Enzymatic and spectroscopic properties of a thermostable [NiFe]-hydrogenase performing H2-driven NAD\(^+\)-reduction in the presence of O\(_2\)

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**A B S T R A C T**

Biocatalysts that mediate the H\(_2\)-dependent reduction of NAD\(^+\) to NADH are attractive from both a fundamental and applied perspective. Here we present the first biochemical and spectroscopic characterization of an NAD\(^+\)-reducing [NiFe]-hydrogenase that sustains catalytic activity at high temperatures and in the presence of O\(_2\), which usually acts as an inhibitor. We isolated and sequenced the four structural genes, hoxFUYH, encoding the soluble NAD\(^+\)-reducing [NiFe]-hydrogenase (SH) from the thermophilic betaproteobacterium, *Hydrogenophilus thermoluteolus* TH-1\(^{1}\) (TH). The HSH was recombinantly overproduced in a hydrogenase-free mutant of the well-studied, H\(_2\)-oxidizing, betaproteobacterium *Ralstonia eutropha* H16 (Re). The enzyme was purified and characterized with various biochemical and spectroscopic techniques. Highest H\(_2\)-mediated NAD\(^+\) reduction activity was observed at 80 °C and pH 6.5, and catalytic activity was found to be sustained at low O\(_2\) concentrations.

Infrared spectroscopic analyses revealed a spectral pattern for as-isolated HSH that is remarkably different from those of the closely related RsH and other [NiFe]-hydrogenases. This indicates an unusual configuration of the oxidized catalytic center in HSH. Complementary electron paramagnetic resonance spectroscopic analyses revealed spectral signatures similar to related NAD\(^+\)-reducing [NiFe]-hydrogenases. This study lays the groundwork for structural and functional analyses of the HSH as well as application of this enzyme for H\(_2\)-driven cofactor recycling under oxic conditions at elevated temperatures.

**1. Introduction**

Enzymatic oxidation of dihydrogen (H\(_2\)) is a widespread trait in the microbial world and is used by many microbes to gain metabolic energy [1,2]. The reversible cleavage of H\(_2\) into protons and electrons is mediated by complex metalloenzymes designated as hydrogenases [3]. In particular, the coupling of H\(_2\) oxidation with aerobic respiration, i.e. the controlled Knallgas reaction (H\(_2\) + \(\frac{1}{2}\) O\(_2\) → H\(_2\)O), releases a high yield of free energy of \(ΔG^\circ = -237.2\) kJ per mol of H\(_2\). Aerobic H\(_2\) oxidation, however, requires hydrogenases that withstand the toxic effect of O\(_2\). Among the different hydrogenase types, there is only one subclass that sustains H\(_2\) oxidation in the presence O\(_2\), namely the O\(_2\)-tolerant [NiFe]-hydrogenases [4]. One prominent member is the soluble NAD\(^+\)-reducing [NiFe]-hydrogenase (SH) from the betaproteobacterium *Ralstonia eutropha* H16 (Re), which is a well-known Knallgas bacterium possessing an H\(_2\)-driven chemolithioautotrophic metabolism [5]. ReSH directly couples H\(_2\) oxidation with the reduction of NAD\(^+\), thereby producing NADH, which is used both for energy conservation (through Complex I and the respiratory chain) and for CO\(_2\) fixation via the Calvin cycle.

The ReSH is a bimodular enzyme consisting of four essential subunits, HoxFUYH, that harbor the [NiFe] active site, where H\(_2\) conversion takes place, and the catalytic center for NAD\(^+\) reduction, which carries a flavin mononucleotide (FMN) [6]. Electron transfer between the two active sites is mediated by four [4Fe4S] clusters and one [2Fe2S] site. Another FMN group has been suggested to be located close to the [NiFe] active site [7]. Two copies of the non-essential HoxI protein, whose function remains so far elusive, are also integral part of the ReSH [8]. The overall subunit composition as well as the cofactor arrangement of NAD\(^+\)-reducing [NiFe]-hydrogenases are reminiscent of their counterparts in [NiFe]-hydrogenases from Clostridium pasteurianum and *Heliobacterium modesticum* [9,10].

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of the situation in the peripheral arm of Complex I. In fact, it is anticipated that the SH represents a phylogenetic ancestor of Complex I [9,10], for which crystal structures are available [11]. Unfortunately, the ReSH has so far defied crystallization.

Three of four highly conserved cysteines coordinating the [NiFe] active site metal ions in the HoxH subunit are missing in the homologous subunit of Complex I (Nqo4 in case of Thermus thermophilus). According to amino acid sequence comparisons and numerous spectroscopic studies, the ReSH carries a [NiFe] center similar to that of canonical [NiFe]-hydrogenases [6,12,13]. Two of the four conserved cysteines serve as terminal nickel ligands, while the remaining two coordinate both the nickel and the iron ions. The iron is further equipped with one carbon monoxide and two cyanide ligands, which are supposed to maintain a low-spin Fe³⁺ state throughout the catalytic cycle. The nickel ion, however, changes its redox state during H₂/H⁺ turnover [6]. As the ReSH is catalytically active under aerobic conditions, a contact of the active site with O₂ is a very likely event. Nonetheless, the H₂ turnover rate remains at almost 100% even in the presence of 20% O₂, which makes ReSH the “world record holder” among O₂-tolerant, energy-converting [NiFe]-hydrogenases [14,15]. Moreover, the ReSH represents the first hydrogenase, for which a catalytic conversion of O₂ into water has been demonstrated [15]. The exceptional O₂ tolerance and the high turnover rates of the ReSH attracted scientists to employ the enzyme both in vitro and in vivo for H₂-driven NADP(H) cofactor regeneration in biotechnologically relevant applications [16–19]. Though very efficient in NADH recycling, however, the ReSH has the disadvantage of being temperature-sensitive [20]. Both the lack of a crystal structure of an NAD(P)⁺-reducing [NiFe]-hydrogenase and the limited temperature stability of ReSH have prompted us to seek out a thermostable version of this enzyme.

*Hydrogenophilus thermoluteolus* TH-1² (HtSH) has been described as an aerobic, facultatively chemolithooautotrophic, hydrogen-oxidizing microorganism, which – like *R. eutrophus* – belongs to the phylogenetic class of betaproteobacteria [21]. It shows optimal chemolithooautotrophic growth with a H₂/O₂/CO₂ gas mixture of 7:2:1 at a temperature of 52 °C [22]. This suggests the presence of at least one O₂-tolerant [NiFe]-hydrogenase. Indeed, a recent study confirmed the presence of an SH-like enzyme in the moderate thermophile [23]. However, neither the corresponding genetic information nor a physiological or spectroscopic characterization of the HtSH is so far available.

