

Understanding Hydrogenases by Ultrafast and 2D-IR Spectroscopy: Proof-of-Concept, Recent Advances, and Future Avenues

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Abstract

Here we report insights into the structure, function, and dynamics of hydrogenases – complex metalloenzymes that catalyse the reversible cleavage of molecular hydrogen – by ultrafast pump-probe and 2D-IR spectroscopy experiments performed using ULTRA. Utilizing O₂-tolerant [NiFe] hydrogenases as model systems, we illustrate how these techniques can be used to explore bond properties, equilibrium and vibrational dynamics, and complex mixtures of molecular states. Fundamental insights will be discussed together with recent progress and perspectives.

Introduction

Molecular hydrogen (H₂) is an ideally clean fuel that releases large amounts of energy but no greenhouse gases upon combustion. So far, however, energy conversion approaches involving H₂ are, *inter alia*, limited by efficient and sustainable strategies for H₂ activation and evolution. Hydrogenases are metalloenzymes that can facilitate these processes by catalysing the reversible cleavage of H₂ using sophisticated active sites that contain cheap and earth-abundant base metals only.^{1,2} Thus, these metalloenzymes represent valuable targets for biotechnological applications and bioinspired catalyst design. However, their rational utilization is still limited by (1) their general sensitivity towards industrially relevant conditions, e.g. the presence of oxygen (O₂) or elevated temperatures, and (2) a lack of mechanistic understanding with respect to key reaction steps and the relation between structure, function, and dynamics.

Several hydrogenases of the [NiFe] subtype are able to sustain catalytic H₂ cycling in the presence of ambient O₂ levels and, in some cases, also at elevated temperatures.^{2,3} Consequently, these O₂-tolerant [NiFe] hydrogenases are promising candidates for a green H₂ economy. Besides other cofactors, they contain a catalytic site composed of two metal ions, Ni and Fe, which are coordinated by four cysteines (Fig. 1).⁴ In addition, the Fe is equipped with three biologically uncommon ligands, one carbon monoxide (CO) and two cyanides (CN⁻),⁵ which are relevant for two reasons. First, these (Lewis-amphoteric) ligands tune the properties of the catalytic site in a complex and not yet fully understood manner,^{2,6} thereby enabling biocatalytic H₂ conversion.⁷ Secondly, they absorb IR radiation – at frequencies that are highly sensitive to structural and electronic changes and in a spectral regime where typical biomolecules are otherwise trans-

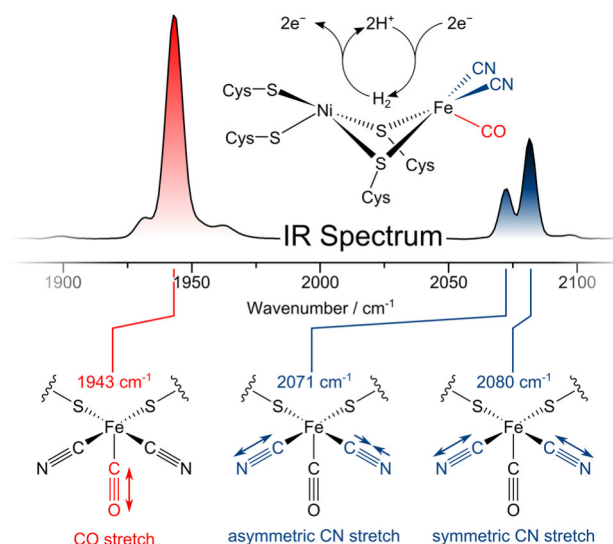


Fig. 1: IR absorption spectrum and active site structure of oxidized ReRH in the Ni_a-S state. CO (red) and CN (blue) stretching modes and frequencies are indicated. Reproduced from Ref. 9 with permission from the Royal Society of Chemistry.

parent (Fig. 1).⁸ Thus, they represent ideal IR chromophores, so that IR spectroscopy has been one of the most important techniques in hydrogenase research.

Until recently, IR spectroscopy of hydrogenases has been limited to linear absorption experiments and the interpretation of fundamental vibrational transition frequencies. Moreover, most studies have focused on time-averaged insights from equilibrium and steady-state studies. Based on experiments performed with the ULTRA laser system, we have recently introduced ultrafast pump-probe and two-dimensional (2D) IR spectroscopy to hydrogenase research to overcome this limitation and gain insights into structural details and dynamics.⁹ Using O₂-tolerant [NiFe] hydrogenases as model systems, we will summarize key results from our first proof-of-concept study (see Ref. 9 for details and experimental procedures) together with recent advances and future perspectives. Due to the COVID-19 pandemic, experiments scheduled for March 2020 were interrupted, so that only preliminary statements can be made regarding latest results.

