Hydrogenases

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Observation of the Fe–CN and Fe–CO Vibrations in the Active Site of [NiFe] Hydrogenase by Nuclear Resonance Vibrational Spectroscopy**

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Hydrogenases (H_2ases) are key enzymes that catalyze both the production and consumption of molecular hydrogen.[1] They have attracted interest for potential applications in a hydrogen economy as enzymes themselves or as aspirational targets for biomimetic catalysts.[2] In the [NiFe] H_2ases, catalysis takes place at a Ni–Fe dimetallic center.[3] In this active site, Fe is linked to Ni by a pair of cysteine thiolate ligands. Additionally, depending on the redox poise or degree of aerobic deactivation, the Fe and Ni centers are linked by hydride, oxo/hydroxo, and possibly hydroperoxo ligands.[4] The unique Fe site also carries one CO and a pair of CN^- ligands, the first naturally occurring center of its kind that was discovered.[5] [NiFe] H_2ases invariably possess additional FeS clusters that relay electrons to and from the active site (Supporting Information, Figure S1).

Although [NiFe] H_2ases have been extensively characterized by crystallography,[3,4a] and more than ten different chemical states have been distinguished by infrared (IR) spectroscopy,[3,6] many questions remain about the identity and structure of these species. Furthermore, apart from $Fe(CN)_2CO$ ligation (Figure 3 left), structures involving $Fe(CN)_3CO$[7] and $Fe(SO)_x$[8] ligation have been proposed. An understanding of the structural transformations involved in conversion between different states is obviously critical to elucidating the mechanism of this H_2ase. Herein we describe the application of a relatively new technique, nuclear resonance vibrational spectroscopy (NRVS), which is also called nuclear inelastic scattering (NIS) or nuclear resonant inelastic X-ray scattering (NRIXS), by which the structure and dynamics of the redox chain and active site of [NiFe] H_2ase can be addressed.

NRVS has emerged as a powerful technique for probing the dynamics of Fe in metalloproteins.[9] The measurement involves scanning a highly monochromatic (meV) X-ray beam through a nuclear resonance. Unlike Mössbauer spectroscopy, which detects recoil-free nuclear transitions, NRVS involves inelastic events with the creation or destruction of phonons, thus allowing vibrational fine structure to be observed. The resulting spectrum is similar to a conventional IR or Raman spectrum[10] but with different selection rules and intensity mechanisms. Specifically, the NRVS intensity for a given normal mode is related to the motion of the resonant nucleus (in this case ^{57}Fe) along the direction of the incident X-ray beam.[9d,11] The validity of this NRVS interpretation has already been exhaustively checked by comparison with IR and Raman spectra of FeS model compounds,[12] small Fe proteins,[13] and model compounds or proteins with CO and CN ligands.[14]

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Unlike IR and Raman spectroscopy, NRVS is immune to interference from water or protein modes, can probe any oxidation state, and is impervious to sample fluorescence. With its particular elemental and isotopic specificity, NRVS provides a valuable complement to more conventional spectroscopic methods. In complex metalloenzymes such as H_2ases, the question is whether detection of specific vibrations of the ligands attached to the active site iron in the various states of the catalytic cycle is possible in the presence of many other FeS clusters of the protein. In this study, we answer that question by presenting the first NRVS results on a catalytic [NiFe] H_2ase obtained from *Desulfovibrio vulgaris* Miyazaki F (DvMF). Despite strong contributions from the three different FeS clusters in the electron transport chain, high-frequency bands are observed that can be assigned to the Fe–CO and Fe–CN bending and stretching modes within the [NiFe] active site. The features are assigned by comparison with model compounds and smaller FeS proteins.

