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Nuclear Resonance Vibrational Spectroscopy (NRVS) of Fe–S model compounds, Fe–S proteins, and nitrogenase

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Abstract We have used nuclear resonance vibrational spectroscopy (NRVS) to examine the nature of the Fe–S unit. Specifically, vibrational characteristics have been determined, and through incremental steps in model system complexity, applied to analysis of the enzyme nitrogenase. This stepwise strategy demonstrates NRVS as a viable bioinorganic tool, and will undoubtedly increase the application of synchrotron spectroscopy to biological problems.

Key words ferredoxin · rubredoxin · vibrational spectroscopy · synchrotron · Mössbauer

1 Introduction

Iron–sulfur species are ubiquitous in nature [1]; among their responsibilities are the enzymatic redox transformations of CO_2, H_2, and N_2 [2]. These substrates, being likely foundations of Earth's early atmosphere, have led Huber and Wächterhäuser to postulate that life may have evolved via the catalytic function of iron–sulfur metalloclusters [3]. The base component of these metalloclusters is the 2Fe2S rhombus, from which many of the larger clusters can be constructed, not just stereochemically, but also literally, as in the case of nitrogenase (N_2ase), the enzyme responsible for the reduction of dinitrogen to ammonia [4]. N_2 fixation is the key step in the nitrogen cycle [5, 6], and this biological ammonia synthesis is responsible for about half of the protein available for human consumption. In *Azotobacter vinelandii* (*Av*) the Mo-dependent N_2ase that accomplishes this reaction uses electrons from an Fe_4S_4 cluster in a 63 kDa Fe protein (Av2) to reduce a 230 kDa $\alpha_2\beta_2$ MoFe protein (Av1). Within Av1, an Fe_8S_7 'P-cluster' supplies electrons to the active site $MoFe_7S_9$ 'FeMo-cofactor', sometimes called the 'M-center.' This is extractable into organic

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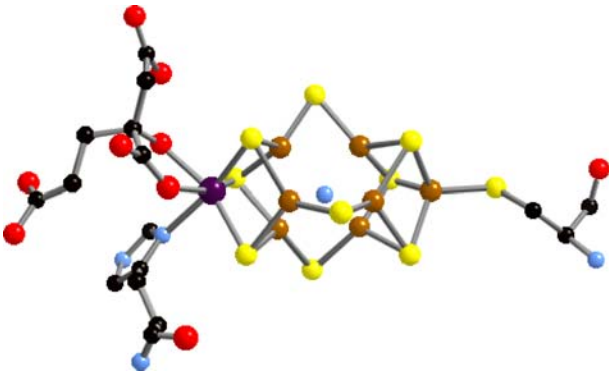

Fig. 1 *Ball and stick* representations of *A. vinelandii* N_2ase FeMo-cofactor, illustrating central location of proposed light atom (PDB code 1M1N) [11]

solvents as 'FeMoco' (Fig. 1) [7]. Protein-bound FeMo-cofactor is ligated by a cysteine on one end, and on the other by histidine and homocitrate ligands [8–10].

We have examined model systems and iron–sulfur proteins (including N_2ase and FeMoco) by the technique of nuclear resonance vibrational spectroscopy (NRVS). The theory behind this technique is described in detail in other articles in this issue, however it is suffice to say here that the above technique is element specific, and through suitable isotopic labeling allows for focus upon the metal atoms within the catalytic machinery. With this spectroscopic tool we hope to address questions that are beyond the reach of protein crystallography; furthermore, important catalytic intermediates are not always the species that can be crystallized, and crystallized species may not necessarily be part of the catalytic cycle. To validate our NRVS data collection and analysis procedures, we initially recorded spectra for a number of model compounds. We took an *aufbau* approach, starting with simple systems and gradually building up to complex clusters. The early work simulated the obtained spectra using Urey–Bradley force fields; the later studies were complemented by DFT calculations. Model compound and small protein studies remain critical for (1) improving experimental methods, (2) refining transferable UBFF force fields, (3) testing DFT predictions, and (4) expanding a database for interpretation of enzyme spectra.

