Enzymatic and spectroscopic properties of a thermostable [NiFe]-hydrogenase performing H_2-driven NAD^+-reduction in the presence of O_2

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KEYWORDS

Hydrogenase; hydrogen; oxyhydrogen reaction; nickel; iron; respiratory Complex I, flavin; iron-sulfur cluster; pyridine nucleotide; enzyme kinetics; infrared vibrational spectroscopy, electron paramagnetic resonance spectroscopy; nuclear resonance vibrational spectroscopy; biotechnology; cofactor recycling

ABSTRACT

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^T (*Ht*). The *Ht*SH 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 *Ht*SH that is remarkably different from those of the closely related *Re*SH and other [NiFe]-hydrogenases. This indicates an unusual configuration of the oxidized catalytic center in *Ht*SH. 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 *Ht*SH as well as application of this enzyme for H_2-driven cofactor recycling under oxic conditions at elevated temperatures.

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 ($H_2 + ½ O_2 \rightarrow H_2O$), releases a high yield of free energy of $\Delta G° = –237.2$ 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 chemolithoautotrophic metabolism [5]. *Re*SH 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 *Re*SH is a bi-modular 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 *Re*SH [8]. The overall subunit composition as well as the cofactor arrangement of NAD^+-reducing [NiFe]-hydrogenases are reminiscent 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 *Re*SH 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 *Re*SH 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 Fe^{II} state throughout the catalytic cycle. The nickel ion, however, changes its redox state during H_2/H^+ turnover [6]. As the *Re*SH is catalytically active under aerobic conditions, a contact of the active site with O_2 is a very likely event. Nonetheless, the H_2 turnover rate remains at almost 100 % even in the presence of 20 % O_2, which makes *Re*SH the "world record holder" among O_2-tolerant, energy-converting [NiFe]-hydrogenases [14,15]. Moreover, the *Re*SH represents the first hydrogenase, for which a catalytic conversion of O_2 into water has been demonstrated [15]. The exceptional O_2 tolerance and the high turnover rates of the *Re*SH attracted scientists to employ the enzyme both *in vitro* and *in vivo* for H_2-driven NAD(P)H cofactor regeneration in biotechnologically relevant applications [16–19]. Though very efficient in NADH recycling, however, the *Re*SH 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 *Re*SH have prompted us to seek out a thermostable version of this enzyme.

Hydrogenophilus thermoluteolus TH-1^T (*Ht*) has been described as an aerobic, facultatively chemolithoautotrophic, hydrogen-oxidizing microorganism, which – like *R. eutropha* – belongs to the phylogenetic class of betaproteobacteria [21]. It shows optimal chemolithoautotrophic growth with a H_2:O_2:CO_2 gas mixture of 7:2:l at a temperature of 52 °C [22]. This suggests the presence of at least one O_2-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 *Ht*SH is so far available.

In this study, we present the DNA sequence of the structural genes of the four *Ht*SH subunits in addition to the gene encoding the *Ht*SH-specific endopeptidase. The *Ht*SH was recombinantly overproduced in *R. eutropha* and – upon purification – characterized by means of biochemical and spectroscopic methods. It turned out to be the first characterized [NiFe]-hydrogenase that performs H_2-driven NAD^+ reduction at elevated temperatures and in the presence of O_2.

RESULTS AND DISCUSSION

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

The draft sequence (published elsewhere) of the *H. thermoluteolus* TH-1^T genome revealed the *Ht*SH-related genes, *hoxF*, *hoxU*, *hoxY*, *hoxH*, and *hoxW*, which are apparently arranged as an operon (Fig. 1). Pairwise alignments of *Ht*SH and *Re*SH 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-1^T genome does not contain a copy of the gene encoding the HoxI protein, which is a constituent of the *Re*SH [24].

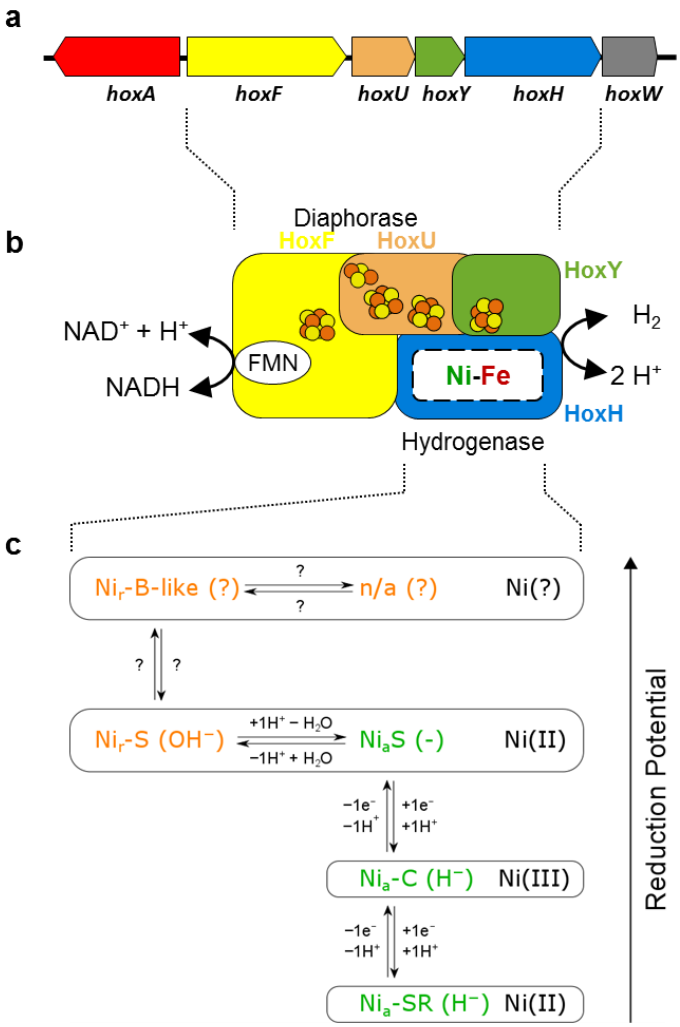


Fig. 1. Arrangement of the *Ht*SH-related genes (**a**), proposed subunit/cofactor composition (**b**), and observed active site redox states of *Ht*SH (**c**). Genes *hoxF*, *U*, *Y*, and *H* encode the subunits of the SH protein, while *hoxA* has presumably a regulatory function. Upon insertion of the [NiFe] active site, the *hoxW* gene product mediates cleavage of a C-terminal extension of the HoxH subunit. The proposed cofactor composition in **b** is derived from amino acid sequence comparisons with the corresponding subunits of *Re*SH and Complex I from *Thermus thermophilus* (see Fig. S1) and analogies to the well-characterized *Re*SH. The assignment of active site species and their interconversions shown in **c** is based on IR and EPR spectroscopic analyses (see below). Redox states highlighted in green belong to the catalytic conversion of H_2, while the orange ones represent inactive states that – except for Ni_r-S – require reductive treatment to be converted into the Ni_a-S state. The unassigned oxidized state labelled with n/a is unprecedented (see below).

Heterologous overproduction and purification of functional *Ht*SH

For heterologous overproduction of the *Ht*SH in *R. eutropha* and subsequent purification, the *hoxFUYHW* genes were amplified by PCR and put under the control of the native SH promoter of *R. eutropha* 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

129 synthetic *hox*$_{strep}$*FUYHW* operon was inserted into the broad-host range vector pEDY309
130 resulting in plasmid pJP09, encoding *Strep*-tagged *Ht*SH.

131 For enzyme purification, plasmid pJP09 was transferred into strain *R. eutropha* HF1054,
132 in which the native *hoxFUYHWI* genes as well as *hoxG* encoding the large subunit of the
133 membrane-bound [NiFe]-hydrogenases have been eliminated by isogenic in-frame deletions.
134 This prevented any "subunit mixing" between *Ht*SH and *Re*SH proteins. The transconjugant
135 strain *R. eutropha* HF1054 (pJP09) was cultivated heterotrophically under oxygen-limited
136 conditions as described previously [15,25]. In a first experiment, the H_2-driven NAD^+
137 reduction activity was measured in soluble extract of the recombinant cells. The activity was
138 2.50 ± 0.12 U mg^{-1} of protein (Table 1), suggesting the presence of functional *Ht*SH proteins.
139 This result also demonstrates that the general [NiFe]-hydrogenase maturation machinery of *R.*
140 *eutropha* [26–28] is able to synthesize and to deliver the active site constituents for the HoxH
141 subunit of *Ht*SH.

142 The *Ht*SH protein was then purified to homogeneity by *Strep*-Tactin affinity and size
143 exclusion chromatography as described in materials and methods. From 10 g (wet weight) of
144 cells, we routinely obtained 10–12 mg of protein with a specific H_2-driven NAD^+ reduction
145 activity of 33.4 ± 0.6 U mg^{-1} of protein (measured at 50 °C, Table 1). The reverse reaction,
146 namely NADH-driven H_2 production, was catalyzed with an activity of 1.0 ± 0.3 U mg^{-1} of
147 protein. Using dithionite-reduced methyl viologen (MV) as artificial, low-potential electron
148 donor, the H_2 production activity increased to 30 ± 5 U mg^{-1} of protein. SDS-PAGE
149 performed with the *Ht*SH preparation revealed four protein bands assigned to the subunits
150 HoxFUHY (Fig. 2).

Table 1. Purification of *Ht*SH protein enzyme by affinity chromatography.