In this study, we present the DNA sequence of the structural genes of the four HtSH subunits in addition to the gene encoding the HtSH-specific endopeptidase. The HtSH was recombinantly overproduced in *R. eutrophus* and – upon purification – characterized by means of biochemical and spectroscopic methods. It turned out to be the first characterized [NiFe]-hydrogenase that performs H₂-driven NAD⁺ reductase activity at elevated temperatures and in the presence of O₂.

2. Results and discussion

2.1. Identification of the genes encoding the NAD⁺-reducing [NiFe]-hydrogenase of *H. thermoluteolus*

The draft sequence (published elsewhere) of the *H. thermoluteolus* TH-1² genome revealed the HtSH-related genes, hoxF, hoxU, hoxY, hoxH, and hoxW, which are apparently arranged as an operon (Fig. 1). Pairwise alignments of HtSH and ReSH proteins (Fig. S1) revealed 40%, 37%, 44%, 46%, and 26% identical residues for HoxF, HoxU, HoxY, HoxH, and HoxW, respectively. Notably, the *H. thermoluteolus* TH-1² genome does not contain a copy of the gene encoding the HoxL protein, which is a constituent of the ReSH [24].

2.2. Heterologous overproduction and purification of functional HtSH

For heterologous overproduction of the HtSH in *R. eutrophus* and subsequent purification, the hoxFUHYW genes were amplified by PCR and put under the control of the native SH promoter of *R. eutrophus* as described in Materials and methods. Furthermore, a sequence encoding the Strep-tag II peptide was attached to the 5’ end of the hoxF gene. The resulting synthetic *hoxFUHYW* operon was inserted into the broad-host range vector pEDY309 resulting in plasmid pJP09, encoding Strep-tagged *HtSH*.

For enzyme purification, plasmid pJP09 was transferred into strain *R. eutrophus* HF1054, in which the native *hoxFUHYW* genes as well as *hoxG* encoding the large subunit of the membrane-bound [NiFe]-hydrogenases were eliminated by isogenic in-frame deletions. This prevented any “subunit mixing” between HtSH and ReSH proteins. The transconjugant strain *R. eutrophus* HF1054 (pJP09) was cultivated heterotrophically under oxygen-limited conditions as described previously [15,25]. In a first experiment, the H₂-driven NAD⁺ reduction activity was measured in soluble extract of the recombinant cells. The activity was 2.50 ± 0.12 U mg⁻¹ of protein (Table 1), suggesting the presence of functional HtSH proteins. This result also demonstrates that the general [NiFe]-hydrogenase maturation machinery of *R. eutrophus* [26–28] is able to synthesize and to deliver the active site constituents for the HoxH subunit of HtSH.

The HtSH protein was then purified to homogeneity by Strep-Tactin affinity and size exclusion chromatography as described in Materials and methods. From 10 g (wet weight) of cells, we routinely obtained 10–12 mg of protein with a specific H₂-driven NAD⁺ reduction activity of 33.4 ± 0.6 U mg⁻¹ of protein (measured at 50 °C, Table 1). The reverse reaction, namely NADH-driven H₂ production, was catalyzed with an activity of 1.0 ± 0.3 U mg⁻¹ of protein. Using dithionite-reduced methyl viologen (MV) as artificial, low-potential electron donor, the H₂ production activity increased to 30 ± 5 U mg⁻¹ of protein. SDS-PAGE performed with the HtSH preparation revealed four protein bands assigned to the subunits HoxFUHY (Fig. 2).

2.3. Biochemical characterization of purified HtSH

Based on visual inspection of the protein bands after electrophoretic separation (Fig. 2), a ratio of approximately 1:1 of the two SH modules, HoxFU and HoxYH, was obtained only when Ni²⁺ (0.5 mM) and Mg²⁺ (5 mM) ions were present during the whole purification process. A similar observation has been made previously for the NAD⁺-reducing [NiFe]-hydrogenase from *Rhodococcus opacus* [29]. Consequently, the following activity assays were conducted in the presence of Ni²⁺ and Mg²⁺ ions in addition to 2 μM FMN, the latter of which led to a shortened lag phase but did not change the maximal H₂ oxidation activity (Fig. S2). This suggests that FMN serves as an electron acceptor, and reduced FMN can reactivate those inactive HtSH species which cannot be activated by H₂ alone. This mechanism is similar to the NADH-based reactivation of as-isolated ReSH [24,30]. HtSH-driven NAD⁺-reduction activity for purified HtSH (Fig. 3), however, was observed when the reductant TCEP (0.75 mM) was added in addition to FMN. Activity was maximal after a lag period of ca. 2.5 min. The removal of just TCEP led to a dramatic increase of the lag time (ca. 25 min), and the activity dropped to 25% of the value measured in the presence of TCEP (Fig. 3). The negative effect of the missing TCEP could be partly compensated through addition of catalytic amounts of NADH (5 μM), which led to the recovery of approx. 50% of the maximal activity and a halved lag phase (Fig. 3). This indicates that NADH supports reductive reactivation of aerobically purified HtSH as previously observed for SH from *R. eutrophus* [24,30]. A considerable further shortening of the lag phase was accomplished by increasing the protein concentration in the assay. In the presence of 0.8 μM HtSH and only 2.5 μM NADH, it took only 4 min until full activity was developed (Fig. 3). This suggests that the rate of reductive reactivation can also be accelerated by intermembrane electron transfer between individual HtSH enzymes. The likelihood of electron exchange between HtSH enzymes is of course greater at higher protein concentration.

Based on the knowledge derived from the experiments described
above, NiCl₂, MgSO₄, FMN, and TCEP were added to the following activity assays, unless stated otherwise. Using this standard protocol at a fixed temperature of 50 °C, we first determined the H₂-dependent NAD⁺ reduction activity of purified HtSH at different pH values. This was accomplished with a universal buffer that spanned the entire pH range from pH 4.5–9 (Fig. 4) as well as with three buffers with different pH ranges (Fig. S3). From both experiments, an optimum pH of 6.5 was derived. This is in marked contrast to ReSH that performs best at pH 8.0 [20,30] (Table 2), where the H₂-driven NAD⁺ reduction activity of ReSH ranges from 50 ± 4 U mg⁻¹ of protein (measured at pH 6.5, Fig. 4).

