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A conserved glutamate orchestrates transitions between catalytic intermediates in [NiFe]-hydrogenase

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[NiFe]-hydrogenases catalyze the reversible cleavage of molecular hydrogen with exceptional efficiency under mild conditions and, therefore, serve as powerful blueprints for the development of sustainable, bioinspired H_2-evolving catalysts. While the structure of the $NiFe(CN)_2CO$ active site has been extensively characterized, how outer-sphere residues regulate catalytic dynamics and proton-coupled electron transfer remains poorly understood. Here, we examine the functional role of a strictly conserved glutamate in the second coordination sphere of the regulatory [NiFe]-hydrogenase from *Cupriavidus necator*. Substitution of this glutamate with glutamine results in a dramatic loss (>99%) of catalytic activity. However, comprehensive IR, EPR, and resonance Raman spectroscopic analyses reveal that the residue is not required for the formation or stabilization of the key catalytic intermediates along the Ni_a–S → Ni_a–SR → Ni_a–C → Ni_a–L1 sequence. Notably, low-temperature IR spectroscopy shows that the transition from Ni_a–L1 to Ni_a–L2 is selectively disrupted in the absence of the conserved glutamate. These results identify the Ni_a–L2 state as a *bona fide* catalytic intermediate and demonstrate that the glutamate residue initiates critical outer-sphere rearrangements required to advance the catalytic cycle and enable productive proton transfer. Together, these findings elucidate how the protein matrix actively controls active-site reactivity in [NiFe]-hydrogenases and highlight the importance of second-sphere interactions in tuning catalytic efficiency. This work provides mechanistic principles that are directly relevant to the rational design of synthetic and biomimetic hydrogen-evolving catalysts for sustainable energy conversion.

Broader context

Hydrogen is considered as a key energy carrier for a low-carbon future, but its large-scale production and use require efficient, sustainable, and affordable catalysts. In nature, hydrogenases catalyze the reversible hydrogen conversion with exceptionally high rates and minimal overpotential using only earth-abundant metals, making them powerful sources of inspiration for clean energy technologies. However, translating these biological principles into usable catalysts has been hindered by an incomplete understanding of how enzymes control chemical reactivity beyond the metal active site. This work addresses a key challenge in enzyme-mediated catalysis: how does the surrounding protein matrix enable efficient hydrogen conversion. By studying a hydrogen-processing model enzyme, we demonstrate how a conserved amino acid near the active site nickel ion, which was already known to be essential for activity, controls catalysis by directing subtle interactions between the second coordination sphere and the metal active site. These findings underscore the importance of the protein environment in modulating the reactivity of transition-metal-based biocatalysts and offer mechanistic guidance for the rational design of synthetic and biomimetic catalysts. More broadly, the principles identified here may extend to other energy-converting enzymes that operate through proton-coupled electron transfer, thereby providing guidance for the development of next-generation (bio)catalysts for the sustainable production of fuels and chemicals.

1. Introduction

Hydrogenases use earth-abundant transition metals for fast, efficient, and reversible H_2 cleavage, with almost no overpotential.[1] In the subgroup of [NiFe]-hydrogenases, catalysis takes place at a heterobimetallic [NiFe] cofactor located deep

