

長期利用課題報告

Nuclear Resonance Vibrational Spectroscopy (NRVS) of Iron-Sulfur Enzymes for Hydrogen Metabolism, Nitrogen Fixation, & Photosynthesis

Project Leader: Stephen P. Cramer^{1,2}

Other members: Lifen Yan¹, Hongxin Wang^{1,2}, Devrani Mitra¹, Yisong Guo¹, Christie Dapper³, William E. Newton³, Yuichi Fujita⁴, Hideaki Ogata⁵, Wolfgang Lubitz⁵, Jon M. Kuchenreuther⁶, James R. Swartz⁶, Yoshitaka Yoda⁷

¹ Department of Applied Science, University of California, Davis, CA 95616

² Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720

³ Department of Biochemistry, Virginia Polytechnic Institute, Blacksburg, VA 24061

⁴ School of Agriculture, Nagoya University, Nagoya, Japan

⁵ Max-Planck Institut für Bioorganische Chemie, Mülheim an der Ruhr, Germany

⁶ Department of Chemical Engineering, Stanford University, Stanford, CA 94305

⁷ SPring-8 / JASRI

Introduction

Fe-S proteins serve a wide variety of essential tasks in living systems, including electron transfer within proteins, catalysis of chemical reactions, sensing of the chemical environment, regulation of DNA expression, repair of damaged DNA, and maintenance of molecular structure [1]. The significance of understanding the structure and function of these proteins can hardly be overstated. We have been studying Fe-S enzymes that catalyze some of biology's most important reactions. The fixation of molecular N₂ to two molecules of NH₃ by nitrogenase supplies the chemically reactive N needed for building proteins and nucleic acids. In photosynthetic bacteria and green algae, the reduction of protochlorophyllide to chlorophyllide *a* creates a key light-trapping molecule for biological photosynthesis. This reaction is catalyzed by dark-operative protochlorophyllide reductase (DPOR), an enzyme with significant homology to nitrogenase. The production or consumption of molecular H₂ by hydrogenase is as fast as the best artificial fuel cells, but uses earth-abundant Fe instead of rare and expensive Pt. Overall, Fe-S enzymes help shape our environment and make life as we know it feasible.

NRVS

Mössbauer spectroscopy provides chemical information about a sample from small changes in nuclear transition energies. This is possible because for some events, the *recoilless fraction* f , a γ -ray is absorbed without energy transfer to molecular vibrations or phonons. On the other hand, in the recoil fraction $(1-f)$, nuclear transitions *do* couple to vibrations. Metal-ligand stretching and bending frequencies range from ~ 50 - 2000 cm⁻¹, or ~ 6 - 250 meV. These values are orders of magnitude greater than hyperfine splittings, and vibrations are not directly observed in conventional Mössbauer spectroscopy. However, as shown in Figure 2, vibrational transitions are observed in NRVS experiments at 3rd generation synchrotron radiation sources such as SPring-8. NRVS is exciting for studies of Fe-S enzymes because it provides an isotope selective vibrational spectrum. Only modes that involve displacement of the ⁵⁷Fe nucleus couple to the nuclear transition. The fraction of the excitation probability $S(\)$ that goes into a transition is proportional to the fraction of kinetic energy due to ⁵⁷Fe in the given vibrational mode.

In a typical NRVS experiment, such as at SPring-8 beamline 9, an undulator is the source of x-rays, which are