The NRVS-derived PVDOS (partial vibrational density of states) for "oxidized" and "reduced" states of DvMF [NiFe] H_2ase are presented in Figure 1. Since 11 of 12 Fe in this enzyme are in [3Fe-4S] or [4Fe-4S] centers (Figure 1, top), the spectra are dominated by contributions from these clusters.[12b,13b] The strong features range from protein and cluster torsional modes below 100 cm^{-1}, to bending and breathing modes near 150 cm^{-1}, and Fe–S stretching modes between 250 and about 400 cm^{-1}. For oxidized H_2ase, the highest frequency bands with significant intensity arise from Fe–S stretching involving Cys ligands at about 390 cm^{-1}, as well as Fe–S stretching modes primarily from bridging sulfide near 380 cm^{-1}.[13b] Weak modes associated with S-C-C bending with a small contribution of Fe–S stretching may occur between 430 and 450 cm^{-1}.[12b,13a]

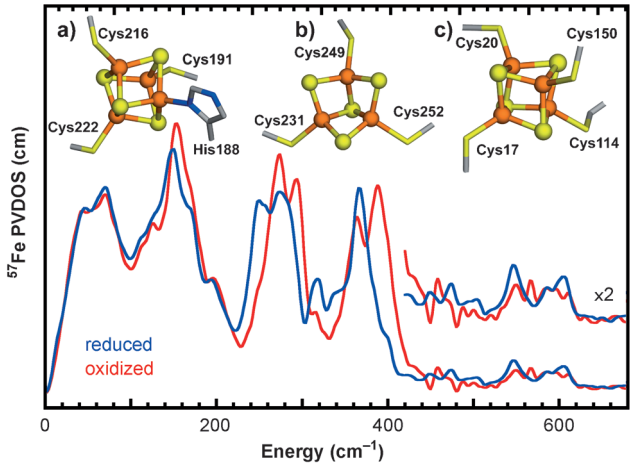

Figure 1. NRVS-derived ^{57}Fe PVDOS for oxidized (——) and reduced DvMF H_2ase (——). For experimental details, see the Supporting Information. Top: a) distal [4Fe-4S] with Cys_3His ligation; b) medial [3Fe-4S] and c) proximal [4Fe-4S] clusters in DvMF [NiFe] H_2ase.

Upon reduction of the protein, the Fe–S stretching bands shift to lower frequencies by 3 or 6% (depending on whether one uses centroid or peak positions). A similar fractional change is observed in the region around 150 cm^{-1} associated with the Fe–S bending modes, but the smaller absolute shift is harder to observe. Comparable redox shifts have been observed for *Pyrococcus furiosus* (*Pf*) mutant D14C ferredoxin (Fd), and it is consistent with reduction of the clusters from the $[4Fe\text{-}4S]^{2+}$ to the $[4Fe\text{-}4S]^{1+}$ redox level in our reduced sample.

For comparison with our [NiFe] H_2ase, at the top of Figure 2 we have included spectra for $[4Fe\text{-}4S]^{2+}$ clusters in three reference proteins: Fe protein of *Azotobacter vinelandii* (*Av*) nitrogenase, *Pf* ferredoxin D14C, and reduced high-potential Fe protein (HiPIP) from *Acidithiobacillus ferrooxidans* (*Af*). These all contain clusters with the same $[4Fe\text{-}4S]^{2+}$ core as oxidized DvMF H_2ase. Although these [4Fe-4S] clusters have similar NRVS band centroids, there are differences in relative peak intensities within the bands for different proteins. Some of these differences can be attributed to variations in the Fe-S-C-C dihedral angles.[13b]

As just one example of the variation of relative peak intensities and positions within these envelopes, in the 330–400 cm^{-1} region, all three proteins have a maximum at about 383 cm^{-1}. However, on the low energy side, D14C *Pf* Fd has a strong peak at 355 cm^{-1}, *Av* nitrogenase (N_2ase) has a smaller peak at 350 cm^{-1}, and *Af* HiPIP has only weak

