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Metalloenzymes

Light-Induced Electron Transfer in a [NiFe] Hydrogenase Opens a Photochemical Shortcut for Catalytic Dihydrogen Cleavage

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Abstract: [NiFe] hydrogenases catalyze the reversible cleavage of molecular hydrogen into protons and electrons. Here, we have studied the impact of temperature and illumination on an oxygen-tolerant and thermostable [NiFe] hydrogenase by IR and EPR spectroscopy. Equilibrium mixtures of two catalytic [NiFe] states, Ni_a-C and Ni_a-SR'', were found to drastically change with temperature, indicating a thermal exchange of electrons between the [NiFe] active site and iron-sulfur clusters of the enzyme. In addition, IR and EPR experiments performed under illumination revealed an unusual photochemical response of the enzyme. Ni_a-SR'', a fully reduced hydride intermediate of the catalytic cycle, was found to be reversibly photoconverted into another catalytic state, Ni_a-L. In contrast to the well-known photolysis of the more oxidized hydride intermediate Ni_a-C, photoconversion of Ni_a-SR'' into Ni_a-L is an active-site redox reaction that involves light-driven electron transfer towards the enzyme's iron-sulfur clusters. Omitting the ground-state intermediate Ni_a-C, this direct interconversion of these two states represents a potential photochemical shortcut of the catalytic cycle that integrates multiple redox sites of the enzyme. In total, our findings reveal the non-local redistribution of electrons via thermal and photochemical reaction channels and the potential of accelerating or controlling [NiFe] hydrogenases by light.

Hydrogenases are metalloenzymes that catalyze the reversible cleavage of dihydrogen into protons and electrons.[1] The catalytic site of [NiFe] hydrogenases contains one Ni and one Fe ion, bridged by two cysteinyl thiolates.[2] Two further cysteines are terminally bound to the Ni, and the Fe is additionally coordinated by one CO and two CN^- ligands.[3–9] Different redox-structural states of the [NiFe] site are distinguishable by infrared (IR) spectroscopy through the stretching vibrations of the diatomic ligands. These vibrations are sensitive towards various structural and electronic aspects of the active site, and the CO stretch vibration represents a convenient identifier for different [NiFe] states.[7,10]

Redox-structural states with an unpaired electron at the nickel (Ni^I and Ni^{III}) can also be probed by electron paramagnetic resonance (EPR) spectroscopy.[1,11–13] Hydride-bound states of the active site play a key role in the catalytic cycle of [NiFe] hydrogenases. One of these hydride-carrying states, called Ni_a-C state (Ni^{III}, $S=1/2$),[1,13,14] is known to be light-sensitive. Illumination results in a dissociation of the bridging hydride, which is transferred as a proton to one of the terminal cysteines of the active site. This tautomerization yields one or more Ni_a-L states (Ni^I, $S=1/2$),[1,12,13,15–22] some of which are believed to be part of the catalytic cycle.[23–25] Ni_a-C is thermodynamically more stable than Ni_a-L, and accumulation of the latter is most easily achieved by illumination of Ni_a-C at cryogenic temperatures.[13–15,20,22] Under such conditions, the rate of the exergonic thermal back reaction is drastically decreased, so that Ni_a-L is kinetically trapped. One-electron reduction of Ni_a-C leads to another set of hydride states, called Ni_a-SR.[1,16,26,27] At least three Ni_a-SR sub-states are known, often designated as Ni_a-

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SR, Ni_a-SR', and Ni_a-SR''.[28] The exact differences between these sub-states and their individual roles in the catalytic cycle are a matter of discussion.[16]

Here, we studied the soluble NAD^+-reducing [NiFe] hydrogenase from *Hydrogenophilus thermoluteolus* TH-1[29–32] (*Ht*SH) using IR and EPR spectroscopy. *Ht*SH is an ideal candidate for biotechnological applications for three reasons. First, *Ht*SH couples the reversible cleavage of H_2 to the redox conversion of NAD, which is an important soluble cofactor in biocatalytic transformations. Thus, this enzyme is a promising candidate for atom-efficient nucleotide cofactor regeneration.[33,34] Second, *Ht*SH retains part of its catalytic activity under mild oxic conditions,[30,31] and at high O_2 partial pressure the active site is protected by adopting a coordinatively saturated configuration stabilized by a high-valent Ni.[31] Finally, this enzyme preserves its catalytic activity in a wide range of (elevated) temperatures, in contrast to most other hydrogenases.[30]

