

Chemical Science

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the [Information for Authors](#).

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard [Terms & Conditions](#) and the [Ethical guidelines](#) still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ARTICLE

Nuclear resonance vibrational spectroscopy reveals the FeS cluster composition and active site vibrational properties of an O₂-tolerant NAD⁺-reducing [NiFe] hydrogenase

Cite this: DOI: 10.1039/x0xx00000x

Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

www.rsc.org/

Lars Lauterbach,^{1,2,*} Hongxin Wang,^{2,3,†} Marius Horch,^{1,†} Leland B. Gee,² Yoshitaka Yoda,⁴ Yoshihito Tanaka,³ Ingo Zebger,¹ Oliver Lenz,¹ Stephen P. Cramer^{2,3,*}

Hydrogenases are complex metalloenzymes that catalyze the reversible splitting of molecular hydrogen into protons and electrons essentially without overpotential. The NAD⁺-reducing soluble hydrogenase (SH) from *Ralstonia eutropha* is capable of H₂ conversion even in the presence of usually toxic dioxygen. The molecular details of the underlying reactions are largely unknown, mainly because of limited knowledge of the structure and function the various metal cofactors present in the enzyme. Here all iron-containing cofactors of the SH were investigated by ⁵⁷Fe specific nuclear resonance vibrational spectroscopy (NRVS). Our data provide experimental evidence for one [2Fe2S] center and four [4Fe4S] clusters, which is consistent with amino acid sequence composition. Only the [2Fe2S] cluster and one of the four [4Fe4S] clusters were reduced upon incubation of the SH with NADH. This finding explains the discrepancy between the large number of FeS clusters and the small amount of FeS cluster-related signals as detected by electron paramagnetic resonance spectroscopic analysis of several NAD⁺-reducing hydrogenases. For the first time, Fe-CO and Fe-CN modes derived from the [NiFe] active site could be distinguished by NRVS through selective ¹³C labeling of the CO ligand. This strategy also revealed the molecular coordinates that dominate the individual Fe-CO modes. The present approach explores the complex vibrational signature of the Fe-S clusters and the hydrogenase active site, thereby showing that NRVS represents a powerful tool for the elucidation of complex biocatalysts containing multiple cofactors.

Introduction

Hydrogenases are nature's catalysts for the reversible cleavage of molecular hydrogen into protons and electrons. In case of [NiFe] hydrogenase, the active site nickel is coordinated to the protein matrix by four cysteinyl thiolates, two of which serve as bridging ligands to iron (Figure 1). The iron is further coordinated by one carbonyl and two cyanide ligands.¹ While most [NiFe] hydrogenase are reversibly inactivated by O₂, the soluble, NAD⁺-reducing [NiFe] hydrogenase (SH) from *Ralstonia eutropha* H16, which couples the reversible oxidation of H₂ to the reduction of NAD⁺, remains highly active even under ambient O₂ concentration.^{2,3}

The SH is therefore a promising enzyme for biotechnological applications, e.g. nucleotide cofactors regeneration.⁴ The O₂ tolerance of the SH relies on its capability to continuously reduce O₂ to water and hydrogen peroxide, allowing for H₂ oxidation in the presence of oxygen.³ Sequence analysis indicates that the [NiFe] active center of this enzyme is connected to the NAD⁺-binding site by one [2Fe-2S] center and four [4Fe-4S] clusters as well as two flavin mononucleotide (FMN) molecules (Figure 1).⁵ While the FMN cofactors have been confirmed experimentally, only signals of the [2Fe2S] cluster and one [4Fe4S]

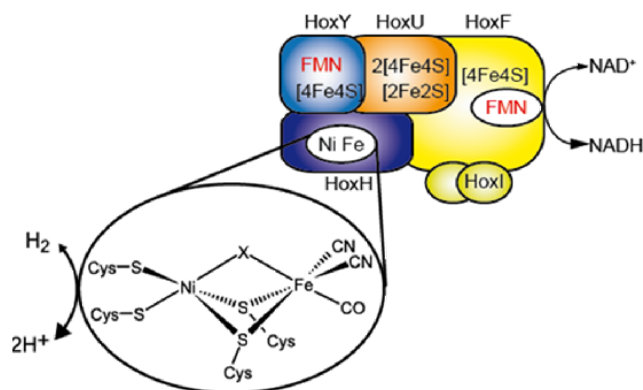


Figure 1. Schematic overview of the cofactor composition of the SH. The [NiFe] active site is shown in the inset, with X denoting the site of hydride or inhibitor binding.