Fraction[a]	Volume (mL)	Protein concentration (mg/mL)	Total protein (mg)	Specific activity (U mg^{-1})[b]	Total activity (U)	Yield (%)	Enrich-ment factor
SE	40	29.2	1168	2.5 ± 0.1	2920	100	1
AC	1.4	29.7	41.6	12.1 ± 0.1	502	17	4.8
SEC	2.4	4.9	11.7	33.4 ± 0.6	391	13	13.4

[a]The *Ht*SH 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 H_2-dependent NAD^+ reduction in 50 mM bis-Tris, pH 6.5, supplemented with 1 mM NAD^+, 0.5 mM $NiCl_2$, 5 mM $MgSO_4$, 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 one minute. Values of a representative purification are shown.

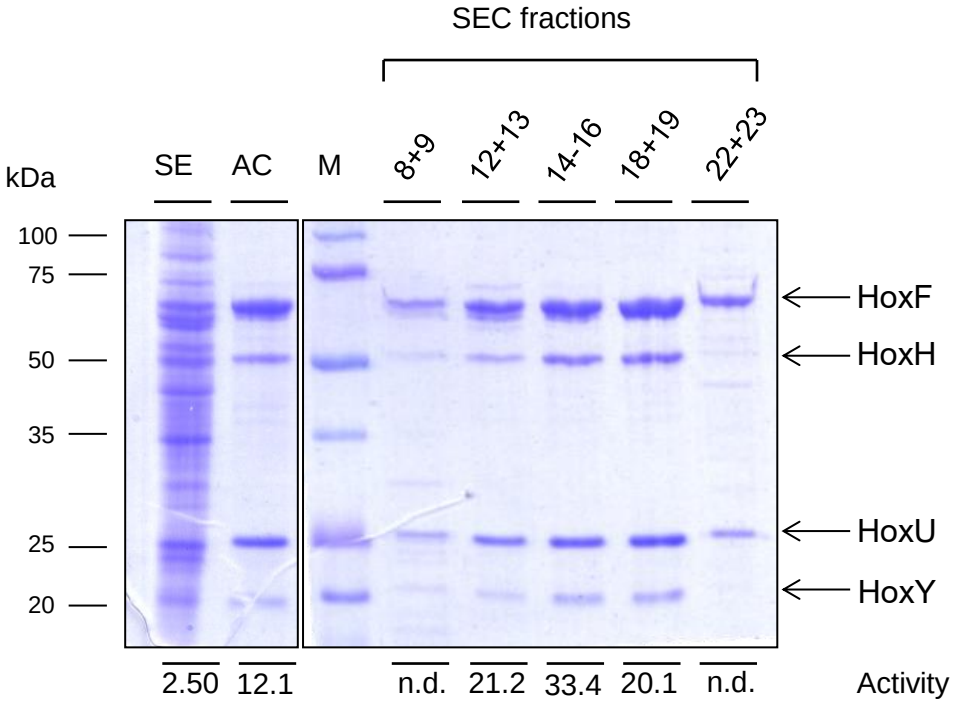


Fig. 2. Purification of the *Ht*SH protein. A protein amount of 30 µg of soluble extract (SE) and 5 µg of *Ht*SH purified by affinity chromatography (AC) and selected fractions (from the subsequent size exclusion chromatography (SEC) were electrophoretically separated on a 12 % SDS-polyacrylamide gel and subsequently stained with Coomassie brilliant blue. The specific H_2-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.

Biochemical characterization of purified *Ht*SH.

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 Ni^{2+} (0.5 mM) and Mg^{2+} (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 Ni^{2+} and Mg^{2+} ions in addition to 2 µM FMN, the latter of which led to a shortened lag phase but did not change the maximal H_2 oxidation activity (Fig. S2). This suggests that FMN serves as an electron acceptor, and reduced FMN can reactivate those inactive *Ht*SH species which cannot be activated by H_2 alone. This mechanism is similar to the NADH-based reactivation of as-isolated *Re*SH [24,30]. Highest H_2-driven NAD^+ reduction activity for purified *Ht*SH (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 *Ht*SH as previously observed for SH from *R. eutropha* [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 *Ht*SH and only 2.5 µM NADH, it took only 4 minutes until full activity was developed (Fig. 3). This suggests that the rate of reductive reactivation can also be accelerated by intermolecular electron transfer between individual *Ht*SH enzymes. The likelihood of electron exchange between *Ht*SH enzymes is of course greater at higher protein concentration.

Based on the knowledge derived from the experiments described above, $NiCl_2$, $MgSO_4$, 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 H_2-dependent NAD^+ reduction activity of purified *Ht*SH 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 *Re*SH that performs best at pH 8.0 [20,30] (Table2), where the *H. thermoluteolus* enzyme showed only about 10 % of the maximal H_2-driven NAD^+ reduction activity of 50 ± 4 U mg^{-1} of protein (measured at pH 6.5, Fig. 4).

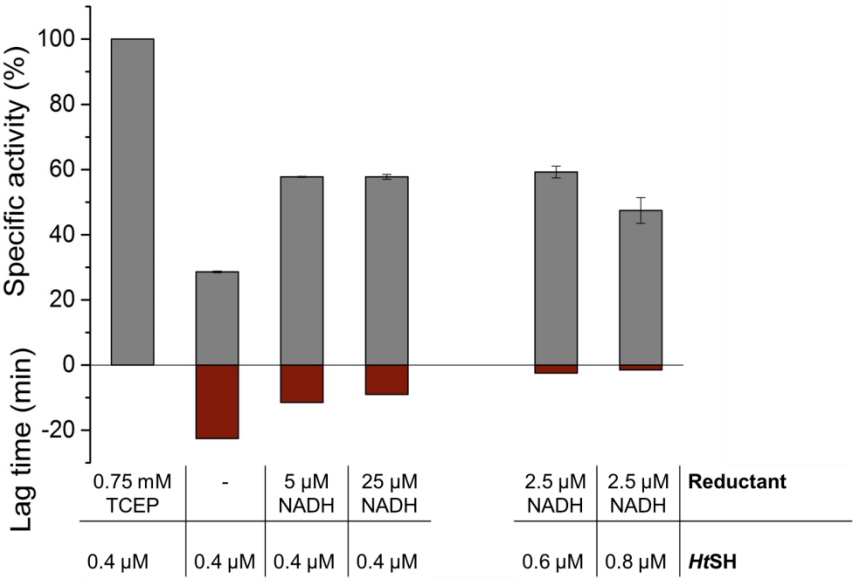


Fig. 3. Dependence of H_2-driven NAD^+ reduction activity of purified *Ht*SH protein on the addition of reductants TCEP and NADH. The assay was performed at 50 °C in 50 mM bis-Tris, pH 6.5, supplemented with 1 mM NAD^+, 0.5 mM $NiCl_2$, 5 mM $MgSO_4$, 2 µM FMN, and varying amounts of TCEP, NADH and *Ht*SH. 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.

In order to elucidate the origin of the unusual pH optimum, the 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 *Re*SH [31]. The H_2: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 *Ht*SH is primarily dictated by the intrinsic bias of the H_2/H^+-cycling module of the holoenzyme.

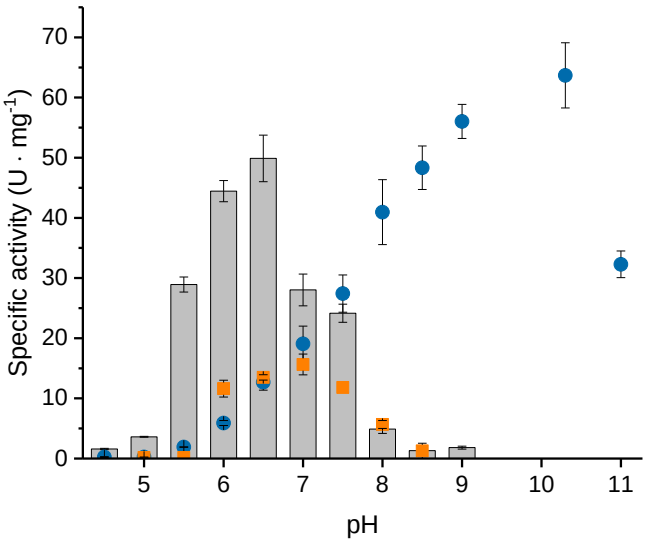


Fig. 4. Activity of purified *Ht*SH protein at different pH values. The graph depicts the H_2-dependent NAD^+ reduction activities of *Ht*SH (grey bars) as well as the H_2:benzyl viologen (orange symbols) and NADH:benzyl viologen (blue symbols) oxidoreductase activities of the individual *Ht*SH modules. The measurements were performed as described in materials and methods with 45 nM of *Ht*SH 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^+, 1mM NADH, or 5 mM benzyl viologen, in addition to 0.5 mM $NiCl_2$, 5 mM $MgSO_4$, 2 µM FMN, and 0.75 mM TCEP.

