

## 2003B期実施開始の長期利用課題研究紹介

財団法人高輝度光科学研究センター  
利用業務部

2003B期に長期利用課題として採択しました2課題につきましては、2006A期に終了し、事後評価が実施され、その評価結果及び成果リストについては、前々回利用者情報 (Vol. 12 No.1) に掲載しました。

今号では、2課題のうち前号の課題に引き続き、  
〔実験課題名〕「Nuclear Resonance Vibrational Spectroscopy (NRVS) of Hydrogen and Oxygen Activation by Biological Systems」を「Nuclear Resonance Vibrational Spectroscopy (NRVS) of Iron-Sulfur Enzymes for Nitrogen Fixation and Hydrogen Metabolism」として掲載いたします。

〔実験責任者〕

Stephen P. Cramer (Department of Applied Science, University of California, Davis and Physical Biosciences

Division, Lawrence Berkeley National Laboratory, Professor)

〔課題名〕

Nuclear Resonance Vibrational Spectroscopy (NRVS) of Hydrogen and Oxygen Activation by Biological Systems

〔課題番号/ビームライン/実施シフト〕

2003B0032-LD3-np	BL09XU	21シフト
2004A2032-LD3-np	BL09XU	21シフト
2004B3032-LD3-np	BL09XU	24シフト
2005A4032-LD3-np	BL09XU	45シフト
2005B0009	BL09XU	21シフト
2006A0009	BL09XU	33シフト

計165シフト

### Nuclear Resonance Vibrational Spectroscopy ( NRVS ) of Iron-Sulfur Enzymes for Nitrogen Fixation and Hydrogen Metabolism

Project Leader :

Stephen P. Cramer (Department of Applied Science, University of California, Davis and Physical Biosciences Division, Lawrence Berkeley National Laboratory, Professor)

Project Member :

Simon J. George (Physical Biosciences Division, Lawrence Berkeley National Laboratory, Research Scientist)

Yisong Guo (Department of Applied Science, University of California, Davis, PhD Student)

Matt C. Smith (Department of Applied Science, University of California, Davis, Postdoctoral)

Hongxin Wang (Department of Applied Science, University of California, Davis, Research Scientist)

Yuming Xiao (Department of Applied Science, University of California, Davis, PhD Student)

## 1 . Introduction

Iron containing metalloproteins play key roles in many important biochemical processes. Our research focuses on two critical iron-sulfur enzymes-nitrogenase and hydrogenase. Nitrogenase ( $N_2$ ase) catalyzes the reduction of dinitrogen to ammonia and this biological ammonia synthesis is responsible for about half of the protein available for human consumption<sup>[1]</sup>. Hydrogenase ( $H_2$ ase) catalyzes the evolution (or consumption) of dihydrogen.  $H_2$  catalysis is crucial for the metabolism of many anaerobic organisms, and knowledge about the mechanism of  $H_2$  evolution may prove critical for a future hydrogen economy<sup>[2]</sup>. Although scientists have studied  $N_2$ ase and  $H_2$ ase for a long time, with significant progress especially after crystal structures of these metalloenzymes came out in 1990s (Figure 1), there are still lots of fundamental questions left such as where substrates bind and interact with these proteins and how structures of active sites change during the course of catalytic cycle.

Nuclear resonance vibrational spectroscopy (NRVS) is a relatively new technique that became available as a spectroscopic method because of third generation synchrotron source and development of x-ray optics with sub-meV resolution<sup>[6]</sup>. It involves scanning an extremely monochromatic x-ray beam through a nuclear resonance. Apart from the familiar 'zero phonon' Mössbauer resonance, there are additional transitions that correspond to nuclear excitation in combination with excitation (Stokes) or de-excitation (anti-Stokes) of vibrational modes. The measurement technique exploits

the relatively long lifetime of the nuclear excitation, along with the pulsed nature of the synchrotron source, by electronically gating on Fe K emission that occurs following internal conversion in between synchrotron pulses. Compared to other well-established vibrational spectroscopic techniques such as infrared spectroscopy and Raman scattering, the biggest advantage of NRVS is its site selectivity. NRVS is only sensitive to vibrations of Mössbauer nuclei (in our case,  $^{57}\text{Fe}$ ). It means now we can observe vibrations of Fe atoms at the active site of  $N_2$ ase and  $H_2$ ase while ignoring interference from other part of proteins.

The goal of our program is to use NRVS to answer structural and dynamical issues of these proteins that are beyond the reach of other methods. We expect to have better understanding of (a) the structure and dynamics of  $N_2$ ase and  $H_2$ ase, (b) how these enzymes are biosynthesized and ultimately (c) their molecular mechanism of catalysis. This information may eventually prove useful for development of synthetic small molecule 'mimics' that can catalyze the same reactions.