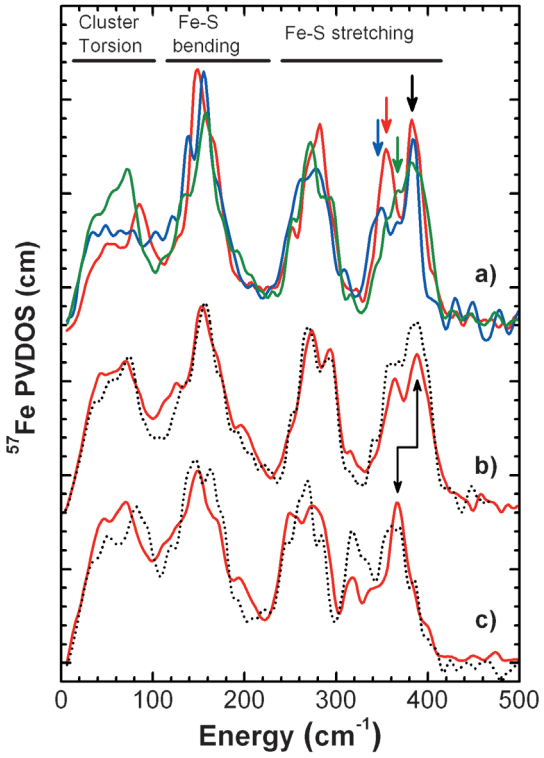

Figure 2. PVDOS from ^{57}Fe NRVS for a) D14C *Pf* Fd (——), *Af* reduced HiPIP (——), and oxidized *Av* N_2ase Fe protein (——), b) oxidized H_2ase (——) and weighted sum of reduced HiPIP plus oxidized [3Fe-4S] D14C *Pf* Fd (•••••), c) reduced H_2ase (——) and weighted sum of reduced Fe protein plus reduced D14C *Pf* Fd plus reduced [3Fe-4S] D14C *Pf* Fd (•••••). Sample preparation and spectral weighting details are found in the Supporting Information.

shoulders (indicated by colored arrows). The detailed structure of the spectra is thus sensitive to the conformation and environment of a given cluster. We note that both reduced HiPIP and the proximal and distal [4Fe-4S] clusters in [NiFe] H_2ase have one or more Fe-S-Cys connections with dihedral angles close to 0° or 180°, where the kinematic coupling between Fe–S motion and SCC side chain motion is greatest.[15] A more detailed analysis of the two distinct $[4Fe\text{-}4S]^{2+}$ cluster contributions to the NRVS is beyond the scope of this work.

Interpretation of the NRVS PVDOS is further complicated by the presence of a "medial" [3Fe-4S] cluster in the H_2ase electron-transfer chain. For comparison, we have recorded the spectrum of a similar cluster in a modified form of the *Pf* Fd protein. In a purely empirical approach, we found that the best match to the spectrum of the oxidized H_2ase came from combining spectra for the reduced HiPIP with the appropriate contribution from the [3Fe-4S] ferredoxin (Figure 2b). In a similar vein, the spectrum for the reduced H_2ase sample was simulated well by combining equal weights of the reduced *Pf* Fd D14C spectrum, the reduced Fe protein spectrum, and the reduced [3Fe-4S] ferredoxin spectrum (Figure 2c). The quality of these empirical simulations allows us to conclude that nearly all of the features below 400 cm^{-1} arise from the 11 out of 12 Fe that are part of the Fe–S cluster electron-transfer chain.

The final and chemically most interesting contribution to the H_2ase NRVS is from the unique Fe in the [NiFe] active site. As this is one low-spin Fe^{2+} center[4b] in the presence of 11 Fe from FeS clusters, the fraction of signal from its Fe–CO and Fe–CN modes is small. Fortunately, from Raman data[16] and also from the NRVS of myoglobin-CO (Mb-CO),[17] LFe-CO models,[14b] and [Fe] H_2ase,[14a] we anticipate that Fe–CO and Fe–CN bands will occur in the 400–650 cm^{-1} region, where FeS clusters have negligible NRVS intensity.