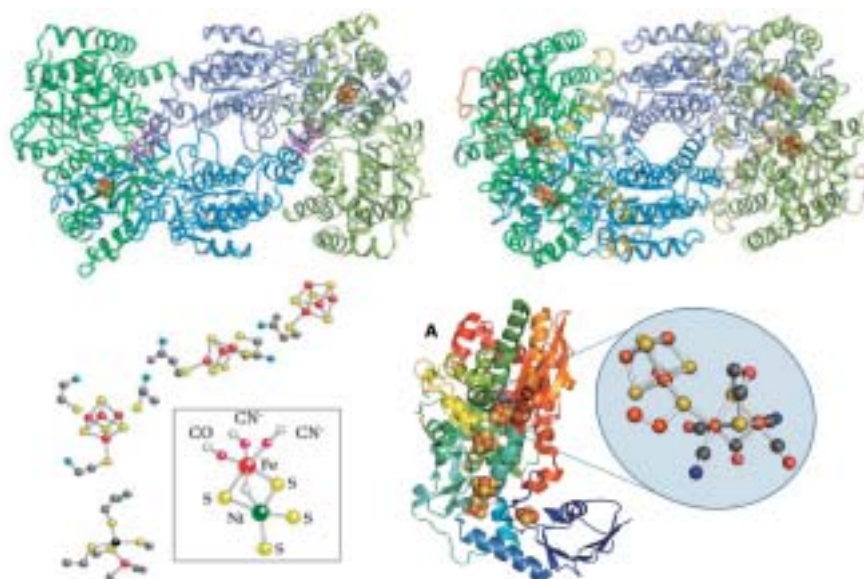


Figure 1 Key enzymes in this study. Top: BchNB (catalytic unit of dark-operative proto-chlorophyllide reductase (left) vs. (right) N₂ase MoFe protein [2]; lower left: clusters in [NiFe] H₂ase and highlighted [NiFe]-cluster, lower right: intact [FeFe] H₂ase and highlighted H-cluster.

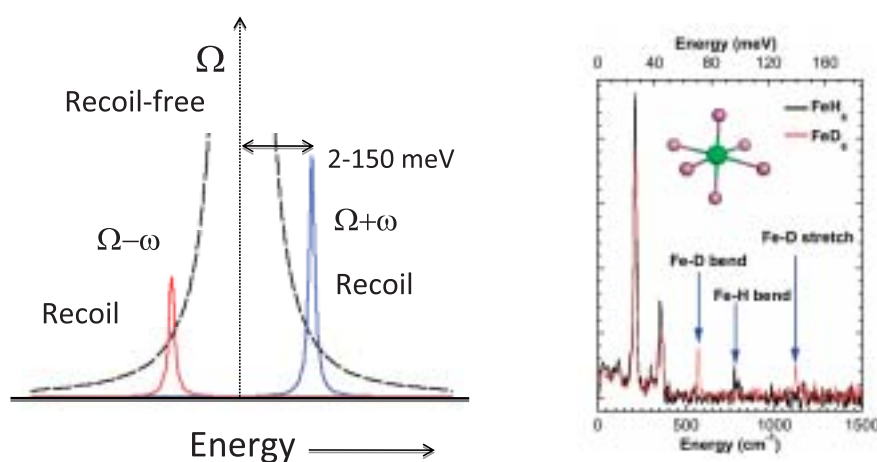


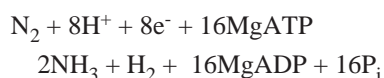
Figure 2 Left: Main NRVS regions. Ordinate is 'counts'. Right: NRVS for Fe-H/D vibrations.

filtered to a bandpass of 1 eV by a premonochromator. A second high-resolution monochromator then narrows the bandpass to ~1 meV. An avalanche photodiode (APD) with ~1 ns time resolution detects delayed Fe fluorescence and distinguishes it from scattered radiation.

Nitrogenase

Biological nitrogen fixation, involving reduction of dinitrogen to ammonia, is the key reaction in the nitrogen cycle and the ultimate source for most N in living

systems, via the reaction [3]:



In *Azotobacter vinelandii* (Av) the Mo-dependent N₂ase that accomplishes this reaction uses electrons from an Fe₄S₄ cluster in an Fe₂ 'Fe protein' (Av2) to reduce a larger Fe₂ 'MoFe protein' (Av1). Within Av1, an Fe₈S₇ 'P-cluster' supplies electrons to the active site MoFe₇S₉ 'FeMo-cofactor' (Figure 1) [4]. There are also N₂-reducing enzymes based on VFe- or even FeFe-

cofactors.

As a prelude to studying the more difficult topic of N_2 binding, we chose to first examine the better-defined complexes of N_2 ase with CO. The inhibition of nitrogen fixation by CO has been known since at least 1941 [5]. The nature of CO interactions with N_2 ase has an added significance because there are (unpublished) reports of hydrocarbon synthesis (Fischer-Tropsch chemistry) during CO reactions with VFe N_2 ase. CO inhibits the reduction of N_2 and other substrates reversibly and non-competitively [6], at micromolar concentrations and on a sub-second time scale consistent with the rate of electron transfer [7]. CO does not, however, inhibit H_2 evolution by N_2 ase so that, under CO, N_2 ase effectively becomes an ATP-dependent hydrogenase.