*Ht*SH exists as a heterotetrameric HoxHYFU complex (Figure 1), which can be divided into two catalytic modules, the hydrogenase module (HoxHY), catalyzing H_2 oxidation, and the diaphorase module (HoxFU), catalyzing NAD^+ reduction.[30] The two active sites in these modules, i.e. the [NiFe] site in HoxH and a flavin mononucleotide (FMN) molecule in HoxF, are separated by 73 Å,[35] so that direct electron transfer is not possible. Thus, the two reaction sites are coupled via an electron relay of iron-sulfur (FeS) clusters. HoxY harbors one [4Fe4S] cluster, HoxU harbors one [2Fe2S] cluster and two [4Fe4S] clusters, and HoxF carries one [4Fe4S] cluster.[35]

*Ht*SH was studied in its H_2-reduced, catalytically active form. IR spectra recorded over a wide temperatures range reveal a temperature-dependent change of the equilibrium between Ni_a-C and one of the Ni_a-SR sub-forms, namely Ni_a-SR'', which suggest an exchange of electrons between the active site and the FeS clusters. Even more, we demonstrate, for the first time that the same Ni_a-SR sub-form can be photoconverted to Ni_a-L. Supported by EPR spectroscopy, this process is shown to involve a net electron transfer across the border of the [NiFe] site, proving a previously proposed light-induced electron transfer pathway in hydrogenases.[18]

After treatment of *Ht*SH with H_2, the IR spectrum recorded at 298 K exhibits a mixture of reduced catalytically active states with characteristic CO stretch frequencies (Figure 2, top, black trace), in particular Ni_a-C (1973 cm^{-1}), Ni_a-SR (1959 cm^{-1}), and Ni_a-SR'' (1936 cm^{-1}). These assignments are in line with previous publications,[30,31] and small deviations of values reported throughout the manuscript can be explained by the impact of temperature on the vibrational frequencies and slight variations across protein batches and/or reduction protocols.

Interestingly, the observed state composition changes during the freezing of the sample (accomplished within 30 min), with drastic variations observed for all mentioned states (Figure 2, top, grey trace). Most notably, the Ni_a-C state dominating at room temperature is clearly diminished at all temperatures below ca. 200 K, while the population of the Ni_a-SR'' state is considerably increased at the expense of all other states (Figure 2, bottom). This observation, exemplarily shown for 110 K, implies that Ni_a-C is partly converted to Ni_a-SR''. Due to different Ni oxidations states in these two catalytic intermediates (see above), their interconversion requires electron translocation between the active site and other redox couples within the enzyme, most likely involving the proximal [4Fe4S] cluster located in the HoxY subunit, 10–13 Å away from the [NiFe] center.

The observation of a thermal equilibration between different redox-structural states is consistent with similar observations on other hydrogenases.[22,36,37] In particular, a temperature-dependent change of the Ni_a-C/Ni_a-SR ratio has also been observed for the regulatory hydrogenase from *Cupriavidus necator* (*Cn*RH).[22] In general, temperature-dependent equilibria between different redox-structural

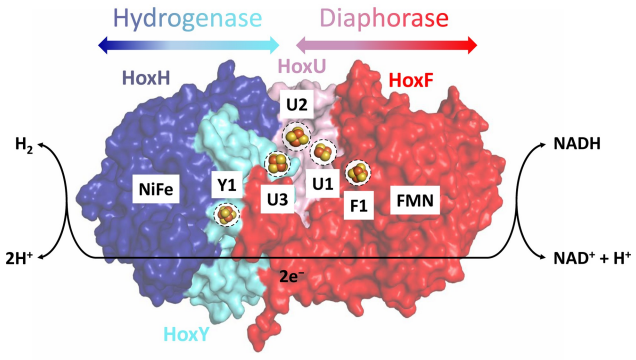

Figure 1. Subunit and cofactor composition of *Ht*SH. HoxH and HoxY, depicted in shades of blue, form the hydrogenase module with HoxH harboring the [NiFe] active site and HoxY containing a [4Fe4S] cluster. HoxF and HoxU, depicted in shades of red, form the NAD^+ reductase (diaphorase) module. HoxF harbors a [4Fe4S] cluster and a flavin mononucleotide (FMN) molecule, while HoxU contains two [4Fe4S] clusters and one [2Fe2S] cluster.

