FTIR study on the light sensitivity of the [NiFe] hydrogenase from *Desulfovibrio vulgaris* Miyazaki F: Ni–C to Ni–L photoconversion, kinetics of proton rebinding and H/D isotope effect

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Received 8th July 2009, Accepted 28th July 2009
First published as an Advance Article on the web 18th August 2009
DOI: 10.1039/b913635e

The light-induced Ni–C to Ni–L transition results in the dissociation of a hydrogenic species, originating from the dihydrogen splitting at the active site. Back conversion in the dark to form Ni–C was investigated by studying the rebinding kinetics of this ligand in protonated (H_2/H_2O) and deuterated (D_2/D_2O) samples using time resolved FTIR spectroscopy.

Introduction

Hydrogenases are bidirectional enzymes that play a key role in the cellular energy metabolism of a wide variety of microorganisms.[1] The [NiFe] hydrogenase from the anaerobic sulfate reducing bacterium *Desulfovibrio* (*D.*) *vulgaris* Miyazaki F is a membrane attached protein consisting of two subunits.[2] *In vivo*, it works preferably as an uptake hydrogenase. Heterolytic splitting of dihydrogen takes place at the [NiFe] active site located in the large subunit, while electrons are transferred to the physiological redox partner cytochrome c_3[2] *via* three FeS centres; one Fe_3S_4 and two Fe_4S_4 clusters, located in the small subunit. The catalytic cycle of the *D. vulgaris* hydrogenase comprises a number of intermediate states,[3] among which, Ni–C is pivotal for the enzymatic mechanism. The Ni–C state (Ni^{3+}, paramagnetic $S = 1/2$) is directly involved in the hydrogen conversion and was shown to carry a hydrogenic species (Fig. 1). Electron nuclear double resonance (ENDOR) and hyperfine sublevel correlation (HYSCORE) spectroscopic experiments on the regulatory hydrogenase from *Ralstonia eutropha*[4] and on *D. vulgaris* Miyazaki F[5] combined with single crystal EPR[6] and theoretical[7] studies detected a hydride ligand (H^-) in the bridging position between the two metals in the Ni–C state. This ligand is absent in the oxidised states of the enzyme and proposed to be derived from the substrate hydrogen that is split heterolytically at the active site.

Illumination at temperatures below 180 K converts Ni–C to the Ni–L state, with concomitant photodissociation of the bridging hydride,[8–10] as shown by EPR studies. This light-induced transition can also be followed by Fourier transform infrared (FTIR) spectroscopy by monitoring the stretching vibrations of the CO and CN^- ligands attached to the Fe[11–13] (Fig. 1), as has been recently shown for the more oxidised intermediate state (Ni–SIr) of this enzyme.[14]

In this work, we report the first infrared investigation of the Ni–C to Ni–L photoconversion for [NiFe] hydrogenases from sulfate reducing bacteria and a novel rapid scan FTIR study on the kinetics of the proton rebinding. The activation barrier for the re-association of the proton was determined and the H/D isotope effect examined over the accessible temperature range.

Experimental

Sample preparation

The [NiFe] hydrogenase was isolated from the sulfate reducing bacterium *D. vulgaris* Miyazaki F and was purified as described previously.[15] The Ni–C state was prepared in 50 mM H_2O (pH 7.4) and D_2O based Tris/Cl (pD 7.4) buffers, respectively. Degassing of the samples (concentration 1 mM) was followed by incubation at room temperature for 50 min with 1 bar of H_2 or D_2 gas, respectively. Transfer of the samples into the infrared cell was carried out anaerobically prior to freezing in liquid N_2.

