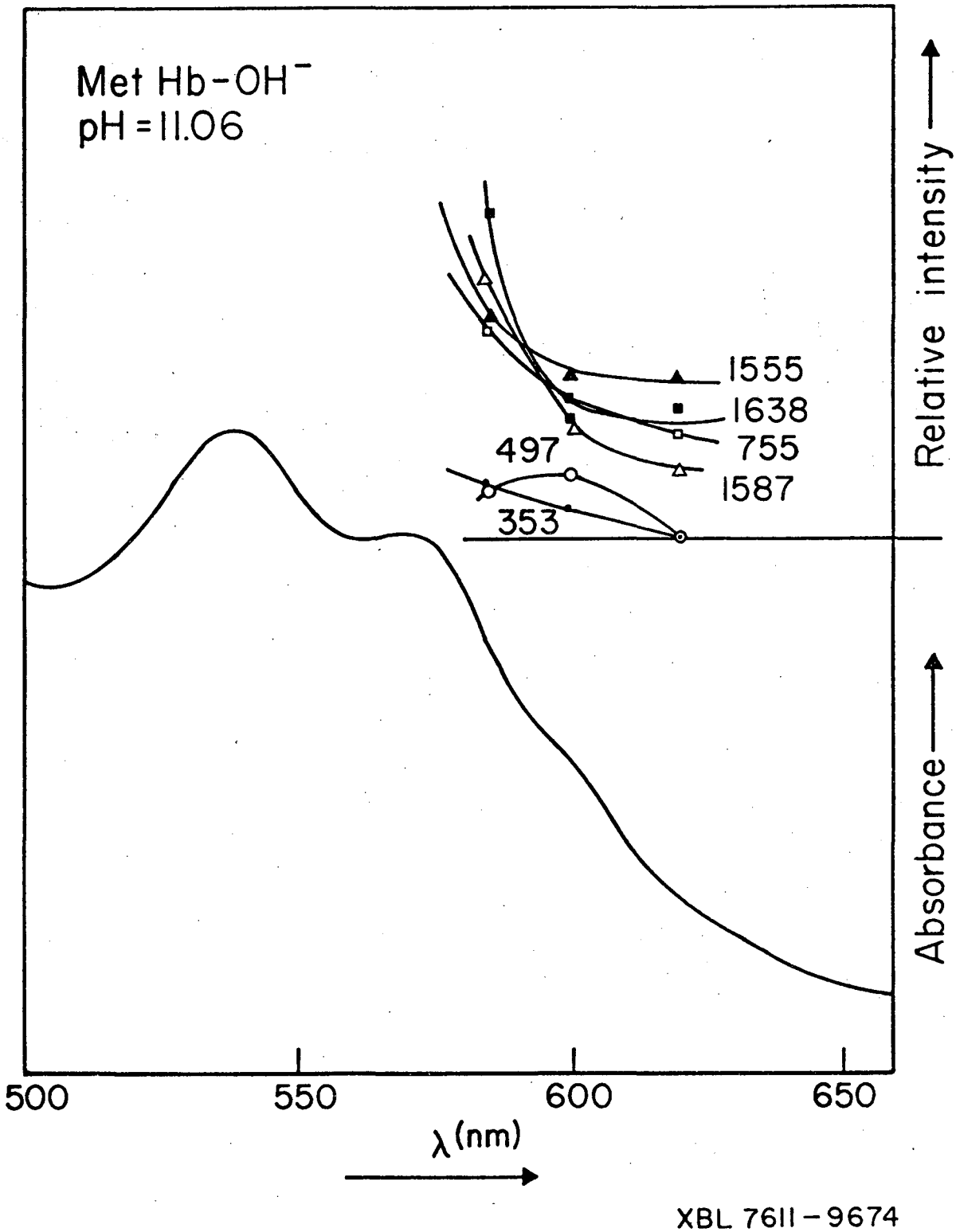

Met Hb-OH⁻
pH = 11.06
1555
1638
755
1587
497
353
Relative intensity
Absorbance
500
550
600
650
λ(nm)
XBL 7611-9674