225 **Table 2.** Comparison of soluble, $NAD(P)^+$-reducing [NiFe] hydrogenases[a]

Organism	*H. thermoluteolus* TH-1^T	*R. eutropha* H16	*Synechocystis* sp. PCC 6803	*Pyrococcus furiosus*
Designation	SH	SH	bidirectional hydrogenase	SH1
Subunit composition	HoxHYFU	$HoxHYFUI_2$ [24]	HoxHYFUE [32]	αδβγ [33]
Molecular weight (kDa)	168	207 [24]	180 [32]	153 [33]
K_M H_2 (µM)	42	37 [30]	11.3 [34][b]	140 [33]
Physiological electron acceptors/donors	NAD^+	NAD^+	$NAD(P)^+$/ NAD(P)H, $ferredoxin_{red}$, $flavodoxin_{red}$ [35,36]	$NAD(P)^+$
K_M $NAD(P)^+$ (µM)	469 (NAD^+)	560 (NAD^+) [30]	n.p.	40 ($NADP^+$) [33]
k_{cat} for H_2-driven $NAD(P)^+$ reduction (s^{-1})	150 s^{-1}	485 s^{-1} [8]	n.p.	99 s^{-1} (NAD^+) [33] 38-89 s^{-1} ($NADP^+$) [37]
v_{max} for NAD(P)H-driven H_2 Production	0.9 U mg^{-1}	1.2 U mg^{-1}	2.81 (U mg^{-1}) [32]	1.5-2 U mg^{-1} (NADPH)
T_{opt}	80°C	35°C [20]	60°C [32]	80°C [38,39]
pH_{opt}	6.5	8 [20,30]	6.3 [32]	8.4 [38]
Behavior towards O_2	moderately O_2-tolerant ~50 % H_2-dependent NAD^+ reduction activity[c] in the presence of 19 µM O_2	O_2-tolerant, ~85% H_2-dependent NAD^+ reduction activity[c] in the presence of 470 µM O_2 [15]	O_2-sensitive, no catalytic activity in the presence of O_2; can be rapidly reactivated under reducing conditions [40]	moderately O_2-tolerant, ~25% of H_2 oxidation activity[d] in the presence of 14 µM O_2 [41]

226 [a]Note that values are only limitedly comparable since the assay conditions were not identical.
227 [b]Value has been determined for the bidirectional hydrogenase from the *Synechocystis* sp. relative,
228 *Anabaena variabilis*.
229 [c]Compared to the activity measured in the absence of O_2. Activities were measured
230 spectrophotometrically in solution.
231 [d]Compared to the activity measured in the absence of O_2. Activities were measured electrochemically
232 with immobilized enzyme at oxidizing potential.
233 n.p.; not published
234

235 Measurements of the H_2-dependent NAD^+ reduction activity of purified *Ht*SH at different
236 temperatures were performed in bis-Tris buffer at pH 6.5 and revealed a maximal activity of
237 71.0 ± 0.3 U mg^{-1} of protein at a temperature of 80 °C (Fig. 5). This is in sharp contrast to
238 *Re*SH, which quickly loses activity at temperatures higher than 35 °C [20] (Table 2). At
239 33 °C, which is the temperature optimum of *Re*SH activity [30], *Ht*SH showed less than 20 %
240 of the maximal activity.

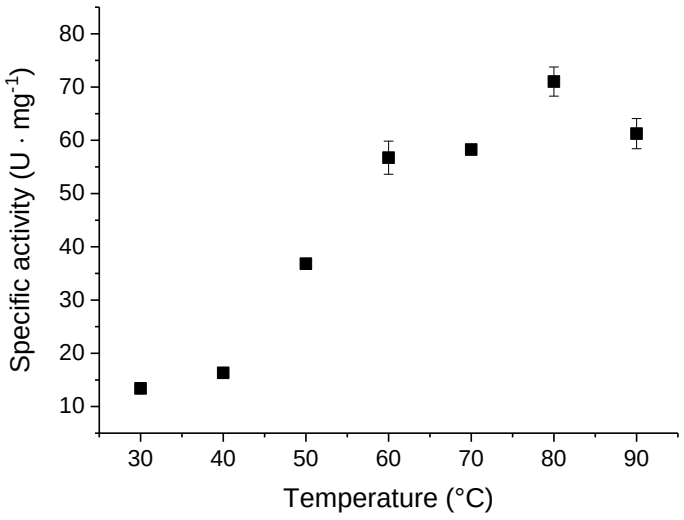


Fig. 5. Temperature dependence of the H_2-dependent NAD^+ reduction activity of purified *Ht*SH protein. The measurements were performed as described in materials and methods with 45 nM of *Ht*SH in 50 mM bis-Tris buffer, pH 6.5, containing 1 mM NAD^+, 0.5 mM $NiCl_2$, 5 mM $MgSO_4$, 2 µM FMN, and 0.75 mM TCEP. If the error bars are not visible, they are equal or smaller than the symbol size.

In a next series of experiments, we determined the Michaelis-Menten constants (K_M) for the natural substrates of the *Ht*SH. The K_M value for NAD^+ was evaluated based on the H_2-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 *Re*SH [30]. Activity measurements of the *Ht*SH-mediated benzyl viologen reduction activity in the presence of various NADH concentrations resulted in a K_M^{NADH} of 1.2 mM (Fig. S5), which is surprisingly high when compared to the corresponding value of 80 µM determined for the *Re*SH [30]. This suggests that the main physiological role of *Ht*SH enzyme is H_2-driven NAD^+ reduction.

A value of 42 ± 3 µM was determined for the apparent Michaelis-Menten constant, K_M^{app}, for H_2 during H_2-driven NAD^+ reduction of the enzyme (Fig. S6), which is comparable to that measured for *Re*SH (37 µM, [30], Table 2).

Cofactor content and O_2 tolerance of *Ht*SH

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 *Ht*SH 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 *Ht*SH 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 irons in *Ht*SH, 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 *Re*SH [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 *Ht*SH.

Consistent with the chemolithoautotrophic growth capacity of the host organism under aerobic conditions, the isolated *Ht*SH showed sustained H_2-driven NAD^+ reduction activity in the presence of O_2 (Table 3). However, its O_2 tolerance revealed to be lower than that of the *Re*SH (Table 3, Table 2). While the *Re*SH preserves approximately 100 % activity observed at 20 % O_2 (measured at 30°C in Tris/HCl buffer, pH 8) [14,15], the *H. thermoluteolus* enzyme showed at 10 % O_2 less than 20 % of the activity measured in the absence of O_2. At 2 % O_2, it displayed only 50 % of the activity observed under anaerobic conditions. However, at low O_2 pressure (0.2 %), *Ht*SH activity remained at almost 100 % (Table 3). In this respect, it is noteworthy that the intracellular O_2 concentration in living cells is generally

much lower than the external one. This explains why *H. thermoluteolus* cells grow well with H_2 and CO_2 even at ambient O_2 concentrations, although the isolated enzyme is more O_2 sensitive than the SH from *R. eutropha*.

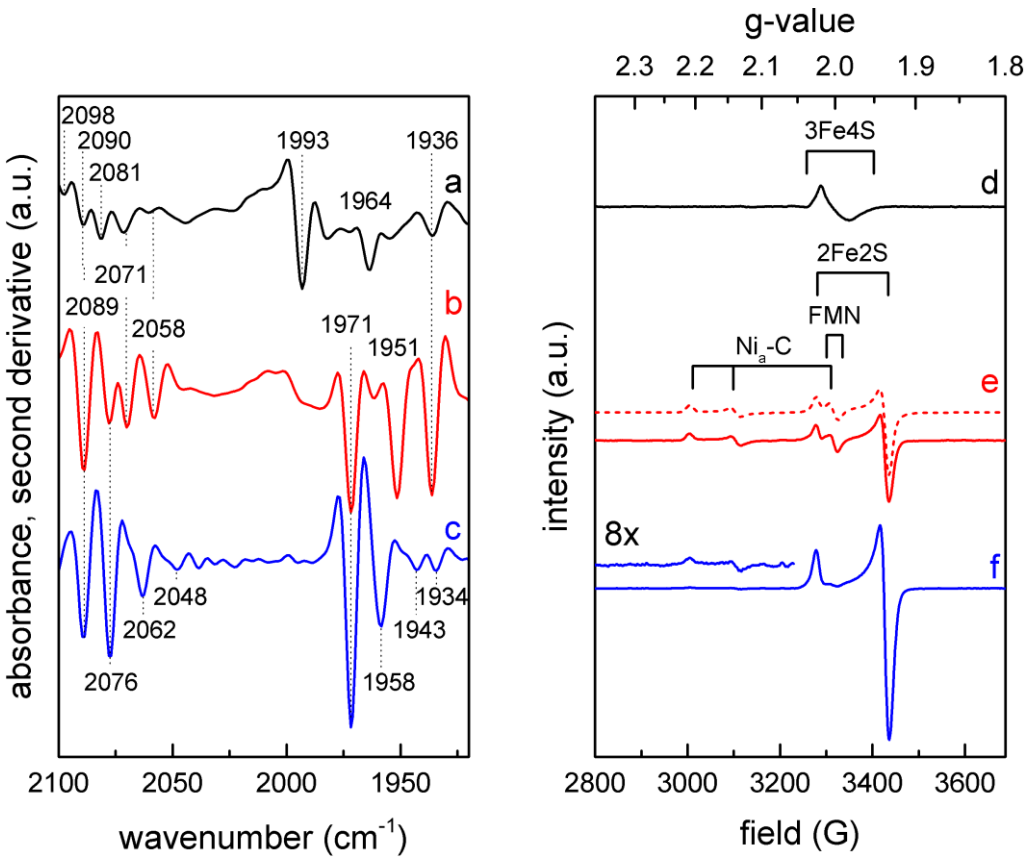
Table 3. H_2-driven NAD^+ reduction activity of the *Ht*SH protein[a] in the presence of various O_2 concentrations.

O_2 / H_2 / N_2 fractions[b] (% v/v)	$[O_2]$ (µM)	Hydrogenase activity in the presence of O_2 (U mg^{-1} of protein)[c]	k_{cat} (s^{-1})	Hydrogenase activity (%)
0 / 33.33 / 66.66	0.00	16 ± 2	45.9	100
0.2 / 33.33 / 66.46	1,9	15 ± 4	43.0	94.2
2 / 33.33 / 64.66	18,8	7.7 ± 0.3	21.5	49.8
10 / 33.33 / 56.66	94,0	1.3 ± 0.5	3.6	16.6

[a]*Ht*SH was purified by affinity chromatography as described in materials and methods. [b]For each O_2 concentration, a fixed volume of H_2-saturated buffer was mixed with various proportions of O_2- and N_2-saturated buffers. The gas phase contained the corresponding gas mixtures. [c]H_2-mediated NAD^+ reduction activity was measured at 50 °C and pH 6.5.