## 2 . Experimental

$^{57}\text{Fe}$  NRVS spectra were recorded using published procedures at Beamline 09-XU at SPring-8, Japan<sup>[7]</sup>. During the 3-year period of this long-term proposal, improvements were achieved on both high heat-load pre-monochromator and high resolution monochromator. Experimental resolution was improved from 3.5meV to 1.1meV that is proving sufficient to resolve most of the NRVS details. The flux was  $\sim 3 \times 10^9$  in a 1.1meV

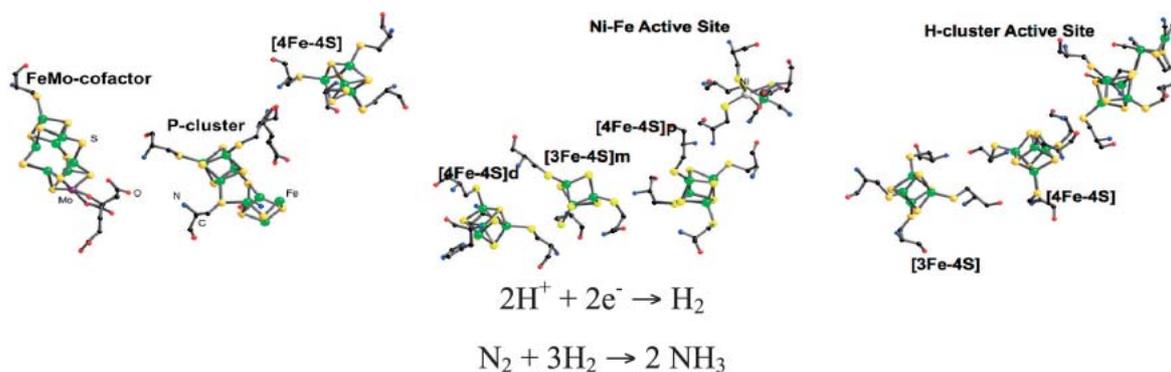


Figure1 . Bioinorganic chemist's view of  $N_2$ ase and  $H_2$ ase. Left to right : clusters in (a) Mo  $N_2$ ase<sup>[3]</sup>, (b) [NiFe]  $H_2$ ase, including 'distal', 'medial', and 'proximal' Fe-S clusters<sup>[4]</sup>, (c) *C. pasteurianum* [FeFe]  $H_2$ ase<sup>[5]</sup>.

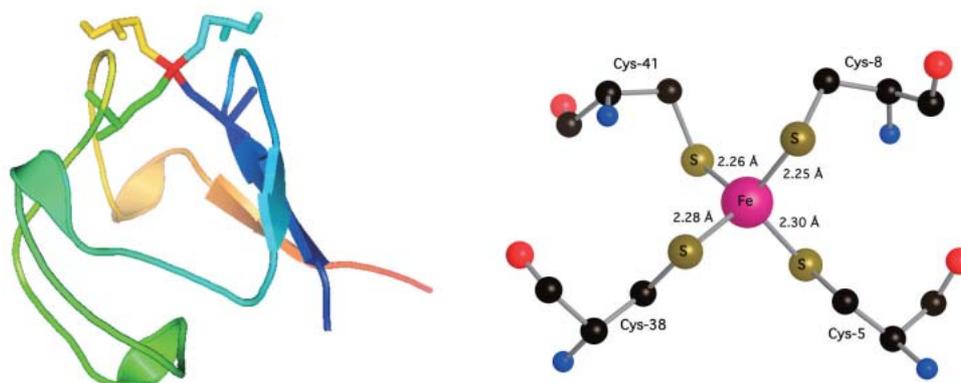


Figure 2 . Left : ' Pymol ' representation of oxidized *Pf* Rd, including sticks for cysteine residues, illustrating exposed location of Fe site (red). Right : ' Crystallmaker ' close-up of Fe site showing slight compression of Fe-SCys8 and Fe-SCys41 bonds and distinction between  $\sim 90^\circ$  FeSCC dihedral angles for ' exposed ' cysteines and  $\sim 180^\circ$  FeSCC dihedral angles for ' buried ' cysteines (PDB code 1BRF).

bandwidth, using a liquid nitrogen-cooled Si(1,1,1) double crystal monochromator followed by asymmetrically cut Ge(4,2,2) and two Si(9,7,5) crystals. During NRVS measurements, samples were maintained at low temperatures using liquid He cryostats. Temperatures were calculated using the ratio of anti-Stokes to Stokes intensity according to:  $S(-E) = S(E)\exp(-E/kT)$ . Nuclear fluorescence and Fe K fluorescence (from internal conversion) were recorded with an APD array at SPring-8 [ 8 ] .

### 3 . Results

#### 3.1 Rubredoxin

Rubredoxins are small ( $\sim 50$  amino acid) electron-transfer proteins that contain a single  $\text{Fe}(\text{S-cys})_4$  redox center [ 9 ] . Apart from their role in specific biological catalytic reactions, rubredoxins serve as model systems for understanding the factors that determine reduction potentials in metalloprotein. High-resolution x-ray crystal structures of different rubredoxins all reveal a roughly tetrahedral  $\text{FeS}_4$  site, often described as approaching  $D_{2d}$  symmetry *via* a compression along an  $S_4$  axis (Figure 2). In *Pf* Rd, this distortion results in 2 compressed ( $\sim 103 \pm 1^\circ$ ) (Cys5S-Fe-SCys41 and Cys8S-Fe-SCys41) and 4 expanded ( $\sim 111$ - $114^\circ$ ) S-Fe-S angles; there are also 2 shorter Fe-S bond lengths (2.25-2.26Å) (Fe-SCys8 and Fe-SCys41) and 2 slightly longer Fe-S bonds (2.28-2.30Å) (Fe-SCys5 and Fe-SCys38) [ 10 ] .