In order to elucidate the origin of the unusual pH optimum, the

Table 1

Purification of HtSH protein enzyme by affinity chromatography.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (mL)</th>
<th>Protein concentration (mg/mL)</th>
<th>Total protein (mg)</th>
<th>Specific activity (U mg⁻¹)b</th>
<th>Total activity (U)</th>
<th>Yield (%)</th>
<th>Enrichment factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>SE</td>
<td>40</td>
<td>29.2</td>
<td>1168</td>
<td>2.5 ± 0.1</td>
<td>2920</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>AC</td>
<td>1.4</td>
<td>29.7</td>
<td>41.6</td>
<td>12.1 ± 0.1</td>
<td>502</td>
<td>17</td>
<td>4.8</td>
</tr>
<tr>
<td>SEC</td>
<td>2.4</td>
<td>4.9</td>
<td>11.7</td>
<td>33.4 ± 0.6</td>
<td>391</td>
<td>13</td>
<td>13.4</td>
</tr>
</tbody>
</table>

a The HtSH protein was purified from soluble cell extracts (SE) by Strep-Tactin affinity chromatography (AC) and subsequent size exclusion chromatography (SEC) as described in Materials and methods.

b Activity was determined by H₂-dependent NAD⁺ reduction in 50 mM bis-Tris, pH 6.5, supplemented with 1 mM NAD⁺, 0.5 mM NiCl₂, 5 mM MgSO₄, 2 μM FMN, and 0.75 mM TCEP at a temperature of 50 °C. One Unit (U) corresponds to the amount of converted substrate (in μmol) in 1 min. Values of a representative purification are shown.
enzymatic reactions of the two SH modules were tested separately in a pH-dependent manner (Fig. 4). First, the HoxFU-catalyzed NADH-benzyl viologen oxidoreductase activity was measured as described in Materials and methods. Maximum activity of 64 ± 5 U mg⁻¹ of protein was reached at approximately pH 10, which is qualitatively consistent with the observations made previously for the HoxFU module of the ReSH [31]. The H₂-benzyl viologen oxidoreductase activity of the HoxHY module, however, was found to be optimal at approximately pH 7.0. These results indicate that the pH optimum of the HšSH is primarily dictated by the intrinsic bias of the H₂/H⁺-cycling module of the holoenzyme.

Measurements of the H₂-dependent NAD⁺ reduction activity of purified HšSH at different temperatures were performed in bis-Tris buffer at pH 6.5 and revealed a maximal activity of 71.0 ± 0.3 U mg⁻¹ of protein at a temperature of 80 °C (Fig. 5). This is in sharp contrast to ReSH, which quickly looses activity at temperatures higher than 35 °C [20] (Table 2). At 33 °C, which is the temperature optimum of ReSH activity [30], HšSH showed less than 20% of the maximal activity.

In a next series of experiments, we determined the Michaelis-Menten constants (K_M) for the natural substrates of the HšSH. The K_M value for NAD⁺ was evaluated based on the H₂-driven NAD⁺ reduction activity of the enzyme and revealed to lie at 469 μM (Fig. S4), which is close to 560 μM, the value determined for ReSH [30]. Activity measurements of the HšSH-mediated benzyl viologen reduction activity in the presence of various NADH concentrations resulted in a K_M of 1.2 mM (Fig. S5), which is surprisingly high when compared to the corresponding value of 80 μM determined for the ReSH [30]. This suggests that the main physiological role of HšSH enzyme is H₂-driven NAD⁺ reduction.

A value of 42 ± 3 μM was determined for the apparent Michaelis-Menten constant, K_M for H₂ during H₂-driven NAD⁺ reduction of the enzyme (Fig. S6), which is comparable to that measured for ReSH (37 μM, [30], Table 2).

2.4. Cofactor content and O₂ tolerance of HšSH

Fluorescence determination revealed 1.07 FMN per SH tetramer. Using inductively coupled plasma optical emission spectrometry, 14.2 ± 0.2 Fe and 2.4 ± 0.1 Ni per SH molecule were detected. On the basis of conserved amino acid residues that are involved in Fe-S cluster coordination in Complex I, 19 iron atoms are expected in addition to one nickel in the catalytic center of the hydrogenase module (Fig. 1, Fig. S1). Additional information on the type of iron-sulfur clusters present in HšSH was obtained by nuclear resonance vibrational spectroscopy (NRVS). NRVS is a synchrotron-based vibrational spectroscopic technique that selectively probes iron-specific normal modes and has been shown to provide details on [NiFe]-hydrogenase cofactor structure and composition [42,43]. The partial vibrational density of states (PVDOS) for oxidized HšSH is presented in Fig. S7. The band at 414 cm⁻¹ is characteristic for the presence of a [2Fe2S] cluster [44], which is supposed to be coordinated by the HoxU subunit. Of the 19 iron ions in HšSH, 16 are expected to be constituents of [4Fe4S] clusters. Indeed, also the spectral pattern between 0 and 400 cm⁻¹ is very similar to that of ReSH [43] and a [4Fe4S] cluster-containing ferredoxin [45] (Fig. S7), which indicates dominant contributions of [4Fe4S] cluster species. Thus, these results support the presence of four [4Fe4S] clusters and one [2Fe2S] species in HšSH.

Consistent with the chemolithoautotrophic growth capacity of the host organism under aerobic conditions, the isolated HšSH showed sustained H₂-driven NAD⁺ reduction activity in the presence of O₂.

![Fig. 2. Purification of the HšSH protein. A protein amount of 30 μg of soluble extract (SE) and 5 μg of HšSH purified by affinity chromatography (AC) and selected fractions (from the subsequent size exclusion chromatography (SEC) were electrophoretically separated on a 12% SDS-polyacrylamide gel and subsequently stained with Coomassie brilliant blue. The specific H₂-driven NAD⁺ reduction activity (U mg⁻¹ of protein) of each fraction is specified below. Lane M contains marker proteins and their corresponding molecular weights are given on the left hand side.](image1)

![Fig. 3. Dependence of H₂-driven NAD⁺ reduction activity of purified HšSH protein on the addition of reductants TCEP and NADH. The assay was performed at 50 °C in 50 mM bis-Tris, pH 6.5, supplemented with 1 mM NAD⁺, 0.5 mM NiCl₂, 5 mM MgSO₄, 2 μM FMN, and varying amounts of TCEP, NADH and HšSH. The lag time refers to the time elapsed from assay start until full activity was achieved. 100% activity refers to 19 U mg⁻¹ of protein.](image2)
in Materials and methods with 45 nM of HtSH in an universal buffer composed of 16 mM citrate, 16 mM Tris, and 16 mM glycine. Activities were measured at a temperature of 50 °C in the presence of either of 1 mM NAD+, 1 mM NADH, or 5 mM benzyl viologen, in addition to 0.5 mM NiCl₂, 5 mM MgSO₄, 2 μM FMN, and 0.75 mM TCEP.

(Table 3). However, its O₂ tolerance revealed to be lower than that of the ReSH (Table 3, Table 2). While the ReSH preserves approximately 100% activity observed at 20% O₂ (measured at 30 °C in Tris/HCl buffer, pH 8) [14,15], the H. thermoluteolus enzyme showed at 10% O₂ less than 20% of the activity measured in the absence of O₂. At 2% O₂, it displayed only 50% of the activity observed under anaerobic conditions. However, at low O₂ pressure (0.2%), HtSH activity remained at almost 100% (Table 3). In this respect, it is noteworthy that the intracellular O₂ concentration in living cells is generally much lower than the external one. This explains why H. thermoluteolus cells grow well with H₂ and CO₂ even at ambient O₂ concentrations, although the isolated enzyme is more O₂ sensitive than the SH from R. eutropha.