When the 400–650 cm^{-1} region is magnified (Figures 1 and 3), peaks emerge that have intensities and frequencies consistent with Fe–CN and Fe–CO stretching and bending modes. The oxidized protein has bands with maxima at around 542 and 582 cm^{-1} that shift to about 547 and 605 cm^{-1} in the reduced sample. From previous work on model compounds and the [Fe]–H_2ase,[14a] as well as precedents from heme–CO derivatives,[18] these bands can be assigned to Fe–CO stretch and Fe-C-O bend modes, respectively. As frequently noted,[14c] strong Fe–CO backbonding pushes Fe–CO bend modes (in this case at 578 cm^{-1}) to a higher frequency than the Fe–CO stretch mode[18] (in this case at 512 cm^{-1}). Density functional theory (DFT) calculations have also shown that the bend mode mixes with a tilt mode.[19]

The small shifts of ν_{FeCO} to higher frequency with reduction are consistent with the well-known inverse correlation between ν_{FeC} and ν_{CO} in FeCO complexes.[10,18,20] For example, Spiro found that for myoglobin-CO, ν_{FeCO} increases by a factor of about 0.75 of the magnitude of the decrease of ν_{CO}.[18] We admit that Mb–CO is not a good structural model for H_2ase, but we assume a similar slope for the inverse correlation. Thus, if the bulk of the "oxidized" sample is in the Ni-A form,[3,4b] with $\nu_{CO} \approx 1956$ cm^{-1} and most of the "reduced" sample is in the Ni-R1 form,[3,4b] with $\nu_{CO} \approx 1948$ cm^{-1}, then the 5 cm^{-1} increase in ν_{FeCO} is the expected magnitude and sign compared to the 8 cm^{-1} decrease in ν_{CO}. At the moment, our ability to further analyze this shift is limited by a number of factors, including the experimental (NRVS) spectral resolution of about 8 cm^{-1}, the presence of splittings in some of the bands, and the likelihood of some sample heterogeneity with respect to the states in the sample.

To aid in interpretation of these features, we also recorded the NRVS for a model compound with an Fe coordination sphere similar to the H_2ase active site: $(NEt_4)_2[^{57}Fe(bdt)(CN)_2(CO)]$ (**1**)[21] (Figure 3, left top) and the toluene-dithiolate analogue of **1** (Supporting Information). For this complex, the Fe-CO stretch and Fe-CO bend modes are assigned respectively to features at 562 and 621 cm^{-1} (Figure 3, right, red trace). The strength of the Fe–CO bond is reflected in the relatively high Fe–CO stretch and bend frequencies, and as an additional consequence of backbonding into the CO π* orbital, there is a relatively low ν_{CO} stretching frequency of 1897 cm^{-1}.[21] For comparison, Mb–CO has lower frequency Fe–CO stretch and bend modes at 512 and 578 cm^{-1}[19,22] and a higher ν_{CO} stretch at about 1945 cm^{-1}.[23]

For the Fe–CN modes in **1**, four Fe–CN modes might be expected (for in-phase and out-of-phase Fe-CN stretches and bends). In Figure 3a, we label four candidate features at 421, 433, 476, and 509 cm^{-1}, but there are other features from about 400 to 530 cm^{-1}. The additional complexity might arise from coupling with the benzenethiolate ring, which also has modes in this region.[12b] Fe–CN dynamics are clearly more

