Assignment of the Fe-N_ε (His) stretching mode in the resonance Raman spectra of a monomeric insect cyanomethaemoglobin

Ellen A. Kerr, Nai-Teng Yu* and Klaus Gersonde+,*

School of Chemistry, Georgia Institute of Technology Atlanta, GA 30332, USA, and +*Abteilung Physiologische Chemie, Rheinisch-Westfälische Technische Hochschule (RWTH), D-5100 Aachen, FRG*

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Resonance Raman (RR) spectra of the monomeric cyanomethaemoglobin CTT III from insect larvae of *Chironomus thummi thummi* are shown for the range of 200–550 cm^{-1}. By iron and cyanide isotope exchange a line varying between 307 cm^{-1} for ^{57}Fe-$^{13}C^{15}N$ and 311 cm^{-1} for ^{54}Fe-$^{12}C^{14}N$, has been assigned to the Fe-N_ε stretching mode of this haem complex. The substitution of ^{54}Fe for ^{57}Fe has no effect on the Fe-C=N bending mode whereas it affects the Fe-CN stretching mode.

Resonance Raman spectroscopy *Cyanomethemoglobin* *Fe-N_ε (His) stretching* *Monomeric hemoglobin*

1. INTRODUCTION

In haemoglobins the invariant amino acid residue His F8, known as the proximal histidine, provides the only covalent link between the haem iron and the globin. It has been suggested first in [1,2] that the proximal histidine plays an essential role for the regulation of haemoglobin function providing the rack mechanism of the proximal histidine. Later we presented the first experimental evidence for the validity of such a mechanism which is based on the *trans*-effect of the imidazole group *trans* to the exogenous ligand [3,4]. Thus, the vibrational frequency associated with the Fe-N_ε (His, proximal) bond has been implicated as fundamentally important for the understanding of allosteric regulation of the haem-ligand reactivity. Recently, resonance Raman studies have made advances by identifying the ν(Fe(II)-N_ε) stretching mode in deoxyhaemoglobin (216 cm^{-1}) and deoxymyoglobin (220 cm^{-1}) [5,6] and in various other high-spin ferrous haemoproteins and model compounds [7]. In addition, the ν(Fe(III)-N_ε) has been identified for the ferric haemoproteins, aquo-met Mb, ferric HRP, and ferric HRP fluoride [8].

We report for the first time the observation and clear assignment of the ν(Fe(III)-N_ε) stretching frequency for a low-spin, 6-coordinated ferric haem complex. The resonance Raman spectrum of the cyanide complex of a monomeric insect met haemoglobin from *Chironomus thummi thummi* (CTT III) reveals a line at ~309 cm^{-1} which exhibits sensitivity to both iron and cyanide isotopic substitutions.

2. MATERIALS AND METHODS

The monomeric haemoglobin III (CTT III) from insect larvae of *C. thummi thummi* was purified as in [9]. The preparation of globin and the reconstitution of CTT III with ^{57}Fe-protoporphyrin-IX (95 atom% ^{57}Fe) and ^{54}Fe-protoporphyrin-IX (99 atom% ^{54}Fe) were performed as in [4,9]. For each sample, the lyophilized met CTT III was dissolved in 0.2 M Tris–HCl buffer (pH 9.2) to give a Hb solution with a haem concentration of about 80 μM. The solution was cleared by centrifugation after the addition of a slight excess of solid

* To whom correspondence should be addressed

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potassium cyanide and then transferred to a Raman cell. For the ligand isotope substitution we employed $K^{13}C^{15}N$ (90 atom% ^{13}C; 92 atom% ^{15}N).

Raman scattering was excited using the 413.1 nm line from a Spectra Physics model 171-01 krypton-ion laser, collected by a 90° geometry, and recorded on a highly sensitive multichannel Raman system described in [10]. Fenchone was used to calibrate all spectra.

3. RESULTS AND DISCUSSION

The low-frequency region (200–550 cm^{-1}) resonance Raman spectra of cyanomet CTT III at pH 9.2 using 413.1 nm excitation are presented in fig.1 for various iron and cyanide isotopes. In each panel, spectra of two samples are compared which were measured under identical instrumental and experimental conditions, i.e., one spectrum was recorded immediately following the other. Each comparison was repeated several times to insure the reproducibility and accuracy of the isotope-induced frequency shifts.

The upper panels of fig.1 (a,d) show the effect of iron isotope substitution. The replacement of ^{57}Fe by ^{54}Fe in cyano-met CTT III (ligated with $^{13}C^{15}N$) leads to a shift of the Raman line from 307 cm^{-1} to 309 cm^{-1}. This frequency shift of 2 cm^{-1} by substitution with iron of smaller mass agrees well with the shifts observed in other ferric and ferrous haemoproteins where ^{56}Fe was replaced with ^{54}Fe in order to identify and assign the Fe-N_ϵ(His) stretching mode [5,6,8].