Fig. 6

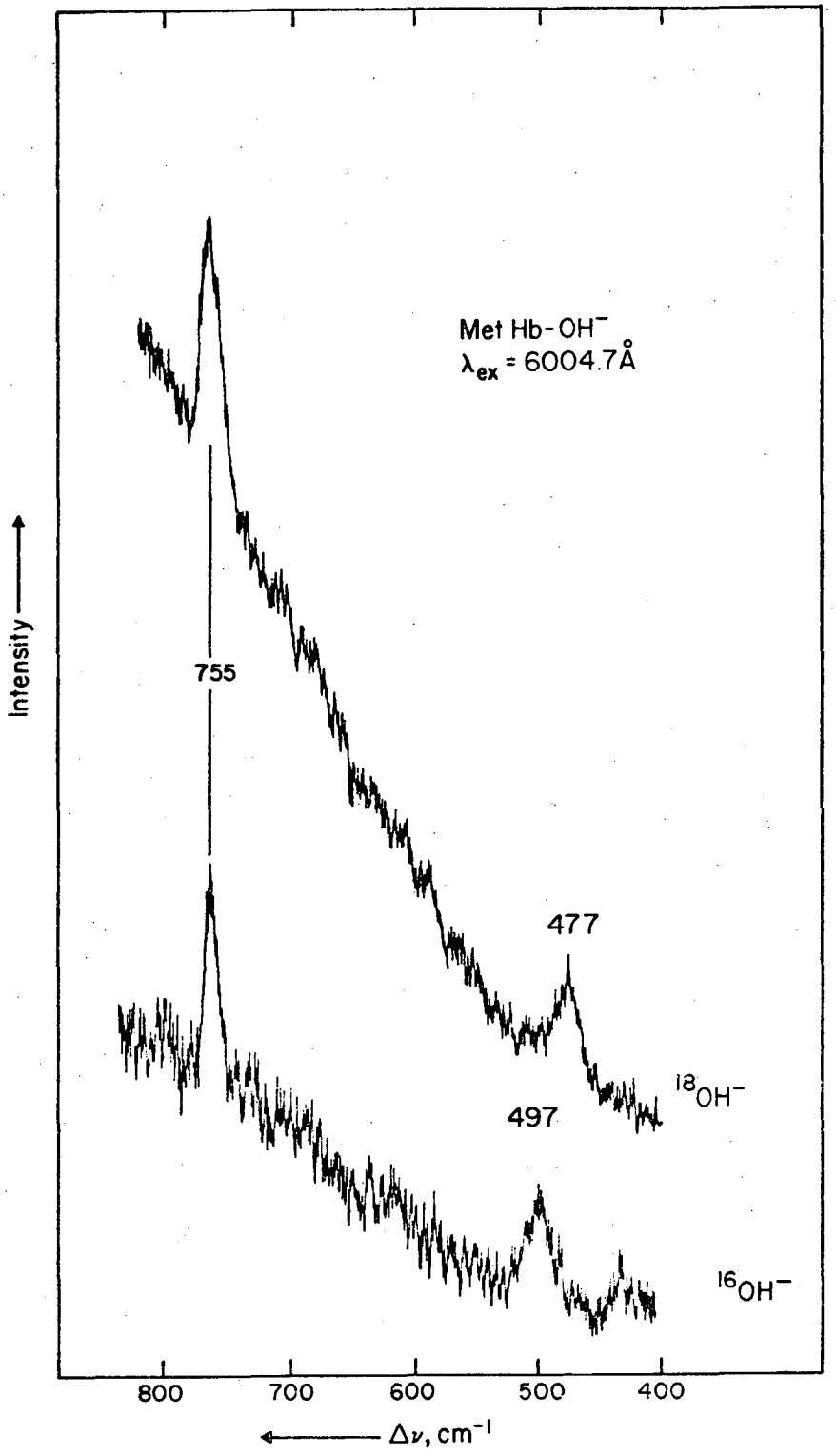

Met Hb-OH⁻
λex = 6004.7Å
Intensity
755
477
497
18OH⁻
16OH⁻
800
700
600
500
400
Δν, cm⁻¹
XBL 7611-9668