The dynamical properties of the oxidized and reduced

Fe sites play an important role in the redox properties of rubredoxins. Previous Resonance Raman work had shown an asymmetric Fe-S stretch region divided into 3 bands near  $350$ - $370\text{cm}^{-1}$  [ 11 ] and in our Raman spectra we observed these and additional bands out to  $440\text{cm}^{-1}$ . The NRVS was very broad in this region, suggesting that stretching modes are strongly coupled with protein side chain motion. A model with 5-atom chains extending from the Fe site was required to quantitatively reproduce the Fe-S stretch region- quite similar to Goddard's ' chromophore in protein ' model. In the reduced rubredoxin, strong asymmetric Fe-S modes were shifted to  $300$ - $320\text{cm}^{-1}$ . This is the first observation of Fe-S stretching modes in a reduced Rd [ 12 ] .

Very recently, we have performed our first single crystal NRVS measurements on a rubredoxin crystal. Large ( $1\text{mm}^2$ ) crystals were grown in collaboration with Prof. Robert Bau (University of Southern California). The data are excellent and clearly show an orientation dependence in the Fe-S stretches (Figure 3). We are proposing that the higher frequency stretching modes are associated with the shorter Fe-S bond lengths.

#### 3.2 Nitrogenase

Biological nitrogen fixation, involving reduction of dinitrogen to ammonia, is the key reaction in the nitrogen cycle [ 1 ] . In *Azotobacter vinelandii* (*Av*) the Mo-dependent nitrogenase ( $\text{N}_2\text{ase}$ ) consists of two metalloproteins: Fe protein (*Av2*) and MoFe protein (*Av1*)

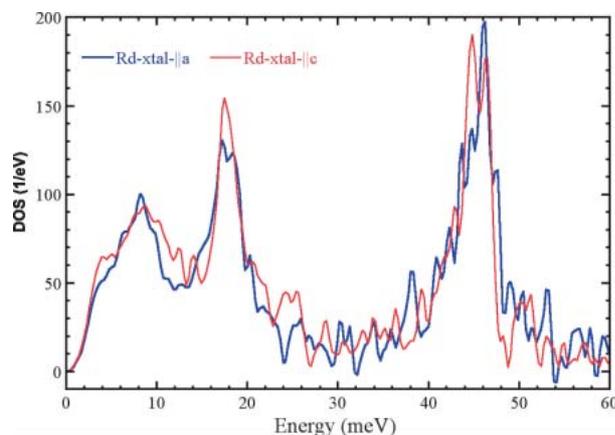


Figure 3 . Orientation dependent NRVS spectra of rubredoxin single crystal.

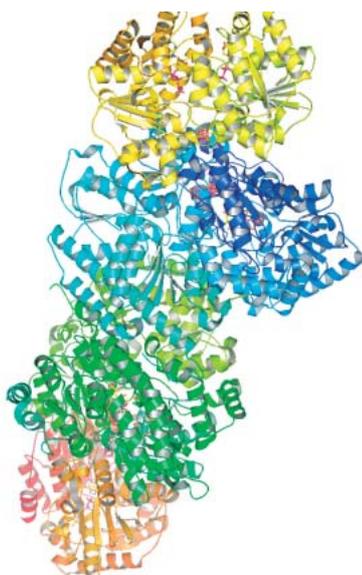


Figure 4 . Cartoon representation of the nitrogenase complex. Top and bottom are Fe proteins (yellow and orange) ; middle is MoFe protein (blue and green). (PDB 1G21)

(Figure 4). The ~ 63kDa Fe protein is a dimer of two identical subunits bridged by a single [4Fe-4S] cluster. In addition to binding the [4Fe-4S] cluster, the second principal functional feature of the Fe-protein is to bind nucleotides, MgATP and MgADP. During catalysis, the Fe protein provides electrons to MoFe protein in a MgATP-dependent reaction and is the only known reductant that will support substrate reduction by the MoFe protein. The ~ 230kDa MoFe protein is composed

of two identical dimers ( $\alpha_2\beta_2$ ) that each consist of two different metal clusters, the iron-molybdenum cofactor (FeMo-co) and the P cluster. The FeMo-cofactor, which locates in a cleft of the  $\alpha$ -subunit, is the active center where substrates bind and react with the enzyme and can be extracted into organic solvent. The P-cluster is buried at the interface between  $\beta$ -subunit and  $\beta'$ -subunit and is believed to be the first electron acceptor from Fe-protein and transport electrons to the FeMo-cofactor [13].