The middle panels of fig.1 (b,e) show the effect of cyanide isotope substitution. Upon increasing the mass of cyanide by 2, i.e., replacement of $^{12}C^{14}N$ with $^{13}C^{15}N$, the Raman line at 311 cm^{-1} shifts to 309 cm^{-1} (see panel b). Therefore, this mode is sensitive to a change of both iron mass and exogeneous ligand mass which indicates that it arises from the bond between iron and the trans axial ligand, i.e., proximal imidazole. The shift toward lower frequency is expected with increasing mass because the ν(Fe-N_ϵ) is expected to be vibrationally coupled with the ν(Fe-CN^-). Authors in [11] used this type of coupling as the basis of their search for the Fe-imidazole mode in oxy Mb. Using $^{18}O_2$ substitution, they tentatively identified the ν(Fe-N_ϵ) in oxy Mb as a shoulder at 272 cm^{-1}.

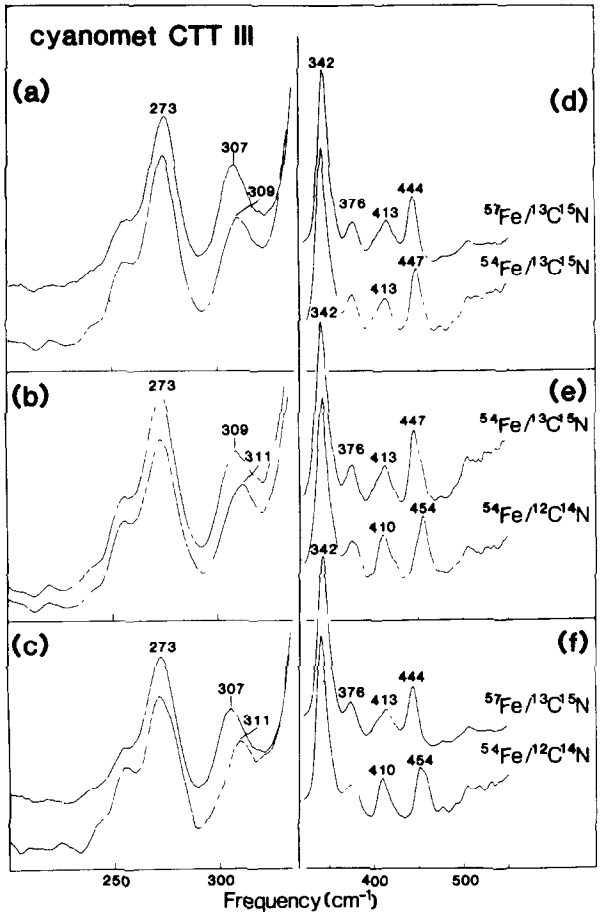

Fig.1. Effects of iron and cyanide isotopes on the resonance Raman spectra of cyano-met haemoglobin CTT III. Excitation wavelength, λ = 413 nm; power, 15 mW; haem concentration, ~80 μM; buffer, 0.2 M Tris–HCl (pH 9.2). The spectra of the 200–325 cm^{-1} region (a–c) are expanded 2.5 times relative to those spectra of the 325–550 cm^{-1} region (d–f). Isotope substitution as indicated in the panels.

The effect of changing simultaneously both iron and cyanide isotopes, is shown in the lower panels c and f. In panel c, the 307 cm^{-1} line of the ^{57}Fe haem-reconstituted CTT III ligated with $^{13}C^{15}N$ exhibits a frequency shift to 311 cm^{-1} when compared with the ^{54}Fe haem-reconstituted CTT III ligated with $^{12}C^{14}N$.

The Fe-N_ϵ(His) stretching mode has proven difficult to definitely assign in many haemoprotein complexes since it is expected to fall into the region where other low-frequency vibrational modes can interfere. Raman spectra of deoxy Hb and deoxy

Mb [12] show lines at 302 and 303 cm^{-1}, respectively; however, they are reported to remain unaltered within ± 1 cm^{-1} upon iron isotope substitution. Authors in [8] identified a line at 307 cm^{-1} in aquo-met Mb at pH 7.8 which proved to be sensitive to the acid-base transition. They stated the mode as one dependent upon the spin state of the haem iron and the accompanied structural change of haem. Authors in [11] observed a broad line at 305 cm^{-1} in the spectrum of oxy Mb which they identified as containing two modes, a vinyl bending mode, at 308 cm^{-1}, and a methine out-of-plane deformation mode, at 310 cm^{-1}. It is interesting to note that the latter did lose some intensity by $^{18}O_2$ substitution. Nevertheless, the iron and cyanide isotope shifts described here for the sharp line at 309 cm^{-1} in cyano-met CTT III are significant and show strong evidence for the assignment of this mode as primarily involving the Fe-N_ϵ(His) stretching vibration. To further support this assignment, a normal coordinate analysis has been carried out on the simplified model-imidazole–Fe-C-N system. Details can be found elsewhere [13]. Qualitatively, calculated vibrational frequencies and isotope shift patterns agree well with the experimentally observed values.

The Fe(III)-CN stretching and the Fe(III)-C-N bending vibrations for the native ferric cyanide complexes of CTT III have been previously assigned [14]. Cyanide isotope substitution identified the two lines at 453 and 412 cm^{-1}, respectively. The spectra of panels d–f present the effect on these two lines by both iron and cyanide isotope substitution. Thus, these data provide additional support for our previous assignments [14].

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