Fig. 7

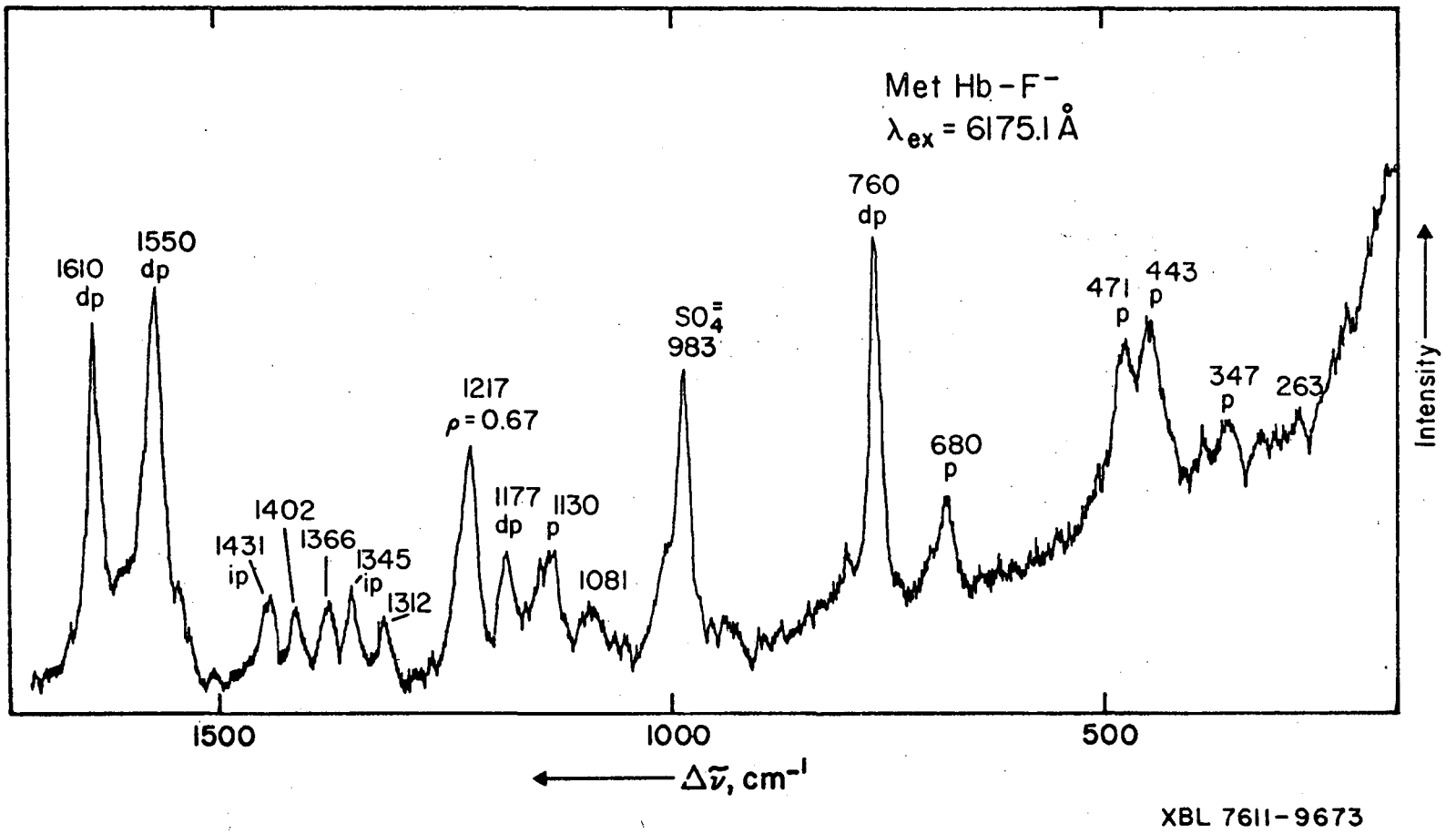

Met Hb-F⁻
λex = 6175.1 Å
1610 dp
1550 dp
1431 ip
1402
1366
1345 ip
1312
1217 ρ = 0.67
1177 dp
1130 p
1081
SO4= 983
760 dp
680 p
471 p
443 p
347 p
263
Intensity
1500
1000
500
Δν̃, cm⁻¹
XBL 7611-9673