Comparison of soluble, NAD(P)+-reducing [NiFe] hydrogenases.

|| Organism | H. thermoluteolus TH-1¹ | R. eutropha H16 | Synechocystis sp. PCC 6803 | Pseudomonas furiosus |
|---|---|---|---|---|
| Designation | SH | SH | Bidirectional hydrogenase | SH |
| Κₛ H₂ (μM) | 42 | 37 [30] | 11.3 (34)² | 140 [33] |
| Κₛ NAD(P)⁺ (μM) | 469 (NAD⁺) | 560 (NAD⁺) [30] | 485 s⁻¹ [8] | NAD(P)⁺ |
| kcat for H₂-driven NAD (P)⁺ reduction (s⁻¹) | 150 s⁻¹ | | | n.p. |
| Vmax for NAD(P)H-driven H₂ production | 0.9 U mg⁻¹ | 1.2 U mg⁻¹ | 2.81 (U mg⁻¹) [32] | 1.5–2 U mg⁻¹ (NADPH) |
| Topt | 80 °C | 35 °C [20] | 60 °C [32] | 80 °C [38,39] |
| ΨH₂ | 6.5 | | 6.3 [32] | 8.4 [38] |
| Behavior towards O₂ | Moderately O₂-tolerant – 50% H₂-dependent NAD⁺ reduction activity³ in the presence of 19 μM O₂ | O₂-tolerant, – 85% H₂-dependent NAD⁺ reduction activity³ in the presence of 470 μM O₂ [15] | O₂-sensitive, no catalytic activity in the presence of O₂; can be rapidly reactivated under reducing conditions [40] | Moderately O₂-tolerant, – 25% of H₂ oxidation activity³ in the presence of 14 μM O₂ [41] |

¹ HtSH was purified by affinity chromatography as described in Materials and methods.
² For each O₂ concentration, a fixed volume of H₂-saturated buffer was mixed with various proportions of O₂- and N₂-saturated buffers. The gas phase contained the corresponding gas mixtures.
³ H₂-mediated NAD⁺ reduction activity was measured at 50 °C and pH 6.5.

![Figure 4](image4.png)

**Fig. 4.** Activity of purified HtSH protein at different pH values. The graph depicts the H₂-dependent NAD⁺ reduction activities of HtSH (grey bars) as well as the H₂-benzyl viologen (orange symbols) and NADH-benzyl viologen (blue symbols) oxidoreductase activities of the individual HtSH modules. The measurements were performed as described in Materials and methods with 45 nM of HtSH in 50 mM bis-Tris buffer, pH 6.5, containing 1 mM NAD⁺, 0.5 mM NiCl₂, 5 mM MgSO₄, 2 μM FMN, and 0.75 mM TCEP. If the error bars are not visible, they are equal or smaller than the symbol size.

![Figure 5](image5.png)

**Fig. 5.** Temperature dependence of the H₂-dependent NAD⁺ reduction activity of purified HtSH protein. The measurements were performed as described in Materials and methods with 45 nM of HtSH in 50 mM bis-Tris buffer, pH 6.5, containing 1 mM NAD⁺, 0.5 mM NiCl₂, 5 mM MgSO₄, 2 μM FMN, and 0.75 mM TCEP. If the error bars are not visible, they are equal or smaller than the symbol size.

**Table 3**

<table>
<thead>
<tr>
<th>O₂/H₂/N₂ fractions (μM)</th>
<th>[O₂] (μM)</th>
<th>Hydrogenase activity in the presence of O₂ (U mg⁻¹ of protein)⁴</th>
<th>kcat (s⁻¹)</th>
<th>Hydrogenase activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0/33.33/66.66</td>
<td>0.00</td>
<td>16 ± 2</td>
<td>45.9</td>
<td>100</td>
</tr>
<tr>
<td>0.2/33.33/64.46</td>
<td>1.9</td>
<td>15 ± 4</td>
<td>43.0</td>
<td>94.2</td>
</tr>
<tr>
<td>2/33.33/64.66</td>
<td>18.8</td>
<td>7.7 ± 0.3</td>
<td>21.5</td>
<td>49.8</td>
</tr>
<tr>
<td>10/33.33/65.66</td>
<td>94.0</td>
<td>1.3 ± 0.5</td>
<td>3.6</td>
<td>16.6</td>
</tr>
</tbody>
</table>

⁴ HtSH was purified by affinity chromatography as described in Materials and methods.
⁵ For each O₂ concentration, a fixed volume of H₂-saturated buffer was mixed with various proportions of O₂- and N₂-saturated buffers. The gas phase contained the corresponding gas mixtures.
⁶ H₂-mediated NAD⁺ reduction activity was measured at 50 °C and pH 6.5.
Table 4
CO and CN stretching frequencies (cm⁻¹) of IR-spectroscopically observed HtSH [NiFe] active site species.

<table>
<thead>
<tr>
<th>Assignment</th>
<th>ν(CO)</th>
<th>ν(CN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n/a⁴</td>
<td>1993</td>
<td>2081</td>
</tr>
<tr>
<td>Ni₄B-like</td>
<td>1964</td>
<td>2087</td>
</tr>
<tr>
<td>Ni₃S</td>
<td>1936</td>
<td>2058</td>
</tr>
<tr>
<td>Ni₃S</td>
<td>1951</td>
<td>2076</td>
</tr>
<tr>
<td>Ni₂C</td>
<td>1971</td>
<td>2076</td>
</tr>
<tr>
<td>Ni₄SR</td>
<td>1958</td>
<td>2062</td>
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<tr>
<td>Ni₃SR'</td>
<td>1943</td>
<td>2048</td>
</tr>
<tr>
<td>Ni₄SR''</td>
<td>1934</td>
<td>2048</td>
</tr>
</tbody>
</table>

⁴ Not assigned. Oxidized active site species of unknown structure.

2.5. Spectroscopic characterization of HtSH

To gain insight into structure and function of the metal cofactors, in particular of the [NiFe] active site, HtSH samples treated with different redox agents were characterized by IR and EPR spectroscopy. For both types of spectroscopic measurements, samples were prepared under identical conditions to guarantee comparability of the results. In addition, IR spectro-electrochemical experiments were performed to provide insight into equilibria between the individual redox states of the [NiFe] active site. All IR data are displayed as second derivative spectra where the maximum of an absorption band appears as a sharp negative peak. Peak positions derived from IR and EPR spectroscopy as well as their assignment to individual cofactors and redox states are summarized in Tables 4 and 5, respectively.

