Progress in the 2D-IR Characterization of Hydrogenases at Ultra

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Introduction

Molecular hydrogen (H_2) is a perfectly clean fuel that releases large amounts of free energy but no green-house gases upon combustion.[1] Employing H_2 as a future fuel requires efficient and sustainable strategies for its production and activation. Hydrogenases are complex metalloenzymes that catalyze the reversible cleavage of H_2 by utilizing sophisticated base-metal actives sites, thereby providing their host organisms with the ability to utilize H_2 as a source of energy or a means for disposing of excess electrons.[2] Hydrogenases might be used for future biotechnological activation or serve as a blueprint for the design of cheap and efficient H_2 conversion catalysts that obviate the usage of expensive and resource-limited noble metals.

Two main classes of hydrogenases with bimetallic active sites can be distinguished (Fig. 1):[2] [NiFe] hydrogenases contain a heterobimetallic active site containing Ni and Fe coordinated by a total of four cysteine thiolates from the protein.[3] [FeFe] hydrogenases, on the other hand, feature a more complex active site, called the H-cluster. This cofactor can be divided into two sub-parts, an ordinary [4Fe4S] cluster and a unique [2Fe] center, that are connected to each other and the protein matrix by a single cysteine residue.[4,5] Catalytic H_2 conversion is accomplished by the [2Fe] subsite, which contains two Fe ions bridged by a 2-azapropane-1,3-dithiolate ligand. Active sites of both classes of hydrogenases additionally feature biologically uncommon inorganic ligands, carbon monoxide (CO) and cyanide (CN^-).[6–12]

A rational application of hydrogenases requires a thorough understanding of their catalytic mechanism and its relation to molecular structure and dynamics. Consequently, spectroscopic techniques play a key role in the investigation of these enzymes.[2] CO and CN^- ligands at their active sites represent ideal vibrational probes whose stretching vibrations give rise to structurally sensitive signals in the infrared (IR) absorption spectrum (Fig. 1). Thus, IR spectroscopy has long been used for studying both [NiFe] and [FeFe] hydrogenases.[2,6–15] While most of these studies have been performed on isolated and purified protein, selected studies have probed hydrogenases within live cells,[16–19] thereby gaining insights into structural and functional aspects under physiologically relevant conditions.

Despite the strength of IR spectroscopy, the informational content of linear IR spectra is inherently limited. We have therefore utilized ULTRA to introduce ultrafast and nonlinear IR techniques to hydrogenase research. Specifically, we have recorded IR_{pump}-IR_{probe} and 2D-IR spectra to provide insights into active-site bond properties, vibrational coupling, energy transfer, and structural dynamics of a well-studied [NiFe] hydrogenases.[20] This proof-of-concept study was later

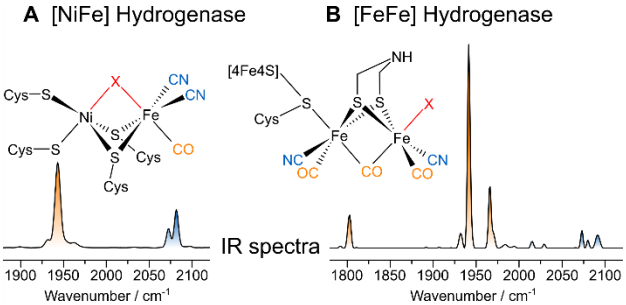

Fig. 1: Active-site structures and linear IR absorption spectra of (A) [NiFe] hydrogenases and (B) [FeFe] hydrogenases.

complemented by computational work and studies on a model complex and a more complex [NiFe] hydrogenase.[21–23] Here we report on the latest extensions of this approach through experiments performed at ULTRA.