Exposure of N_2 ase to CO during turnover elicits species with a variety of EPR signals, namely 'lo-CO', 'hi-CO', and 'hi(5)-CO', depending on the partial pressure of CO ([CO]) [7]. Each of these species and its characteristic EPR signal is described by the [CO] required for its formation [8,9]. The 'hi-CO' species that we have chosen to examine by NRVS has an axial EPR signal (g -values near 2.17 and 2.06) and is formed under a [CO] many times higher than required for substrate inhibition. This 'hi-CO' N_2 ase is reported to contain two CO molecules bound to the FeMo-cofactor [10].

As illustrated in Figure 3, we have been able to observe distinct changes in N_2 ase NRVS upon treatment to produce the 'hi-CO' species. There was a significant loss

in intensity in the peak around 190 cm^{-1} . In the past we associated this region with cluster 'breathing' modes [11], and it is clear that these are affected by CO binding. More informative (and difficult to see) is the development of a band at 508 cm^{-1} that we associate with Fe-CO stretching. Although there have been numerous indirect studies implicating binding at Fe, the NRVS is the first data to unambiguously prove and characterize the strength of the Fe-CO bonding. In collaborations with theorists Case, Noodleman, and Pelmentschikov, we have pursued a theoretical DFT analysis of the N_2 ase CO interaction, and this has yielded the structure proposed in Figure 3.

BchNB

As illustrated in Figure 1, the enzyme DPOR has a protein structure quite similar to N_2 ase. However, instead of the P-cluster and FeMo cofactor, as part of its active site it uses a special [4Fe-4S] cluster, with 3 cysteine and 1 aspartate ligands (Figure 4). It turns out that we had already studied a small ferredoxin with the same ligation – *Pyrococcus furiosus* ferredoxin (*Pf Fd*). Although there are broad similarities in their NRVS, there are also significant differences, and our goal is to understand what is special about the DPOR cluster. Of particular interest is a band just above 50 meV ($>400\text{ cm}^{-1}$) that may be associated with Fe-O (Asp) stretching. In *Pf Fd* it has been proposed that a 'carboxylate shift' occurs with this residue upon protein reduction [12].

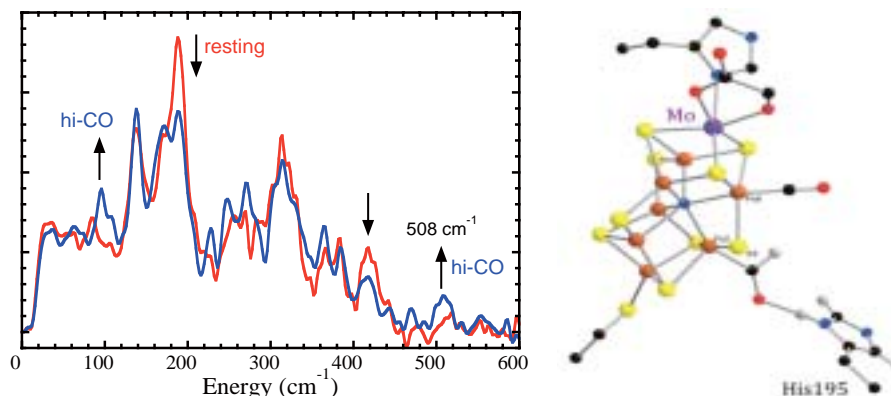


Figure 3 Left: N_2 ase FeMo cofactor NRVS with () and without () CO. Ordinate represents ^{57}Fe partial vibrational density of states. Arrows indicate direction of change with CO. Right: DFT-based structure for proposed CO/CHO complex.