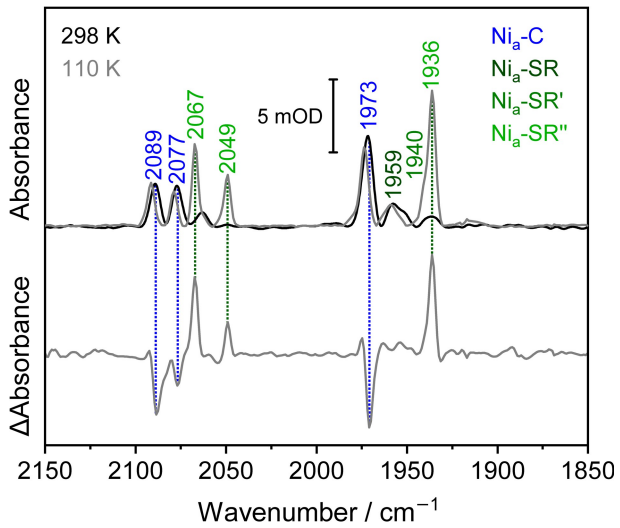

Figure 2. IR absorbance spectra of H_2-reduced *Ht*SH recorded at 298 K (black) and 110 K (grey). The corresponding 110-K-*minus*-298-K difference spectrum is shown below.

[NiFe] states are relevant for multiple reasons. First, they highlight the need of applying IR spectroscopy at different temperatures when comparing the results to other techniques that require measurements at cryogenic temperatures, e.g. EPR spectroscopy, nuclear resonance vibrational spectroscopy (NRVS), resonance Raman (RR) spectroscopy, or X-Ray absorption spectroscopy (XAS). Second, our findings demonstrate that hydrogenases may not be in thermodynamic equilibrium at low temperatures, depending on the rate of temperature change. Hence, even measurements conducted at the same temperature but using different techniques (where the freezing rate is dictated by the apparatus used) may not reflect the same mixture of (kinetically trapped) states.

For the study of photo-inducible species, the H_2-reduced *Ht*SH was rapidly frozen (within 30 min) to temperatures far below the glass transition (110 K). The sample was subsequently illuminated with blue light from an LED (maximum emission wavelength: 460 nm) to photo-convert light-sensitive states. To highlight photo-induced state transitions, IR difference spectra were calculated by subtracting the absorbance spectra recorded in the dark from that recorded under illumination (light-*minus*-dark). In these difference spectra, negative peaks represent the depletion of dark-adapted parent states, and positive ones correspond to the formation of photo-induced species.

Surprisingly, two negative and one positive CO stretching bands are detected in the light-dependent IR difference spectrum recorded at 110 K (Figure 3A, bottom). The positions and intensities of the negative signals equal those observed for Ni_a-C and Ni_a-SR'' in the IR absorbance spectrum of the dark-adapted state (Figure 3A, top, black trace). This demonstrates that Ni_a-C and Ni_a-SR'' states are completely converted to a single active-site state by illumination with blue light. This product state is assigned to Ni_a-L, based on a positive CO stretching band at 1922 cm^{-1} and two CN stretching bands at 2047 and 2062 cm^{-1}.[15,16,20,21,38] For both parent states, mono-exponential decay curves can be fitted to the time evolution of the CO-stretch signal, while a biexponential saturating exponential curve is necessary to model the photo formation of Ni_a-L via the same approach. Both analyses yielded similar time constants (Figure 3B), confirming the formation of Ni_a-L from both Ni_a-C and Ni_a-SR''. Supported by QM/MM calculations, photoconversion of at least one Ni_a-SR sub-state to Ni_a-L was previously postulated to occur under high photon fluxes, e.g. during RR experiments.[18] Photoconversion of Ni_a-SR to Ni_a-L was also claimed by Bagley, according to articles by Pandelia et al. and Lubitz et al., but no data was shown.[1,39] Thus, clear experimental evidence for this process and its feasibility under moderate photon fluxes was missing so far, so that our observations represent the first direct proof for the photochemical conversion of a fully reduced state of [NiFe] hydrogenase. Since all Ni_a-SR sub-states differ from Ni_a-L by one electron (see above), the photoconversion of Ni_a-SR'' to Ni_a-L requires net electron transfer from the [NiFe] site towards the enzyme's FeS clusters. This is in sharp contrast to the well-known transformation of Ni_a-C to Ni_a-L, which represents a local tautomerization of the [NiFe]