IR spectra of as-isolated HtSH exhibit up to three distinct bands at 1993, 1964, and 1936 cm⁻¹ (Fig. 6, trace a). Signals in this spectral region are generally associated with the stretching vibration of the intrinsic CO ligand of the [NiFe] active site, and different vibrational frequencies reflect distinct redox/structural states of this cofactor [3,46–49]. The three individual CO stretching vibrations of oxidized HtSH are separated by approximately 30 cm⁻¹, which is exceptional for active site species of oxidized [NiFe] hydrogenases. This observation suggests that the active site of as-isolated HtSH can adopt three configurations that strongly differ in terms of structural and/or electronic properties. The signal at 1964 cm⁻¹ may reflect the apparently EPR-silent “Ni₄B-like” state (Fig. 1), which was previously detected for ReSH and other NAD(P)⁺-reducing [NiFe] hydrogenases [6,8,12,40,50,51], and the band at 1936 cm⁻¹ is assigned to the Ni₃S state (see below). The signal at 1993 cm⁻¹, however, is unprecedented and absent in as-isolated ReSH [8,50,52–54]. According to relative intensities of the CO stretching bands, the contributions of the three different states varied across different as-isolated HtSH preparations. The unusual signal at 1993 cm⁻¹, however, generally represented the dominant species. To the best of our knowledge, such a high CO stretching frequency has not been observed for any [NiFe] hydrogenase to date. This suggests unusually high oxidation states of the metal ions, e.g. formation of ferric iron [55], or unusual structural features at or in close vicinity of the [NiFe] active site. In general, such observations and the appearance of multiple oxidized states may result from the contact with O₂ during and after protein isolation [50,56]. Importantly, all IR-spectroscopically detected oxidized species of the HtSH active site can be activated under reducing conditions (Fig. 6, traces b and c), as observed previously for, e.g., ReSH [50]. This indicates that the modifications reflected by the unusual signal at 1993 cm⁻¹ are reversible and not related to oxidative damage.

The EPR spectrum of as-isolated HtSH was measured at 10 K (Fig. 6, trace d) and exhibits a minor signal, presumably related to a [3Fe4S] cluster. Since no such cofactor is expected for native HtSH, this feature likely reflects the (partial) oxidative damage of one or more [4Fe4S] clusters, which is in line with preparation-dependent variations of the signal intensity. This situation is reminiscent of ReSH and the related NAD⁺-reducing hydrogenase from Rhodococcus opacus (Ro), both of which exhibit similar signals related to (non-native) [3Fe4S] species [8,13,53,57–60]. Furthermore, a weak rhombic signal, detected at 35 K, (Fig. S8, trace a) is presumably related to a paramagnetic [NiFe] active site state of as-isolated HtSH. Signals related to typical active site species of oxidized “standard” [NiFe] hydrogenases, however, were not detected, which is consistent with previous findings for NAD(P)⁺-reducing hydrogenases from other organisms [6,8,12,13,40,53,57–61].

Upon addition of the mild reducing agents TCEP and NADH to as-isolated HtSH, bands at 1993 and 1964 cm⁻¹ disappeared from the IR spectrum in favor of two new absorption features at 1971 and 1951 cm⁻¹ (Fig. 6, trace b). The former is ascribed to the Ni₄C state of the enzyme, which is in line with previous studies showing that Ni₄C exhibits the highest CO stretching frequency among all catalytically active [NiFe] species [3]. The second band, observed at 1951 cm⁻¹, is assigned to the one-electron more oxidized Ni₃S state, consistent with an intensity decrease upon hydrogen incubation of the enzyme (see below and Fig. 6, trace c). In ReSH and soluble hydrogenase 1 (SH1) from the hyperthermophilic organism Pyrococcus furiosus (Pf), this state corresponds to signals at 1946 cm⁻¹ [50] and 1950 cm⁻¹ [51], respectively (note that PSH1 differs from ReSH and ReS in terms of its subunit and cofactor composition [6]). The band at 1936 cm⁻¹ gains intensity upon incubation of as-isolated HtSH with TCEP and NADH (Fig. 6, traces a and b) indicating that it reflects a partially reduced [NiFe] species with a formal Ni³⁺ oxidation state. Since this CO stretching frequency is clearly lower than those observed for most other HtSH [NiFe] active site species, we tentatively assign this intermediate to the deprotonated Ni₃S subspecies, which features a bridging OH⁻ ligand.

The corresponding EPR spectrum of TCEP/NADH-reduced HtSH was recorded at 35 K and clearly shows the hydride-containing Ni₄C state (Ni⁵⁺, S = 1/2), consistent with the corresponding assignment of the strong IR absorbance at 1971 cm⁻¹. Moreover, signals attributed to a [2Fe2S] cluster (consistent with the results obtained by NRVS, Fig. S7) and a flavin radical species were detected (Fig. 6, trace e). These assignments are supported by simulation and subsequent summation of the individual components (Fig. 6, trace e, dashed line) and consonant with previous assignments for ReSH and RoSH [8,12,13,40,53,57–60,62]. Measurements performed at 10 K (Fig. S8, trace b) revealed an additional broad signal at g = 1.85, possibly reflecting a [4Fe4S] cluster.

Upon incubation of HtSH with H₂ (in the presence of TCEP and NADH), the 1971 cm⁻¹ band, assigned to the Ni₄C state, becomes the most intense signal of the IR spectrum, and corresponding CN stretching vibrations of this catalytic intermediate can be identified at 2076 and 2089 cm⁻¹ (Fig. 6, trace c). Moreover, a new redox species is formed as indicated by the appearance of an absorption band at 1958 cm⁻¹ (Fig. 6, trace c). According to spectro-electrochemical measurements (Fig. S9, traces b and c), an enrichment of this species requires lower potentials than that of the Ni₄C state. Therefore, we attribute this signal to the fully reduced Ni₄SR species with corresponding CN stretching bands at 2076 and 2062 cm⁻¹, which is in line with band assignments for PSH1 [51]. In case of ReSH, a similar set of signals, including an identical CO stretching band at 1958 cm⁻¹, has been assigned to the Ni₄SR2 state [6,12,50]. In the current case,
however, this assignment is less plausible since CO stretching bands of \textit{HisH} active site redox states appear to be generally higher in frequency than their counterparts in ReSH. Two further weak bands at 1943 and 1934 cm$^{-1}$ (Fig. 6, trace c) might reflect Ni$_5$SR$^-$ and Ni$_5$SR$^+$ subspecies of the reduced state [12,50]. Consistently, these states were observed as bands at 1940 (Ni$_5$SR$^-$) and 1931 cm$^{-1}$ (Ni$_5$SR$^+$) for \textit{PfSH}, which also exhibits generally higher CO stretching frequencies than ReSH [51]. Observation of these two subspecies provides further support for the assignment of the 1958 cm$^{-1}$ band to Ni$_5$SR as there is no other signal in the IR spectrum of \textit{HisH} that could be attributed to the main component of this species.

