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PROJECT ADMINISTRATION DATA SHEET

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Project No. G-33-G11 Q5169-1A0 GIT DATE 9/17/86

Project Director: Dr. Nai-Teng Yu School/[illegible] Chemistry

Sponsor: DHHS/PHS/NIH/NIGMS

Type Agreement: Grant No. 5 ROI GM18894-16

Award Period: From 9/1/86 To 8/31/87 (Performance) 11/30/87 (Reports)

Sponsor Amount:	This Change	Total to Date
Estimated:	$	$ 121,797
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Cost Sharing Amount: $ _____ Cost Sharing No: _____

Title: Laser-Excited Raman Spectroscopy of Biopolymers

ADMINISTRATIVE DATA

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Defense Priority Rating: N/A Military Security Classification: N/A

(or) Company/Industrial Proprietary: _____

RESTRICTIONS

See Attached NIH Supplemental Information Sheet for Additional Requirements.

Travel: Foreign travel must have prior approval – Contact OCA in each case. Domestic travel requires sponsor approval where total will exceed greater of $500 or 125% of approved proposal budget category.

Equipment: Title vests with GIT

COMMENTS:

Continuation of G-33-G10 (Final Report not due under previous project).

SPONSOR'S I. D. NO. 01.108.001.86.042

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GEORGIA INSTITUTE OF TECHNOLOGY OFFICE OF CONTRACT ADMINISTRATION

SPONSORED PROJECT TERMINATION/CLOSEOUT SHEET

Date Sept 17, 1987

Project No. G-33-G11 School/~~BRK~~ Chemistry

Includes Subproject No.(s) N/A

Project Director(s) Dr. Nai- Teng YU ~~XXXXX~~/ GIT

Sponsor DHHS/PHS/NIH/NIGMS

Title Laser-Excited Raman Spectroscopy of Biopolymers

Effective Completion Date: 8/31/87 (Performance) 11/30/87 (Reports)

Grant/Contract Closeout Actions Remaining:

- [ ] None
- [X] Final Invoice or Final Fiscal Report
- [ ] Closing Documents
- [ ] Final Report of Inventions
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Continues Project No. G-33-G10 Continued by Project No. G-33-G12

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FORM OCA 69.285

C. Progress Report/Preliminary Studies

(i) Period: September 1, 1982 - October 15, 1986

(ii) Professional Personnel who have worked on the project:

Yu, Nai-Teng, Professor (Principal Investigator), 09/01/82-present, 35%

Benko, B.,	Research Associate,	09/01/82 - 08/31/83,	100%
Kerr, E. A.,	Grad. Res. Assist.,	09/01/82 - 12/31/84,	100%
Mackin, H. C.,	Grad. Res. Assist.,	09/01/82 - 03/20/85,	100%
Tanaka, T.,	Grad. Res. Assist.,	09/01/82 - present,	100%
Lin, S. H.,	Grad. Res. Assist.,	09/01/82 - present,	100%
Liang, J.,	Research Technician,	01/01/86 - 08/31/86	100%

Gersonde, K., Collaborator (Professor, Dept. of Physiological Chemistry, University of Aachen (RWTH), West Germany)

Chang, C. K., Collaborator (Professor of Chemistry, Michigan State University)

Nagai, K., Collaborator (Lab. of Molecular Biology, Medical Research Council, Cambridge, England)

Tsubaki, M., Summer Research Associate and Collaborator (Assistant Professor, Kagawa Medical School)

(iii) Brief Summary of Previous Application's Specific Aims: (1) To investigate the factors affecting the strength of iron-ligand bonds such as different proximal bases, distal side nonbonding steric effects, tilting of the proximal base etc., (2) To test the applicability of various hypotheses for protein modulation/regulation of heme reactivity; (3) To establish correlations for determing the Fe-C-O geometry in carbonmonoxy hemoproteins; (4) To identify new axial ligand-associated vibrations; (5) To investigate ligand binding to abnormal Hb/Mb; (6) To study allosteric control mechanisms in monomeric insect Hbs from Chironomus thummi thummi; (7) To obtain significant structural information in cytochrome P-450$_{scc}$ catalytic cycle; (8) To study RR excitation profiles of axial ligand vibrations; and (9) To obtain information related to the structural events accompanying the photolysis of MbCO, HbCO and model heme-CO complexes.