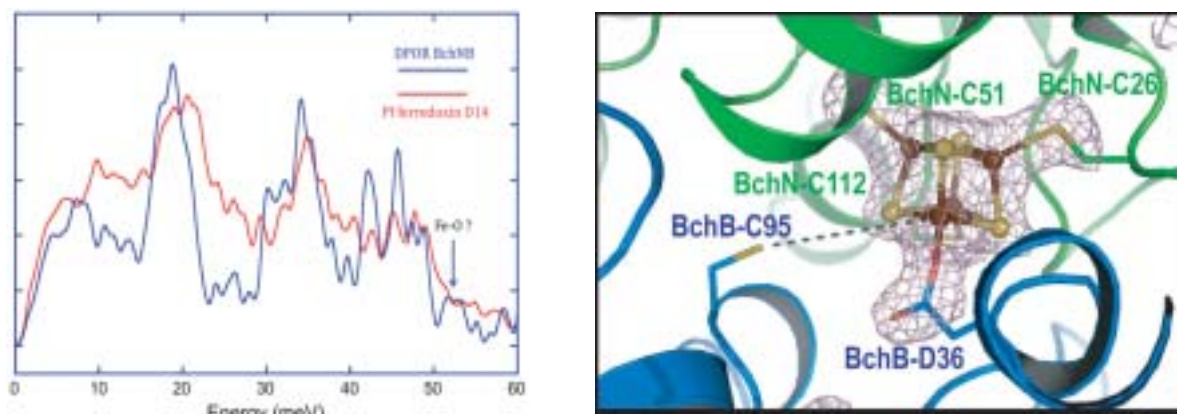
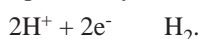


Figure 4 Left: Comparison of NRVS for *Pyrococcus furiosus* ferredoxin () and DPOR BchNB protein (). Right: Structure of the [4Fe-4S] cluster, showing special sidechain (D36) [2].

Hydrogenases

In living organisms, H₂ uptake and evolution is accomplished by the enzyme H₂ase [13]:



There are three phylogenetically unrelated H₂ases [14]: [NiFe] [15], [FeFe] [16], and [Fe] H₂ases [17]. Apart from their biochemical significance, these enzymes have generated intense technological interest because of their relevance for a future H₂ economy [13]. [NiFe] H₂ases are found in many bacteria and archaea, and in these enzymes, catalysis occurs at an unusual [Ni-Fe] cluster (Figure 5) [18]. The catalytic sites of [FeFe] H₂ases contain an ‘H-cluster’, comprised of a [4Fe-4S] cluster bridged to a unique [2Fe]_H subcluster (Figure 5). These [FeFe] H₂ases are among the most efficient H₂ catalysts known, with K_{cat} values that range up to 6000 molecules of H₂ s⁻¹ [19]. Crystal structures of two types of [FeFe]

H₂ase, CpI from *C. pasteurianum* and DdH from *D. desulfuricans*, have been solved [16,20]. [Fe] H₂ase, also known as Hmd, catalyzes the transfer of a hydride ion from H₂ to methenyl-H₄MPT⁺, yielding methenyl-H₄MPT⁺. Originally thought to be ‘metal-free’, it is now known to contain an Fe bound by 2 CO molecules and an organic cofactor (Figure 5) [17].

During the course of our project we examined all three types of H₂ase. Since Hmd [Fe] H₂ase has only one type of Fe, with two CO ligands, it presented especially strong NRVS in the high frequency region (Figure 6). Although we originally expected 4 Fe-CO modes (from two coupled Fe-CO bend and two coupled Fe-CO stretch modes), we saw a more complicated pattern with at least 6 strong bands. We now believe the additional features arise from coupling with Fe-CO modes for the acyl coordination recently discovered in the revised crystal structure (Figure 5). We