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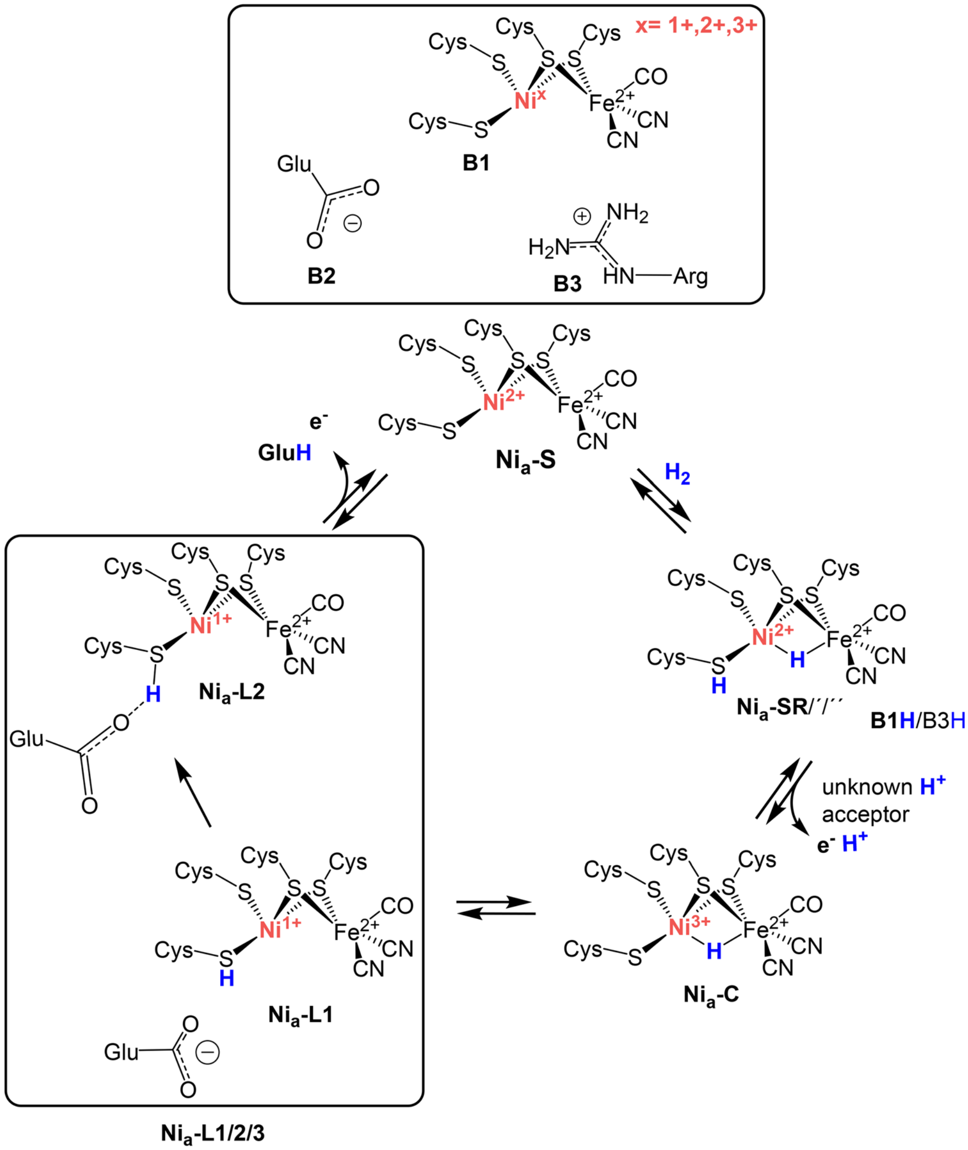

Fig. 1 Proposed catalytic cycle of [NiFe]-hydrogenase. Schematic representation of the active site of [NiFe]-hydrogenases (top) and the proposed catalytic cycle comprising the four key intermediates Ni$_a$–S, Ni$_a$–SR, Ni$_a$–C, and Ni$_a$–L. Throughout the cycle, only nickel changes its oxidation state (1+, 2+, 3+), while iron remains in the low-spin Fe^{2+} state. The Ni$_a$–SR and Ni$_a$–L states each consist of at least three sub-forms. The proposed proton acceptors in the vicinity of the [NiFe] site are a Ni-bound terminal cysteine (Cys479 in RH, B1), a nearby glutamate (Glu13 in RH, B2), and an arginine (Arg411 in RH, B3), which are displayed in the top panel in their protonated/deprotonated forms according to the physiological conditions. A recent study has elucidated the structural details of the Ni$_a$–L1 and Ni$_a$–L2 species, both of which feature a proton covalently bound to a terminal nickel-coordinating cysteine residue (Cys479 in, RH).[29] Ni$_a$–L2 is further stabilized by a hydrogen bond between the protonated cysteine (Cys479 in RH) thiolate and a nearby deprotonated glutamate (Glu13 in RH). The catalytic relevance of the Ni$_a$–L1 and Ni$_a$–L2 intermediates is discussed in detail in this manuscript.