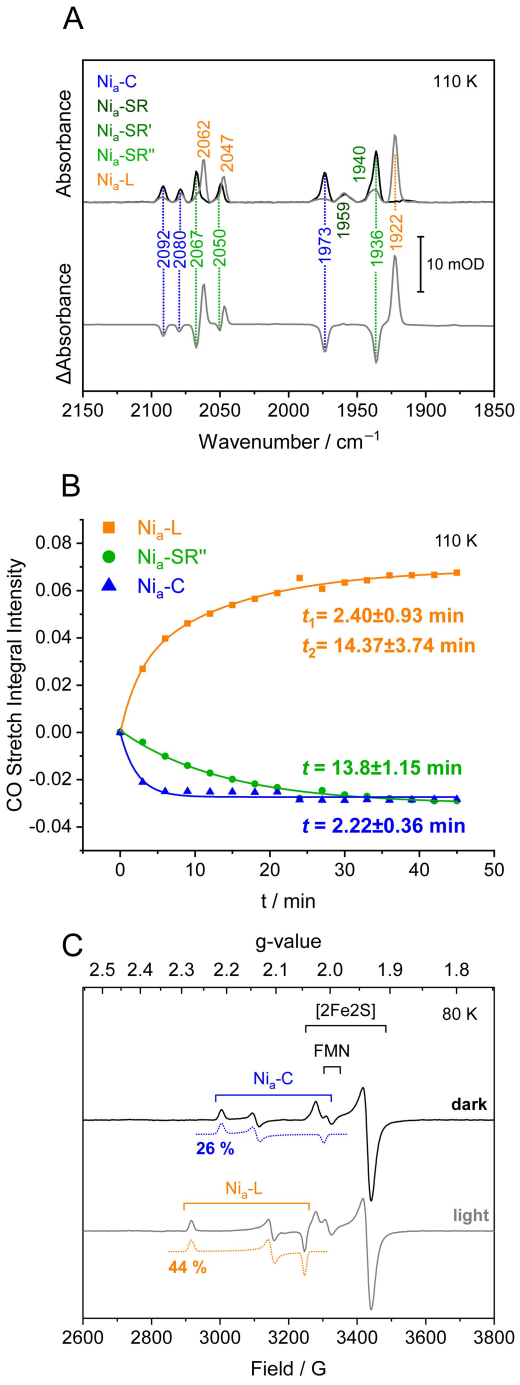

Figure 3. Photoconversion of H_2-reduced *Ht*SH. (A) IR absorbance spectra recorded at 110 K before (black) and after (grey) illumination. The corresponding light-*minus*-dark difference spectrum is shown below. (B) Time evolution of different [NiFe] states following illumination at the same temperature (spectra and time traces obtained at other temperatures are shown in Figure S1). State populations are quantified via the integral intensity of the associated CO-stretch absorption band. A biexponential saturating exponential curve is fitted to the Ni_a-L data (orange), while monoexponential decay curves are fitted to Ni_a-C (blue) and Ni_a-SR'' (green) time series. (C) EPR spectra recorded before illumination (dark) and after 2 hours of illumination (light) at 80 K. Ni_a-C and Ni_a-L states were quantified relative to the signal intensity of the [2Fe2S] cluster, which does not change during illumination. The simulations of the complete spectra are displayed in Figure S2.

center only. Further confirmation of this newly detected photo process was obtained by EPR spectroscopy. EPR spectra recorded at 80 K in the dark (Figure 3C, top) exhibit characteristic signals of the paramagnetic Ni_a-C state ($g_x = 2.211$, $g_y = 2.139$, $g_z = 2.010$), an FMN radical ($g = 2.003$), and a reduced [2Fe2S] cluster ($g_{\parallel} = 2.027$, $g_{\perp} = 1.934$), in line with previous studies.[1,52] Consistent with the IR data, the Ni_a-C state is completely converted to Ni_a-L ($g_x = 2.278$, $g_y = 2.110$, $g_z = 2.046$) during illumination, while the signals from the FMN cofactor and the [2Fe2S] cluster remain unchanged (Figure 3C, bottom). Since the intensity of the [2Fe2S] cluster signal was unaffected by illumination, it was used as an internal standard for relative quantification of Ni_a-C and Ni_a-L signals. Using this approach, Ni_a-L and Ni_a-C were found to equal 44 % and 26 %, respectively, of the [2Fe2S] signal intensity. This finding demonstrates that the amount of Ni_a-L formed is much higher than that of the Ni_a-C state observed in the dark-adapted spectrum. This confirms the partial formation of Ni_a-L from another, diamagnetic parent state, assigned to Ni_a-SR'' based on the IR data (Figure 3A, bottom).