Spectroscopic characterization of *Ht*SH

To gain insight into structure and function of the metal cofactors, in particular of the [NiFe] active site, *Ht*SH 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 Table 4 and Table 5, respectively.



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308 **Fig. 6.** IR (left) and EPR (right) spectra of *Ht*SH recorded under different redox conditions. Samples
309 were prepared as described in materials and methods and measured in the as-isolated, oxidized state
310 (black spectra) or in their reduced states (red spectra: samples reduced with TCEP and NADH; blue
311 spectra: samples reduced with TCEP, NADH, and H_2). IR spectra were acquired at 10 °C, while EPR
312 spectra were recorded at either 10 K (d) or 35 K (e, f).
313

314 **Table 4.** CO and CN stretching frequencies (cm^{-1}) of IR-spectroscopically observed *Ht*SH [NiFe]
315 active site species.

Assignment	v(CO)	v(CN)	
n/a[a]	1993	2081	2090
Ni_r-B-like	1964	2087	2098
Ni_r-S	1936	2058	2071
Ni_a-S	1951	2076	2089
Ni_a-C	1971	2076	2089
Ni_a-SR	1958	2062	2076
Ni_a-SR'	1943	2048	2062
Ni_a-SR''	1934	2048	2062

[a]Not assigned. Oxidized active site species of unknown structure

316

317

Table 5. g-values of *Ht*SH cofactor species observed by EPR spectroscopy.

Assignment	g_1	g_2	g_3
[3Fe4S]	2.004	1.982	
[2Fe2S]	2.026	1.935	
[NiFe]: Ni_a-C	2.210	2.139	2.013
[NiFe]: n/a[a]	2.260	2.127	2.034
FMN	2.003		

[a]Not assigned.

IR spectra of as-isolated *Ht*SH 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 *Ht*SH 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 *Ht*SH 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 "Ni_r-B-like" state (Fig. 1), which was previously detected for *Re*SH and other $NAD(P)^+$-reducing [NiFe] hydrogenases [6,8,12,40,50,51], and the band at 1936 cm^{-1} is assigned to the Ni_r-S state (see below). The signal at 1993 cm^{-1}, however, is unprecedented and absent in as-isolated *Re*SH [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 *Ht*SH 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

O_2 during and after protein isolation [50,56]. Importantly, all IR-spectroscopically detected oxidized species of the *Ht*SH active site can be activated under reducing conditions (Fig. 6, traces b and c), as observed previously for, e.g., *Re*SH [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 *Ht*SH 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 *Ht*SH, 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 *Re*SH 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 *Ht*SH. 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 *Ht*SH, 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 Ni_a-C state of the enzyme, which is in line with previous studies showing that Ni_a-C 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 Ni_a-S state, consistent with an intensity decrease upon hydrogen incubation of the enzyme (see below and Fig. 6, trace c). In *Re*SH and soluble hydrogenase I (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 *Pf*SH1 differs from *Ht*SH and *Re*SH in terms of its subunit and cofactor composition [6]). The band at 1936 cm^{-1} gains intensity upon incubation of as-isolated *Ht*SH with TCEP and NADH (Fig. 6, traces a and b) indicating that it reflects a partially reduced [NiFe] species with a formal Ni^{II} oxidation state. Since this CO stretching frequency is clearly lower than those observed for most other *Ht*SH [NiFe] active site species, we tentatively assign this intermediate to the deprotonated Ni_r-S subspecies, which features a bridging OH^- ligand.

The corresponding EPR spectrum of TCEP/NADH-reduced *Ht*SH was recorded at 35 K and clearly shows the hydride-containing Ni_a-C state (Ni^{III}, $S = 1/2$), consistent with the corresponding assignment of the strong IR absorbance at 1971 cm^{-1}. Moreover, signals attributed to a [2Fe2S] cluster (constituent 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 *Re*SH and *Ro*SH [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 *Ht*SH with H_2 (in the presence of TCEP and NADH), the 1971 cm^{-1} band, assigned to the Ni_a-C 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 Ni_a-C state. Therefore, we attribute this signal to the fully reduced Ni_a-SR species with corresponding CN stretching bands at 2076 and 2062 cm^{-1}, which is in line with band assignments for *Pf*SH1 [51]. In case of *Re*SH, a similar set of

signals, including an identical CO stretching band at 1958 cm^{-1}, has been assigned to the Ni_a-SR2 state [6,12,50]. In the current case, however, this assignment is less plausible since CO stretching bands of *Ht*SH active site redox states appear to be generally higher in frequency than their counterparts in *Re*SH. Two further weak bands at 1943 and 1934 cm^{-1} (Fig. 6, trace c) might reflect Ni_a-SR' and Ni_a-SR'' subspecies of the reduced state [12,50]. Consistently, these states were observed as bands at 1940 (Ni_a-SR') and 1931 cm^{-1} (Ni_a-SR'') for *Pf*SH1, which also exhibits generally higher CO stretching frequencies than *Re*SH [51]. Observation of these two subspecies provides further support for the assignment of the 1958 cm^{-1} band to Ni_a-SR as there is no other signal in the IR spectrum of *Ht*SH that could be attributed to the main component of this species.

The EPR spectrum of H_2-incubated *Ht*SH, 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_a-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_a-C state, indicating strong magnetic coupling of the active site with another paramagnetic species. This temperature dependence of the Ni_a-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_a-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 *Pf*SH1 [39], *Pyrococcus furiosus* ferredoxin [65], and individual clusters of homologous respiratory Complex I [66–68]. For the Ni_a-C state of *Ht*SH, this effect appears to be most pronounced for the NADH/TCEP/H_2-treated sample. Assuming 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 *Ht*SH, initial IR spectro-electrochemical 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 *Ht*SH 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.

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 *Hydrogenophilus thermoluteolus* TH-1^T [21], and its corresponding structural genes were heterologously overexpressed in the mesophilic host *Ralstonia eutropha* H16. This procedure resulted in the formation of catalytically active *Ht*SH protein, which clearly shows that the hydrogenase-specific maturation machinery from *R. eutropha* [5] is capable of synthesizing and inserting the $NiFe(CN)_2CO$ cofactor into the large hydrogenase subunit of *Ht*SH. Taking into account the successful heterologous

overproduction of SH from *Rhodococcus opacus* [69], *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.

Table 2 shows biochemical and structural properties of the *Ht*SH in comparison with those of other soluble $NAD(P)^+$-reducing [NiFe]-hydrogenases. The isolated *Ht*SH 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 °C. In terms of biotechnologically relevant cofactor regeneration [19], the *Ht*SH is complementary to *Pf*SH1, which preferably reduces $NADP^+$ in a H_2-dependent manner at high temperature [33]. Although to a lesser extent when compared to *Re*SH, *Ht*SH shows catalytic H_2-mediated NAD^+ reduction in the presence of O_2 in solution assays. For *Pf*SH1, O_2-tolerant H_2 oxidation (but not $NAD(P)^+$ reduction) has so far only be shown electrochemically with immobilized enzyme [41]. Though phylogenetically closely related to *Ht*SH and *Re*SH, 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 *Re*SH, 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 *Ht*SH 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 *Ht*SH protein revealed the occurrence of FMN, [2Fe2S], 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_r-B-like state that is not directly involved in H_2/H_+ cycling as well as the Ni_a-S, Ni_a-C, and Ni_a-SR states which are generally accepted to be intermediates of the catalytic cycle. While the Ni_a-C state was identified both by IR and EPR spectroscopy, all other states are

464 EPR-silent and were assigned based on IR spectroscopic analyses only. Interestingly, the
465 Ni_a-C signal in the EPR spectrum of H_2-treated *Ht*SH was mainly observed at temperatures
466 below 10 K, presumably due to fast spin-lattice relaxation related to magnetic coupling with
467 another cofactor that is paramagnetic under these reducing conditions. This observation
468 represents an important finding that could explain why Ni_a-C and other paramagnetic active
469 site species have often not been observed for $NAD(P)^+$-reducing [NiFe] hydrogenases [6,40].
470 Furthermore, the as-isolated, oxidized *Ht*SH exhibits a CO stretching vibration at 1993 cm^{-1},
471 which is extremely high in frequency and so far unprecedented for [NiFe]-hydrogenases. This
472 unusual vibrational band most likely reflects an alternative geometry and/or coordination
473 environment of the hetero bimetallic active site. Since no crystallographic data is available
474 yet, further spectroscopic investigations are currently in progress to gain detailed insight into
475 the structure this novel species.
476

Materials and methods

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

The *Ht*SH-derived gene cluster containing *hoxFUYHW* was amplified by PCR using the primers

5’-agaacctgtacttccagggcgcaacacgaggaggaggaac-3’

and

5’-ctcggtacccggggatccatacctcctcttcgtgggtgaaaaaac-3’,

and genomic DNA from *Hydrogenophilus thermoluteolus* TH-1^T as the template. The underlined bases of the primers are complementary to plasmid pGE837, which is a pCM66 [70] derivative carrying a XbaI-BamHI-cut fragment from plasmid pGE770 [15] with P_{SH}-$_{Strep}$*hoxF* from *Ralstonia eutropha* H16 followed by a sequence encoding a GGGENLYFQG linker with a TEV cleavage site (underlined residues). Plasmid pGE837 was linearized by inverted PCR using primers 5’-atggatccccgggtaccga-3’ and 5’-gccctggaagtacaggttctcg-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 *hoxF* gene was equipped with a linker sequence and a *Strep*-tag II-encoding sequence. A P_{SH}-*hox*$_{Strep}$*FUHYW* fragment was cut out with Eco53KI and XbaI, and the resulting 5.7 kb fragment was inserted into the ScaI-XbaI-cut vector pEDY309 [72]. This yielded plasmid pJP09, which was subsequently transferred by conjugation to *R. eutropha* HF1054, which is a HF424 [73] derivative carrying an additional in-frame deletion in the *hoxI* gene.