within the large subunit of the heterodimeric functional unit of the enzyme.[2] The bimetallic center is coordinated by four highly conserved cysteine residues—two bind exclusively to the nickel ion and two act as bridging ligands between the nickel and iron ions.[3,4] The Fe is additionally coordinated by one carbon monoxide (CO) and two cyanide (CN^-) strong-field ligands (Fig. 1). An open coordination site is typically present between the two metals, which may be either empty or occupied by *e.g.*, hydroxide or hydride ligands.[5,6] The electrons released from the splitting of H_2 are conducted to the physiological redox partners *via* an array of closely spaced [Fe–S] clusters located in the hydrogenase small subunit – in some cases additional prosthetic groups such as FAD (flavin adenine dinucleotide) or FMN (flavin mononucleotide) are involved in electron transfer.[7,8] [NiFe]-hydrogenases exhibit remarkable catalytic diversity (in terms of rates and bias), which is essentially determined by the protein scaffold, which, in addition to the electron relay, also provides gas channels for H_2 to access/leave the active site as well as a dedicated pathway consisting of amino acids and water molecules to efficiently transport protons.[9–11] Decades of research by various groups contributed to a consensus mechanism of reversible H_2 cleavage (Fig. 1).[12–14] In brief, the catalytic cycle is presumably initiated by the binding of H_2 to the Ni_a–S intermediate (Ni^{2+}–[ ]–Fe^{2+}),[15–17] which is followed by the cleavage of the H_2 molecule, yielding the bridging hydride species of the Ni_a–SR (Ni^{2+}–H–Fe^{2+}) intermediate.[18] Subsequent removal of one electron and one proton results in the formation of the Ni_a–C state (Ni^{3+}–H–Fe^{2+}).[19–21] Ni_a–C then tautomerizes to the Ni_a–L form through proton translocation from the bridging hydride to one of the terminal cysteine residues, accompanied by reduction of the nickel center to the formal Ni^{1+} state.[17,22,23] Finally, the removal of the cysteine-bound proton and an electron from the nickel ion regenerates the original Ni_a–S intermediate, completing the catalytic cycle.[24,25]

Among the various intermediates, both Ni_a–SR and Ni_a–L exist as multiple isoelectronic subforms (Fig. 1).[12,13] Although some of these species are thought to be involved in H_2 activation, their exact structure and individual roles in catalysis remain experimentally unclear. The O_2-tolerant regulatory [NiFe]-hydrogenase from *Cupriavidus necator* (CnRH) has already been employed in several studies as a model system for probing the electronic and molecular structures of various catalytic intermediates.[20,21,26–28]

Using IR difference spectroscopy at low temperature, we have recently identified key structural features of two distinct Ni_a–L subforms.[29] The results indicated that Ni_a–C converts to a metastable Ni_a–L1 intermediate (observable at $T < 130$ K) prior to the formation of Ni_a–L2, with both subforms containing a proton covalently bound to the terminal cysteine coordinated to Ni (Cys479 in RH, Fig. 1). IR difference spectroscopy also revealed structural rearrangements in the vicinity of the active site, including a hydrogen bond between Glu13 and the protonated Cys479 in Ni_a–L2, supporting a role for Glu13 in proton transfer (PT). Although prior studies have underscored the importance of this conserved glutamate,[30–32] the conformational/structural dynamics of the residues surrounding the [NiFe] center remain poorly understood. To assess whether the recently reported outer-sphere spectral features of RH are intrinsically linked to the Ni_a–C → Ni_a–L1 → Ni_a–L2 progression within the catalytic cycle, we substituted Glu13 with glutamine, a structurally conservative yet non-protonatable analogue. Using a combination of low-temperature IR, resonance Raman, and electron paramagnetic resonance (EPR) spectroscopy, we demonstrate that Glu13—strictly conserved across [NiFe]-hydrogenases—is essential for enabling the Ni_a–L1 → Ni_a–L2 transition. Beyond confirming the involvement of this conserved glutamate in the proton-transfer network, consistent with observations in other hydrogenase classes, our results reveal how second-sphere residues actively modulate active-site reactivity by driving critical outer-sphere protein rearrangements that promote catalytic turnover.