cluster^{5,6} have been detected by electron paramagnetic resonance (EPR) spectroscopy, and similar results have been obtained for other NAD⁺-reducing [NiFe] hydrogenases.^{7,8} Until now, the reason for

the discrepancy between the number of potential binding motifs and EPR-detected FeS clusters is unclear and, thus, experimental evidence for the proposed set of clusters is still missing.

Nuclear resonance vibrational spectroscopy (NRVS) is a synchrotron-based vibrational spectroscopic technique that selectively probes iron-specific normal modes and the related molecular coordinates, therefore it is an ideal technique for the investigation of iron-containing metalloproteins in terms of both structure and dynamics. In the case of [NiFe] hydrogenase, NRVS has first been successfully applied in 2013.⁹ Likewise, resonance Raman (RR) spectroscopy represents a comparable method to gain complementary insights.^{10,11} In the case of the SH, however, the application of RR is strongly impeded by the FMN-derived fluorescence. Moreover, no RR signal was found for the [NiFe] active site of oxidized SH with several excitation wavelengths (data not shown), demonstrating the necessity of NRVS for the characterization of this particular enzyme.

Experimental

Sample Preparation

Ralstonia eutropha was cultured and NAD⁺-reducing hydrogenase (SH) was purified according to³ except that 18 μM ⁵⁷FeCl₂ was used in the minimal medium. For labelling of the SH with ¹³CO, 5000 ppm ¹³CO gas was added into a 16 L desiccator, which contained atmospheric gas. The NADH-treated samples were prepared by 30 min anaerobic incubation of 1mM SH with 50mM NADH. The concentration of oxidized SH samples was 1mM.

NRVS Measurements

Nuclear resonance vibrational spectroscopy (NRVS) measurements were conducted at Spring-8 BL09XU and BL19LXU. A ¹N₂-cooled Si (1, 1, 1) high heat load monochromator was used to produce an incident beam with ~1 eV resolution, followed by a high resolution monochromators with an asymmetrically cut Ge (3,3,1) and two nested Si (9, 7, 5) crystals to achieve an ~0.8 meV energy resolution at 14.4125 keV. A 4-channel avalanche photo diode detector array was used to detect the K-fluorescence X-ray from internal conversion and the nuclear gamma ray emission, by ⁵⁷Fe atoms. The detectors were placed as close as possible to the surface of the sample. All measurements were performed at 10 K in the cryostat base. The real sample temperatures, as obtained from the spectral analysis, was 30 – 60 K.

To enhance the S/N ration in the Fe–CN, Fe–CO range of the spectra, sectional measurements of these regions were performed. While in the usual scans the time for every point is 5 s, in the case of sectional scans the time for points in the region of interest, i.e. from 400 to 680 cm⁻¹ was 10 - 20 s and the rest was 1-3 s depending on measurements.

The raw NRVS data were analyzed by the PHOENIX software¹² to extract the single-phonon iron partial vibrational density of states (⁵⁷Fe PVDOS). The resonance peak position for each scan was aligned in the PHOENIX analysis while the energy scale was calibrated with an external reference ([NEt₄][FeCl₄]) periodically.

To produce an empirical NRVS spectral simulation, *Rhodobacter capsulatus* (*Rc*) [2Fe2S] ferredoxin NRVS¹³ was used as a representative model for [2Fe2S] clusters while the *Pyrococcus furiosus* (*Pf*) [4Fe4S] ferredoxin NRVS was used as a model for [4Fe4S] clusters (both in their oxidized (ox) and reduced (red) states).¹⁴ These spectra were first auto-normalized to 3, representing spectra averaged for one iron (subscript “norm”). For [4Fe4S] clusters, the *Pf* ferredoxin NRVS was then multiplied by 4 to get a weighed NRVS spectrum considering that the cluster has 4 irons, while for [2Fe2S] clusters, the *Rc ferredoxin* NRVS was multiplied by 2 for the same reason. Such spectra were then multiplied by the numbers of the same type of clusters included in the SH, the total