Our experimental findings suggest a photoconversion of a Ni_a-SR intermediate to a Ni_a-L species, but they could, in principle, also be compatible with a slow thermal conversion of Ni_a-SR'' to Ni_a-C, followed by a faster photoconversion of Ni_a-C to Ni_a-L. In the latter case, the conversion of Ni_a-SR'' to Ni_a-C could either be a thermal equilibration due to local heating following photon absorption or conversion based on Le Chatelier's principle, where the photo-depletion of Ni_a-C would be restored by thermal conversion of Ni_a-SR''. However, both possibilities are equally unlikely since equilibration between Ni_a-SR'' and Ni_a-C is negligible below 200 K (see Figures S1 and S3). Thus, excluding a dominating thermal process, we conclude that a genuine photoconversion of Ni_a-SR'' to Ni_a-L is observed. This could either involve a photoconversion of Ni_a-SR'' to Ni_a-C followed by the typical photo-formation of Ni_a-L or the direct conversion of Ni_a-SR'' to Ni_a-L. The latter reaction seems more likely, due to the alleged photosensitivity of the bridging hydride in Ni_a-SR'' (analogous to that of Ni_a-C) and the lack of indications for an intermediary formation of Ni_a-C.

Utilizing the NAD^+-reducing [NiFe] hydrogenase from *Hydrogenophilus thermoluteolus* as a model enzyme, this IR and EPR study revealed a pronounced temperature-dependent change of the ratio between two hydride-bound catalytic intermediates, Ni_a-C and Ni_a-SR''. Such changes need to be taken into account when interpreting spectroscopic data recorded at low temperatures, and they may also be relevant for the comparative analysis of hydrogenases from hyperthermophilic and mesophilic host organisms. Moreover, we could demonstrate, for the first time, the light-induced conversion of a fully reduced catalytic intermediate of a [NiFe] hydrogenase. This reaction was found to transform the hydride state Ni_a-SR'' to a Ni_a-L state with a free coordination site between the two metals (Figure 4). Both processes involving Ni_a-SR'' require the net electron transfer from the active site towards FeS clusters of the enzyme. Notably, neither of these processes was observed for other Ni_a-SR substates of *Ht*SH. This could indicate that Ni_a-SR''

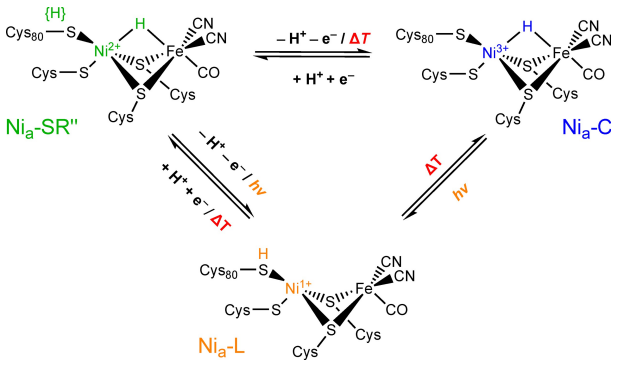

Figure 4. Thermal and photochemical reactions between the main species discussed in the text.

(electron at the active site, hole at the FeS cluster relay) is thermodynamically destabilized relative to Ni_a-C and Ni_a-L (hole at the active site, electron at the FeS relay). We propose that this is due to a lack of charge compensation at the [NiFe] site in this state since the proton derived from H_2 cleavage is likely located furthest from this cofactor in Ni_a-SR'', which features the lowest CO stretching frequency of all Ni_a-SR sub-forms.

In total, our results highlight the diversity of redox-structural [NiFe] states that can be formed under catalytically relevant conditions and the various thermal and photochemical reaction channels that connect them. It remains to be elucidated, which of these states and transformations are functionally relevant and, whether this relevance is conserved across [NiFe] hydrogenases from different classes or organisms. In particular, the pronounced temperature dependence of the Ni_a-C⇌Ni_a-SR'' equilibrium and the photoconversion of Ni_a-SR'' have not been reported for hydrogenases from mesophilic organisms, raising the question whether these features are more prevalent in thermostable enzymes. Another open question concerns the intermittent and final electron acceptors involved in the light- and temperature-dependent transformations of Ni_a-SR'': While the enzyme's [4Fe4S] clusters are expected to play a key role, more detailed information is missing so far, due to the difficulty of detecting clusters of this type in group-3 hydrogenases. Irrespective of these details, the discovered light-driven Ni_a-SR''→Ni_a-L reaction represents a potential photochemical shortcut of the catalytic cycle that skips the Ni_a-C intermediate between them. Together with the light-driven electron transfer discussed above, this finding provides new perspectives for the manipulation of hydrogenases with light.

Supporting Information

The authors have cited an additional reference within the Supporting Information (Ref. [40]).

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

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