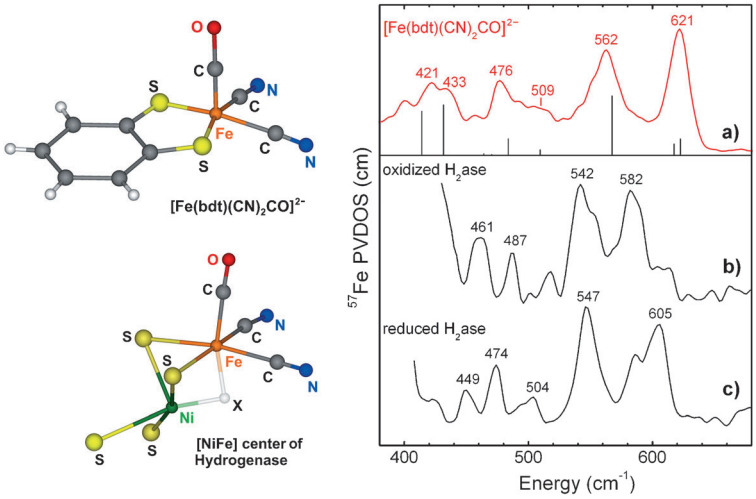

Figure 3. Left: Structure of model complex **1** and the [NiFe] active site of DvMF [NiFe] H_2ase. bdt = benzenedithiolate. Right: High-frequency region of the ^{57}Fe PVDOS for a) $(NEt_4)_2[^{57}Fe(bdt)(CN)_2(CO)]$ (——), b) oxidized [NiFe] H_2ase, and c) reduced [NiFe] H_2ase from DvMF. The H_2ase traces are slightly different from Figure 1, because the above data is from scans that were heavily weighted for the time of data collection in the high frequency region.

complicated compared to the straightforward Fe–CO bend and stretch modes. The heme–CN literature suggests that weaker backbonding in the Fe–CN case leads to more complex patterns of mode ordering and mixing of Fe–CN stretch and bend motions. For linear Fe–CN groups, the Fe–CN bend frequency is higher than the Fe–CN stretch, while for bent Fe–CN groups, the opposite holds.[16b]

In the NRVS of the DvMF H_2ase, several bands are observed in the 400–500 cm^{-1} range where Fe–CN modes are expected. For the oxidized sample, there are reproducible peaks at 461 and 487 cm^{-1}, whereas in the reduced spectrum, the significant peaks are at 449, 474, and 504 cm^{-1}. However, it is possible that additional Fe–CN modes are obscured at both ends of this frequency range. At the low end, FeS modes mixed with cysteine side chain modes can produce a weak NRVS signal, while on the high frequency side Fe–CN features might be buried under or coupled with stronger Fe–CO structure. A more complete assignment awaits the combination of better resolution, isotopic labeling, and supporting DFT calculations.

In conclusion, we have reported the first NRVS measurements for a [NiFe] H_2ase. The spectrum from 0–400 cm^{-1} is dominated by contributions from the 11 out of 12 Fe present in FeS clusters of the electron transport chain, and this FeS cluster region for H_2ase compares well with a reduced HiPIP spectrum. An H_2ase model compound spectrum indicated that the Fe–CN and Fe–CO modes of the H_2ase [NiFe] active site should fortunately have frequencies at around 420 to 620 cm^{-1}, which is outside the FeS cluster region. We assigned the stronger H_2ase bands in the 540–605 cm^{-1} region to Fe–CO stretching and bending modes, and relegated the weaker features between 450 and 505 cm^{-1} to Fe–CN stretching and bending modes.

One of the most exciting future applications of NRVS to H_2ases will likely be the observation of Fe-H/D related normal modes, as they would help characterize the geometry of the bridge and the nature of hydrogen activation. The primary obstacle to such chemically informative experiments is the limited flux and resolution of current facilities. However, already-built beamlines are becoming available with 5-fold[24] or even 10-fold[25] increases in flux, and in the not too distant future four additional orders of magnitude will be available from seeded free electron laser sources.[26] These improvements should transform biological NRVS experiments from heroic to routine.

Experimental Section

According to FTIR and EPR spectra, the oxidized ^{57}Fe-labeled H_2ase contained mostly Ni-A, the H_2-reduced sample a mixture of Ni-C, and Ni-R1 and some Ni-SIa.[3,4b] Details of protein and sample preparations are also given in the Supporting Information. ^{57}Fe labeling and preparation of *Acidithiobacillus ferrooxidans* HiPIP and *Pyrococcus furiosus* ferredoxin (D14C mutant), and the synthesis of the studied ^{57}Fe model compounds are given in the Supporting Information. The NRVS measurements were conducted either at SPring-8 BL09XU or at the Advanced Photon Source (APS), Beamline 3-ID, at $T = 10$ K (cryostat base) using monochromators with circa 1 meV resolution and a 1 cm^2 APD detector. Details of the measurements and further analysis of the raw data are collected in the Supporting Information.

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