sum was calculated, and divided by 18 in order to account the number of considered Fe ions. For example:

$$SH_{ox} = [1 * 2 * Rc \text{ ferredoxin}(ox)_{norm} + 4 * 4 * Pf \text{ ferredoxin}(ox)_{norm}] / 18, \text{ while}$$

$$SH_{red} = [0.5 * 2 * Rc \text{ ferredoxin}(ox)_{norm} + 0.5 * 2 * Rc \text{ ferredoxin}(red)_{norm} + 3 * 4 * Pf \text{ ferredoxin}(ox)_{norm} + 1 * 4 * Pf \text{ ferredoxin}(red)_{norm}] / 18.$$

To evaluate integral intensities in the spectral range of FeS cluster normal modes (0 – 450 cm⁻¹), spectra were first re-normalized with respect to the integral intensities of the Fe–CO/CN centered modes (450 – 650 cm⁻¹), assuming that these signals are comparable for different [NiFe] hydrogenases. Using this procedure, spectra were corrected for preparation - and acquisition related differences - such that integral FeS intensities of the normalized spectra can be directly compared. The FeS integral intensities were then determined as a marker for the relative amount of Fe ions involved in FeS clusters of different [NiFe] hydrogenases. The whole procedure was performed by using the Bruker OPUS software.

IR Measurements

Infrared (IR) spectra with a spectral resolution of 2 cm⁻¹ were recorded on a Bruker Tensor 27 spectrometer, equipped with a liquid nitrogen-cooled mercury cadmium telluride (MCT) detector. The sample compartment was purged with dried air and the sample was held in a temperature-controlled (283 K) gas-tight IR-cell for liquid samples (volume ~ 7 μL, optical path length = 50 μm), equipped with CaF₂ windows. Spectra were processed and baseline-corrected by using the Bruker OPUS software.

Results and discussion

FeS Cluster Composition

In this communication, we report a combined NRVS and infrared spectroscopic characterization of SH in its as-isolated (oxidized) and NADH-reduced state. The partial vibrational density of states (PVDOS) for oxidized and NADH-reduced SH as detected by NRVS is presented in Figure 2A. As 16 out of 19 expected Fe atoms

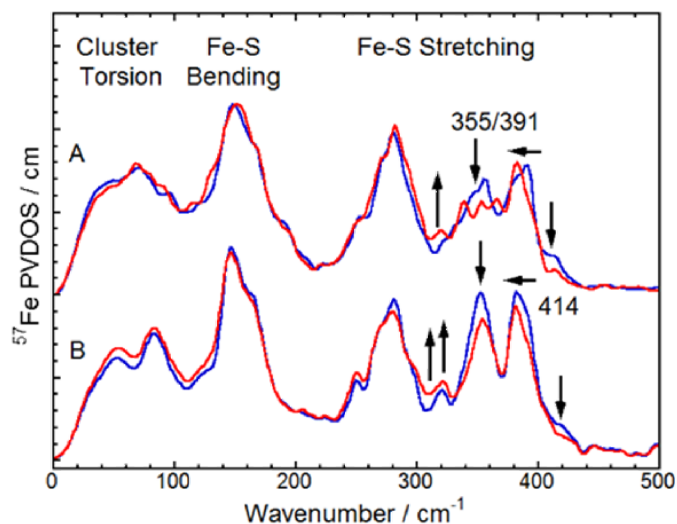


Figure 2. NRVS-derived ⁵⁷Fe PVDOS (in the FeS cluster mode region). A: oxidized (blue), NADH-reduced (red) SH. B: a weighted sum of one oxidized [2Fe2S] of *Rc* ferredoxin plus four oxidized [4Fe4S] clusters of *Pf* ferredoxin (red) vs. a weighted sum of one 50% reduced / 50% oxidized [2Fe2S] cluster of *Rc* ferredoxin plus one reduced and three oxidized [4Fe4S] clusters of *Pf* ferredoxin (blue). NRVS data on *Rc* and *Pf* ferredoxins have been published previously.^{13,14} Arrows indicate the changes between the spectra in each pair.