We have achieved most of the specific aims and generated a total of 19 publications and 3 manuscripts. The relations between Specific Aims and papers are as follows: Aim #1 → Papers #1, 2, 3, 5, 17 & 20; Aim #2 → Papers #10, 13, 15, 16, & 19; Aim #3 → Paper #1 & 6; Aim #4 → Papers #8, 13, 14, 15, 18, 21 and unpublished preliminary results; Aim #5 → Paper #9 and unpublished preliminary results; Aim #6 → Papers #4, 8, 10, 13, 15, 16, 18 & 19 and unpublished preliminary results. Our productive collaborations with other scientists was not envisaged in the original proposal (p. 33). The unanticipated development (vigorus collaborations with K. Gersonde of RWTH-Aachen, West Germany and K. S. Smith of UC Davis) has opened up exciting avenues for further study. The Aim #7 has been pursued by the P.I.'s close associate Dr. M. Tsubaki after he embarked on an independent research career at Kagawa Medical School (see Tsubaki, M.

and Ichikawa, Y. (1985) Biochem. Biophys. Acta, 827, 268; Tsubaki, M., Hiwatashi, A. and Ichikawa, Y. (1986) Biochemistry, 25, 3503). #8 we have obtained RR excitation profile of ν(Co-NO), ν(Mn-His) and ν(Mn-NO) stretching modes in the nitrosyl complexes of Co- and Mn-substituted CTT III. The originally suggested ν (Fe-S) mode has not been pursued because of the excellent work published by P. M. Champion (Northeastern Univ.). Finally, the specific Aim #9 were postponed because of the unanticipated extra efforts put into the collaborations with Prof. Klaus Gersonde and Kelvin S. Smith. Furthermore, elegant work on the subject has been carried out by Spiro (Princeton) and Friedman (AT&T Bel Laboratories). Overall, we have effectively utilized the available limited resources and accomplished all of the major specific aims. Important findings and new insight into the mechanisms of protein control of heme affinity are summarized in the following section.

(iv) Summary of Progress and Significant Findings:

(a) Correlations between ν(Fe-CO) and ν(C-O) stretching vibrations

Two sets of inverse relations between ν(Fe-CO) and ν(C-O) have been found (see Fig. 1). The set of points labeled 1 through 15 represent a wide variety of carbonmonoxy heme complexes, which all possess very similar neutral nitrogenous bases as a trans ligand. These complexes exhibit a strong inverse correlation between ν(Fe-CO) and ν(C-O) . Another set of points labeled 18 through 21, which represent a variety of cytochrome P-450 enzymes each with a thiolate sulfur as the trans ligand of heme iron, also exist an inverse linear relation between ν(Fe-CO) and ν(C-O). Thus, it is the nature of the trans ligand which determines the relation between ν(Fe-CO) and ν(C-O). The point 16 is from Fe(II)(TpivPP)(THF)(CO). The weak trans ligand (i.e., tetrahydrofuran) leads to an unusually high ν(Fe-CO). The trans ligand in carbonmonoxy cytochrome c oxidase (point 17) is not known; however, Fig. 1 suggests that it may be unusually weak just like THF. See paper #12 and Tsubaki et al. (1986).

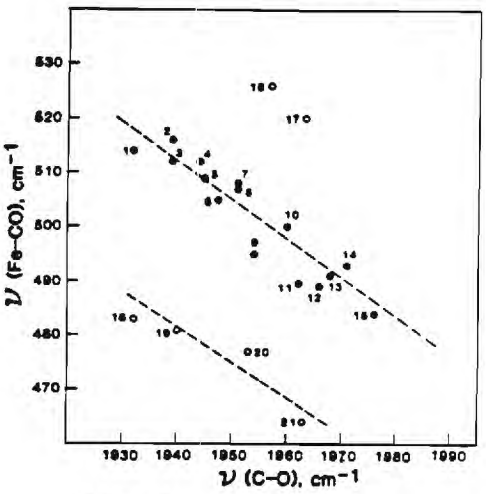

Fig. 1

(b) Enhancement of δ(Fe-C-O) bending mode; Inverse relation between CO binding affinity and ν(Fe-CO) frequency in sterically hindered "strapped hemes"

We have made an important observation: the δ(Fe-C-O) bending vibration becomes greatly resonance-enhanced (via Soret-excitation) relative to the ν(Fe-CO) stretching mode when the Fe-C-O linkage is distorted (later shown to be primarily tilted). The greater the Fe-C-O distortion, the greater the δ(Fe-C-O)/ν(Fe-CO) intensity ratio. In the absence of steric effect, the Fe-C-O linkage is linear and

perpendicular to the heme plane; the δ(Fe-C-O) bending mode is too weak to be detected (or the δ(Fe-C-O)/ν(Fe-CO) intensity ratio is zero). These results were obtained from CO complexes of a series of synthetic hemes equipped with a varying degree of steric hindrance provided by a hydrocarbon chain (of varying chain length) strapped across one face of the heme (see Paper #1).