Fig. 8

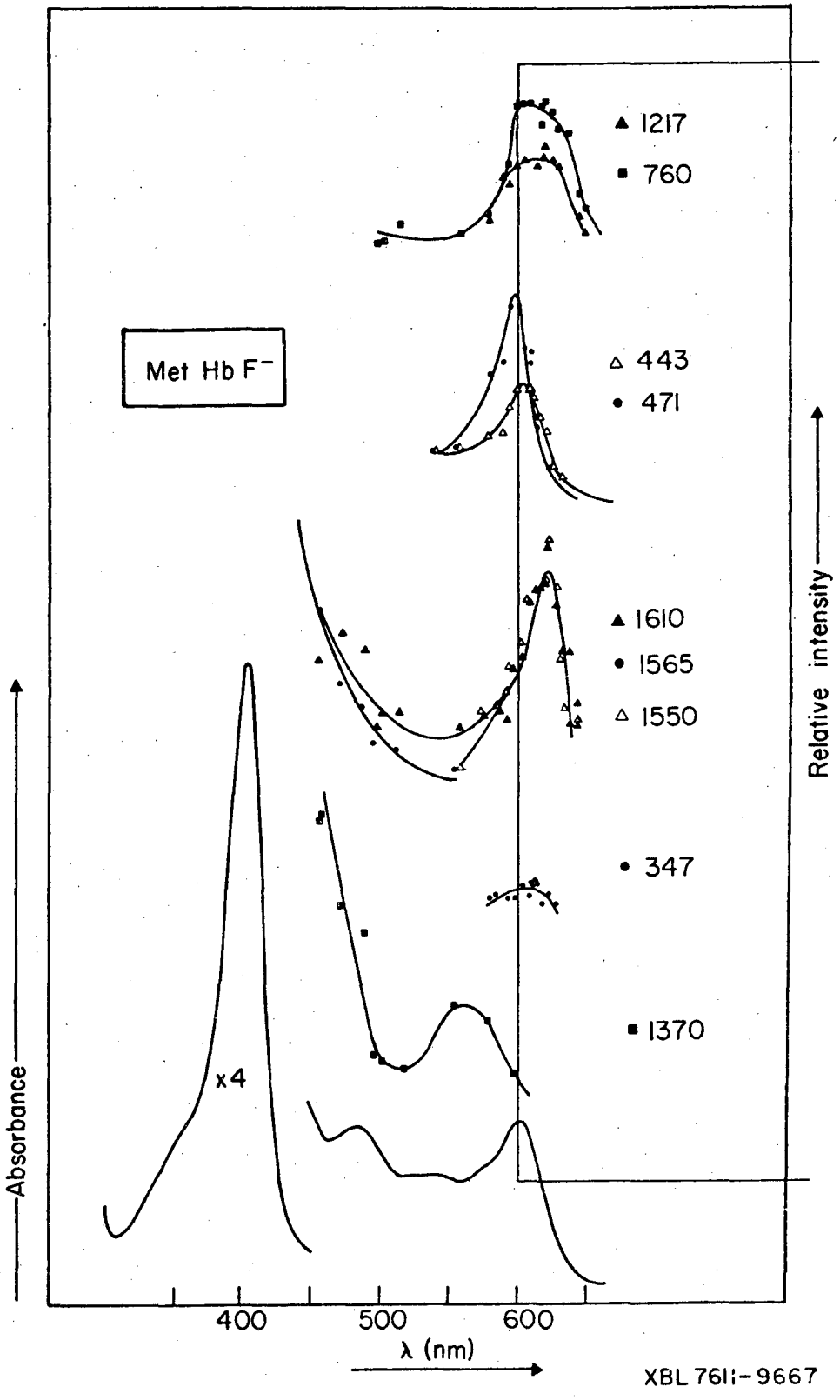

Met Hb F⁻
▲ 1217
■ 760
△ 443
● 471
▲ 1610
● 1565
△ 1550
● 347
■ 1370
x4
Absorbance
Relative intensity
400
500
600
λ (nm)
XBL 7611-9667