A recent structure for *Av1* at 1.16Å resolution [3] revealed electron density at the center of the trigonal prismatic cage of Fe atoms in the FeMo-cofactor, and hence an overall  $\text{MoFe}_7\text{S}_9\text{X}$  core cluster composition. The electron density is consistent with a light (C, N, or O) atom. Characterization of the interstitial atom is essential for understanding both the biosynthesis of the FeMo-cofactor and the mechanism of nitrogenase. We have used NRVS to study the dynamics of the Fe-S clusters in nitrogenase. The catalytic site FeMo-cofactor exhibits a strong signal near  $190\text{cm}^{-1}$ , where conventional Fe-S clusters have weak NRVS (Figure 5). This intensity is ascribed to cluster breathing modes whose frequency is raised by an interstitial atom. A variety of Fe-S stretching modes are also observed between  $250$  and  $400\text{cm}^{-1}$ . This work is the first spectroscopic information about the vibrational

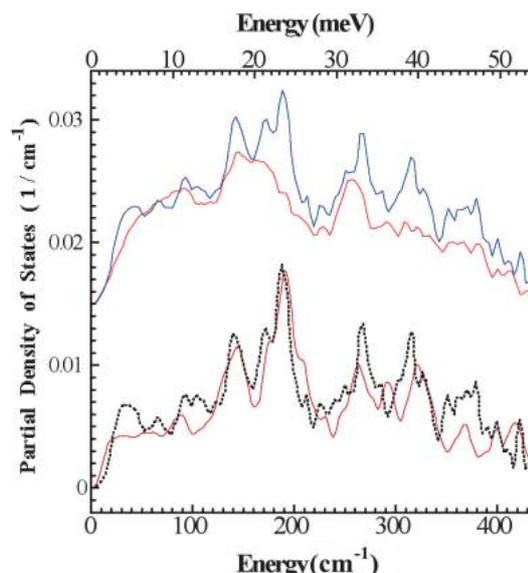


Figure 5 . Experimental  $^{57}\text{Fe}$  PVDOS functions,  $D_{\text{Fe}}(\omega)$ , for (top to bottom) (a) *Av1* (—) vs. *nifE Av1* (---) ; (b) *Av1-nifE:Av1* difference spectrum (—) vs. isolated FeMoco (.....).

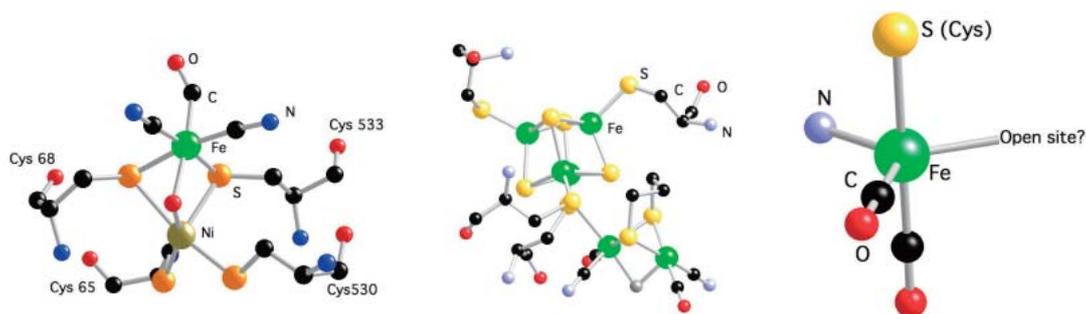


Figure 6 . Active site structure of NiFe H<sub>2</sub>ase from *D. gigas* (left); Active site structure of FeFe H<sub>2</sub>ase from *D. deculfuricans* (middle); and Model of the active site structure of Hmd H<sub>2</sub>ase from *M. marburgensis* (right).

modes of the intact nitrogenase FeMo-cofactor and P-cluster and support the presence of an interstitial atom in both isolated FeMoco and in the *AvI*-bound FeMoco-cofactor [14].

### 3.3 Hydrogenase

Hydrogen (H<sub>2</sub>) metabolism occurs in a large variety of micro-organisms, such as methanogenic, sulfate-reducing, fermentative, nitrogen-fixing, photosynthetic bacteria, where H<sub>2</sub> activation is catalyzed by hydrogenases (H<sub>2</sub>ases) following the reaction:  $H_2 \rightleftharpoons 2H^+ + 2e^-$ . H<sub>2</sub>ases are among the most efficient H<sub>2</sub> catalysts known, with turn over rate ranging up to 6000 molecules of H<sub>2</sub> per second [15]. There are three classes of H<sub>2</sub>ases - [NiFe] H<sub>2</sub>ases [4] (including a [NiFeSe] subset), [FeFe] H<sub>2</sub>ases [5], and Fe-S cluster-free H<sub>2</sub>ases (Hmd) [16]. [NiFe] H<sub>2</sub>ases, which contain Ni-Fe dinuclear catalytic center, are mostly involved in H<sub>2</sub> oxidation, while [FeFe] H<sub>2</sub>ases, which contain Fe-Fe dinuclear catalytic center, are mostly involved in H<sub>2</sub> production. Hmd H<sub>2</sub>ases, which were thought to be 'metal free', are now found to have a Fe mononuclear catalytic center. The enzymes catalyze the reversible reduction of methenyltetrahydromethanopterin (methenyl-H<sub>4</sub>MPT) to methylene-H<sub>4</sub>MPT using H<sub>2</sub>. The comparison of the active site structures (Figure 6) of the three types of H<sub>2</sub>ases has revealed common features, which is the Fe sites are all terminally bound with nonprotein hexogenous diatomic ligands CO and/or CN. This is an indication of convergent evolution, and the structure similarities are most probably essential for an efficient activation of H<sub>2</sub>. During this long-term proposal period, we have studied the active sites of these three types of H<sub>2</sub>ases in different enzyme states using NRVS

along with some inorganic model complexes.