When the CO binding affinity decreases by decreasing the chain length, the ν(Fe-CO) frequency increases. Heme 5 (unstrapped) 495 cm^{-1}; FeSP-15, 509 cm^{-1}; FeSP-14, 512 cm^{-1}; FeSP-13, 514 cm^{-1}. The important correlations established for the strapped heme system have been found useful by others for the interpretations of resonance Raman spectra of the CO complexes of human hemoglobin (Rousseau et al., 1983), cytochrome oxidase (Argade et al., 1984), Cytochrome P-450 (Uno et al., 1985; Tsubaki and Ichikawa, 1985; Tsubaki et al., 1986; Anzenbacher, Kirkup & Spiro, 1984), horseradish peroxidase (Evangelista-Kirkup et al., 1986), cytochrome c peroxidase (Smulevich et al., 1986), cytochrome b_{562}-O complex (Uno et al., 1985) and Glycera dibranchiata hemoglobin (Carson et al., 1985; Armstrong et al., 1986) and tryptophan 2,3-dioxygenase (Uno et al., 1986).

In contrast with CO complexes, the ν(Fe-CN) frequency decreases as the CN^- binding affinity decreases. The interpretation about the difference between CO and CN^- complexes has been given in Paper #20. Although the δ(Fe-C-N) bending mode was not enhanced in the strapped hemes, it has been clearly identified at ~410 cm^{-1} in cyanomet CTT III (Paper #6 and 19).

(c) Direct demonstration of trans effect: a tension on Fe-Im bond (Imidazole in-plane tilt and Fe-Im bond weakening) decreases the CO binding affinity, but increases the Fe-CO bond strength

We have studied the Fe-CO bonding interactions in iron "picket fence" porphyrin complexes with sterically unhindered (N-MeIm) and hindered (1,2-Me_2Im) axial bases. The 2-methyl group of 1,2-Me_2Im provides restraint to the motion of the axial base toward the porphyrin, causing the Im in-plane tilt and the consequent weakening of the Fe-Im bond (hence the Fe-Im bond lengthening). While the CO affinity of Fe(II)(TpivPP)(1,2-Me_2Im) is ~400 times lower than that of the unconstrained complex, the ν(Fe-CO) frequency for the (1,2-Me_2Im) complex at 496 cm^{-1} is higher than that for the (N-MeIm) complex at 489 cm^{-1}.

Therefore, the Fe-CO bond is stronger for the lower affinity complex. If the formation of the Fe-C bond were an isolated event involving no changes in other parts of the complex, the Fe-C bond in the low affinity (1,2-Me_2Im) complex must be weaker than that in the high affinity (N-MeIm) complex. Since the reverse relation was observed, the extra chemical bond energy gained in the Fe-C bond in the low affinity complex must be compensated by a greater energy loss in other parts of the complex. This energy compensation is the result of charge redistribution upon ligand binding through the σ and π bonding network of the axial ligands and porphyrin macrocycle. An unusually short Fe-CO bond (1.706Å) in Fe(II)(deutero

heme)(THF)CO also corresponds to a weak CO binding affinity when compared with the Fe-CO bond length (1.77Å) in Fe(II)TPP(pyridine)CO where the CO binding affinity is much greater (for detailed discussion, see paper #3). Other examples include Mn(II)-NO bond (1.644Å) which is much shorter (hence stronger) than Fe(II)-CO bond (1.77Å) or Fe(II)-NO bond (1.743Å); yet the NO binding to Mn(II) heme is much weaker. Our preliminary results locate the Mn(II)-NO stretching frequency at ~631 cm^{-1} in Mn(II) mesoheme CTT IV · NO complex, which is much higher than Fe(II)-NO stretching (~550 cm^{-1}) and Fe(II)-CO stretching (~500 cm^{-1}) in insect Hb(CTT IV) system.