Fig. 9

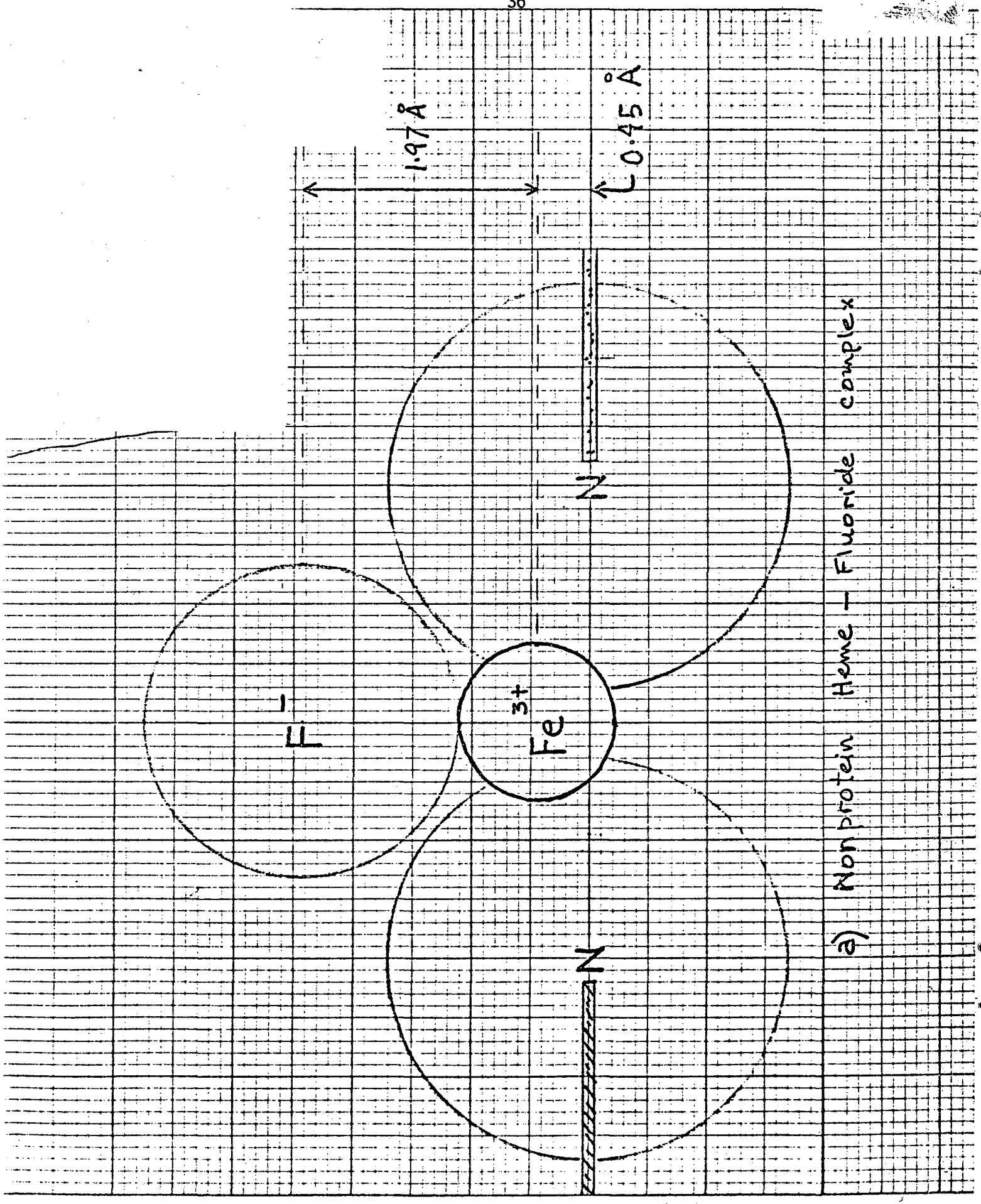


Fig. 10A

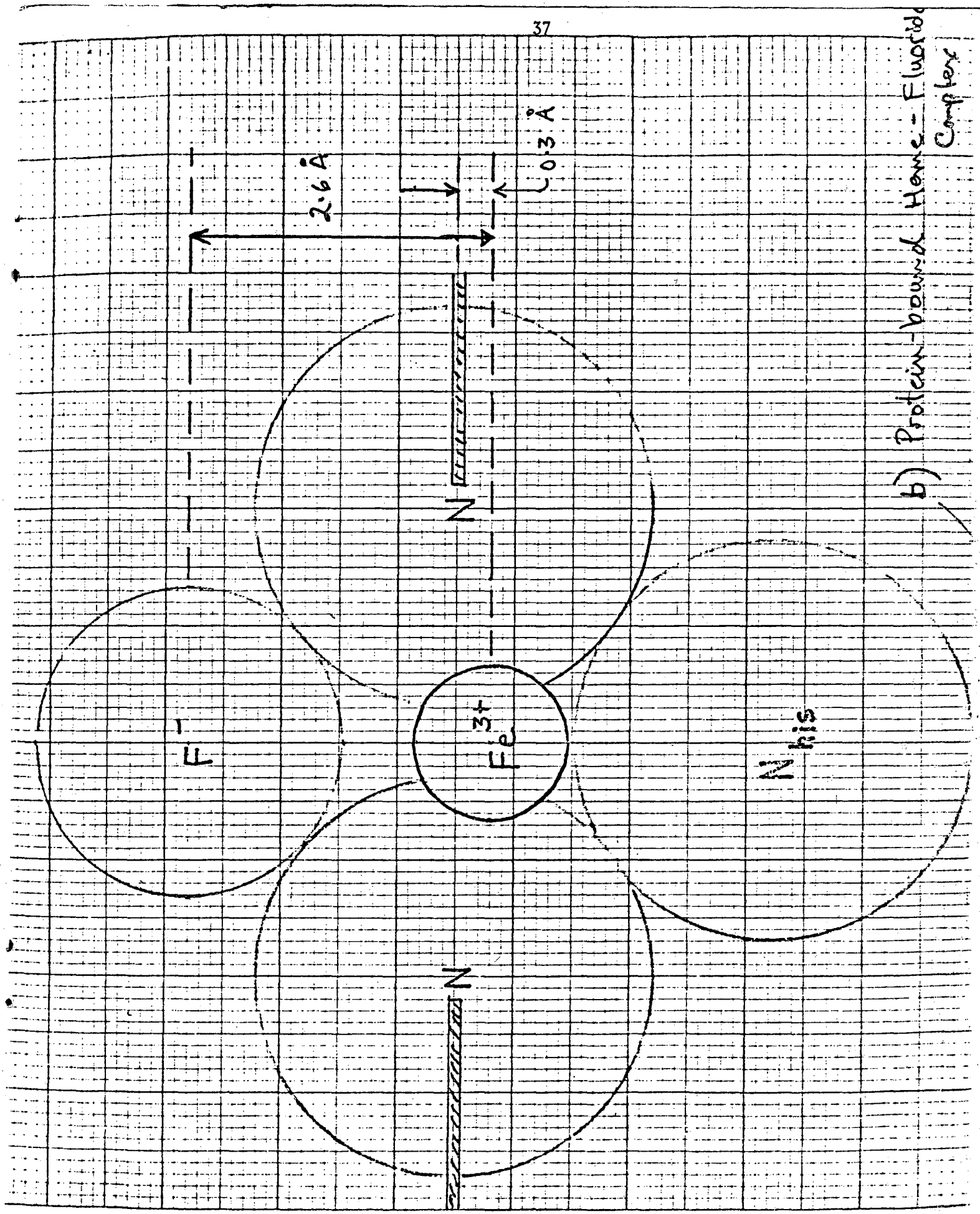

b) Protein-bound Heme-Fluoride Complex
2.6 Å
0.3 Å
F⁻
N
N
N
N
Fe3+
N his

Fig. 10B

This report was done with support from the Department of Energy. Any conclusions or opinions expressed in this report represent solely those of the author(s) and not necessarily those of The Regents of the University of California, the Lawrence Berkeley Laboratory or the Department of Energy.

TECHNICAL INFORMATION DEPARTMENT
LAWRENCE BERKELEY LABORATORY
UNIVERSITY OF CALIFORNIA
BERKELEY, CALIFORNIA 94720