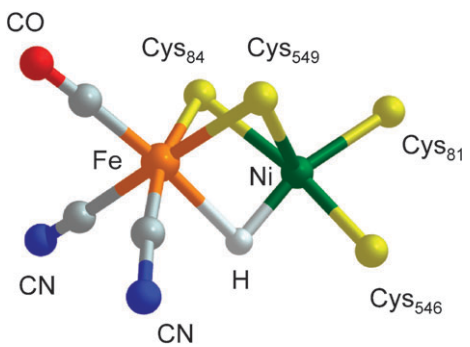

Fig. 1 The active site of the *D. vulgaris* Miyazaki F [NiFe] hydrogenase in the Ni–C state. Ni and Fe are bridged by two cysteinyl residues. Fe is further coordinated by three inorganic ligands, one carbonyl and two cyanides. Ni is additionally coordinated by two terminally bound thiolate ligands. The substrate in the form of a hydride (H^-) is located in the bridging position between the two metals.

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FTIR spectroscopy

The spectra were recorded on a Bruker IFS 66v/S FTIR spectrometer equipped with a MCT photoconductive detector (Kolmar Technologies). A continuous flow cryostat system (Optistat CF, Oxford Instruments) with an ITC 503S controller (Oxford Instruments) was used. The spectral resolution was 2 cm^{-1}. The samples were illuminated *in situ* for 3 min (halogen lamp 250 W, 24 V) prior to measuring the recombination kinetics in the rapid scan mode. The time resolution of the rapid scan measurements was in the order of one second.

Results and discussion

Infrared spectroscopy probes the [NiFe] active site by monitoring the vibrational frequencies of the diatomic ligands at the Fe, *i.e.* one carbonyl and two cyanides.[16] These frequencies are reliable sensors for changes in the electron density, coordination number on the Fe as well as changes in the hydrogen bonding.[17]

Fig. 2a shows a light-minus-dark difference infrared spectrum of the H_2 reduced hydrogenase at 150 K. In such a spectrum the light sensitive states (educts) appear as negative bands and the light-induced states (products) as positive bands. The Ni–C state is described by three negative bands, one in the low frequency region that is associated with the CO vibration (1963 cm^{-1}) and two in the high frequency region associated with the coupled CN^- oscillators (2077, 2087 cm^{-1}). Conversion from Ni–C to Ni–L upon illumination results in a shift of all bands towards lower frequencies, similar to what has been observed for the *Allochromatium* (*A.*) *vinosum* hydrogenase.[11] The spectrum of Ni–L consists of a CO band at 1911 cm^{-1} and two conjugate CN^- bands at 2048 and 2061 cm^{-1}. Such a uniform shift of all three vibrational frequencies shows an enhancement of the π-back bonding character of the CN^- and the CO ligands to Fe.[18] Lower vibrational frequencies of the diatomic ligands therefore suggest an increase in the electron density at the Fe as a result of the dissociation of the bridging ligand.

A wavelength dependent EPR study of the Ni–C to Ni–L conversion showed a correlation with absorption bands in the visible spectrum that could be associated with electronic transitions of the [NiFe] centre.[9] Therefore, upon illumination, electronic excitation of the Ni–Fe site results in the dissociation of the bridging ligand. Based on results from previous theoretical studies,[19] the chemical form of the dissociated ligand is proposed to be a proton. This suggests that the complex becomes fairly acidic resulting in its light-induced deprotonation facilitated presumably by a nearby base. In the current work no SH vibrations in Ni–L were detected in the range between 2520–2590 cm^{-1}, which would indicate a protonation of one of the coordinating cysteinyl residues.[20] However, the absence of SH stretching bands in the present work does not exclude their existence since they may be too weak to be detected. Fig. 2b shows a light-minus-dark FTIR difference spectrum of the deuterated (D_2/D_2O) form of the reduced hydrogenase. Apart from slight shifts (0–1 cm^{-1}) of the infrared bands no other changes were detected.

Conversion from Ni–C to Ni–L was observed to be reversible in the dark,[8] involving dissociation of the bridging hydrogenic species upon illumination and its rebinding to the active site as a proton during dark adaptation.

Recombination rate constants for this process were studied using rapid scan FTIR. Fig. 3 shows a three-dimensional representation of the back conversion kinetics at 150 K. The first slice of the spectrum corresponds to the time $t = 0$, where the amount of hydrogenase molecules in the Ni–L state is maximum. At subsequent times the Ni–C state reappears, as rebinding of the ligand occurs.