(d) Method of quantitative estimation of Fe-C-O bond angle

We have derived the following expression for estimating the Fe-C-O bond angle in heme · CO complexes (see paper #1 and 12):

$$\sin^2\theta = \frac{1/M_i^2 - B/M^2}{B/m_2^2 - 1/m_{2i}^2}$$

where $B = \left(\frac{\nu_{1i}\ \nu_{2i}}{\nu_1\ \nu_2}\right)^2$, $M^2 = \frac{m_1\ m_2\ m_3}{m_1 + m_2 + m_3}$; $M_i^2 = \frac{m_1\ m_{2i}\ m_3}{m_1 + m_{2i} + m_3}$

Here, m_1, m_2 and m_3 are masses of Fe, C and O, respectively; upon isotopic substitution at the carbon atom, m_2 becomes m_{2i} and ν_1 and ν_2 become ν_{1i} and ν_{2i}, respectively; ν_1 and ν_2 are the frequencies (in cm^{-1}) of Fe-CO and C-O stretching vibrations. This method has been found useful by other researchers (e.g., Carson et al., 1985; Rousseau et al., 1984; Argade et al., 1984). The application of the method to various carbonmonoxy hemoproteins has led to the conclusion that the Fe-CO distortion caused by the protein is primarily tilting, with a small degree of bending (the Fe-C-O angle $\geq$ 170°). For example, we found that the Fe-C-O angle for Mb·CO is 175±5°, in disagreement with the 135° estimated originally by Norvell et al., (1975) from neutron diffraction data, but in agreement with the x-ray crystallographic data of Hb·CO by Baldwin (1980).

(e) Comparison of bonding interactions between Fe(III)-NO and Fe(II)-CO, two isoelectronic species.

In general, the geometry of M-X-Y linkage (where M = metal; XY = diatomic ligand) can be predicted on the basis of the sum of the d electrons on the metal together with only those electrons on the XY ligand which occupy the π^* or σ^* levels. As the sum of electrons becomes greater than six, a progressively more bent end-on configuration of M-X-Y linkage is observed (see paper #12).

The Fe(III)-NO moiety is isoelectronic with Fe(II)-CO, having a total of 6 ($d + \pi^*$) electrons. Therefore, linear geometry is expectd for both species. The Fe(III)-NO stretching mode has been detected, for the first time, at 595 cm^{-1} in ferric Mb·NO (see paper #5), which is higher than the Fe(II)-CO stretch at 512 cm^{-1} in Mb·CO. This indicates that the Fe(III)-NO bond is stronger than the Fe(II)-CO

bond. Recently, Scheidt et al. (1984) found that the crystal structures of nitrosyl complexes of ferric porphyrins have unusually short Fe(III)-NO bond lengths: 1.652(5) A for $[Fe(TPP)(NO)H_2O]IO_4$ and 1.644(3) A for $[Fe(OEP)(NO)]IO_4$, compared to the 1.77(3) A value found in Fe(II)(TPP)(Py)(CO). We also have identified the Fe(III)-N-O bedning mode at ~578 cm-1 for the first time (see paper #5). Other ferric nitrosyl hemeproteins studied include ferric Hb•NO and ferric HRP•NO. The ferric HRP•NO is unusually stable and its Fe(III)-NO stretching frequency at 604 cm^{-1} is higher than 595 cm^{-1} in ferric Mb•NO or 594 cm^{-1} in ferric Hb•NO. This may imply some unusual interaction involving distal histidine in HRP, which stabilizes the unpaired electron on NO from shifting into the Fe d-orbitals. The third isoelectronic species, Mn(II)-NO, has an even higher Mn(II)-NO stretching frequency ~631 cm^{-1}, indicating an unusually short Mn(II)-CO bond length (see Preliminary Results.)

(f) A new sensitive probe for distal histidine-ligand interactions: the Co(II)-NO stretching vibration.

We have identified, for the first time, the Co-NO stretchiny vibration in the (550-580 cm^{-1}) region in NO complexes of Co-substituted hemoproteins. Monomeric Hbs with a distal histidine (sperm whale Mb and leghemoglobin) exhibit this virbration at 573-575 cm^{-1}, whereas the Hbs without a distal histidine (elephant Mb and insect Hb CTT III) show this vibration in the (553-558 cm^{-1}) range. In contrast, the Fe-NO vibration (550-556 cm^{-1}) does not sensitively reflect the distal histidine-ligand interactions. More detailed discussion is given in paper #18.