in this protein are located in [4Fe-4S] centers (Figure 1), the spectra are most likely dominated by contributions from these cofactors. Consistently, the entire pattern between 0 and 400 cm^{-1} is very similar to the NRVS of the [4Fe4S] cluster in *Pf* ferredoxin and not far from that of the “standard” [NiFe]-hydrogenase from *Desulfovibrio vulgaris* (*Dv*) Miyazaki F, which contains the [NiFe] site, one [3Fe4S] center and two [4Fe4S] clusters.^{14,9} However, in contrast to *Dv* hydrogenase, integral intensities in this spectral range are strongly increased by a factor of about 1.5, if the two spectra are normalized with respect to the Fe-CO/CN intensities of the active site (Figure 3). This observation is in line with the higher amount of FeS clusters in the SH (18 Fe ions) as compared to “standard” [NiFe] hydrogenases (11 Fe ions). Thus, the present study provides the first spectroscopic evidence for the large number of FeS clusters in the SH.

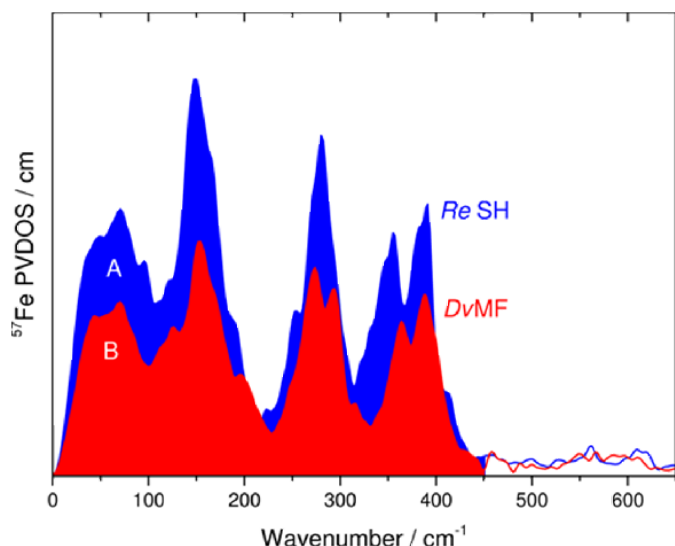


Figure 3. Comparison of integral intensities for the FeS cluster related ^{57}Fe PVDOS in the 0 – 450 cm^{-1} range of (A) oxidized SH and (B) oxidized *Dv* hydrogenase. Spectra were normalized with respect to the integral intensities of the Fe(CO)(CN)₂ moiety (450 – 650 cm^{-1}).

In general, pronounced spectral features from FeS clusters range from (delocalized) torsional modes below 100 cm^{-1} via breathing and bending modes near 150 cm^{-1} to structurally sensitive stretching vibrations between 250 and about 420 cm^{-1} (Figure 2A). The oxidized SH shows two intensive bands with peak maxima around 355 cm^{-1} and 391 cm^{-1} , which reflect normal modes with predominant Fe–S stretching character. While both modes are dominated by displacements of the bridging sulfides (Fe–S^b stretching), the band at 355 cm^{-1} may also include increased movements of terminal cysteines (Fe–S^t stretching).¹⁵ Furthermore, oxidized SH shows a band at 414 cm^{-1} , which is characteristic for [2Fe2S] clusters of ferredoxins (e.g. those from *Rhodobacter capsulatus* (*Rc*) and *Aquifex aeolicus* (*Aa*)).¹³ The *Dv* hydrogenase does not contain a [2Fe2S] cluster, and, thus, no corresponding NRVS signal is observed.⁹ Remarkably, a weighted sum of one oxidized [2Fe2S] cluster of *Rc* ferredoxin¹³ and four oxidized [4Fe4S] clusters of *Pf* ferredoxin¹⁴ provides an ideal fit for the NRVS of as-isolated SH (Figure 2B, red and Figure S1C/E). Including an oxidized [3Fe4S] cluster (*Pf* ferredoxin)⁹ into the simulation instead of one of the [4Fe4S] clusters does not improve the agreement with the experimental spectrum (Figure S1D). In contrast, the weighted sum spectra would exhibit obvious band splitting at around 150 and 290 cm^{-1} , the latter of which is also

observed for [3Fe4S] cluster-containing *Dv* hydrogenase (Figure 3B and figure S1B). No such splitting is observed for the SH, suggesting that the previously proposed presence of a native [3Fe4S] cluster in enzymes of this type is unlikely.^{5,16}