Strain *R. eutropha* HF1054 (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, 5mM $MgCl_2$, 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 *Ht*SH 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 *Ht*SH protein with homogenous subunit stoichiometry, size exclusion chromatography was conducted after affinity chromatography. An amount of 200 µL of the concentrated *Ht*SH 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 *Ht*SH 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).

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 *Ht*SH 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 *Ht*SH 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_i 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 N_2 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)

552 was tested at 50 °C in buffers with different pH values (composition see above). Prior to use,
553 the buffers were saturated with H_2.
554 In order to determine affinity constants for NAD^+ or NADH, the initial reaction velocities for
555 H_2-dependent NAD^+ and NADH-dependent BV reduction, respectively, were measured at
556 50 °C and varying substrate concentrations. The recorded slopes were plotted against the
557 substrate concentration and fitted to the Michaelis-Menten kinetic using the program Origin
558 2016.
559 Determination of affinity towards H_2 was performed amperometrically by mixing different
560 volumes of H_2- and N_2-saturated buffers (50 mM bis-Tris, pH 6.5, 5 mM $MgCl_2$, 0.5 mM
561 $NiCl_2$) to a total volume of 1.3 mL in the reaction chamber of a modified Clark electrode. The
562 assay contained further the natural electron acceptor, NAD^+ (1 mM), in addition to 0.75 mM
563 TCEP, and 2 µM FMN. The reaction was started by enzyme addition, and the resulting
564 current change was recorded. The derived reaction velocities were plotted against the H_2
565 concentration and fitted to the Hill equation using Origin 2016.
566 **Protein, iron, and FMN determination.** The protein concentration was determined with the
567 BCA™ Protein Assay Reagent Kit (Pierce, USA) using bovine serum albumin as the
568 standard. The flavin mononucleotide concentration in protein samples was analyzed
569 fluorometrically as described previously [30,31] Iron and nickel contents of purified *Ht*SH
570 samples were analyzed by inductively coupled plasma optical emission spectroscopy (ICP-
571 OES) as previously described [76]. Final numbers were derived from two biological
572 replicates, while each sample was measured three times (three technical replicates).
573 **Sample preparation for IR and EPR spectroscopy**. For the characterization of as-isolated
574 *Ht*SH, protein fractions were concentrated to approx. 0.3 mM using Amicon Ultra 0.5 mL
575 Centrifugal Filters (Merck KGaA) and measured without further treatment. Samples of
576 reduced *Ht*SH 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 *Ht*SH 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 *Ht*SH, 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_2O 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.

IR spectroscopy. IR spectra of 0.3 mM solutions of as-isolated and chemically reduced *Ht*SH 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.

IR spectro-electrochemical experiments. IR spectro-electrochemical experiments were performed on ca. 0.3 mM solutions of *Ht*SH, 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), anthraquinone-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 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.

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).

NRVS spectroscopy. For nuclear resonance vibrational spectroscopy (NRVS), *R. eutropha* HF1054 (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 *Ht*SH was purified

via *Strep*-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 ^{57}Fe partial vibrational density of states (PVDOS) by the PHOENIX software [80], while the energy scale was calibrated with an external reference ($[NEt_4][FeCl_4]$). For the *Ht*SH protein sample (22 µl, 0.8 mM), the accumulation time was 21 h.

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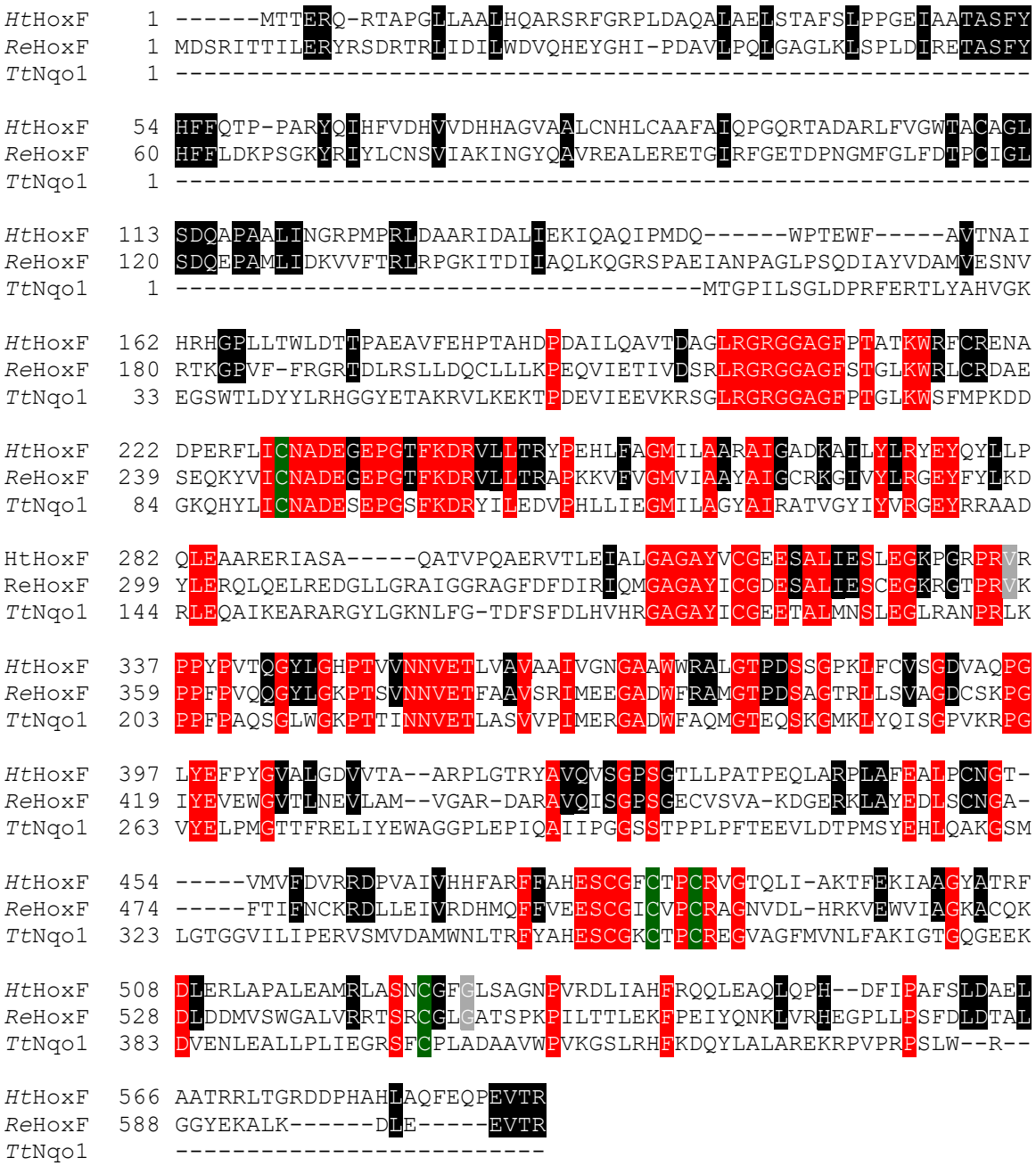
Supplementary Information

(supplementary figures S1-10)



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TtNQO2     1 MGFFDDKQDFLEETFAKYPPEGRRA--AIMPLLRRVQQEEGWIRPE-RIEEIARLVGTTP

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ReHoxF   169 AYVDAM
TtNQO2   177 HEVEV


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ReHoxH  216 YDFHQKHRAQVDSEADVPALSMCLVGDDDNVDYYHGRLRIIDDDKH-IVREFDYHDYLDH
TtNqo4  205 EALFAESPIFYERARGVGVIPPE---VAIDLGLTGGSLRASGVNYD-VRKAYPYSGYETY

HtHoxH  283 LVEAVRPWSYMKFPYLKAYGEPDGFYRVGPSARLINCDRLTTARAEAARQRFLTFDQGTV
ReHoxH  275 FSEAVEEWSYMKFPYLKELGREQGSVRVGPLGRMNVTKSLPTPLAQEALERFHAYTKGRT
TtNqo4  259 TFDVPLGERGDVFDRM-----------------------------------------------

HtHoxH  343 AHSTLGYHWARLIEMLHCAELIEALLTD---ADLEGGELRAR---GQRQHRGVG------
ReHoxH  335 NNMTLHTNWARAIEILHAAEVVKELLHD---PDLQKDQLVLTPPPNAWTGEGVG------
TtNqo4  277 --------LVRIREMRESVKIIKQALERLEPGPVRDPNPQITPPPRHLLETSMEAVIYHF

HtHoxH  391 ----------------VIEAPRGTLIHHYEVGDDDLITYCNLIVSTTHNNAVMNQAVTTA
ReHoxH  386 ----------------VVEAPRGTLLHHYRADERGNITFANLVVATTQNNQVMNRTVRSV
TtNqo4  329 KHYTEGFHPPKGEVYVPTESARGE-LGYYIVSDGGSMPYRVKVRAP--------------

HtHoxH  435 AKAFLSGV-TLTEALLNHIEVAVRAFDPCLSCATHALGQMPLVVSLHHKDVPTPIDMLVR
ReHoxH  430 AEDYLGGHGEITEGMMNAIEVGIRAYDPCLSCATHALGQMPLVVSVFDAAGRL-IDERAR
TtNqo4  374 --SFVNLQ-------------------SLPYACKGEQVPDMVAIIASLDPVMGDVD-----