(g) Heme-rotational disorder monitored by resonance Raman

The "heme disorder" was first discovered in the proton NMR spectra of cyanomet insect hemoglobins (LaMar et al., 1978). It is due to rotation of the heme group about a symmetry axis (i.e., α,γ-meso axis). Gersonde et al. (1986) have resolved the individual O_2 kinetic behavior of the two heme-rotational components in monomeric insect hemoglobins. The two components exhibit remarkable differences in off-rate constants and Bohr effects, suggesting that heme disorder may have functional and regulatory relevance.

We have obtained the first Raman evidence for the existence of heme-rotational disorder in the cyanide complexes of insect Hb system. The two Fe(III)-C-N bending modes corresponding to the two heme-rotational components have been identified at 407 and 427 cm^{-1} in cyanomet deutero CTT IV labeled with ^{57}Fe at pH 9.5 (see Fig. 2). The high-frequency component exhibits pH-sensitivity whereas the low-frequency one in pH-insensitive. The unsymmetric protoheme IX, if rotated by 180°, exerts different interactions with the protein environment. This causes significant changes in the distal side of the heme groups as reflected by the variation in the O_2 off-rate constant, and different Fe(III)-C-N bending modes. The Fe(III)-CN stretching mode near 446 cm^{-1} also indicates two componets, although they are not well-resolved.

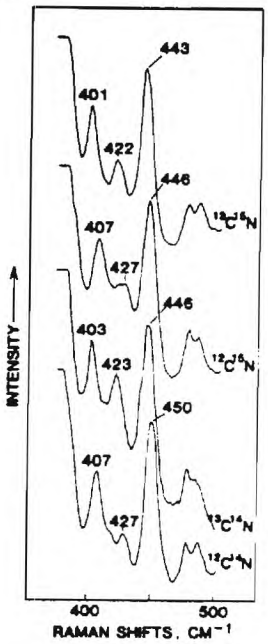

Fig. 2

Replacing the unsymmetrical protoheme IX (2,4-divinyl) with the "symmetrical" protoheme III (2,3-divinyl) we eliminate the heme disorder. Sharpening of the Fe-N (His) (at 313 cm^{-1}) and Fe-CN (at 453 cm^{-1}) stretching modes was observed and a single Fe-C-N bending mode (at 412 cm^{-1}) appeared.

(h) Iron-carbon bond in CO and CN^- complexes of CTT III: critical comparison between resonance Raman and X-ray.

Through use of x-ray structural and Raman spectral data available for Fe(II)(TPP)(Py)(CO) and Fe(II)(deutero P)(THF)(CO), a relation known as Badger's rule has been established for the (Fe, C) pair of carbonmonoxy heme systems as: $\gamma_e = 0.75 + 1.38K^{-1/3}$. In addition, the influence of the Fe-C-O bond angle on the ν(Fe-CO) frequency has been determined using normal coordinate analysis. Based on these relations we have established a scheme for estimating the Fe-C bond length and the nature of Fe-C-O distortion in carbonmonoxy CTT III. It should be of interest to make a critical comparison between our resonance Raman results and x-ray structural determinations. X-ray studies at 1.4Å resolution on carbonmonoxy CTT III (Steigeman and Weber, 1979) reported the Fe-C bond length as 2.4Å , which is unusually large compared to the values found in carbonmonoxy human HbA (i.e., 1.80Å) (Baldwin, 1980) and the heme complex Fe(II)(TPP)(Py)(CO)(i.e., 1.77Å) (Peng & Ibers, 1976). Other relevant structural parameters from x-ray include the Fe-C-O bond angle of 161°±5° and a tilt angle of 8°. Based on resonance Raman methodology, we obtain a structure where the CO is tilted, the Fe-C-O linkage is bent to ~170°, and the Fe-C bond length lies between 1.80 and 1.82 Å well within the range of model compounds and other hemoproteins. Since the protein crystals contain large amounts of water resembling highly concentrated solution, the large discrepancies in Fe-C bond length (1.81Å vs. 2.4Å) between our resonance Raman results and the x-ray structural data point to the possibility that the x-ray analysis by Steigeman and

Weber (1979) must be in error. Furthermore, we have also estimated the Fe-C bond length in cyanomet CTT III as ~1.91Å, which also disagrees with the 2.2Å value by Steigeman and Weber (1979). (See paper #6 and 12).