The EPR spectrum of H$_2$-incubated \textit{HisH}, recorded at 35 K, is dominated by the signal of the [2Fe2S] cluster (Fig. 6, trace f) confirming further enzyme reduction. In contrast to the IR data, this EPR spectrum exhibits only trace amounts of the Ni$_6$C state. However, in addition to broad features at positions typical for reduced [4Fe4S] cofactors (g = 1.83), an EPR spectrum recorded at 6.5 K (see Fig. S8, trace c) reveals pronounced broadened signals in the field range characteristic for the Ni$_6$C state, indicating strong magnetic coupling of the active site with another paramagnetic species. This temperature dependence of the Ni$_6$C signal pattern can be explained by fast spin-lattice relaxation of an Fe-S cluster near the [NiFe] site, leading to enhanced relaxation and broadening of the Ni$_6$C signal until its disappearance at higher temperatures. Similar magnetic interactions have been described in detail for “standard” [NiFe] hydrogenases [63,64], and particularly pronounced coupling effects were also reported for \textit{PfSH} [39], \textit{Pyrococcus furiosus} ferredoxin [65], and individual clusters of homologous respiratory Complex I [66–68]. For the Ni$_6$C state of \textit{HisH}, this effect appears to be most pronounced for the NADH/TCEP/H$_2$-treated sample. Assumed that unspecific, preparation-dependent effects can be excluded, this observation suggests that spin-lattice relaxation is accelerated by coupling to a paramagnetic cofactor ([4Fe4S] species) that is barely reduced by TCEP/NADH alone.

To support band assignments and gain insight into the reversibility of redox reactions at the [NiFe] active site of \textit{HisH}, initial IR spectroelectrochemical measurements and gas-exchange experiments were performed (Fig. S9, Fig. S10). As summarized in Table 4, these studies allowed a preliminary assignment of the CN stretching bands for all detected [NiFe] active site states. The monitored interconversions also confirmed the above-made assignments of the individual [NiFe] active site species, and the corresponding redox equilibria could be established (Fig. 1c). Remarkably, after reduction of as-isolated \textit{HisH} and subsequent re-oxidation, the [NiFe] active site species reflected by the unusual 1993 cm$^{-1}$ band did not re-appear (Fig. S9, Fig. S10). Thus, we propose that the reaction resulting in this particular species is kinetically hindered, suggesting a pronounced structural reorganization. In line with the unusually high CO stretching frequency, this observation supports the idea that this oxidized state differs considerably from other typical [NiFe] active site intermediates.

### 3. Conclusion

Here, we provide the first combined biochemical and spectroscopic characterization of a NAD$^+$-reducing [NiFe]-hydrogenase that is both thermostable and O$_2$-tolerant. The enzyme originates from the thermophile \textit{Hydrogenophilus thermoluteolus} TH-1$^+$ [21], and its corresponding structural genes were heterologously overexpressed in the mesophilic host \textit{Ralstonia eutropha} H16. This procedure resulted in the formation of catalytically active \textit{HisH} protein, which clearly shows that the hydrogenase-specific maturation machinery from \textit{R. eutropha} [5] is capable of synthesizing and inserting the NiFe(CN)$_2$CO cofactor into the large hydrogenase subunit of \textit{HisH}. Taking into account the successful heterologous overproduction of SH from \textit{Rhodococcus opacus} opacu$|$ [69], \textit{R. eutropha} seems to be an excellent host for synthesis and isolation of catalytically active SH proteins from bacterial species that are so far unamenable to genetic engineering.

\textbf{Table 2} shows biochemical and structural properties of the \textit{HisH} in comparison with those of other soluble NAD(P)$^+$-reducing [NiFe]-hydrogenases. The isolated \textit{HisH} is a heterotetrameric enzyme with a turnover frequency of ca. 150 s$^{-1}$ for H$_2$-driven reduction of NAD$^+$ at pH 6.5 and 50$^\circ$C. In terms of biotechnologically relevant cofactor regeneration [19], the \textit{HisH} is complementary to \textit{PfSH}, which preferably reduces NADP$^+$ in a H$_2$-dependent manner at high temperature [33]. Although to a lesser extent when compared to ReSH, \textit{HisH} shows catalytic H$_2$-mediated NAD$^+$ reduction in the presence of O$_2$ in solution assays. For \textit{PfSH}, O$_2$-tolerant H$_2$ oxidation (but not NAD(P)$^+$ reduction) has so far only been shown electrochemically with immobilized enzyme [41]. Though phylogenetically closely related to \textit{HisH} and...
ReSH, the purified bidirectional [NiFe]-hydrogenase from *Synechocystis* sp. seems to be rather unstable and is rapidly inactivated by O$_2$. The well-characterized and extraordinary O$_2$-tolerant ReSH, in contrast, shows good stability and highest activity at moderate temperatures and pH 8, but quickly loses activity at temperatures above 35 °C [20]. In summary, the HtSH represents an attractive candidate for biotechnological applications, e.g., as an NADH regeneration catalyst in enzymatic cascades that rely on high temperatures and O$_2$ as a co-substrate.

EPR, IR and NRV spectroscopic analyses of the HtSH protein revealed the occurrence of FMN, [FeS$_2$] and [4Fe4S] cluster species as well as typical active site states that have been observed for other soluble NAD(P)$^+$-reducing [NiFe] hydrogenases [6,40,51]. These include the Ni$_{A}$-B-like state that is not directly involved in H$_2$/H$^+$ cycling as well as the Ni$_{A}$-S, Ni$_{C}$-S, and Ni$_{A}$-SR states which are generally accepted as intermediates of the catalytic cycle. While the Ni$_{A}$-C state was identified both by IR and EPR spectroscopy, all other states are EPR-silent and were assigned based on IR spectroscopic analyses only. Interestingly, the Ni$_{A}$-C signal in the EPR spectrum of Ht-treated HtSH was mainly observed at temperatures below 10 K, presumably due to fast spin-lattice relaxation related to magnetic coupling with another cofactor that is paramagnetic under these reducing conditions. This observation represents an important finding that could explain why Ni$_{A}$-C and other paramagnetic active site species have often not been observed for NAD(P)$^+$-reducing [NiFe] hydrogenases [6,40]. Furthermore, the as-isolated, oxidized HtSH exhibits a CO stretching vibration at 1993 cm$^{-1}$, which is extremely high in frequency and so far unprecedented for [NiFe]-hydrogenases. This unusual vibrational band most likely reflects an alternative geometry and/or coordination environment of the hetero bimetallic active site. Since no crystallographic data is available yet, further spectroscopic investigations are currently in progress to gain detailed insight into the structure this novel species.