Nonlinear IR Spectroscopy of [FeFe] Hydrogenases

So far, IR_{pump}-IR_{probe} and 2D-IR studies on hydrogenases have been limited to the [NiFe] class. Synthetic di-iron complexes mimicking selected features of the [2Fe] subsite of [FeFe] hydrogenases were previously studied by these techniques as well, also at ULTRA.[24–28] However, nonlinear IR spectra have not been obtained from [FeFe] hydrogenases, which feature a much more complex vibrational manifold than [NiFe] hydrogenases. Probing these enzymes by nonlinear IR

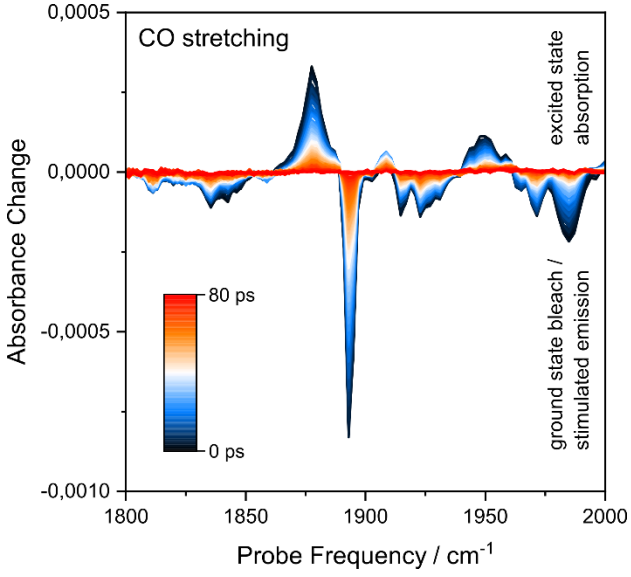

Fig. 2: IR_{pump}-IR_{probe} spectrum of reduced [FeFe] hydrogenase, showing the spectral region typical for CO stretching vibrations.

techniques is technically challenging, due to a manifold of fundamental transition with narrow linewidths of ca. 5 cm^{-1} that extend over a broad frequency range of ca. 300 cm^{-1}. Utilizing the exceptional qualities of ULTRA laser and detection systems, we have recorded IR_{pump}-IR_{probe} and 2D-IR spectra of various catalytic and non-catalytic states of multiple [FeFe] hydrogenases. Fig. 2 shows an exemplary dataset depicting a time series of IR_{pump}-IR_{probe} spectra obtained from an [FeFe] hydrogenase in its reduced state, illustrating the feasibility of the approach. Ground-state bleaching and stimulated emission (negative) as well as excite-state absorption (positive) is observed for both CO and CN stretching modes (the latter are not shown in Fig. 2). Due to the complexity of the spectra, related to the presence of at least five CO/CN oscillators, complementary computational work is necessary to understand the encoded structural and dynamical information.

Towards *in vivo* 2D-IR Spectroscopy of [NiFe] hydrogenases

IR spectroscopy is a powerful technique for studying biological molecules under native conditions. CO and CN stretching vibrations associated with the inorganic ligands at hydrogenase active site (Fig. 1) appear in a spectral regime that is free of other spectral contributions from biological molecules. Thus, these CO and CN^- ligands represent ideal IR spectroscopic probes for studies in complex biological environments. A unique NAD^+-reducing hydrogenase from the bacterium *Cupriavidus necator* (formerly *Ralstonia eutropha*) was previously studied by IR absorption spectroscopy within live cells.[19] This study revealed the native active-site structure of the enzyme and disproved a previous model for explaining its oxygen tolerance. Utilizing a unique approach for probing strongly scattering samples, recently established at ULTRA,[29] we have attempted to record IR_{pump}-IR_{probe} and 2D-IR of this NAD^+-reducing hydrogenase within live cells of *C. necator*. Preliminary data indicate that this approach is feasible, promising valuable insights into the molecular properties of hydrogenases in their native cellular environment.

Conclusions

In conclusion, we have utilized the exceptional qualities of ULTRA to extend the application of ultrafast and nonlinear IR techniques in hydrogenase research. On the one hand, we have applied IR_{pump}-IR_{probe} and 2D-IR spectroscopies to [FeFe] hydrogenases, which significantly exceed [NiFe] hydrogenases in terms of catalytic efficiency and active-site complexity. Comparison of these two classes of hydrogenases will provide insights into their individual properties as well as the structural and dynamical aspects that set them apart. Complementary computational work is necessary to fully understand the vibrational complexity of [FeFe] hydrogenases and its relation to molecular geometry and electronic structure. On the other hand, we have obtained preliminary data of a [NiFe] hydrogenase within live cells, illustrating the general possibility to probe these enzymes *in vivo*. Planned work at ULTRA will be dedicated to technical improvements and extended applications of this approach to gain insights into the impact of the cytoplasmic environment on the native structural and dynamical properties of these enzymes.

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