(i) Allosteric control mechanism for O_2 binding to Co-substituted insect hemoglobins

Substitution of cobalt for iron in CTT II and CTT III does not modify the Bohr-effect, but permits the resonance Raman detection of Co-O_2 stretching, O-O stretching and Co-O-O bending vibrations. Both Co CTT II and Co CTT III exhibit a large Bohr-effect due to a pH-induced conformational transition: t (low affinity, low pH) $\rightleftharpoons$ r (high affinity, high pH). Using $^{16}O_2/^{18}O_2$ isotope substitution the O-O and Co-O_2 stretching and Co-O-O bending mode have been assigned to the two affinity states. For oxy Co CTT II: ν(O-O) changes from 1152 cm^{-1} (pH 5.5; t conformation) to about 1120 cm^{-1} (or 1134 cm^{-1}, depending on interpretations) (pH 9.5, r conformation), ν(Co-O_2) from 512 cm^{-1} (pH 5.5) to 537 cm^{-1} (pH 9.5) and δ(Co-O-O) from 378 cm^{-1} (pH 5.5) to 390 cm^{-1} (pH 9.5). The Co-N (His) stretching mode has also be assigned tentatively at 313 cm^{-1} (pH 5.5) to 307 cm^{-1} (pH 9.5). For the first time, reciprocal behavior between the Co-N and Co-O_2 bonds and between the Co-O_2 and the O-O bonds in an allosteric hemoglobin are demonstrated. It is interesting to note that resonance Raman spectra of deoxy Co CTT II at both low and high pH values are identical. Thus, the protein conformational changes do not affect the heme structure in the deoxy state. In other words, prior to binding, the O_2 ligand "see" identical binding sites. Therefore, the allosteric control occurs primarily in the ligated state, in agreement with the kinetic studies (K. Gersonde, unpublished results) which show that the Bohr-effect is controlled by O_2 off-rate, but not by O_2 on-rate. (See paper #15 and 16).

(j) Detection of Nickel-Histidine Bond in Ni-Substituted Hb and Mb

In collaboration with J. Shelnutt, K. Alston, T. Yamamoto and J. M. Rifkind (see paper #14), the ν(Ni-His) stretching mode has been identified, for the first time, at 236 cm^{-1} (Ni-Hb) and 241 cm^{-1} (Ni-Mb) via $^{58.7}N \rightarrow {}^{64}Ni$ isotope substitution. The frequencies of ν(Ni-His) stretch are higher than those of ν(Fe-His) in deoxy Hb (220 cm^{-1}) and deoxy Mb (224 cm^{-1}) even though the binding of histidine to Ni is less stable. The strong Ni-His bond is compensated for energetically by the weakening of the Ni-N (pyrrole) bonds and the destabilized porphyrin ring as evidenced by the large decreases (up to 40 cm^{-1}) in the frequencies of the core-size marker lines. Distinct sets of Ramn lines have been found as good indicators of 4-, 5-, and 6-coordinate Ni porphyrins. Dramatic spectral changes indicating the conversion of 6- to 4-coordinate species upon the intercalation of Ni-porphyrin into DNA bases have recently been demonstrted by Nakamoto and coworkers (see Blom et al., 1986).

(k) Effects of His(E7) → Gln replacement (elephant Mb) on CO and O_2 binding

We have investigated the effects of His(E7) → Gln replacement on ν(Fe-N$_\varepsilon$), ν(Fe-CO), δ(Fe-C-O), ν(C-O), δ(Fe-O-O)/or ν(Fe-O_2) and ν(O-O) vibrational frequencies and relative intensities. Attempts were made to correlate these results with kinetic data. The Co on-rates for sperm whale and elephant Mbs are similar and we found that the ν(Fe-N$_\varepsilon$) frequencies in these deoxy Mbs are identical (i.e., at 221 cm^{-1}). However, elephant Mb has a CO dissociation rate about five times slower than that of sperm whale Mb. Interestingly, we observed a higher ν(Fe-CO) frequency (i.e., 516 cm^{-1}) for elephant Mb·CO, indicative of a greater geometric distortion. This distortion is also manifested in a strong resonance Raman enhancement of the Fe-C-O bending mode (at 579 cm^{-1}). The Fe-C-O bond angle is estimated from isotope shift data as 175°±5°. This implies that the Fe-C-O moiety is essentially linear and the primary geometric distortion is tilting rather than bending.