#### 3.3.1 Hmd H<sub>2</sub>ase

The Hmd H<sub>2</sub>ase we studied is from the methanogenic archaeon *Methanothermobacter marburgensis* (DSMZ2133). The structure of the active site is not yet known, the recent IR [17], Mössbauer [18], and EXAFS [19] spectra revealed that the active site contains one low spin Fe (Fe(0) or Fe(-)), one S from cysteine, two CO ligands and one N from pyridone cofactor, also a possible vacant site.

We have examined the as-isolated Hmd H<sub>2</sub>ase at pH8, the H<sub>2</sub><sup>18</sup>O exchanged Hmd H<sub>2</sub>ase at pH8, and the Hmd H<sub>2</sub>ase under the presence of H<sub>2</sub> and methenyl-H<sub>4</sub>MPT+ at pH6. The pH8 state is thought to be enzyme resting state; the pH6 state is thought to be active state. The NRVS spectra are shown in Figure 7, they have been interpreted by comparison with a *cis*-(CO)<sub>2</sub>-ligated Fe model complex (Figure 7) as well as by normal mode simulations of a plausible 5-coordinated active site structure. The simulation on the as-isolated pH8 Hmd supports a *cis*-(CO)<sub>2</sub> geometry for the active site of Hmd protein, also it gives further insight into the dynamics of the Fe site, revealing Fe-CO stretch and Fe-CO bend modes at 494, 562, 590, and 648cm<sup>-1</sup>. The NRVS also reveals a band assigned to Fe-S stretching motion at ~311cm<sup>-1</sup>, which is observed in all Hmd samples we examined. A peak at ~379cm<sup>-1</sup> is tentatively assigned to a bound water or hydroxide ligand., which is clearly seen in the pH8 and pH6 spectra, but it is significantly weaker in the H<sub>2</sub><sup>18</sup>O spectrum. We also find from the simulations that the cysteine and the pyridone ring motions definitely have noticeable contributions to the

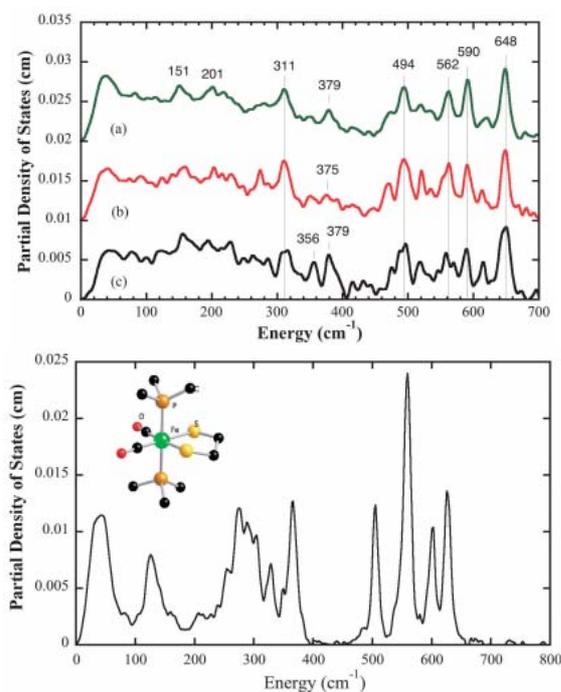


Figure 7 . Top : NRVS PVDOS for (a) as-isolated Hmd in 50mM Tricine/NaOH buffer at pH8, (b) as-isolated Hmd 50mM Tricine/NaOH buffer exchanged in  $^{18}\text{O}$  water at pH8, (c) Hmd in 50mM Mes-NaOH buffer under the presence of  $\text{H}_2$  and methenyl-H4MPT $^+$  at pH6.0; Bottom: NRVS PVDOS of mononuclear cis-(CO) $_2$  complex (Inset is the structure of this complex).

NRVS spectrum, and can influence the overall shape of the spectrum, especially for the low frequency region.

Application of the NRVS technique to the Hmd protein has allowed us for the first time to observe the dynamics of the Fe-CO bending and stretching motion. However, since we do not have exact structure for the active site at this moment, we will wait for the crystal structure of Hmd holo-enzyme to obtain detail simulations on the Hmd NRVS.

### 3.3.2 [NiFe] and [FeFe] H<sub>2</sub>ases

For [NiFe] and [FeFe] H<sub>2</sub>ases study, we still focused on revealing structure and vibrational dynamics on the Fe center of the active centers. We started from two dinuclear Fe model complexes of the [FeFe] H<sub>2</sub>ase active site,  $[\text{NEt}_4][\text{Fe}(\text{S}_2\text{C}_3\text{H}_6)(^{12}\text{CN})_2(\text{CO})_4]$  and  $[\text{NEt}_4][\text{Fe}(\text{S}_2\text{C}_3\text{H}_6)(^{13}\text{CN})_2(\text{CO})_4]$  [20] (Figure 8). The features between 500cm<sup>-1</sup> and 670cm<sup>-1</sup> in both NRVS spectra were contributed mainly from Fe-CO stretching and bending motions. The clearly shift of the features

between 400cm<sup>-1</sup> and 500cm<sup>-1</sup> was due to  $^{12}\text{C}/^{13}\text{C}$  isotope shift on CN- ligands, also we observed Fe-S motions around 300cm<sup>-1</sup> and Fe-Fe stretching motion at ~ 200cm<sup>-1</sup>. These findings are consistent with the published resonant Raman spectra of the same model complexes [21].