2. Experimental section

2.1. Construction of the recombinant *C. necator* strain overproducing RH^{E13Q}

Strain *C. necator* HF574(pGE567) was used for overproduction and purification of "native" RH, which is a variant of the original RH consisting of two copies of the HoxB and HoxC subunits ($HoxB_2C_2$). Truncation of a C-terminal peptide of HoxB and insertion of a Strep-Tag II peptide in its place prevents dimerization and interaction with cognate histidine protein kinase.[33] The Strep-Tag II allows easy purification of the catalytically active HoxBC protein (hereinafter referred to as native RH). The E13Q amino acid exchange in HoxC was created by site-directed mutagenesis and Gibson assembly. The mutation was introduced into plasmid pCH1124 (SI, Table S1), carrying the synthetic operon P_{SH}-*hoxB*$_{stop}$-Strep-Tag II-*hoxC*. The plasmid was amplified as two overlapping fragments using the primers 2_#60_fw and 4_#59_rev, carrying the alternative codon (Table S1), together with the primers SFP95 and SFP96. After the Gibson assembly reaction, the resulting plasmid was checked by sequencing, digested with HindIII and SpeI and the hydrogenase-encoding fragment was ligated into the HindIII/SpeI-cut broad-host-range vector pEDY309.[34] The resulting plasmid pJS98 was transferred by conjugation from *E. coli* S17-1[35] to *C. necator* strain HF574,[36] yielding HF574(pJS98).

2.2. Strain cultivation

Recombinant *C. necator* strains carrying plasmids for overproduction of native RH and its E13Q variant (RH^{E13Q}) were cultivated in a basic mineral medium containing fructose and glycerol as the carbon and energy sources.[37] When the bacterial cultures reached an optical density at 436 nm of 11–13, the cells were harvested by centrifugation (11 500 × *g*, 4 °C, 15 min), and the cell pellet was flash frozen in liquid nitrogen and stored at −80 °C until further use.

2.3. Proteins purification

Native RH and RH^{E13Q} were purified according to the following procedure. Cell pellets of the recombinant strains were resuspended in lysis buffer (5 mL of buffer consisting of 50 mM Tris-HCl, pH 8.0 (at 4 °C), 150 mM NaCl, protease inhibitor cocktail (complete EDTA-free, Roche) and DNase I (Roche) per gram of wet cell paste). The cells were subsequently disrupted at 125 MPa in a French pressure cell (G. Heinemann Ultraschall and Labortechnik, Schwäbisch Gmünd, Germany). Crude extracts were ultracentrifuged for 40 min at 100 000 × *g* and 4 °C, and the resulting soluble extract was loaded onto a Strep-Tactin® high-capacity column (IBA, Göttingen, Germany). The column was washed with ten bed volumes of washing buffer (50 mM Tris-HCl, pH 8.0 (at 4 °C), 150 mM NaCl), and the proteins were eluted with 4 bed volumes of washing buffer containing 3 mM D-desthiobiotin. The elution fraction was concentrated by ultrafiltration (4000 × *g*, 4 °C) using Amicon Ultracel concentrators (Millipore) with a 30 kDa cut-off. The resulting concentrate was diluted 20-fold with washing buffer and again re-concentrated by ultrafiltration. The final protein concentrate was flash-frozen and stored in liquid nitrogen until further use. The protein concentration was determined using a Pierce BCA Protein Assay kit (Thermo Scientific) using bovine serum albumin (BSA) as standard. The purity of the RH^{E13Q} variant was assessed by SDS-PAGE (Fig. S1 in SI).

2.4. Hydrogenase activity assay

H_2-mediated reduction of methylene blue (MB, $\varepsilon(MB)_{570nm}$ = 13.1 mM^{-1} cm^{-1}) by RH was investigated spectrophotometrically using a UV-vis spectrophotometer (Cary 50, Varian, Agilent, Santa Clara, California) as previously described.[16] The 2.0-mL cuvette contained a buffer mixture of 50 mM K_2HPO_4, 100 mM citric acid (pH 7.0) and 0.2 mM MB. The solution was saturated with H_2 gas and then enzyme samples were injected using a gas-tight Hamilton syringe. Measurements were performed with three biological replicates. Activities are reported as U mg^{-1} where 1 U corresponds to 1 μmol of H_2 oxidized per minute.