To account for the slower CO off-rate and higher ν(Fe-CO) frequency (greater geometric distortion) we propose that for elephant Mb there is a ligand-induced conformational change which makes it more difficult for the carbon monoxide to escape. Bound CO interacts with the heme pocket environment (e.g., distal glutamine) causing a greater CO tilting. One must consider the factors such as interactions between the amide oxygen of Gln(E7) and the carbon of the CO ligand, as well as H-bonding between the amide hydrogen and the oxygen of the ligand. In short, after binding the barrier for CO to escape must be heightened resulting in the enhanced CO affinity.

The Co-O_2 stretching vibration of oxy Co elephant Mb was detected at 529 cm^{-1}, which is 10 cm^{-1} less than the Co-O_2 stretch in oxy Co sperm whale Mb (539 cm^{-1}) (Tsubaki and Yu, 1981) and 13 cm^{-1} higher than the 516 cm^{-1} value found for oxy Co "picket fence" porphyrin with N-MeIm as axial base (Mackin et al., 1983). The strong O-O stretching mode appears at 1146 cm^{-1}, which is 9 cm^{-1} lower than the 1155 cm^{-1} value found for oxy Co "picket Fence" porphyrin. However, resonance Raman studies on oxy Mb (Kerr et al., 1985) showed no significant frequency differences of the isotope sensitive line at ~570 cm^{-1} between elephant Mb and sperm whale Mb. This indicates that oxy Co hemoproteins may be a more sensitive model system for detecting changes within the heme pocket. We also have shown that Co-NO stretching mode in Co-substituted hemoproteins is more sensitive to the distal histidine-NO interaction than does the Fe-NO stretching mode in Fe hemoproteins (see section (f) and paper #18).

(l) Detection of manganese-nitrogen triple bond. Formation of nitridomanganese (V) protoporphyrin IX in Mn-substituted Mb

A new complex is formed by adding hypochlorite to Mn-substituted Mb solution (pH 10) containing ammonium ion. This complex is characterized by split α band (585 and 574 nm), β band (544 nm), and a single Soret band (430 nm). Resonance Raman spectra (excited at 441.6 nm) of the complex exhibit two lines at 1010 and 2006 cm^{-1} in addition to the porphyrin ring modes. When the Mn heme was extracted by using

2-butanone, the protein-free heme in alkaline aqueous solution gave a sharp Raman line at 1046 cm^{-1} which is similar to the 1050 cm^{-1} IR band assigned to Mn(V)-N stretching frequency of $MnN(OEPMe_2)$. This serves to confirm that the new complex is nitridomanganese(V) protoporphyrin IX, i.e., Mn(V)≡N PP IX. It is of interest to note that the Mn(V)≡N stretching frequency in the protein is 36 cm^{-1} lower. Previously we demonstrated that the resonance Raman spectrum of Mn(III) $Mb \cdot N_3^-$ excited at 406.7 nm exhibits two azide isotope-sensitive lines at 1010 and 2006 cm^{-1} in addition to the bound azide modes (650 cm^{-1}, bending and 2039 cm^{-1}, antisymmetric stretch) (Yu and Tsubaki, 1980). We now identified these two lines as the Mn(V)≡N stretch and its first overtone. Apparently, laser light at 406.7 nm induces photodecomposition of bound azide to generate nitridomanganese(V).

(m) Raman evidence for reciprocal changes in N$_\varepsilon$-Fe-C-O bonds induced by allosteric transitions

Our resonance Raman data provides direct evidence that the Fe-N$_\varepsilon$ (His) bonds are identical between the two affinity states in monomeric CTT Hb system (paper #10), in contrast with the tetrameric Hb where the Fe-N$_\varepsilon$ (His) bond is weaker in the lower affinity (T) deoxy state than in the high affinity (R) deoxy state (Kitagawa, 1984).

Since the Fe-N$_\varepsilon$ (His) bond in the deoxy forms of Hb CTT III and IV is not affected by the pH-induced structural changes, the ligand, prior to binding, must "see" identical binding sites in tense (t) and relaxed (r) states. One must then expect that the difference in the electronic structure of the heme iron between the two affinity states occurs only in the ligated form. The ligand, after binding, induces a conformational change which allows the conformational information to be transferred into the heme-ligand bonds, thereby modulating the ligand dissociation. In other words, the difference in affinity between the two states is reflected only in the dissociation process. This is consistant with the fact that the on-rate of ligand binding to CTTs III and IV is pH-independent, whereas the off-rate is pH-dependent.