2 Results

Our work in the area of NRVS is summarized below, beginning with the more simple model systems and culminating in the more involved enzymatic constructs. These latter investigations are complicated by (1) the relative diluteness of the iron–sulfur centers within the proteinous environment, (2) the presence of several species, for example [4Fe–4S] clusters that serve as electron conduits, (3) and the greater complexity afforded by a decrease in symmetry, that is often concomitant with the increased number of atoms in the active site. However, even with these factors, evidence is presented that clearly demonstrates that NRVS is entirely applicable as a method for biological studies.

2.1 Model compounds

$[FeH(D)_6]^{4-}$ This was our first NRVS project, conducted largely as a feasibility study, with an eye on hydrogenase projects in the future. We compared these isotopomers to see if

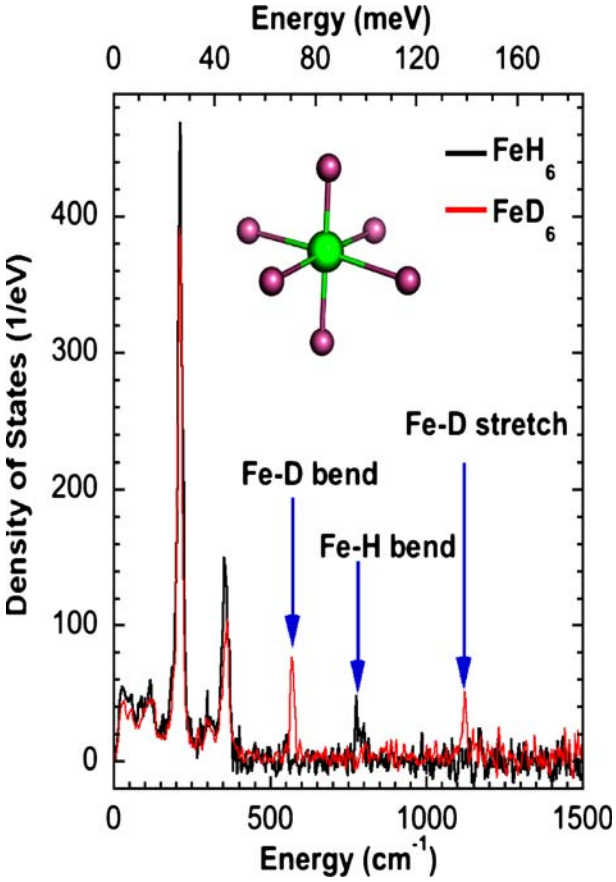

Fig. 2 NRVS of $[Fe(H/D)_6]^{4-}$

Fe-H/D stretches, which involve little Fe motion, would be visible by NRVS. As shown in Fig. 2, Fe–D modes were visible in 2002 [12]. Of note was the surprising strength of the H–Fe–H and D–Fe–D bend modes.

R(FeCl₄) and [NEt₄]₂[Fe₂S₂Cl₄] These compounds were analyzed by a combination of NRVS, Raman, and IR spectroscopies (data not shown). The D_{2h} symmetry of the $[Fe_2S_2Cl_4]^{2-}$ anion with its centre of inversion makes the IR and Raman modes mutually exclusive, thus we were able to observe and assign 16 of the 18 normal modes for this species. Additionally, we were able to observe a change in $[FeCl_4]^-$ symmetry with counterion, and the acoustic phonon modes for all of these samples [13].

$[Fe_4S_4(SPh)_4]^{2-}$ This cluster was examined as a 'simple' model for 4Fe ferredoxins, and as a test for our ability to observe ^{36}S isotope effects. Owing to the low symmetry and a solid-state phase transition at 233 K, the NRVS spectra turned out to be surprisingly complex. Incorporation of ^{36}S into the bridging S positions produced ~9 cm^{-1} shifts in some NRVS bands (Fig. 3). These shifts were reproducible with DFT calculations [14].