Upon reduction of the SH with NADH, a modified band pattern is observed for the spectral region between 300 and 400 cm^{-1} , reflecting (partial) reduction of FeS clusters. Moreover, a band at 414 cm^{-1} , assigned to the oxidized [2Fe2S] cluster, decreases in intensity by around 50% (Figure 2A and Figure S1E). This observation indicates partial reduction of the [2Fe2S] cluster. Consistently, a lowered intensity has also been observed for the corresponding band upon reduction of [2Fe2S] cluster-containing ferredoxins from *Rc* and *Aa*.¹³ In general, band positions and intensities between 0 and 300 cm^{-1} show only marginal changes after reduction of the SH while drastic changes have been observed for *Pf* ferredoxin and *Dv* hydrogenase.^{13,9} This indicates that not all FeS clusters in SH were reduced under the experimental conditions chosen. Indeed, a weighted sum of a 50% reduced / 50% oxidized [2Fe2S] cluster (from *Rc* ferredoxin) plus one reduced and three oxidized [4Fe4S] clusters (from *Pf* ferredoxin) provides the best fit to the experimental spectrum of NADH-reduced SH (Figure 2B red, Figure S1C/E). This finding is consistent with previous EPR spectroscopic studies, revealing signals for the reduced (paramagnetic) states of the [2Fe2S] cluster and in total only one of the four [4Fe4S] clusters.^{5,6} Therefore, this observation can be clearly related to incomplete reduction of FeS clusters, as also observed for respiratory Complex I whose water-soluble domain is homologous to the SH.¹⁷ Consequently, pronounced antiferromagnetic coupling, high-spin ground states or very fast spin relaxation can now be excluded as alternative explanations for the absence of EPR-detectable clusters. In summary, we have probed for the first time the entirety of FeS clusters of an NAD⁺-reducing [NiFe] hydrogenase in its oxidized and reduced states, thereby disentangling the discrepancy between predicted and EPR-detectable FeS clusters in this enzyme type.⁵

While the NRVS of oxidized and reduced SH are well simulated by the corresponding weighted sums of one *Rc* ferredoxin-derived [2Fe2S] cluster and four [4Fe4S] clusters of *Pf* ferredoxin, clear deviations can still be observed in the structural marker region between 300 and 400 cm^{-1} (Figure 2A). Relative intensities of bands in this region have been proposed as structural markers for Fe–S–C–C dihedral angles. These dihedral angles affect contributions of S–C–C bending coordinates to normal modes with predominant Fe–S stretching character, thereby influencing iron-displacement and NRVS intensities.⁹ In this respect, the balanced doublets in the weighted sum spectra suggest little contributions from S–C–C bending coordinates, i.e. Fe–S–C–C dihedral angles close to 90°. The SH NRVS exhibits a pattern that is similar to *Pf* ferredoxin and the weighted sum spectrum (Figure 2A vs. 2B), indicating that most Fe–S–C–C dihedral angles are close to 90° in SH, too. However, an intensity drop of the low-frequency feature around 355 cm^{-1} is indicative of the co-existence of [4Fe4S] clusters with Fe–S–C–C dihedral angles approaching 0/180° (Figure 2A).¹⁴

There is no crystal structure available for the SH; however, dihedral angles might be similar to those in Complex I. Thus, the SH-related NRVS might be well explained by the atomic coordinates derived from the crystal structure of Complex I. Indeed 14 out of 20 (70%) Fe–S–C–C dihedrals in Complex I approach 90° ($\pm 24^\circ$) while the remaining six (30%) assume values close to 0/180° ($\pm 31^\circ$).¹⁷ This finding explains the SH NRVS and supports the structural similarity between Complex I and NAD⁺-reducing [NiFe] hydrogenases.