2.5. IR spectroscopy

For IR measurements at 298 and 90 K, the RH samples were prepared in 50 mM Tris-HCl (pH 8.0 at 277 K) buffer containing 150 mM NaCl and 25% glycerol and concentrated to *ca.* 1.2 mM. The glycerol ensures the formation of a transparent glass in the frozen state. The sample was subsequently reduced by exposure to humidified 100% H_2 or D_2 gas in an anaerobic chamber operating with forming gas (95% N_2 : 5% H_2). The samples were transferred into a gas-tight microcuvette for cryogenic measurements consisting of two CaF_2 windows with an optical path length of 4 μm. The cell was then transferred into a homemade cryostat cooled with liquid-nitrogen, which was mounted in the sample chamber of a Tensor 27 FTIR spectrometer (Bruker), equipped with a liquid-nitrogen cooled mercury cadmium telluride (MCT) detector. The cell compartment was purged with dried air. Data acquisition, spectral analysis, and Gaussian fitting of the CO/CN bands were performed using Bruker OPUS version 7.8. Spectra with a resolution of 2 cm^{-1} were recorded by averaging 200 scans. Absorbance spectra were calculated from averaged single channel spectra of the sample using the correspoding buffer spectrum as reference. Light-minus-dark IR difference spectra were calculated accordingly using the corresponding dark single spectra as reference.[29] The Ni_a–C → Ni_a–L transformation can be induced over a broad temperature range, from 90 K (the lower limit of our liquid N_2 cryostat) up to approximately 160–180 K. Above this range, thermal back-conversion to the initial Ni_a–C state(s) usually takes place. Notably, the Ni_a–L1 → Ni_a–L2 transition is extremely slow below 130 K, while at temperatures between 160 and 180 K it proceeds at rates comparable to the light-induced Ni_a–C → Ni_a–L1 reaction. This results in an apparent direct conversion of Ni_a–C to Ni_a–L2, when illuminating at these temperatures. Therefore, careful adjustment of the experimentally accessible temperature window is essential to selectively resolve the individual active site species.

2.6. EPR spectroscopy

RH^{E13Q} samples with a concentration range of 0.2–0.3 mM and a volume of 100 μL were transferred into quartz EPR tubes (4 mm diameter), frozen in cold ethanol (193 K), and stored in liquid nitrogen for further analysis. EPR samples were illuminated during the experiments using the focused light of a collimated 455 nm LED. A Bruker EMXplus spectrometer combined with an ER 4122SHQE resonator, an Oxford EPR 900 helium flow cryostat, and an Oxford ITC4 temperature controller was used for the EPR experiments. Baseline correction of the experimental spectra was done by subtracting a spectrum of buffer solution measured with the same experimental parameters. Broad background fluctuations were additionally corrected by using a polynomial or spline function. If not otherwise noted, the following experimental parameters were used: 1 mW microwave power, 9.29 GHz microwave frequency, 10 G modulation amplitude, and 100 kHz modulation frequency.

2.7. Resonance Raman spectroscopy

For resonance Raman (RR) spectroscopic investigations, the 568 line of a Kr^+ gas laser (Coherent) was used. Spectra were recorded at 80 K, using a liquid-nitrogen cooled cryostat (Linkam Scientific Instruments), in back-scattering geometry utilizing a confocal setup (Horiba Scientific LabRam). The laser beam was focused by a Nikon 20× objective to the surface of the sample resulting in a spot size of $<$10 μm. Data acquisition was accomplished with a Peltier-cooled CCD array (213 K, Oxford Instruments Andor). The laser power at the sample was adjusted to 1.5 mW using a neutral density glass filter (Schott AG). All samples were directly transferred from liquid nitrogen to the sample chamber (Linkam THMS600 freezing microscope stage) with minimal exposure to air. Individual spectra were recorded for 180 s, and repetitive scans were accumulated to accomplish overall acquisition times of 1.5–3 h per measurement spot, depending on the quality of the corresponding spectra. To calibrate the probe frequency axis, spectra of toluene and acetonitrile (external standard, marker bands at 522 cm^{-1} and 2254 cm^{-1}) were recorded before or after individual sample measurements. For each solution sample, at least two spots on different drops were measured and evaluated separately. Data evaluation of the RR spectra was done using the Bruker OPUS software version 6.5 or higher.