HtHoxH  494 HSDGTIERPTAAPALGTKGT
ReHoxH  489 --------------------
TtNqo4  404 --------------------
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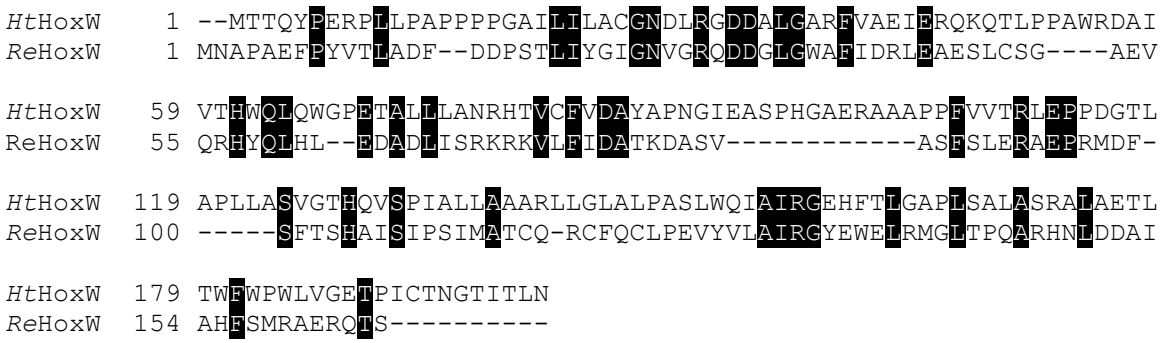

Fig. S1. Alignments of the *Ht*SH subunits, HoxF, U, Y, and H, with those of *Re*SH, and the corresponding Nqo1/2, 3, 6, and 4 subunits of Complex I from *Thermus thermophilus* (*Tt*). Also given is the alignment of the SH-specific endopedidases (HoxW) of *Ht* and *Re*. Amino acid residues that are conserved in all three species are highlighted in red, those conserved only among *Ht* and *Re* are boxed in black. Amino acid residues supposed to be involved in the coordination of Fe-S clusters are indicated in green, those involved in the coordination of the NiFe catalytic center are shown in blue. HoxF represents a fusion protein of Nqo2 and Nqo1 from *Tt*. Therefore, two separate alignments were made. One shows the alignment of the complete *Ht* and *Re* HoxF proteins with NqoI (revealing similarities in a large C-terminal region), and the other one aligns the N termini of the two HoxF proteins with Nqo2. The Nqo3 subunit of *Thermus thermophilus* consists of 783 amino acid residues. As the HoxU subunits of *Ht* and *Re* represent a truncated form of Nqo3, only the first 281 amino acid residues of Nqo3 were used for the alignment.

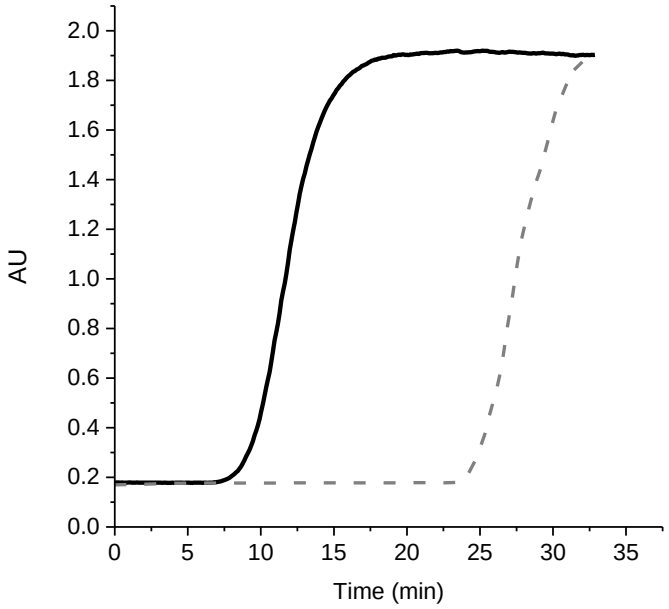

Fig. S2. Kinetics of H_2-dependent NAD^+-reduction (given in absorption units, AU, at 365 nm) catalyzed by *Ht*SH with (solid line) and without (dashed line) the addition of FMN (2 µM). The assay was performed with 0.5 µM *Ht*SH at 50 °C in 50 mM bis-Tris, pH 6.5, supplemented with 1 mM NAD^+, 0.5 mM $NiCl_2$, 5 mM $MgSO_4$, and 0.75 mM TCEP.

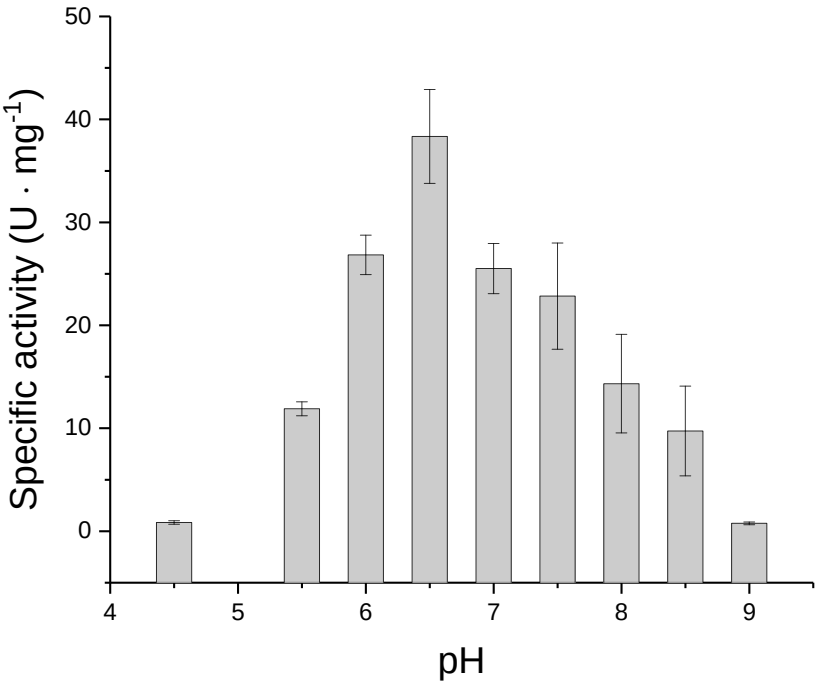

Fig. S3. pH dependence of the H_2-dependent NAD^+ reduction activity of purified *Ht*SH protein on different pH values. The assay contained 60 nM *Ht*SH in one of the following buffers: pH 4.5–7.0, 50 mM citrate; pH 7.5–8.0, 50 mM Tris/HCl; pH 8.5–9.0, 50 mM glycine. Activity was measured in the presence of 1 mM NAD^+, 0.5 mM $NiCl_2$, 5 mM $MgSO_4$, 2 µM FMN, and 0.75 mM TCEP at a temperature of 50 °C.

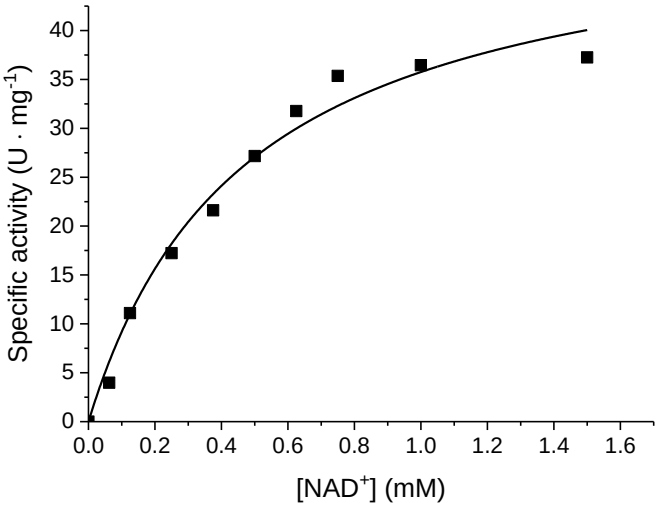

Fig. S4. Determination of the K_M^{NAD+} by measuring *Ht*SH-mediated H_2 oxidation at different NAD^+ concentrations. Activity was measured at a temperature of 50 °C in 50 mM H_2-saturated bis-Tris buffer, pH 6.5, 0.125-1.5 mM NAD^+, 0.5 mM $NiCl_2$, 5 mM $MgSO_4$, 2 µM FMN, and 0.75 mM TCEP. The depicted values (squares) represent the means derived from at least two measurements of one protein preparation. The Michaelis-Menten constant and the corresponding v_{max} value were calculated by non-linear regression. From three biological replicates, a K_M of 469 µM with a coefficient of variation (CV) of 9.8 % was derived. The v_{max} value was 52.7 U mg^{-1}, with a CV of 3.4 %, resulting in a turnover frequency (k_{cat}) of (155 ± 5) s^{-1} (assuming a molecular weight of 167.8 kDa for the heteroterameric *Ht*SH).