For the real enzyme samples, we have measured the first spectra of the as-isolated [NiFe] H<sub>2</sub>ase from *Desulfovibrio vulgaris Miyazaki F* and the as-isolated [FeFe] H<sub>2</sub>ase from *Clostridium acetobutylicum* using NRVS (Figure 8). Both [NiFe] and [FeFe] H<sub>2</sub>ases have more than 10 Fe atoms in each protein molecule. Only one Fe atom for [NiFe] H<sub>2</sub>ase and two Fe atoms for [FeFe] H<sub>2</sub>ase at the active centers, other Fe atoms within each molecule are belong to FeS clusters, which involve in electron transfer pathway during the enzyme catalysis. From Figure 8, we can see that the features with large intensities between 100cm<sup>-1</sup> and 420cm<sup>-1</sup> were mainly contributed from those FeS clusters, which can be compared with our NRVS spectrum of 4Fe ferridoxin. 4Fe ferridoxin contains one 4Fe4S cluster in each molecule. The tremendous Fe sites prevent us from detail study on the active site Fe center of both [NiFe] and [FeFe] H<sub>2</sub>ases.

However, the current results do provide us with useful information. Comparing with the model complex studies

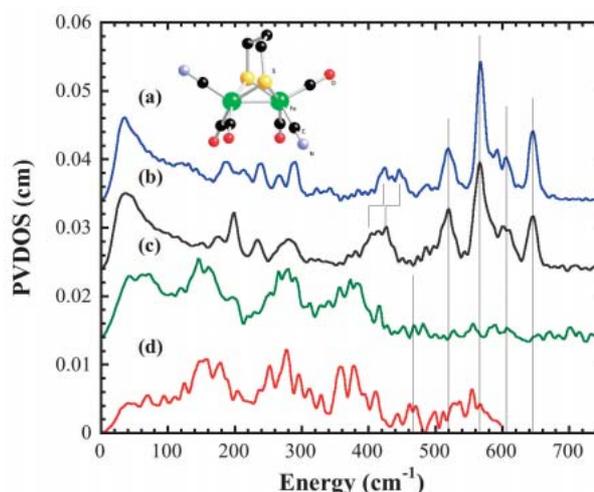


Figure 8 . NRVS PVDOS of (a)  $[\text{NEt}_4][\text{Fe}(\text{S}_2\text{C}_3\text{H}_6)(^{12}\text{CN})_2(\text{CO})_4]$ , (b)  $[\text{NEt}_4][\text{Fe}(\text{S}_2\text{C}_3\text{H}_6)(^{13}\text{CN})_2(\text{CO})_4]$ , (c) as-isolated NiFe H<sub>2</sub>ase from *Desulfovibrio vulgaris Miyazaki F*, (d) as-isolated FeFe H<sub>2</sub>ase from *Clostridium acetobutylicum*; Inset is the structure of  $\text{Fe}(\text{S}_2\text{C}_3\text{H}_6)(\text{CN})_2(\text{CO})_4$ .

and Hmd H<sub>2</sub>ase studies, the features between 500cm<sup>-1</sup> and 620cm<sup>-1</sup> in [NiFe] H<sub>2</sub>ase spectrum and the features between 480cm<sup>-1</sup> and 600cm<sup>-1</sup> in [FeFe] H<sub>2</sub>ase spectrum were possible Fe-CO stretching and bending motions contributed from Fe center of the active sites, while the features between 420cm<sup>-1</sup> and 500cm<sup>-1</sup> in [NiFe] H<sub>2</sub>ase spectrum and the features between 420cm<sup>-1</sup> and 480cm<sup>-1</sup> in [FeFe] H<sub>2</sub>ase spectrum were possible Fe-CN stretching and bending motions.

Since we have already obtained promising results on rubredoxin crystals using NRVS mentioned in Section 3.1, we are now trying to get H<sub>2</sub>ase crystals to perform crystal NRVS. In this way, we can selectively excite the vibrational modes from Fe centers at the H<sub>2</sub>ase active sites, then more clear spectra on the Fe centers can be obtained, and detailed studies can be conducted. Combined with isotopic labeling of ligands at the active sites, characterization on the structure and dynamics of the H<sub>2</sub>ase active sites using NRVS is well possible.

#### 4 . Summary

The results presented above illustrate that NRVS has a role to play in the ever increasingly complex attack on unraveling the secrets of metalloenzymes, and no doubt with continued future development will become more routine and readily available. In the future, we will pursue our single crystal work on nitrogenase and NiFe hydrogenase as well as FeMoco biosynthesis work. As and when it becomes available on BL09XU, we intend to use <sup>61</sup>Ni NRVS of appropriate Ni models and eventually NiFe H<sub>2</sub>ase and other Ni proteins. A site-selective probe of Ni center vibration modes will be very useful and should allow major advances in understanding Ni biochemistry.