$[Fe_6N(CO)_{15}]^{3-}$ This cluster was examined as one of the few models available for interstitial N in a 6-Fe cage, and as a test for our ability to observe $^{15}N/^{14}N$ isotope effects.

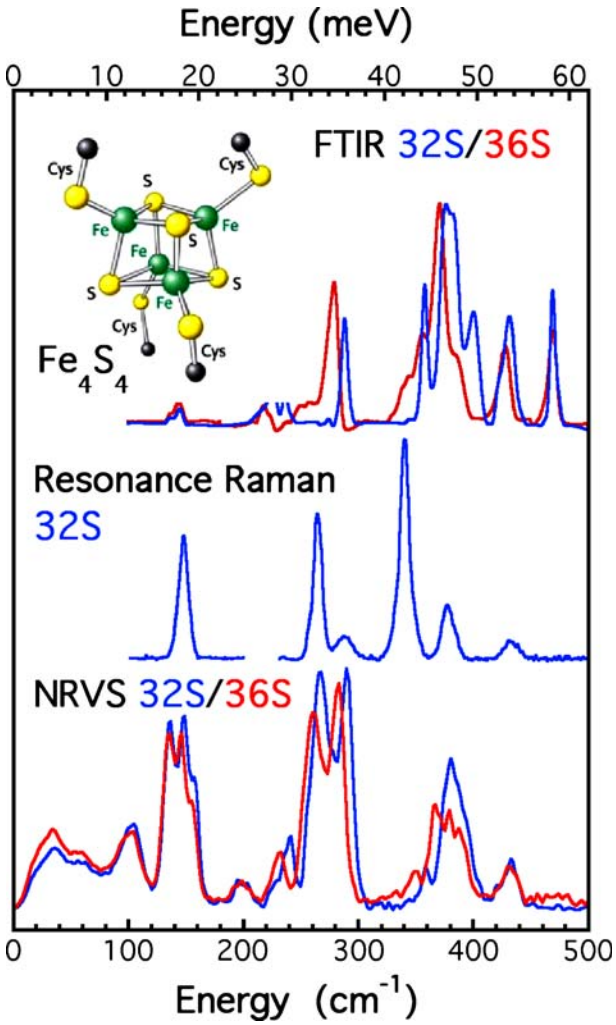

Fig. 3 NRVS (*bottom*) of $[Fe_4S_4(SPh)_4]^{2-}$ with ^{32}S (*blue*) or ^{36}S (*red*) vs. Raman (*middle*) and IR (*top*)

We observed several strong breathing modes for the 6Fe prism, as well as the expected Fe–C stretching bands. A special characteristic of complexes with interstitial 'X' is the presence of 'shake' modes for the X vibrating within the metal cage. The shake modes are very strong in the IR, and give rise to large $^{15}N/^{14}N$ shifts (Fig. 4). We have now seen these shifts in the NRVS as well.

2.2 Protein studies

Rubredoxin We chose *Pyrococcus furiosus* Rd as our first protein because of its 'simple' single FeS_4 center (Fig. 5) [15]. The results turned out to be more complex than expected; there has been a long-standing debate over delocalization of Fe–S modes in Rd. Resonance Raman work had shown an asymmetric Fe–S stretch region divided into three bands near 350–370 cm^{-1} [16]; in our Raman spectra we observed these and additional bands out to 440 cm^{-1}.