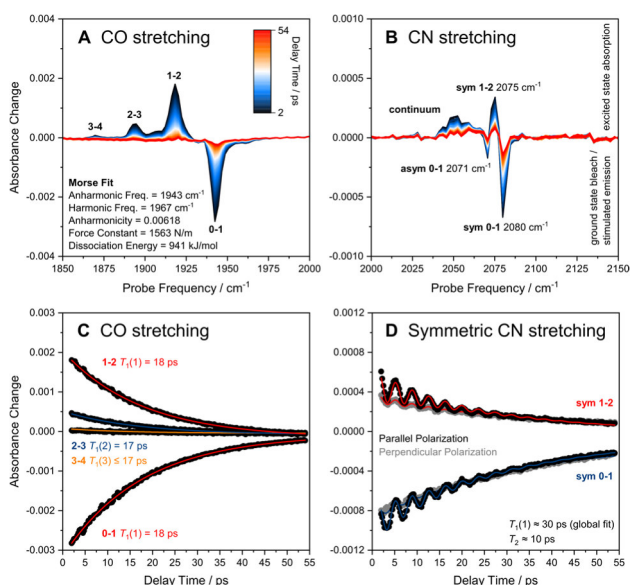


Fig. 2: IR pump-probe spectra (A and B) and time evolution of selected signals (C and D) as obtained for oxidized ReRH in the Ni_a-S state. Unless indicated otherwise, data were acquired with parallel pump-probe polarization. Transitions between vibrational eigenstates $|m\rangle$ and $|n\rangle$ are labelled as $m-n$. For the CO stretch mode (C), vibrational lifetimes $T_1(n)$ of eigenstates $|n\rangle$ were obtained by fitting monoexponential decay curves (coloured lines) to experimental $m-n$ time traces (black dots). For the symmetric CN stretch mode (D), $T_1(1)$ and T_2 were determined by globally fitting the sum of a monoexponential decay curve and a monoexponentially damped sine function (coloured lines) to experimental 0-1 and 1-2 time traces obtained with both parallel (black dots) and perpendicular (grey dots) polarization. sym, symmetric CN stretch mode; asym, asymmetric CN stretch mode. Reproduced from Ref. 9 with permission from the Royal Society of Chemistry.

Results & Discussion

Despite sharing a conserved active-site architecture, individual [NiFe] hydrogenases from different organisms differ considerably, and numerous redox-structural states (catalytic intermediates) of the active site can be observed.^{1,2} For our first proof-of-concept study, we chose the O₂-tolerant¹⁰⁻¹² regulatory [NiFe] hydrogenase from the ‘Knallgas’ bacterium *Ralstonia eutropha* (ReRH) as a simple model system since its air-oxidized form adopts a single redox-structural state,¹¹⁻¹⁴ which also represents the catalytic intermediate involved in the initial binding of H₂.^{7,14,15,16} This state (Fig. 1), called Ni_a-S, will be analysed in the following.

Mapping Chemical Bonds Below 2000 cm⁻¹. The pump-probe spectrum of oxidized ReRH exhibits a sequence of signals that can be assigned to the excitation of the active-site CO stretching mode up to the fourth vibrational level (Fig. 2A).⁹ Given the bond-localized nature of this mode and a constant signal spacing of about 24 cm⁻¹, a Morse potential¹⁷ can be fit to the transition energies. Thereby, solution-phase insights into otherwise inaccessible CO bond properties can be obtained, including the equilibrium bond strength (as reflected by the force constant, $f = 1563$ N m⁻¹) and the overall bond strength (as reflected by the dissociation energy, $D_0 = 941$ kJ mol⁻¹).⁹ Using this approach, ongoing analyses of bond properties in different hydrogenases and various catalytic states will help to rationalise previous observations from conventional IR absorption experiments and provide detailed insights into metal carbonyl bonding. These aspects are crucial, e.g., for understanding biological hydrogen conversion by [NiFe] hydrogenases.

Weaker signals pertaining to CN stretching modes can also be observed in the pump-probe spectrum at frequencies above 2000 cm⁻¹ (Fig. 2B),⁹ but their analysis in terms of bond properties is so-far limited by strong overlap and the inclusion of both CN bond coordinates in either of the two modes. This situation leaves further challenges for future experimental and theoretical studies.