All the kinetics have a single-exponential behaviour (see insert of Fig. 4 and the example in the graphical abstract). This shows that the rebinding is a first-order process and no transient intermediates were detected within the time resolution of these experiments.

The temperature dependence of the rate constants (k) follows the Arrhenius equation $k = A_0 \exp(-E_a/RT)$[21]

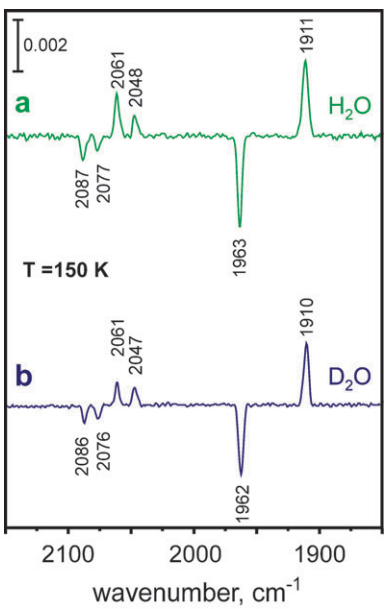

Fig. 2 Light-minus-dark difference FTIR spectra of the *D. vulgaris* Miyazaki F hydrogenase H_2 reduced in H_2O (a) and D_2 reduced in D_2O (b) at 150 K.

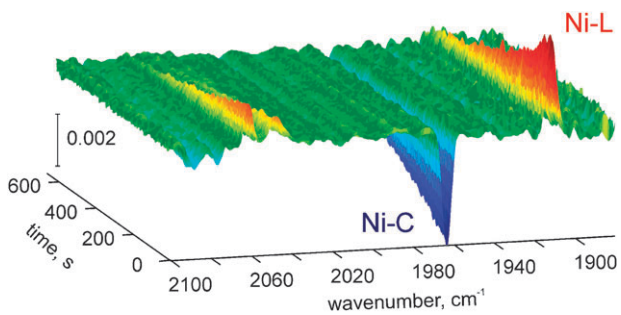

Fig. 3 Three dimensional plot of the time evolution of the light-minus-dark FTIR difference spectra of the H_2 reduced hydrogenase from *D. vulgaris* Miyazaki F at 150 K after switching off the light. The positive bands correspond to Ni–L, negative bands to Ni–C.

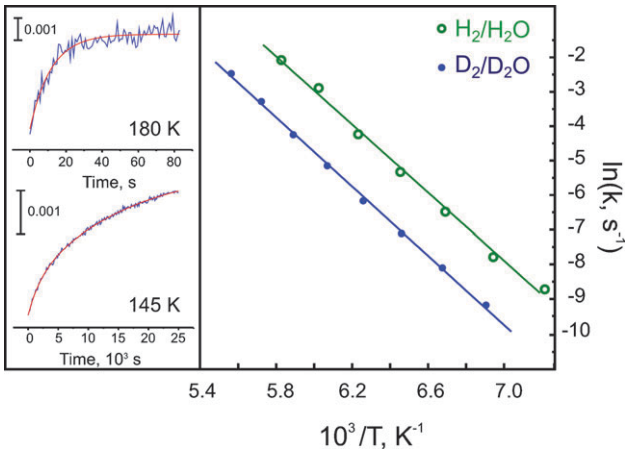

Fig. 4 Temperature dependence of the rate constants for the back-conversion from Ni–L to Ni–C in H_2 (open circles) and D_2 reduced (full circles) samples. Insert: kinetics of the recovery of the Ni–C state (D_2 reduced) at 180 and 145 K by following the absorption band at 1962 cm^{-1}.