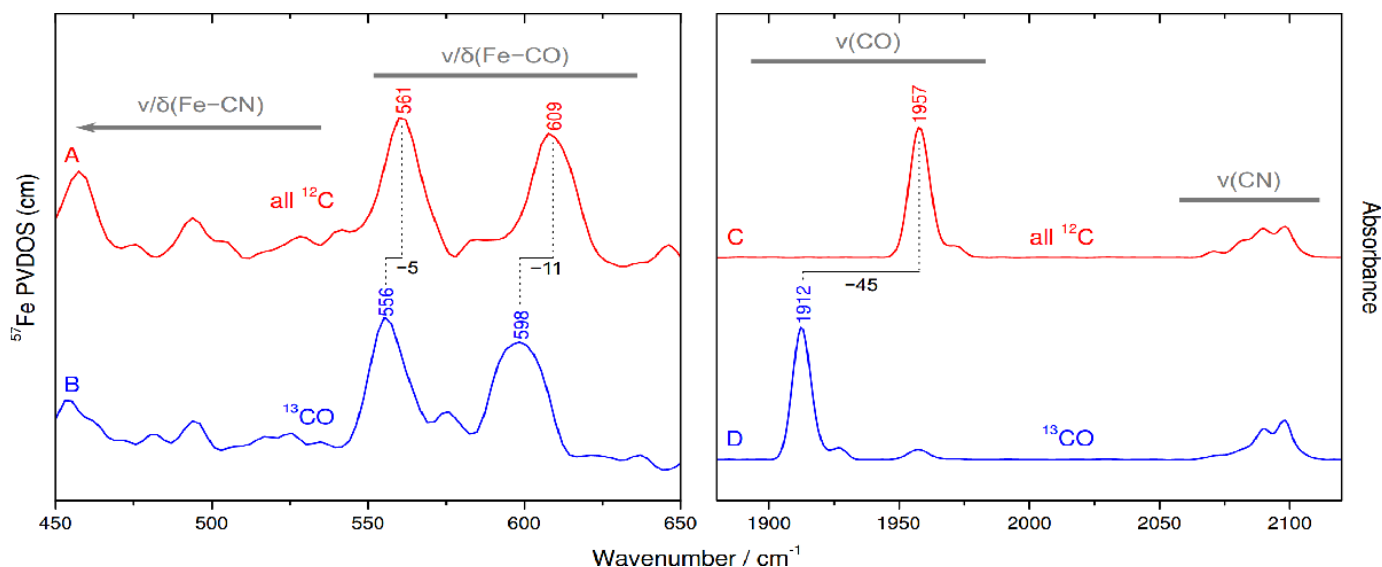


Figure 4. Vibrational signature for the $\text{Fe}(\text{CO})(\text{CN})_2$ moiety of the oxidized SH active site. Left panel: Fe-CO/CN metal-ligand modes as revealed by NRVS. Right panel: CO/CN stretching modes probed by IR spectroscopy. A/C: non-labeled SH (red). B/D ^{13}CO -labeled SH (blue).

Active Site Vibrational Properties

Despite the lower spectral intensity of the active site (only 1 Fe ion compared to 18 located in FeS clusters), NRVS also allows probing Fe-CO and Fe-CN stretching and bending modes (400 - 650 cm^{-1}),⁹ which provide valuable information on structural and electronic properties of the catalytic [NiFe] center (Figure 4A, S2).^{10,11} However, a detailed knowledge of the molecular coordinates included in these modes is essential for the understanding of the complex signature of the $\text{Fe}(\text{CO})(\text{CN})_2$ moiety.

To disentangle this vibrational manifold, we have used selective ^{13}C labeling of the CO ligand by isolating SH from cells grown in a ^{13}CO -enriched atmosphere. To verify selective ^{13}CO labeling, we have first recorded IR spectra from both unlabeled and ^{13}CO -containing as-isolated SH (Figure 4C and D). While unlabeled SH exhibits the typical CO stretching band at 1957 cm^{-1} , this feature is considerably displaced by -45 cm^{-1} in case of ^{13}CO -labeled SH, reflecting the larger mass of ^{13}C . Moreover, both spectra exhibit the same set of four CN stretching bands at around 2098, 2090, 2081 and 2072 cm^{-1} , as reported previously.¹⁹ These observations demonstrate that the ^{13}CO ligand of the [NiFe] site originates from externally supplied gaseous ^{13}CO , which is consistent with observations previously made for the regulatory hydrogenase from *R. eutropha*.²⁰