#### Acknowledgment

We would like to thank Dr. Yoshitaka Yoda for his dedication on developing BL09XU at SPring-8, and his experience and help during the three-year period of this long-term proposal. We also would like to thank all the collaborators, who have been continuously providing us excellent samples. They are Dr. Francis E. Jenney, Jr. and Prof. Michael W. W. Adams at University of Georgia, US for rubredoxin samples, Dr. Karl Fisher and

Prof. William E. Newton at Virginia Tech, US for N<sub>2</sub>ase samples, Prof. Seigo Shima and Prof. Rolf K. Thauer at the Max Planck Institute for Terrestrial Microbiology, Germany for Hmd H<sub>2</sub>ase, Prof. Yoshiki Higuchi at University of Hyogo, Japan for NiFe H<sub>2</sub>ase, Dr. Paul King from National Renewable Energy Laboratory, US For FeFe H<sub>2</sub>ase, and Prof. Thomas B. Rauchfuss from University of Illinois, Urbana-Champaign, US for H<sub>2</sub>ase active site model complexes. Finally, we would like to thank our previous group members, Mr. Philip Titler and Mr. Gregory Mcnerney for assistance with our experiments. This work was funded by NIH GM-65440 (SPC), EB-001962 (SPC), and the DOE Office of Biological and Environmental Research (SPC).

#### Publication List

- 1 . Y. Xiao, H. Wang, S. J. George, M. C. Smith, M. W. W. Adams, J. Francis E. Jenney, W. Sturhahn, E. E. Alp, J. Zhao, Y. Yoda, A. Dey, E. I. Solomon and S. P. Cramer: Normal Mode Analysis of *Pyrococcus furiosus* Rubredoxin via Nuclear Resonant Vibrational Spectroscopy (NRVS) and Resonance Raman Spectroscopy. *Journal of the American Chemical Society* **127**(2005) 14596 - 14606.
- 2 . Y. Xiao, K. Fischer, M. C. Smith, W. Newton, D. A. Case, S. J. George, H. Wang, W. Sturhahn, E. E. Alp, J. Zhao, Y. Yoda and S. P. Cramer: How Nitrogenase Shakes - Initial Information about P-Cluster and FeMo-Cofactor Normal Modes from Nuclear Resonance Vibrational Spectroscopy (NRVS). *Journal of the American Chemical Society* **128** (2006) 7608-7612.
- 3 . M. Tan, A. R. Bizzarri, Y. Xiao, S. Cannistraro, T. Ichiye, C. Manzoni, G. Cerullo, M. W. W. Adams, J. Francis E. Jenney and S. P. Cramer: Observation of Terahertz Vibrations in *Pyrococcus furiosus* Rubredoxin via Impulsive Coherent Vibrational Spectroscopy and Nuclear Vibrational Spectroscopy-Interpretation by Molecular Mechanics. *Journal of Inorganic Biochemistry* **101**(2006) 375-384.

#### References

- [ 1 ] B. E. Smith, R. L. Richards and W. E. Newton : Catalysis for Nitrogen Fixation - Nitrogenases,