**4. Materials and methods**

**4.1. Construction of the synthetic P$_{SH}$-hox$_{ar}$.FUYHW operon, growth conditions, and protein purification**

The HtSH-derived gene cluster containing hoxFUYHW was amplified by PCR using the primers

5′-agaacctgtacctccgcgagctaaacaccagggaggaggaac-3′

and

5′-ctcgctacccggaggatcatactcctcttcgtgggtgaaaaaac-3′

and genomic DNA from *Hydrogenophiles thermocellularis* TH$^{-1}$ as a template. The underlined bases of the primers are complementary to plasmid pGE837, which is a pCM66 [70] derivative carrying a *XbaI*-BamHI-cutter fragment from plasmid pGE770 [15] with the $^{Sh}$-strep$h$oxF from *Raiotalia eutropha* H16 followed by a sequence encoding a GG-ScaI linker with a TEV cleavage site (underlined residues). Plasmid pGE837 was linearized by inverted PCR using primers 5′-atggattcgctgccgtcgg-3′ and 5′-gcgctgatcagtcggtcgg-3′, and the 7.9 kb product served as recipient of the *Ht hoxFUYHW* PCR amplicon, which was inserted according to the Gibson Assembly* manual (New England BioLabs). The resulting plasmid carries the *Ht hoxFUYHW* genes under control of the SH promoter of *R. eutropha* [71], whereby the 5′ end of the hox$^F$ gene was equipped with a linker sequence and a Strep-tag II-encoding sequence. A *P$_{SH}$-hox$_{ar}$.FUYHW* fragment was cut out with *Eco53K* and *XbaI*, and the resulting 5.7 kb fragment was inserted into the *Scal*-BamHI-cutter vector pEDY309 [72]. This yielded plasmid pJP09, which was subsequently transferred by conjugation to *R. eutropha* HFI054, which is a HF424 [73] derivative carrying an additional in-frame deletion in the *hox$^F*$ gene.

Strain *R. eutropha* HFI054 (pJP09) was grown heterotrophically in a mineral salts medium containing a mixture of 0.05% (w/v) fructose and 0.4% (v/v) glycerol (FGN medium) at 30 °C as described previously [25]. Upon reaching an optical density at 436 nm of 9–11, the culture was collected, and the cells were harvested by centrifugation at 8850 × g for 15 min at 4 °C. The cell pellet was resuspended in 50 mM KPO$_4$, pH 7.2, containing 15–20% (v/v) glycerol, 5 mM MgCl$_2$, 0.5 mM NiCl$_2$, and protease-inhibitor cocktail (EDTA-free Protease Inhibitor, Roche). The extract was furthermore supplemented with 5 mM NAD$^+$ in order to keep the HtSH in the oxidized state, which is thought to prevent extensive oxidative damage through reactive oxygen species [15]. After two passages through a chilled French press cell at a pressure of 125 MPa, the soluble extract was separated from solid cell constituents by centrifugation at 72500 × g for 45 min. The supernatant was loaded onto a 2 mL Strep-Tactin Superflow column (IBA), which was previously equilibrated with resuspension buffer. After washing with at least 6 column volumes of resuspension buffer, the protein was eluted in resuspension buffer containing 5 mM desthiobiotin. A final concentration of 20–30 mg mL$^{-1}$ of purified protein was achieved after concentration with Ultra Centrifugal Filter Units (Amicon).

In order to obtain HtSH protein with homogenous subunit stoichiometry, size exclusion chromatography was conducted after affinity chromatography. An amount of 200 μL of the concentrated HtSH eluate was loaded onto a Superdex 200 10/300 GL column which was previously equilibrated with the same buffer used for affinity chromatography. Using an ÄKTA pure system, the flow rate was held at 0.2 mL min$^{-1}$, and protein elution occurred at approximately 0.3 column volumes as observed by an UV/vis absorption increase at 280 nm and 420 nm. Protein fractions of 0.4 mL were collected, and the HtSH subunit composition was checked by SDS-PAGE according to Laemmli et al. [74]. After determining the H$_2$-dependent reduction of NAD$^+$ activity, fractions with highest specific activities and homogeneity were pooled and again concentrated using Ultra Centrifugal Filter Units (molecular weight cut-off of 100 kDa).

**4.1.1. Enzyme assays**

All enzyme measurements were performed in the presence of defined gas mixtures unless stated otherwise. Prior to use in enzyme assays, the buffers were bubbled with the respective gases. Buffers with 100% gas-saturation (1 bar, 50 °C) contained 720 μM H$_2$, 940 μM O$_2$ or 483 μM N$_2$. Buffers containing gas mixtures were prepared by mixing individual buffers with 100% gas saturation. The head space of the reaction vessels was kept as small as possible to avoid degassing of solutions. H$_2$-driven NAD$^+$ reduction of purified HtSH in soluble extracts was determined at 50 °C in a buffer-filled, rubber-stoppered cuvette. The reactions were started by the addition of enzyme, and the absorbance increase at 365 nm due to NADH accumulation was monitored spectrophotometrically with a Cary 50 (Varian). The pH-dependent HtSH activity was measured by using two different strategies. First, to minimize the influence of different buffer components on SH activity, a broad-range buffer system (pH 4.5–9) composed of 16 mM citrate, 16 mM Tris, and 16 mM glycine was used. The buffer system was adjusted at 50 °C with appropriate acids or bases to the desired pH values. Second, SH activity was also tested in the individual buffers mentioned above. Temperature-dependent activity measurements were performed in 50 mM bis-Tris, pH 6.5, containing 0.75 mM TCEP (replacing DTT), 0.5 mM NiCl$_2$, 5 mM MgCl$_2$, and 2 μM FMN. This owes to the fact that DTT precipitates in NiCl$_2$ and MgCl$_2$-containing 50 mM KPO$_4$, buffer at temperatures above 40 °C.

NADH-driven H$_2$ production was measured with a modified Clark-type electrode [75] at 50 °C in 50 mM bis-Tris, pH 6.5, containing 5 mM MgCl$_2$, 0.5 mM NiCl$_2$, 0.75 mM TCEP, 2 μM FMN and 1 mM NADH. The buffers as well as the additives were gassed with air before mixing, and the reaction was started by the addition of enzyme. Diaphorase activity of the SH was recorded spectrophotometrically as NADH-dependent benzyl viologen reduction at 50 °C in buffers with different pH values (composition see above), containing 5 mM benzyl viologen (BV), 1 mM NADH, and 90 μM dithionite. H$_2$-dependent reduction of BV (5 mM) was tested at 50 °C in buffers with different pH values (composition see above). Prior to use, the buffers were saturated with H$_2$. 
In order to determine affinity constants for NAD$^+$ or NADH, the initial reaction velocities for H$_2$-dependent NAD$^+$ and NADH-dependent BV reduction, respectively, were measured at 50 °C and varying substrate concentrations. The recorded slopes were plotted against the substrate concentration and fitted to the Michaelis-Menten kinetic using the program Origin 2016.