Therefore, we investigated the resonance Raman spectra of the two affinity states of the CO-ligated CTTs III and IV. We have identified (via $^{54}Fe/^{57}Fe$ and $^{13}C^{18}O/^{12}C^{16}O$ isotope exchange) the Fe-N$_\varepsilon$ (His) stretching mode at 329 cm^{-1} (pH 5.5) which changes to 317 cm^{-1} (pH 9.5) reflecting the pH-induced confromational transition. The Fe-CO stretching mode is also pH-sensitive changing from 483 cm^{-1} (pH 5.2) to 485 cm^{-1} (pH 9.2) in ^{57}Fe CTT III $\cdot$ $^{13}C^{18}O$ complex. These data provide direct evidence that the so-called "trans-effect" is operative as a control mechanism for ligand-binding in monomeric allosteric insect hemoglobins. In going from the low-affinity to the high-affinity state the Fe-N$_\varepsilon$ (His) bond becomes weaker, whereas the Fe-CO bond becomes stronger (hence the off-rate is smaller).

In summary, we have made important contributions to our understanding of the mechanisms by which various hemoproteins regulate (or control) their heme reactivities for exogenous ligands such as dioxygen (O_2), carbon monoxide (CO), nitric oxide (NO) and cyanide (CN^-). To facilitate the detection of extremely weak Raman signals from the vibrations of metal-ligand moieties, we have relied on our highly sensitive multichannel Raman scattering system which has been considered as one of the best in this country (see previous NIH BBCB Study Section's Summary Statement). We have succeeded in the detection and monitoring of the metal-ligand bonds in allosteric monomeric insect hemoglobins during conformational transitions. We have identified, for the first time, the following important ligand vibrations during the present grand period: ν(Co-NO), ν(Fe-CN^-), δ(Fe-C-N), ν(Fe(III)-NO), δ(Co-O-O), ν(Fe(II)-His) for CO-ligated state, ν(Fe(III)-His) for CN^- ligated state, ν(Ni-His), δ(Fe(III)-N-O, ν(Mn(V)$\equiv$N) , ν(Mn(II)-NO) and tentatively ν(Mn(II)-His) and ν(Co-His) in O_2 ligated state. Previously, we have already identified for the first time, the following vibrations: ν(Fe-CO), δ(Fe-C-O), ν(C-O), ν(Co-O_2), ν(O-O) for Co hemes, ν(Fe(III)-N_3^-) for low- and high-spin, and δ(N=N=N). For correct interpretations of the complex resonance Raman spectra of carbonmonoxy and cyanomet hemoproteins, we have studied the heme model compounds such as "picket fence" porphyrins, imidazole appended hemes, "strapped" hemes and adamantane porphyrin-6,6-cyclophane. We have established a correlation between the Fe-C-O distortion and the resonance Raman enhancement of the δ(Fe-C-O) bending mode. The magnitude and direction of the shifts ν(Fe-CO) and ν(Fe-CN^-) stretching vibrations as a result of Fe-C-O and Fe-C-N^- tilting have also been determined. We have developed a quantitative method for estimating the Fe-C-O bond angle. We have found correlations between ν(Fe-CO) and ν(C-O) stretching vibrations. These results have formed the most important basis for spectral interpretations by others in the CO complexes of various hemoproteins.

More interesting is the discovery of an unusual inverse relationship between affinity and the strength of iron-carbon bond. A stronger iron-carbon bond can correspond to a weaker CO binding affinity. Furthermore, the reciprocal relationship between the iron-N_ε(His) bond and the iron-carbon bond has been demonstrated.

Recently, we challenged the validity of the results of X-ray diffraction studies of carbonmonoxy and cyanomet insect hemoglobin CTT III by Steigeman and Weber. Our resonance Raman studies concluded that the iron-carbon bond length in CTT III·CO cannot be greater than 1.82Å , in disagreement with the 2.4Å value reported by Steigeman and Weber. In addition, we found that the Fe(III)-CN^- bond is longer than the Fe(II)-CO bond, contrary to the results of x-ray diffraction studies.

Studies of cobalt-substituted dimeric insect hemoglobin (Co CTT II) have led to the discovery of the mechanism of the control of dioxygen binding in this allosteric Hb. For the first time, reciprocal behavior between the Co-N and Co-O_2 bonds and between the Co-O_2 and O-O bonds are demonstrated. The results from oxy Co-hemes have been very useful for understanding the structural implications of ν(O-O) resonance enhancement in the oxy Fe cytochrome P_{450}.

In addition, we have demonstrated that heme-rotational disorder in cyanomet CTT Hb's can be monitored by resonance Raman spectroscopy.