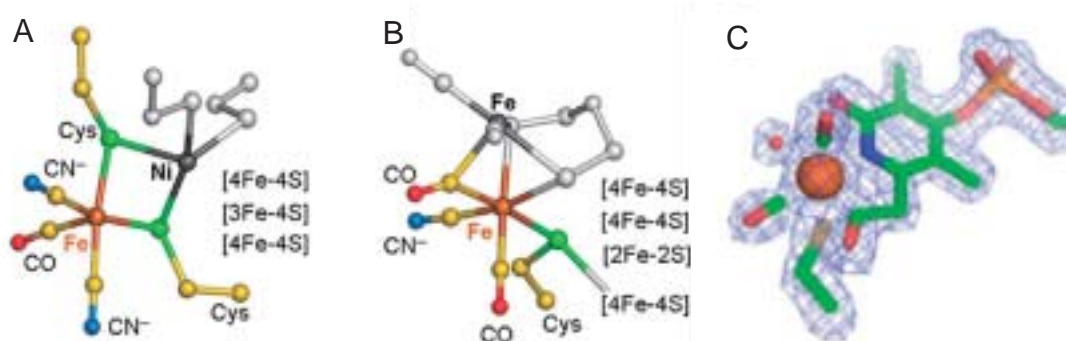


Figure 5 Left to right: (A) [NiFe] H₂ase active site structure. (B) [FeFe] H₂ase structure. (C) Recently revised structure of Hmd [Fe] H₂ase, highlighting Fe-acyl coordination [17].

also saw evidence for an exchangeable water ligand with Fe-O mode at 379 cm^{-1} .

We next examined the more difficult [NiFe] and [FeFe] H_2 ases. Although the [NiFe] H_2 ase has only one active site Fe in the presence of 11 other Fe-S cluster Fe (Figure 1). We were still able to see very weak features between 500 and 600 cm^{-1} due to Fe-CO bend and stretch modes (Figure 6). Even more promising, when ^{57}Fe is selectively incorporated into the H-cluster of CPI H_2 ase, the Fe-CN and Fe-CO modes are clearly visible. We hope to use NRVS on both systems, both to characterize catalytic intermediates and also (using isotopic labeling of biosynthetic precursors) to track and define the biosynthesis of the active sites.

Summary & Conclusion

We have found NRVS to be an extremely valuable spectroscopic probe of Fe-S proteins. Our preliminary surveys have shown that weak Fe-ligand interactions can be seen in the presence of the dominant Fe-S cluster signals. In the future, with more flux and better resolution, we hope to use NRVS to define the catalytic mechanisms of many of these important enzymes.

Acknowledgements

This work was funded by NIH GM-65440, NSF CHE-0745353, and the DOE Office of Biological and Environmental Research. We thank SPring-8 and JASRI

for support of beam time.

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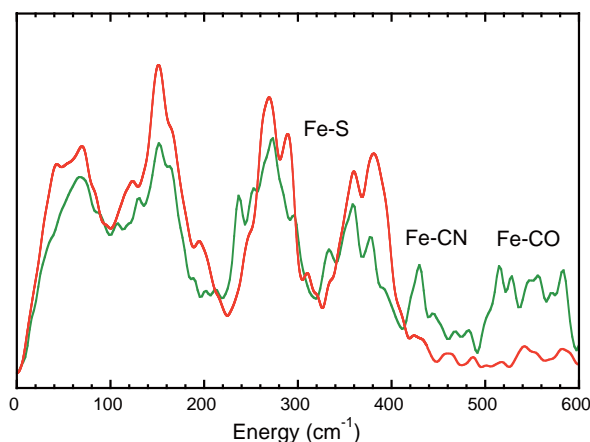
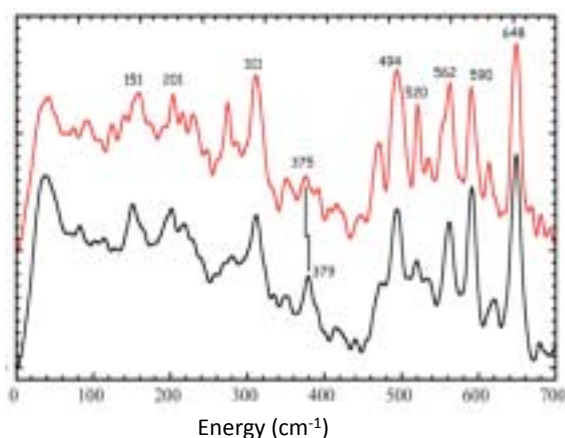


Figure 6 Left: The strong Fe-CO bend and stretch modes (494-649 cm^{-1}) in [Fe] H_2 ase. The 379-375 cm^{-1} shift is assigned to ^{18}O -substituted H_2O in the latter sample. Right: Fe-CX features in NRVS of fully enriched [NiFe] H_2 ase vs. selectively enriched [FeFe] H_2 ase. In both figures ordinates represent ^{57}Fe partial vibrational density of states.

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