Following [FeFe] Hydrogenase Active Site Intermediates by Time-Resolved Mid-IR Spectroscopy

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Supporting Information

ABSTRACT: Time-resolved nanosecond mid-infrared spectroscopy is for the first time employed to study the [FeFe] hydrogenase from Chlamydomonas reinhardtii and to investigate relevant intermediates of the enzyme active site. An actinic 355 nm, 10 ns laser flash triggered photodissociation of a carbonyl group from the CO-inhibited state Hox-CO to form the state Hox which is an intermediate of the catalytic proton reduction cycle. Time-resolved infrared spectroscopy allowed us to directly follow the subsequent rebinding of the carbonyl, re-forming Hox-CO, and determine the reaction half-life to be $t_{1/2} \approx 13 \pm 5$ ms at room temperature. This gives direct information on the dynamics of CO inhibition of the enzyme.

[FeFe] hydrogenases are the most efficient proton-reducing enzymes with turnover frequencies (TOFs) of up to several thousand per second.¹ Much work has been devoted to understanding the mechanisms of their operation and of competing inhibitory reactions.² Detailed understanding and comparison of hydrogenases from different organisms is of fundamental interest and may also guide efforts toward photobiological and photochemical hydrogen production using engineered microorganisms or bioinspired molecular catalysts.³−¹⁸ The high maximum TOF of these enzymes warrants the need for transient techniques that are on the same time scale or faster, so that transient intermediates of the catalytic cycle can be resolved.¹⁹ Until now, only equilibrium states have been detected under non-turnover conditions, and many measurements have been performed at very low temperatures, which is far from the physiological conditions under which these enzymes operate.²,²⁰ Time-resolved spectroscopic techniques may both resolve the kinetics between the known equilibrium states as well as discover new transient intermediates.

The current study was performed on an [FeFe] hydrogenase originating from Chlamydomonas reinhardtii (CrHydA1), which was obtained by overexpressing the apoenzyme in Escherichia coli²¹ and subsequent artificial maturation with a synthetic co-factor.²²,²³ To follow the intermediates of CrHydA1 transiently, the sample was excited with a laser pulse (fwhm: 10 ns; λ: 355 nm) to trigger photodissociation of a carbonyl group from a CO-inhibited state "Hox-CO" and generate a state, "Hox", that is part of the catalytic cycle. The released carbon monoxide then rebinds to the Hox state to re-form the CO-inhibited state Hox-CO. The reaction is followed with transient absorption spectroscopy at room temperature using mid-IR laser light, which is an excellent technique to follow changes of the 3–4 carbonyl and 2 cyanide ligands in the active site. The FTIR spectra of most of the states are known, which allows for safe assignments.²⁴,²⁵ CO inhibition of [FeFe] hydrogenases and its relation to O₂ inactivation have been investigated with voltammetry and spectroscopic methods, which do not give direct information on the CO binding dynamics.²⁰,²⁶–²⁸ Research on specific CO binding dynamics of enzymes has in the past involved proteins with a heme group, such as cytochrome c and myoglobin. Step-scan FTIR measurements have been used to obtain information on their CO dissociation after photolysis.²⁹,³⁰ We know of no step-scan FTIR studies of CO binding and inhibition in hydrogenases. This might be due to the limitation of the step-scan technique requiring a sample that is stable during >10⁴ laser flashes and that give rise to large signals.³¹ In the present work, we instead measure the kinetics in single-wavenumber traces using an average of only ~10 laser flashes. This method has recently been used to study intermediates of a [NiFe] hydrogenase (TOF = 62 s⁻¹) following laser-flash generation of reducing equivalents.³² Because the measurements were performed at ambient, anaerobic conditions in an aqueous solution, they reflect the natural behavior of the enzyme. Water absorbs well in the mid-IR region, and therefore, the thickness of the IR cell was chosen to be only 50 μm. This also means that a high concentration of CrHydA1 was needed to have sufficient absorption to observe the changes occurring after excitation. The concentration of CrHydA1 was chosen to be 2.5 mM in all measurements.