The comparison of NRVS signatures between 550 - 650 cm^{-1} obtained from unlabeled and ^{13}CO -labeled SH reveals isotopic shifts of 5 - 11 cm^{-1} , indicating that major contributions of these bands are due to Fe-CO stretching and bending (Figure 4A and B). In contrast, bands at lower frequencies are largely unaffected by ^{13}CO labeling, demonstrating their affiliation with normal modes that are dominated by Fe-CN coordinates. In addition, closer inspection of ^{13}CO isotopic shifts may also yield information about the character of individual Fe-CO modes in terms of predominant stretching and bending contributions.²¹ The larger ^{13}C isotopic shift, as observed for the band at 609 cm^{-1} ($\Delta v = -11 \text{ cm}^{-1}$), likely reflects more pronounced Fe-C-O bending contributions, while the smaller ^{13}C effects on the band at 561 cm^{-1} ($\Delta v = -5 \text{ cm}^{-1}$) is indicative of a predominant Fe-CO stretching character. In line with previous computational studies,^{10,11} these observations provide first experimental insights into normal mode compositions (Fe-CO vs. Fe-CN) of the [NiFe] hydrogenase active site. Interestingly, Fe-CO modes of the SH, as probed by NRVS, appear at slightly higher frequencies compared to oxidized *Dv* hydrogenase while the IR-

traceable CO stretching vibrations are nearly the same. This observation may provide further details on structural and electronic properties of the SH active site.

Conclusions

In conclusion, the first combined NRVS and IR characterization of oxygen-tolerant [NiFe]-hydrogenase revealed the content and redox behavior of FeS clusters in the NAD^+ -reducing enzyme from *Ralstonia eutropha* H16. We demonstrated the presence of one [2Fe2S] center and four [4Fe4S] clusters, which are only partly reduced upon incubation of the enzyme with the native electron donor NADH. This finding unravels the discrepancy between the number of predicted and EPR-detected FeS clusters in SH and related proteins, including Complex I. This finding underscores the benefit of NRVS for the characterization of complex metalloproteins. Furthermore, our combined approach of NRVS and IR spectroscopy provides access to the entire vibrational signature of the $\text{Fe}(\text{CO})(\text{CN})_2$ moiety of the hydrogenase active site. For the first time, selective isotope labeling with gaseous ^{13}CO allowed the experimental discrimination between Fe-CO and Fe-CN normal modes. Moreover, bands with predominant Fe-CO stretching and bending character could be identified. In summary, vibrational spectroscopy in conjunction with selective isotope labeling allows detailed insights into molecular coordinates of the active site in hydrogenase. An extension of this approach in terms of other site-specific isotope exchanges will likely provide further valuable information about functional and structural aspects of hydrogen-converting biocatalysts.

Acknowledgements

The SH biochemistry and IR work (LL, MH, IZ and OL) were supported by the Deutsche Forschungsgemeinschaft (DFG, Cluster of Excellence UniCat). LL received a short-term EMBO scholarship for performing the work in this study. HW, LG and SPC were supported by National Institutes of Health (GM-65440 to SPC). The NRVS experiments were performed at BL09XU and BL19LXU of SPring-8 with the approval of JASRI and RIKEN (Proposal No. 2013B0103 and 20133892).

Notes and references

¹ Institute of Chemistry, Technische Universität Berlin, Straße des 17. Juni 135, 10623 Berlin, Germany;

² Department of Chemistry, University of California, One Shields Ave, Davis CA 95616, USA;

³ Physical Biosciences Division, Lawrence Berkeley National Laboratory, One Cyclotron Road, Berkeley CA 94720, USA;

⁴ JASRI, SPring-8, 1-1-1 Kouto, Mikazuki-cho, Sayo-gun, Hyogo 679-5198, Japan;

⁵ RIKEN, SPring-8, 1-1-1 Kouto, Mikazuki-cho, Sayo-gun, Hyogo 679-5198, Japan

† Corresponding Authors: lars.lauterbach@tu-berlin.de and spcramer@lbl.gov

[‡] These authors contributed equally.