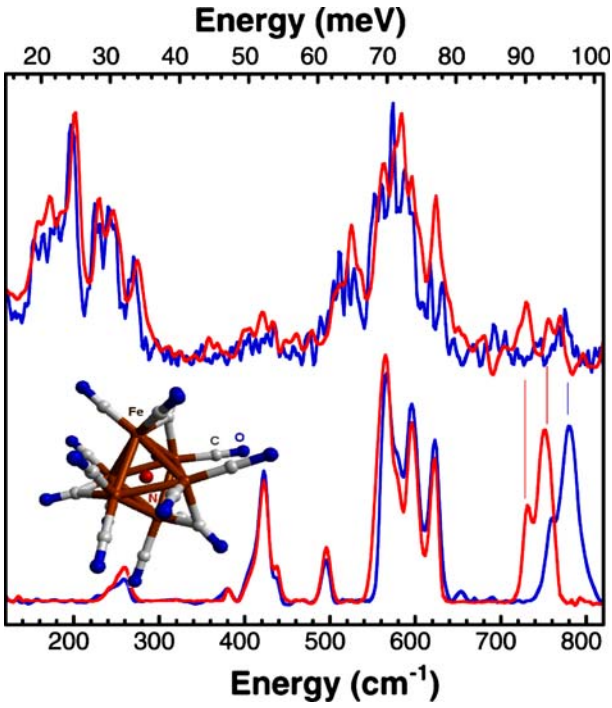

Fig. 4 NRVS (*top*) and IR (*bottom*) for ^{14}N (–) vs. ^{15}N (–) $[Fe_6N(CO)_{15}]^{3-}$

The NRVS was also very broad in this region, suggesting that stretching modes are strongly coupled with protein side chain motion. A model with five-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 [17].

2Fe and 4Fe ferredoxins We recorded NRVS and resonance Raman for *Aquifex aeolicus* (*Aa5*) and *Rhodobacter capsulatus* (*Rc6*) 2Fe Fds. The Fe PVDOS reveals a strong and broad low frequency region (Fig. 5), suggesting that the Fd bend modes are highly delocalized and mixed with larger scale peptide motions. Raman spectra for $^{57}Fe/^{36}S$-substituted *Rc6* exhibit isotope shifts that provide additional constraints on the normal mode analyses. We followed with measurements on a *Pf* 4Fe Fd using the D14C mutant with all Cys side chains [18]. Compared to 2Fe Fds, the 4Fe cluster shows less coupling with the protein matrix.

Nitrogenase and FeMoco Together with Bill Newton and Karl Fisher, we examined samples of ^{57}Fe-enriched Av1, a Δ*nifE* Av1 mutant containing only P-cluster, and isolated FeMoco (Fig. 6). The difference spectrum between Av1 and Δ*nifE* Av1 represents the protein-bound FeMo-cofactor. In this data and in the FeMoco NRVS, the catalytic site exhibited a strong signal near 190 cm^{-1}, where conventional Fe–S clusters have weak NRVS. This intensity was 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 cm^{-1}. The spectra were reasonably well simulated both by empirical UBFF force fields and by DFT calculations [19].