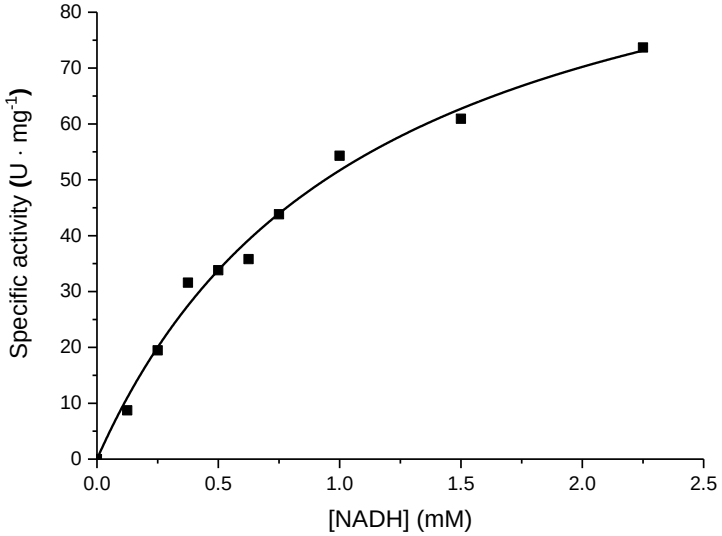

Fig. S5. Determination of the K_M^{NADH} by measuring the *Ht*SH-mediated benzyl viologen reduction activity (squares) at different NADH concentrations. Activity was measured at a temperature of 50 °C in 50 mM bis-Tris buffer, pH 6.5, containing 5 mM benzyl viologen, 0.125–2.25 mM NADH, 0.5 mM $NiCl_2$, 5 mM $MgSO_4$, 2 µM FMN, and 0.75 mM TCEP. The Michaelis-Menten constant and the corresponding v_{max} value were calculated by non-linear regression. From two biological replicates, a K_M of 1.17 mM with a CV of 4.8% was derived. The v_{max} value was 64.9 U mg^{-1}, with a CV of 3.5 %, resulting in a turnover frequency (k_{cat}) of (179 ± 6) s^{-1} (assuming a molecular weight of 167.8 kDa for the heteroterameric *Ht*SH).

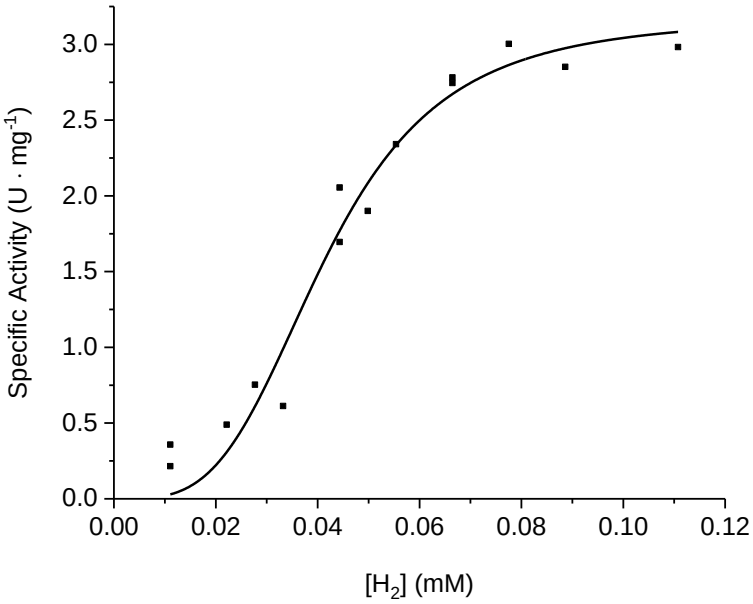

Fig. S6. Determination of the K_M^{app} for H_2 by measuring *Ht*SH-mediated NAD^+ reduction (squares) at different H_2 concentrations. Activity was measured at a temperature of 50 °C in 50 mM bis-Tris buffer, pH 6.5, containing 1 mM NAD^+, 0.5 mM $NiCl_2$, 5 mM $MgSO_4$, 2 µM FMN, and 0.75 mM TCEP. The resulting data were not compatible with a classical Michaelis-Menten fit. Therefore, the K_M^{app} for H_2 (ligand concentration, at which half the ligand-binding sites are occupied) was calculated by non-linear regression (R^2 = 0.959) and revealed to be 41.6 ± 2.5 µM with a Hill coefficient of 2.87 ± 0.8. The origin of the apparent cooperativity is unclear.

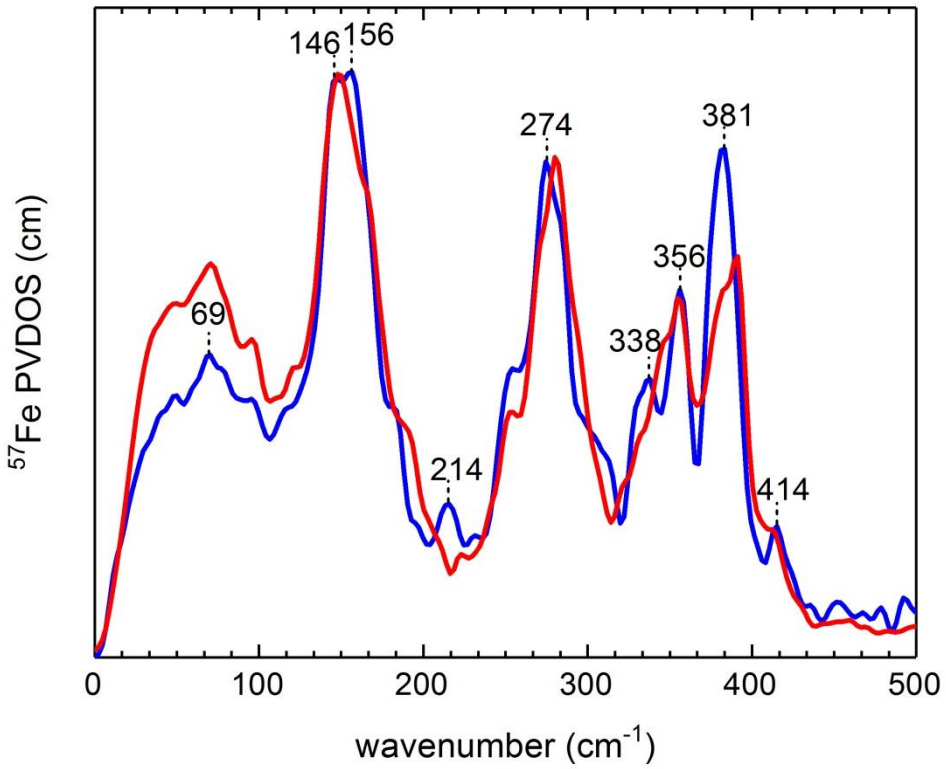

Fig. S7. NRVS-derived ^{57}Fe PVDOS in the spectral region reflecting Fe-S cluster modes. The spectra of as-isolated, oxidized *Ht*SH and *Re*SH (the latter was taken from reference [43]) are shown as blue and red traces, respectively.

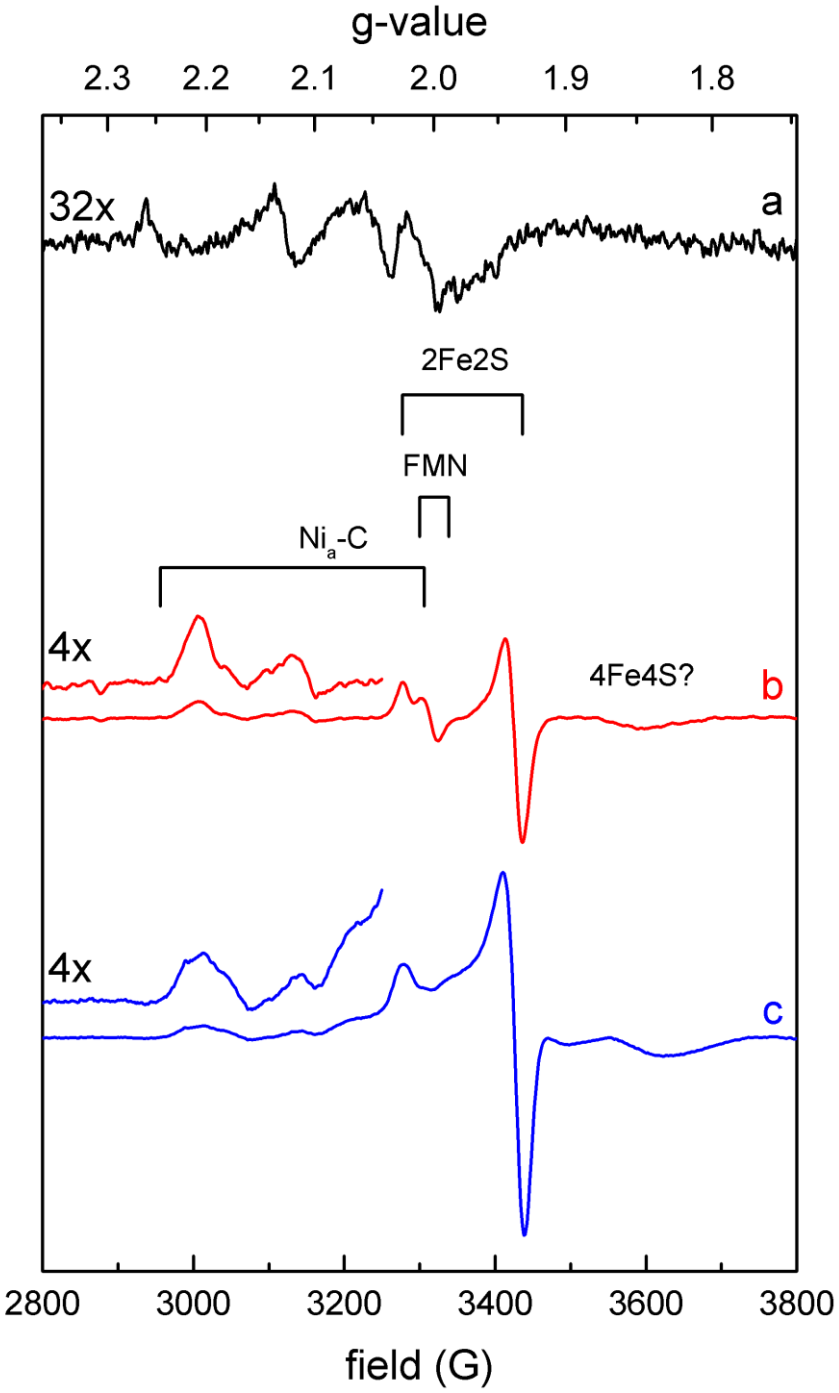

Fig. S8. EPR spectra of (a) as-isolated, (b) TCEP/NADH-reduced and (c) TCEP/NADH/H_2-reduced *Ht*SH. Spectra were recorded at 35 K (a), 10 K (b), and 6.5 K (c).