Electronic Supplementary Information available: representations of data analyses (Fig. S1 and S2). See DOI: 10.1039/b000000x/

1. J. C. Fontecilla-Camps, A. Volbeda, C. Cavazza and Y. Nicolet, *Chem Rev*, 2007, **107**, 4273-4303.
2. K. Schneider and H. G. Schlegel, *Biochemical Journal*, 1981, **193**, 99-107.
3. L. Lauterbach and O. Lenz, *J Am Chem Soc*, 2013, **135**, 17897-17905.
4. L. Lauterbach, O. Lenz and K. A. Vincent, *The FEBS journal*, 2013, **280**, 3058-3068.
5. M. Horch, L. Lauterbach, O. Lenz, P. Hildebrandt and I. Zebger, *FEBS Lett*, 2012, **586**, 545-556.
6. M. Horch, L. Lauterbach, M. Saggi, P. Hildebrandt, F. Lenzian, R. Bittl, O. Lenz and I. Zebger, *Angew Chem Int Edit*, 2010, **49**, 8026-8029.
7. F. Germer, I. Zebger, M. Saggi, F. Lenzian, R. Schulz and J. Appel, *J Biol Chem*, 2009, **284**, 36462-36472.
8. K. Schneider, R. Cammack and H. G. Schlegel, *Eur J Biochem*, 1984, **142**, 75-84.
9. S. Kamali, H. Wang, D. Mitra, H. Ogata, W. Lubitz, B. C. Manor, T. B. Rauchfuss, D. Byrne, V. Bonnefoy, F. E. Jenney, Jr., M. W. Adams, Y. Yoda, E. Alp, J. Zhao and S. P. Cramer, *Angew Chem Int Ed Engl*, 2013, **52**, 724-728.
10. M. Horch, J. Schoknecht, M. A. Mroginski, O. Lenz, P. Hildebrandt and I. Zebger, *J Am Chem Soc*, 2014, **136**, 9870-9873.
11. E. Siebert, M. Horch, Y. Rippers, J. Fritsch, S. Frielingsdorf, O. Lenz, F. V. Escobar, F. Siebert, L. Paasche, U. Kuhlmann, F. Lenzian, M. A. Mroginski, I. Zebger and P. Hildebrandt, *Angew Chem Int Edit*, 2013, **52**, 5162-5165.
12. W. Sturhahn, *Hyperfine Interactions* 2000, **125**, 149.
13. Y. Xiao, M. L. Tan, T. Ichiye, H. Wang, Y. Guo, M. C. Smith, J. Meyer, W. Sturhahn, E. E. Alp, J. Zhao, Y. Yoda and S. P. Cramer, *Biochemistry*, 2008, **47**, 6612-6627.
14. D. Mitra, V. Pelmeshnikov, Y. Guo, D. A. Case, H. Wang, W. Dong, M. L. Tan, T. Ichiye, F. E. Jenney, M. W. Adams, Y. Yoda, J. Zhao and S. P. Cramer, *Biochemistry*, 2011, **50**, 5220-5235.
15. D. Mitra, S. J. George, Y. Guo, S. Kamali, S. Keable, J. W. Peters, V. Pelmeshnikov, D. A. Case and S. P. Cramer, *J Am Chem Soc*, 2013, **135**, 2530-2543.
16. C. Zaborosch, M. Koster, E. Bill, K. Schneider, H. G. Schlegel and A. X. Trautwein, *Biometals*, 1995, **8**, 149-162.
17. H. R. Bridges, E. Bill and J. Hirst, *Biochemistry*, 2012, **51**, 149-158.
18. L. A. Sazanov and P. Hinchliffe, *Science*, 2006, **311**, 1430-1436.
19. R. P. Happe, W. Roseboom, G. Egert, C. G. Friedrich, C. Massanz, B. Friedrich and S. P. J. Albracht, *FEBS letters*, 2000, **466**, 259-263.
20. I. Bürstel, P. Hummel, E. Siebert, N. Wisitruangsakul, I. Zebger, B. Friedrich and O. Lenz, *J Biol Chem*, 2011, **286**, 44937-44944.
21. M. Tsubaki, R. B. Srivastava and N. T. Yu, *Biochemistry*, 1982, **21**, 1132-1140.