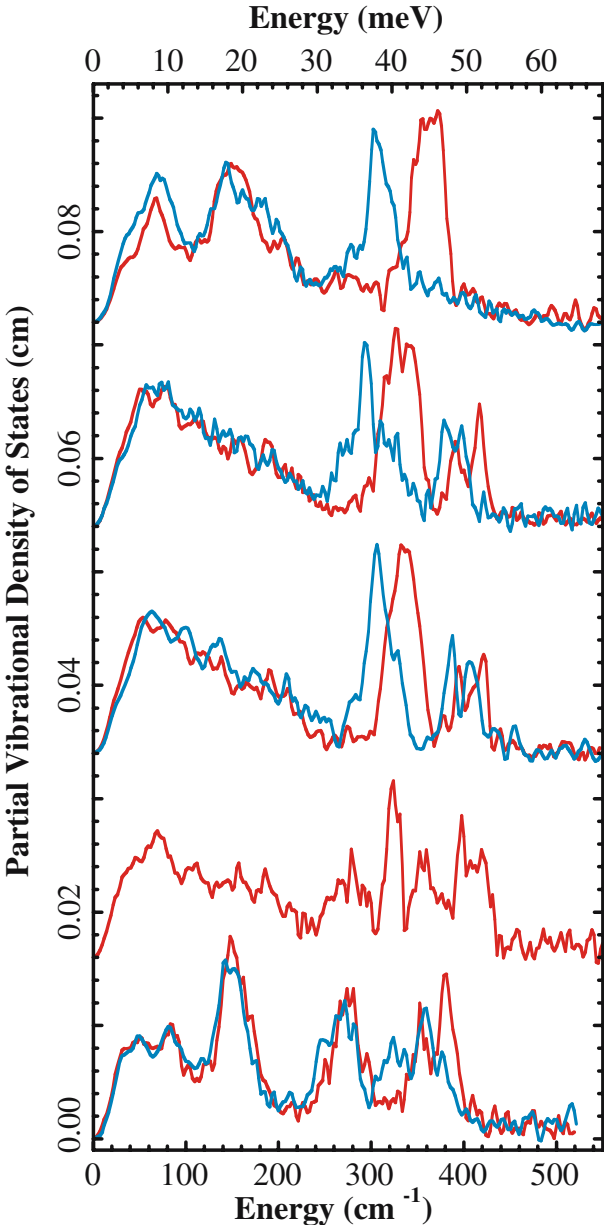

Fig. 5 NRVS of Fe–S proteins. *Top to bottom*, oxidized (red) vs. reduced (blue): (a) *Pf* Rd, (b) 2Fe *Aa5* Fd, (c) 2Fe *Rc6* Fd, (d) 2Fe Rieske protein, (e) 4Fe D14C *Pf* Fd

It is worth emphasizing that this is the first vibrational information ever obtained about the FeMo-cofactor or P-cluster metals. There is a wealth of information to be obtained from these spectra, especially when combined with DFT calculations and isotopic labeling. We are obviously quite excited about the potential of these experiments.

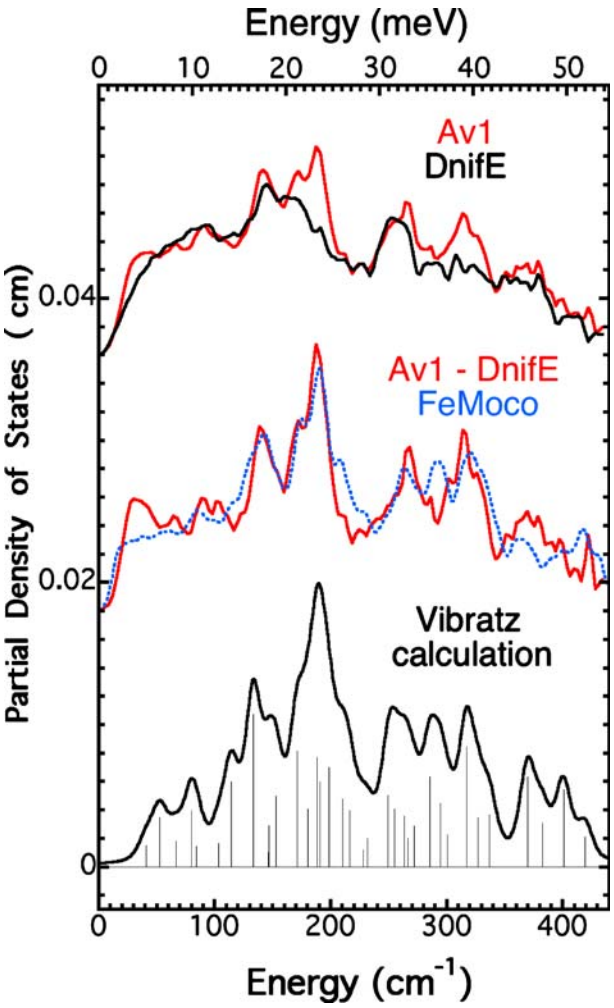

Fig. 6 NRVS of MoFe N_2ase vs. the P-cluster only ΔNifE, MoFe *minus* ΔNifE vs. FeMoco. Vibratz simulation of FeMoco

2.3 Summary

The results presented above unequivocally illustrate that synchrotron spectroscopies have 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.

3 Experimental

NRVS spectra were recorded at beamline 3-ID at the APS, Illinois, and at beamline 09-XU at SPring-8, Japan as previously described [13, 15]. PVDOS were calculated using PHOENIX [20].

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