Determination of affinity towards H$_2$ was performed amperometrically by mixing different volumes of H$_2$- and N$_2$-saturated buffers (50 mM bis-Tris, pH 6.5, 5 mM MgCl$_2$, 0.5 mM NiCl$_2$) to a total volume of 1.3 mL in the reaction chamber of a modified Clark electrode. The assay contained further the natural electron acceptor, NAD$^+$ (1 mM), in addition to 0.75 mM TCEP, and 2 μM FMN. The reaction was started by enzyme addition, and the resulting current change was recorded. The derived reaction velocities were plotted against the H$_2$ concentration and fitted to the Hill equation using Origin 2016.

4.1.2. Protein, iron, and FMN determination

The protein concentration was determined with the BCA™ Protein Assay Reagent Kit (Pierce, USA) using bovine serum albumin as the standard. The flavin mononucleotide concentration in protein samples was analyzed fluorometrically as described previously [30,31]. Iron and nickel contents of purified HiSH samples were analyzed by inductively coupled plasma optical emission spectroscopy (ICP-OES) as previously described [76]. Final numbers were derived from two biological replicates, while each sample was measured three times (three technical replicates).

4.1.3. Sample preparation for IR and EPR spectroscopy

For the characterization of as-isolated HiSH, protein fractions were concentrated to approx. 0.3 mM using Amicon Ultra 0.5 mL Centrifugal filters (Merck KGaA) and measured without further treatment. Samples of reduced HiSH were prepared using different procedures. Prior to all reductive treatments, buffers were purged with Ar for 30 min, and O$_2$ was removed from protein samples by ten consecutive cycles of Ar purging and vacuum exertion. Partial reduction of the enzyme was achieved by 30 min incubation of 0.03 mM HiSH with 2 mM TCEP and 5 mM NADH at 50 °C in an anaerobic, N$_2$-filled glovebox. After these treatments, the samples were concentrated to approx. 0.3 mM, and IR transmission cells and EPR tubes were purged with N$_2$ prior to loading. To further reduce HiSH, solutions containing 0.03 mM of protein were incubated with 2 mM TCEP, 5 mM NADH, and 1 bar O$_2$-free H$_2$ (O$_2$ was removed using a Varian Gas Clean Oxygen Filter PIN CP17970) in H$_2$-saturated buffer at 50 °C for 30 min in an anaerobic chamber (95% N$_2$, 5% H$_2$). The H$_2$ stream was enriched with H$_2$O to avoid sample drying. Prior to measurements, samples were concentrated to ~0.3 mM, and IR transmission cells and EPR tubes were purged with H$_2$. Aliquots of all samples were directly injected into an IR transmission cell for subsequent characterization, while the remainder was transferred to EPR tubes, quenched in cold ethanol (ca. 210 K) and stored in liquid nitrogen for further analysis.

4.1.4. IR spectroscopy

IR spectra of 0.3 mM solutions of as-isolated and chemically reduced HiSH were recorded with a spectral resolution of 2 cm$^{-1}$ using a Bruker Tensor 27 FTIR spectrometer, equipped with a liquid nitrogen-cooled MCT detector. The sample compartment was purged with dry air, and the sample was held in a temperature-controlled (10 °C) gas-tight IR transmission cell for liquid samples (volume: 10 μL, optical path length: 50 μm), equipped with CaF$_2$ windows. The Bruker OPUS software, version 5.5 or higher, was used for data acquisition and evaluation.

4.1.5. IR spectro-electrochemical experiments

IR spectro-electrochemical experiments were performed on ca. 0.3 mM solutions of HiSH, activated anaerobically with 2 mM TCEP, using an Optically Transparent Thin Layer Electrochemical (OTTLE) cell [77] with an optical path length below 10 μm. In order to avoid protein adsorption, the gold mesh working electrode was incubated anaerobically with a mixed self-assembling monolayer of 1 mM cysteamine and 1 mM mercaptopropionic acid, solved in ethanol, for 30 min. Preparation of the OTTLE cell was performed anaerobically in an Ar-filled box. The following redox mediators were added to the protein solution in order to ensure fast equilibration at the applied potentials (0.5 mM each, potential vs. SHE): TMPPO (+262 mV), 1,2-naphthoquinone (+145 mV), 1,4-naphthoquinone (+60 mV), methylene blue (+11 mV), indigo trisulfate (−80 mV), indigo disulfate (−130 mV), 2-hydroxy-1,2-naphthoquinone (−139 mV), resorufin (−195 mV), antarhquione-2-sulfonate (−225 mV), safranin T (−290 mV), benzyl viologen (−358 mV), methyl viologen (−446 mV) [77–79]. Potential-dependent IR spectra with a resolution of 2 cm$^{-1}$ were recorded at 30 °C using a Bruker IFS 66 v/s FTIR spectrometer equipped with a liquid nitrogen-cooled MCT detector. The Bruker OPUS software, version 5.5 or higher, was used for data acquisition and evaluation. Potential control was accomplished using a Model 263A Potentiostat (Princeton Applied Science) and the PARControl 1.05 software. Samples were equilibrated at all potentials for at least 3 min until the corresponding IR spectrum remained unchanged.

4.1.6. EPR spectroscopy

A Bruker EMXplus spectrometer equipped with an ER 4122 SHQE resonators and an Oxford EPR 900 helium flow cryostat with temperature control (Oxford ITC4) between 5 and 310 K was used in the experiments. Spectra were baseline-corrected by subtracting a background spectrum obtained from buffer solution using the same experimental parameters. Experimental conditions: 1 mW microwave power, microwave frequency: 9.29 GHz, 1 mT modulation amplitude, 100 kHz modulation frequency. Spectra simulations were performed using the MATLAB toolbox EasySpin (version 5.1.7).

4.1.7. NRVS spectroscopy

For nuclear resonance vibrational spectroscopy (NRVS), R. eutropha H161054 (pJP09) was cultured as described above, with the exception that 18 μM $^{57}$FeCl$_2$ instead of $^{56}$FeCl$_2$ was used as the iron source. The resulting $^{57}$Fe-labelled HiSH was purified via Srep-Tactin affinity chromatography. NRVS was performed at SPRing-8 BL09XU with a 0.8 meV (6.5 cm$^{-1}$) energy resolution at 14.4125 keV as described previously [43]. The beam size at BL09XU was 1.1 mm (horizontal) × 0.6 mm (vertical). A 4-element avalanche photo diode detector array was used to measure delayed K shell fluorescence and nuclear fluorescence by $^{57}$Fe atoms. All measurements were performed in the cryostat base that was cooled to 10 K. The real sample temperature was 30–60 K, as obtained from the spectral analysis. The raw NRVS data was converted to a $^{56}$Fe partial vibrational density of states (PVDS) by the PHOENIX software [80], while the energy scale was calibrated with an external reference ([NEt$_4$][FeCl$_4$]). For the HiSH protein sample (22 μL, 0.8 mM), the accumulation time was 21 h.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Appendix A  Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbabio.2017.09.006.

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