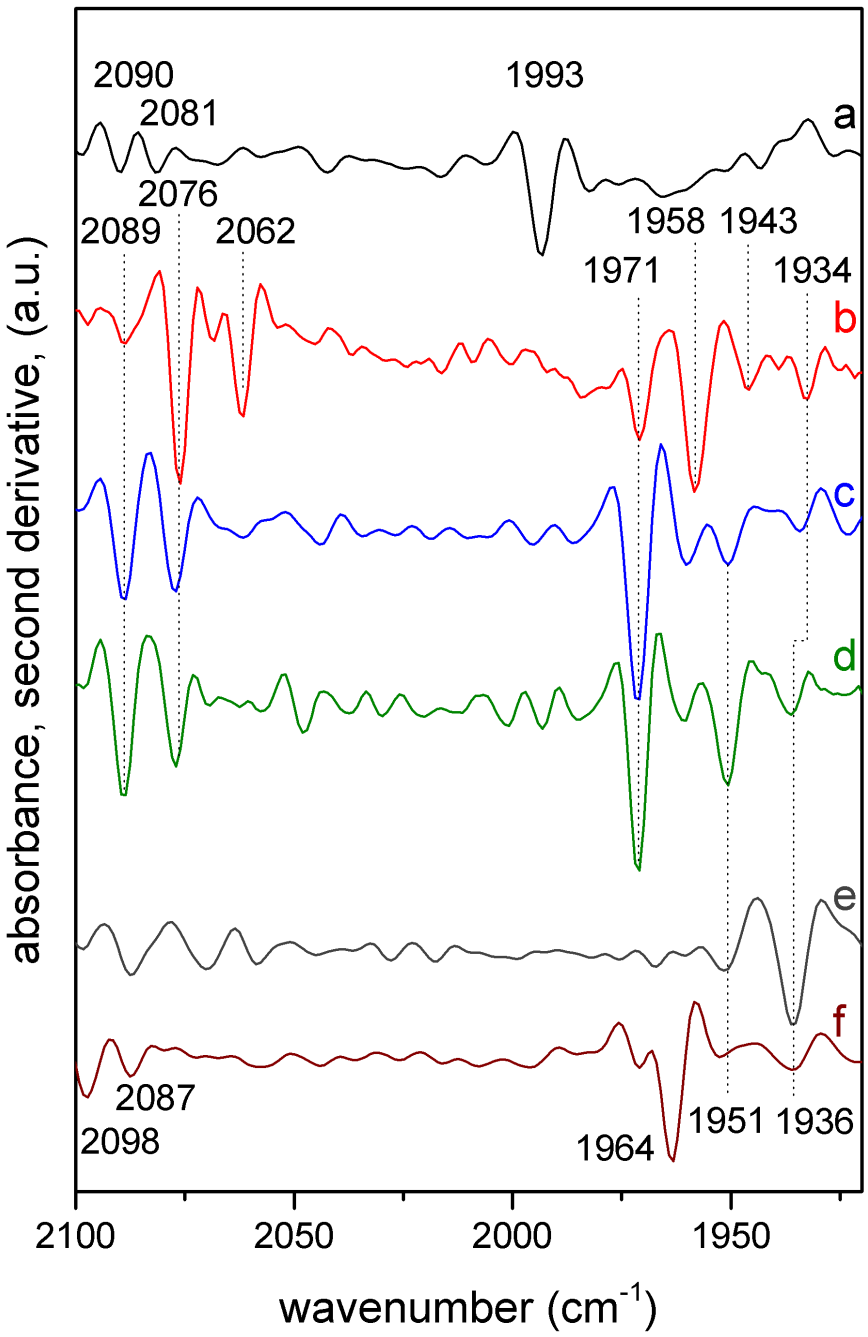

Fig. S9. IR spectra of *Ht*SH from spectro-electrochemical measurements. Spectra were recorded at a) open circuit potential (+270 mV), b) –440 mV, c) –350 mV, d) –310 mV, e) –190 mV, and f) +460 mV vs. SHE. The spectro-electrochemical data essentially confirm the band assignments made above, and the enrichment of Ni_r-S is found to require higher potentials than the formation of Ni_a-S (traces d and e). Moreover, CN stretching frequencies of Ni_a-S, Ni_r-B-like, and the 1993 cm^{-1} species can be tentatively assigned to signals at 2089/2076, 2098/2087, and 2090/2081 cm^{-1}, respectively (traces a, d, and f). While CN stretching bands of Ni_r-S cannot be clearly identified in the spectro-electrochemical data (trace e), the entirety of all previous assignments leaves the two bands at 2071 and 2058 cm^{-1} (Fig. 6, traces a and b) as the most plausible candidates.

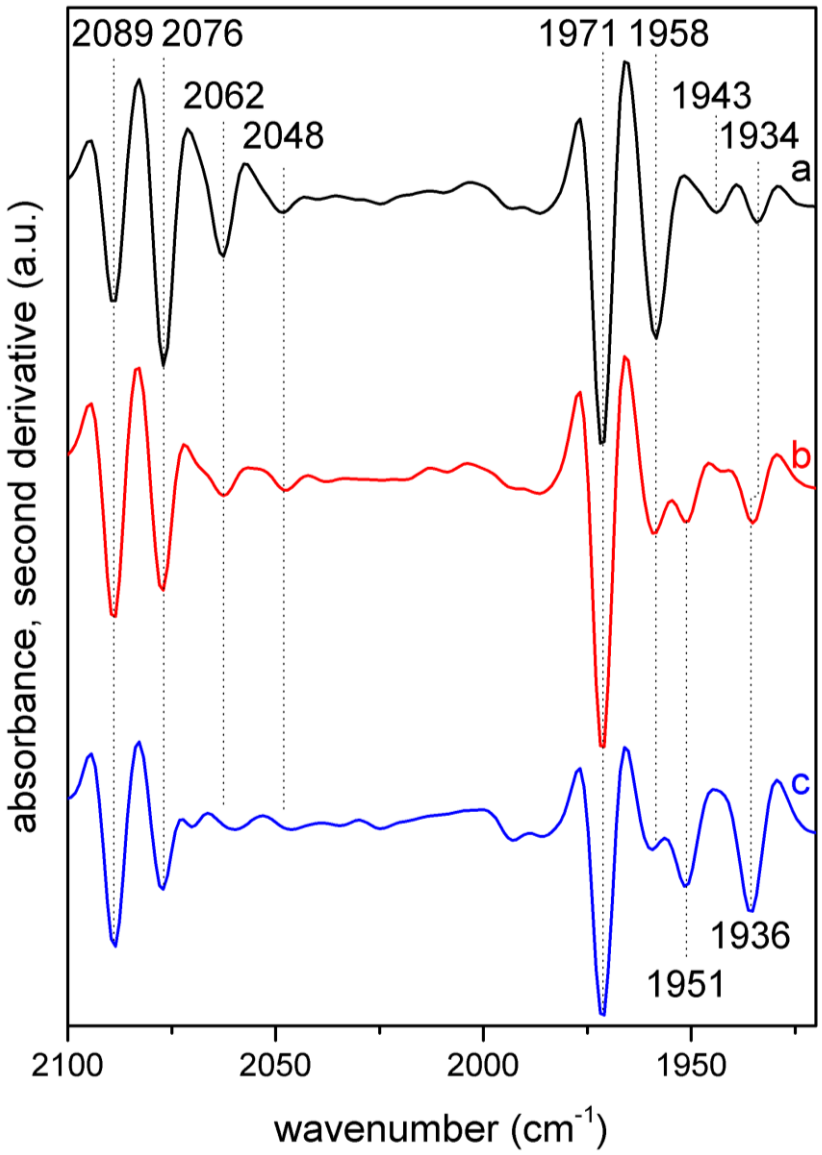

Fig. S10. IR spectra of TCEP/NADH/H_2-treated *Ht*SH, recorded at 10 °C after slow re-oxidation of the enzyme, achieved by diffusion of air for 0.5 h (a), 5 h (b), and 8 h (c) into the IR cell. Trace a represents the spectrum of *Ht*SH recorded directly after hydrogen incubation (analogous to Fig. 6, trace c). After approximately 5 h, intensities of the bands at 1934, 1943, and especially 1958 cm^{-1} decreased in favor of the signals at 1971 and 1951 cm^{-1} (trace b), which indicates conversion of the fully reduced states to Ni_a-C (and Ni_a-S). Consistently, the intensity of CN stretching bands assigned to Ni_a-SR (2076 and 2062 cm^{-1}) decreased as well, revealing a faint doublet at ca. 2062 and 2048 cm^{-1}, which may reflect residual amounts of the Ni_a-SR' and/or Ni_a-SR'' subspecies [12,50]. Upon further re-oxidation (8 h of air diffusion into the IR cell, trace c), the band at 1936 cm^{-1} re-appeared, and the signal at 1951 cm^{-1} increased in intensity. The obtained spectrum resembles the one recorded from the TCEP/NADH-reduced sample, reflecting conversion of Ni_a-C into Ni_r-S and Ni_a-S (Fig. 6 trace c). Thus, findings from slow re-oxidation experiments support the IR assignment of typical [NiFe] species detected for *Ht*SH and their interrelations.