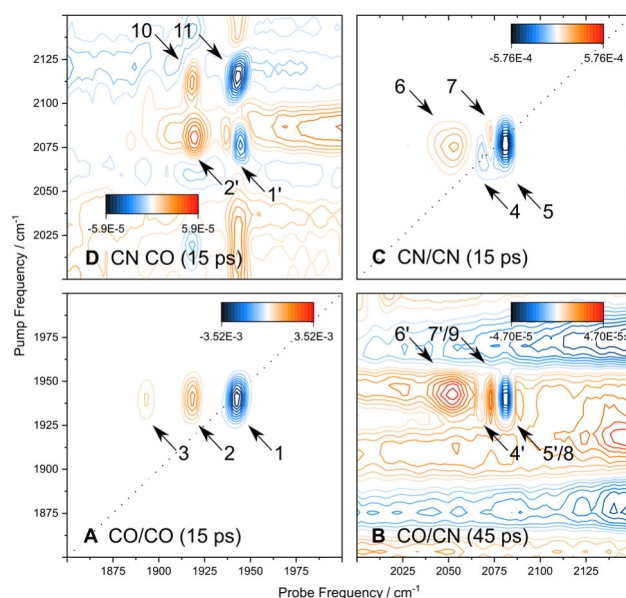


Fig. 3: 2D-IR spectrum of oxidized ReRH in the Ni_a-S state, recorded with parallel polarization. Quadrants A to D reflect different pump and probe regimes, as indicated. Waiting times T_w are listed for each quadrant. Signals are numbered, and those reflecting vibrational energy transfer are marked with a prime. Reproduced from Ref. 9 with permission from the Royal Society of Chemistry.

Relaxation and Energy Transfer Vibrational lifetimes T_1 were determined for both CO and CN stretch modes from time series of pump-probe spectra (Fig. 2C and D).⁹ In both cases, vibrationally excited states decay quickly within few tens of picoseconds – faster than for comparable metal carbonyl or cyanido compounds that were previously probed in weakly interacting hydrophobic solvents.^{18,19,20} This finding indicates a functional role of the protein matrix in dissipating energy from the active site on ultrashort timescales *via* a common route that is shared by relaxation pathways of both vibrational modes. This statement is further supported by waiting-time-dependent CO/CN stretch cross peaks in the 2D-IR spectra (Fig. 3B and D; signals 1' – 7'), indicating fast energy redistribution between both sets of ligands.⁹ In contrast to small-molecule analogues, efficient energy transfer from the deeply buried catalytic site of [NiFe] hydrogenases is not trivial *per se* and, thus, potentially relevant to prevent barrier recrossing of ‘hot’ molecules in the product well from limiting the catalytic rate. Ongoing experiments aim to shed more light on these aspects, *inter alia*, by extending experiments towards [FeFe] hydrogenases, which will allow direct comparison with small-molecule [FeFe] models whose relaxation properties were previously characterized using ULTRA.^{18,20}

Molecular Connectivity and Equilibrium Dynamics Based on both pump-probe and 2D-IR data, we have explored the interaction of the three ligand stretching modes with each other and the protein environment of the [NiFe] site. Fundamental transitions of symmetric and antisymmetric CN stretching modes are separated by only 9 cm⁻¹, complicating the detection of cross peaks between them in the 2D-IR spectrum. Nevertheless, the presence of a CN/CN cross peak can be inferred from an elongation of the main diagonal peak (pertaining to bleaching / stimulated emission of the higher-frequency, symmetric CN stretch mode) towards lower pump frequencies (Fig. 3C, signal 5).⁹ In recent experiments, we could improve the pump frequency resolution, thereby allowing to distinguish such close-lying cross peaks even in mixtures of different redox-structural [NiFe] states. Anharmonic coupling of the CN stretch modes is also proven by the observation of quantum beats with a frequency of 9 cm⁻¹ in the associated relaxation traces (Fig. 2D).⁹ Individual frequency components can be extracted from these quantum-beat patterns by Fourier transformation, thereby providing an additional tool for assigning pairs of signals in complex component spectra.⁹ In addition, a dephasing time of $T_2 \approx 10$ ps can be extracted from the amplitude decay of this oscillatory modulation. Comparison

with the inverse CN stretch linewidths indicates that these are inhomogeneously broadened.⁹ The associated frequency distribution is very narrow (6 cm⁻¹), indicating that the [NiFe] site is structurally constrained – in line with other studies and the functional relevance of entatic states for hydrogenase catalysis.^{7,14,15}

Parts of the CO/CN cross peak signals (Fig. 3B, signals 8 and 9) are present from early waiting times on (250 fs), indicating that CO and CN stretch modes are coupled as well.⁹ In addition, we have observed cross peaks (Fig. 3D, signals 10 and 11) matching the probe frequencies of CO stretch transitions (Fig. 3A,) at a pump frequency above 2100 cm⁻¹ that cannot be associated with any of the diagonal signals (Fig. 3C). These signals were identified as nonlinear pulse shaper artefacts in a recently performed power-dependent study. The possibility of such artefacts should be considered in similar studies.

Conclusions & Outlook

Here we have summarized key findings from ultrafast pump-probe and 2D-IR experiments performed at ULTRA. Based on a proof-of-concept study⁹ and more recent experiments, we have highlighted the potential of these techniques and the ULTRA laser system for studying the structure and dynamics of hydrogenases and other complex bioinorganic and organometallic targets at an unrivalled level of detail. Experimental improvements and potential pitfalls have been illustrated together with future challenges in advanced bioinorganic IR spectroscopy.

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