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A steady-state mid-FTIR spectrum was obtained before and after the 355 nm light excitation experiments, showing no significant changes after flashing with 355 nm light (Figure S1). When overlapping the \( H_{\alpha}\)-CO steady-state spectrum with the transient spectrum (Figure 1), there is perfect correspondence between the bleach features in the transient spectrum (in black) and the peaks in the steady-state spectrum (in red), showing that by flashing the sample a reversible chemical conversion is occurring. The infrared detection capabilities are limited to that by probe light intensity. The cyanide peaks between 2070 and 2150 cm\(^{-1}\) are detected; the 1964 cm\(^{-1}\) peak is overlapping with another peak. Replacing the \( ^{12}\text{CO} \) with \( ^{13}\text{CO} \) in the \([\text{FeFe}]\) complex, as is the case with myoglobin and cytochrome \( c\), \( ^{13}\text{CO} \) may reach several sites with various rebinding rate constants, giving a multiexponential decay (that will be difficult to distinguish from a second-order decay), or possibly completely dissociate to the bulk solution, which could then give a true second-order rebinding. The recombination rate \( (t_{1/2} \approx 13 \pm 5 \text{ ms}) \) compares well to similar rebinding experiments such as myoglobin–CO where the half-life for rebinding of CO at room temperature after photolysis is on the order of \( 10^{-2} \text{ ms} \) or cytochrome \( c\)–CO with a rebinding time constant on the order of \( 10^{-3} \text{ ms} \) at room temperature.

CrHydA1 has been tested for hydrogen evolution and can reach TOFs of 500–1000 s\(^{-1}\). This means that each turnover is completed within 1–2 ms. This opens the way to use the photodissociation of the \( H_{\alpha}\)-CO state as trigger to start a catalytic cycle. The long lifetime of the \( H_{\alpha}\) state \( (t_{1/2} \approx 13 \pm 5 \text{ ms}) \) determined here means that this catalytically active species has time to complete several catalytic cycles before it is inhibited, even in the presence of free CO at ambient temperature.

The present experiments show that time-resolved studies of catalytic intermediates of \([\text{FeFe}]\) hydrogenases are possible using nanosecond laser flash/mid-IR probe spectroscopy. This opens the way for further studies where laser flash dissociation of CO can be a trigger for a \( H_2 \) molecule to enter the catalytic cycle and allows identification of further active site intermediates and the kinetics of their interconversion.

**Experimental Methods**

The gene for the unmatured CrHydA1 was overexpressed in *E. coli* strain BL21(DE3) *ΔiscR* with a pET21(b) plasmid containing the codon-optimized HydA gene with an N-terminal strep-II-tag and TEV cleavage site. Bacteria were grown...
according to Kuchenreuther et al., but without coexpression of maturases and without any sodium selenate. The LB contains 100 mg/L ampicillin, 30 mg/L kanamycin, 2 mM ferric ammonium citrate, 5 g/L glucose, 3.5 mM cysteine, and 0.5 mM of the inductor IPTG for the 20 h anaerobic expression at room temperature. After affinity chromatography purification, the strep-II-tag was cleaved overnight by His6-tagged TEV protease, which was finally removed. The unmutated protein was diluted to 250 μM in 25 mM Tris, 25 mM KCl, pH 8.0, and activated for 1 h at room temperature with an excess of \([\text{Fe}_2(\text{adt})(\text{CO})_4(\text{CN})_2]^{2-}\). After removal of the excess complex, \(\text{CrH}y\text{d}A1-\text{adt}\) was concentrated to 2.5 mM.

For room-temperature (−22 °C) transient absorption measurements, a frequency-tripled Q-switched Nd:YAG laser (Quanta-Ray ProSeries, Spectra-Physics) was employed to obtain 355 nm pump light with 50 mJ/pulse and a fwhm of 10 ns. Probing was done with two continuous-wave quantum cascade (QC) IR lasers with a tuning capability between 1765 and 1925 cm⁻¹ for laser 1 and 1960 and 2150 cm⁻¹ for laser 2 (Daylight Solutions). For IR detection, a liquid-nitrogen-cooled mercury–cadmium–telluride (MCT) detector (KMPV10-1-J2, Kolmar Technologies, Inc.) was used.