Table 1 Inverse rate constants and kinetic isotope effect for the H_2 and D_2 reduced samples (k_H/k_D) in the range from 180 to 140 K

T/K	τ_H/s	τ_D/s	k_H/k_D
180	—	12	—
175	—	27	—
170	10	70	7.0
165	27	175	6.5
160	78	480	6.2
155	210	1240	5.9
150	600	3350	5.6
145	1940	9860	5.1
140	4400	—	—

between 140 and 180 K (Table 1, Fig. 4, open circles), where A_0 is the frequency factor in s^{-1}, E_a is the activation energy in kJ mol^{-1}, R is the universal gas constant 8.3144 J K^{-1} mol^{-1} and T is the temperature in K.

The activation barrier for the rebinding of the proton was estimated by analysing the kinetics of both the Ni–C recovery and the Ni–L disappearance, which were the same within experimental error. The averaged values of the lifetimes (inverse rate constants) at each temperature are included in Table 1. The activation barrier corresponds to $E_a \sim 46$ kJ mol^{-1} (Table 2).

Isotopic substitution of the bridging species in the Ni–C state was carried out as described above. The kinetic isotope effect on the rebinding rate constants is substantial. Recombination of the ligand is 5–7 times slower in the D_2 reduced compared to the H_2 reduced sample (Table 1), in accordance with a primary isotope effect.[22] This shows that the rate-limiting step of the back conversion to Ni–C is the re-association of the proton in the active site. The activation energy for the back conversion from Ni–L to Ni–C in the deuterated sample was found to be somewhat larger and approximately 48 kJ mol^{-1} (Table 2). The zero-point energy of the deuteride ligand is slightly smaller than that of the hydride ligand and a higher activation barrier would thus be anticipated, in accordance with our results. A comparison of the frequency factors (intercept of the Arrhenius plot in the limit where $1/T \rightarrow 0$) showed that the probability for the rebinding of the deuterium and the hydrogen based ligands is very similar.

Table 2 Activation energies (E_a) and frequency factors for the back conversion from Ni–L to Ni–C. The experimental error in the energies is ±7% and the error in the frequency factor is one order of magnitude

	E_a/kJ mol^{-1}	A_0/s^{-1}
H_2 reduced	46	10^{12}
D_2 reduced	48	10^{12}

In our experiments only one light-induced state (Ni–L) for the *D. vulgaris* hydrogenase was observed in the temperature range studied. Therefore, other Ni–L states, which have been described to exist at lower temperatures[9,10] are outside the scope of this work. They probably reflect a different binding position of the H^+/D^+ ion after photodissociation. No marked effect on the yield of the photoproduct was found in our experiments at the given temperatures, indicating the same efficiency of photoconversion for both H_2 or D_2 reduced samples. Assuming a linear Arrhenius dependence, the extrapolated lifetimes for Ni–L at room temperature (297 K) are 57 and 220 μs for H_2 and D_2 reduced samples, respectively.

In this work we report the first FTIR investigation of the Ni–L state for a [NiFe] hydrogenase from a sulfate reducing bacterial organism. The spectra resemble the ones observed previously for the photosynthetic bacterium *A. vinosum*[11] and for the cyanobacterial-like uptake [NiFe] hydrogenase from *Acidithiobacillus ferrooxidans*,[13] showing a similar structure of the light-induced Ni–L state among different organisms. In addition, the present study confirmed, using FTIR spectroscopy and H/D isotope labeling, that the photosensitivity of the catalytically active Ni–C state is related to the dissociation of the bridging hydrogenic species. Rapid scan kinetic measurements showed that rebinding of this substrate ligand as a proton is a first-order process with no intermediates being formed. The activation barrier determined for this process is 46 kJ mol^{-1}. The primary isotope effect on the re-association rate constants demonstrated that the proton transfer is the rate-limiting step during back conversion from Ni–L to the Ni–C state. These results contribute to a better understanding of the character of the metal hydride binding in the catalytic mechanism of the [NiFe] hydrogenases and will help to elucidate its function.

Acknowledgements

Reiner Lomoth (Uppsala University) is gratefully acknowledged for helpful discussions. We thank Patricia Malkowski for the purification of the enzyme. This work was financially supported by the EU/Energy Network project SOLAR-H2 (FP7 contract 212508), by the Max Planck Society and BMBF (Bio H2, 03SF0455C).

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