- Relevant Chemical Models and Commercial Processes. *Springer* (2004).
- [ 2 ] K. A. Vicent, J. A. Cracknell, A. Parkin and F. A. Armstrong : Hydrogen cycling by enzymes : electrocatalysis and implications for future energy technology. *Dalton Trans.* (2005) 3397-3403.
- [ 3 ] O. Einsle, F. A. Tezcan, S. L. A. Andrade, B. Schmid, M. Yoshida, J. B. Howard and D. C. Rees : Nitrogenase MoFe-Protein at 1.16 Å Resolution : A Central Ligand in the FeMo-Cofactor. *Science* **297**(2002) 1696-1700.
- [ 4 ] A. Volbeda, M. H. Charon, C. Piras, E. C. Hatchikian, M. Frey and J. C. Fontecilla-Camps : Crystal Structure of the Nickel-Iron Hydrogenase from *Desulfovibrio gigas*. *Nature* **373**(1995) 580-587.
- [ 5 ] J. W. Peters, W. N. Lanzilotta, B. J. Lemon and L. C. Seefeldt : X-ray Crystal Structure of the Fe-Only Hydrogenase (CpI) from *Clostridium pasteurianum* to 1.8 Å Resolution. *Science* **282**(1998) 1853-1858.
- [ 6 ] J. T. Sage, C. Paxson, G. R. A. Wyllie, W. Sturhahn, S. M. Durbin, P. M. Champion, E. E. Alp and W. R. Scheidt : Nuclear resonance vibrational spectroscopy of a protein active-site mimic. *Journal of Physics : Condensed Matter* **13**(2001) 7707-7722.
- [ 7 ] Y. Yoda, M. Yabashi, K. Izumi, X. W. Zhang, S. Kishimoto, S. Kitao, M. Seto, T. Mitsui, T. Harami, Y. Imai and S. Kikuta : Nuclear resonant scattering beamline at SPring-8. *Nuclear Instruments & Methods in Physics Research Section A - Accelerators Spectrometers Detectors & Associated Equipment* **467**(2001) 715-718.
- [ 8 ] S. Kishimoto, Y. Yoda, M. Seto, S. Kitao, Y. Kobayashi, R. Haruki and T. Harami : Array of avalanche photodiodes as a position-sensitive x-ray detector. *Nuclear Instruments & Methods In Physics Research Section A* **513**(2004) 193-196.
- [ 9 ] J. Meyer, and J.-M. Moulis : Rubredoxin, in *Handbook of Metalloproteins* (A. Messerschmidt and R. Huber, Eds.) Wiley, New York. (2001) pp 505-517.
- [ 10 ] R. Bau, D. C. Rees, D. M. Kurtz, R. A. Scott, H. S. Huang, M. W. W. Adams and M. K. Eidsness : Crystal structure of rubredoxin from *Pyrococcus furiosus* at 0.95 Å resolution, and the structures of N-terminal methionine and formylmethionine variants of PfRd. Contributions of N-terminal interactions to thermostability. *Journal of Biological Inorganic Chemistry* **3**(1998) 484-493.
- [ 11 ] R. S. Czernuszewicz, L. K. Kilpatrick, S. A. Koch and T. G. Spiro : Resonance Raman Spectroscopy of Iron( ) Tetrathiolate Complexes : Implications for the Conformation and Force Field of Rubredoxin. *Journal of the American Chemical Society* **116**(1994) 1134-1141.
- [ 12 ] Y. Xiao, H. Wang, S. J. George, M. C. Smith, M. W. W. Adams, J. Francis E. Jenney, W. Sturhahn, E. E. Alp, J. Zhao, Y. Yoda, A. Dey, E. I. Solomon and S. P. Cramer : Normal Mode Analysis of *Pyrococcus furiosus* Rubredoxin via Nuclear Resonant Vibrational Spectroscopy (NRVS) and Resonance Raman Spectroscopy. *Journal of the American Chemical Society* **127**(2005) 14596 -14606.
- [ 13 ] D. C. Rees, F. A. Tezcan, C. A. Haynes, M. Y. Walton, S. Andrade, O. Einsle and J. B. Howard : Structural basis of biological nitrogen fixation. *Philosophical TRANSACTIONS OF THE ROYAL SOCIETY OF LONDON SERIES A* **363**(2005) 971-984.
- [ 14 ] Y. Xiao, K. Fischer, M. C. Smith, W. Newton, D. A. Case, S. J. George, H. Wang, W. Sturhahn, E. E. Alp, J. Zhao, Y. Yoda and S. P. Cramer : How Nitrogenase Shakes - Initial Information about P-Cluster and FeMo-Cofactor Normal Modes from Nuclear Resonance Vibrational Spectroscopy (NRVS). *Journal of the American Chemical Society* **128**(2006) 7608-7612.
- [ 15 ] M. Frey : Hydrogenases : Hydrogen-Activating Enzymes. *ChemBioChem* **3**(2002) 153-160.
- [ 16 ] S. Shima and S. K. Thauer : A Third Type of Hydrogenase Catalyzing H<sub>2</sub> Activation. *The Chemical Record* **7**(2007) 37-46.
- [ 17 ] E. J. Lyon, S. Shima, R. Boecher, R. K. Thauer, F.-W. Grevels, E. Bill, W. Roseboom and S. P. J. Albracht : Carbon Monoxide as an Intrinsic Ligand to Iron in the Active Site of the Iron-Sulfur-Cluster-Free Hydrogenase H<sub>2</sub>-Forming Methylenetetrahydromethanopterin Dehydrogenase As Revealed by Infrared Spectroscopy. *Journal of the American Chemical Society* **126**(2004) 14239-14248.
- [ 18 ] S. Shima, E. J. Lyon, R. K. Thauer, B. Mienert, and

- E. Bill : Mössbauer Studies of the Iron-Sulfur-Cluster-Free Hydrogenase : The Electronic State of the Mononuclear Fe Active Site. *Journal of the American Chemical Society* **127**(2005) 10430-10435.
- [ 19 ] M. Korbas, S. Vogt, W. Meyer-Klaucke, E. Bill, E. J. Lyon, R. K. Thauer and S. Shima : The iron-sulfur-cluster-free hydrogenase (Hmd) is a metalloenzyme with a novel iron binding motif. (2006).
- [ 20 ] F. Gloaguen, J. D. Lawrence, M. Schmidt, S. R. Wilson and T. B. Rauchfuss : Synthetic and structural studies on  $[\text{Fe}_2(\text{SR})_2(\text{CN})_x(\text{CO})_{(6-x)}](x-)$  as active site models for Fe-only hydrogenases. *Journal of the American Chemical Society* **123**(2001) 12518-12527.
- [ 21 ] A. T. Fiedler and T. C. Brunold : Combined Spectroscopic/Computational Study of Binuclear Fe(I)-Fe(I) Complexes : Implications for the Fully-Reduced Active-Site Cluster of Fe-Only Hydrogenases. *Inorganic Chemistry* **44**(2004) 1794-1809.

Stephen P. Cramer

Department of Applied Science, University of California, Davis  
One Shields Avenue, Davis, CA 95616, USA  
TEL : (+1)530-752-0360 FAX : (+1)530-752-2444  
e-mail : spcramer@ucdavis.edu