The IR probe light was superimposed on the actinic laser beam in a quasi-collinear arrangement at an angle of 25°. Transient absorption traces were acquired with a Tektronix TDS 3052 500 MHz (5GS/s) oscilloscope in connection with the L900 software (Edinburgh Instruments) and processed using Origin 9 software. Samples were kept in a home-built IR glovebox (Unilab, MBraun).

Infrared steady-state spectra were collected on a Bruker IFS 66v/S FTIR spectrometer controlled with OPUS software with a liquid-nitrogen-cooled MCT detector from Kolmar Technologies. The resolution of all measurements was 2 cm⁻¹, and the apodization function used was the Blackman–Harris three-term.

The quantum yield (\(\phi\)) is calculated by estimating the number of CO-photodissociated enzymes (\(N_{\text{diss}}\)) and dividing this value by the number of absorbed photons (\(N_{\text{abs}}\))

\[
\phi = \frac{N_{\text{diss}}}{N_{\text{abs}}}
\]

The number of photodissociated enzymes can be measured by looking at the bleaches of the transient signals (\(\Delta A_{\text{bs}}\)) to estimate the concentration by Lambert–Beer law and multiplying the concentration (\(c\)) by the excitation beam cross section (\(A = 1 \text{ cm}^2\)) and the path length of the cell (\(l = 50 \mu\text{m}\))

\[
c = \frac{\Delta A_{\text{bs}}}{\epsilon l}
\]

and

\[N_{\text{diss}} = N_A c A l\]

yields

\[N_{\text{diss}} = \frac{N_A A \Delta A_{\text{bs}}}{\epsilon} \]

where \(\epsilon\) is the extinction coefficient at the wavenumber of the bleached band in question and \(N_A\) is the Avogadro constant.

The number of photons (\(N_{\text{pho}}\)) can be calculated by measuring the excitation pulse energy (\(E = 50 \text{ mJ}\)) and calculating the number of photons in the pulse by knowing that all photons have a wavelength (\(\lambda\)) of 355 nm

\[N_{\text{pho}} = \frac{E \lambda}{h c_{\text{light}}} \]

where \(h\) is the Planck constant and \(c_{\text{light}}\) is the speed of light. The number of absorbed photons can be determined by knowing the absorbance of the sample at 355 nm (Abs)

\[N_{\text{abs}} = N_{\text{pho}} (1 - 10^{-\text{Abs}}) = \frac{E \lambda (1 - 10^{-\text{Abs}})}{h c_{\text{light}}} \]

Therefore, the quantum yield is then

\[\phi = \frac{N_{\text{diss}}}{N_{\text{abs}}} = \frac{h c_{\text{light}} N_A A \Delta A_{\text{bs}}}{E \lambda (1 - 10^{-\text{Abs}})} \]

The fit equation for the second-order decays in Figure S4 is

\[y = \frac{[A]_0}{1 + [A]_0 k(x - t_0)} + b\]

where \([A]_0\), \(k\), \(t_0\) and \(b\) are constants corresponding to the initial absorbance at time zero, the second-order rate constant, time zero, and a background offset, respectively.

The fit equation for the monoexponential decays in Figure S5 is

\[y = [A]_0 e^{-(x - t_0)/\tau} + b\]

where \([A]_0\), \(\tau\), \(t_0\) and \(b\) are constants corresponding to the initial absorbance at time zero, the lifetime of the process, time zero, and a background offset, respectively.

The three ratios between the \(\chi^2\) values of the monoexponential (first-order) fits and the second-order fits for the 1811, 1968, and 2013 cm⁻¹ wavenumbers are 1.8, 2.0, and 0.52 respectively. This means that the second-order fits for two out of three decay traces are better than the monoexponential (first-order) fits.

### ASSOCIATED CONTENT

Supporting Information
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Additional FTIR and UV–vis spectra and transient IR traces (PDF)

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Notes
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