3. Results and discussion

3.1. Biochemical and basic spectroscopic analysis of RH^{E13Q}

The glutamate-to-glutamine exchange variant of the regulatory [NiFe]-hydrogenase from *C. necator* (RH^{E13Q}) was generated by site-directed mutagenesis (Table S1, methods section) and purified as described previously (Fig. S1).[37] The purified protein showed an H_2 oxidation activity of 0.004 ± 0.001 μmol H_2 mg^{-1} min^{-1}, which corresponds to *ca.* 0.1% of the value of native RH of *C. necator*. The dramatic effect of the Glu-to-Gln exchange on catalysis is consistent with previous studies on the prototypical [NiFe]-hydrogenase from *Solidesulfovibrio fructosivorans* (*Sf*H_2ase),[30] the soluble NAD^+-reducing hydrogenase from *Hydrogenophilus thermoluteolus* (*Ht*SH),[38] and the membrane-bound hydrogenases from *E. coli* Hyd-1 (*Ec*Hyd-1) and Hyd-2 (*Ec*Hyd-2).[31,39] Although this amino acid substitution renders most hydrogenases almost inactive, a few, such as the $NADP^+$-reducing soluble hydrogenase I from *Pyrococcus furiosus* (*Pf*SH) and the MBH-type Hyn from *Thiocapsa roseopersicina* BBS,[40,41] retain considerable activity. This suggests the presence of alternative H^+ transfer pathways and/or rescue mechanisms.

To assess whether replacing Glu with Gln in RH influences the incorporation of the active site metals and/or their redox behavior, we first characterized the as-isolated, oxidized *C. necator* RH^{E13Q} variant using room-temperature IR and native RH as the reference (Fig. 2). The IR spectrum of the as-isolated RH^{E13Q} is dominated by a broad ν_{CO} band centered at 1952 cm^{-1}, along with ν_{CN} bands at 2075 and 2084 cm^{-1} (Fig. 2a, top), and a minor species characterized by a ν_{CO} band at 1969 cm^{-1}. Native RH, on the other hand, essentially shows only one ν_{CO} band at 1943 cm^{-1} with two ν_{CN} bands at 2071 and 2080 cm^{-1} (Fig. 2a, bottom), corresponding to the Ni_a–S intermediate of the active site. To facilitate the assignment of the two active site species in as-isolated RH^{E13Q}, we performed complementary EPR and RR spectroscopy. EPR spectroscopic analysis of RH^{E13Q} (Fig. 2b) revealed signals of a paramagnetic state similar to the Ni_r–B species (Ni^{3+}–OH–Fe^{2+}) of RH, whose *g* values (g_x = 2.20, g_y = 2.15, g_z = 2.02) resemble those observed for the Ni_r–B′ species in the oxidized large subunit HoxC of RH and HoxG of MBH.[3,42] These observations led to the assignment of the 1969 cm^{-1} band to the CO stretching vibration of a Ni_r–B-like state in as-isolated RH^{E13Q}. Based on the weak Ni_r–B signal, we deduce that most of the active site states of the RH^{E13Q} sample are diamagnetic. This became supported by RR spectroscopy, selectively probing metal–ligand vibrations, such as Fe–CO and Fe–CN stretching and bending modes of the active site, which typically occur in the spectral range of 400–650 cm^{-1}. By comparing the RR data (recorded with an 568 nm excitation line) of as-isolated native RH enriched in the Ni_a–S intermediate[17,23,43] with those from as-isolated RH^{E13Q}, we found that the RR spectroscopic signatures of the two proteins are very similar (Fig. 2c).

Additionally, we also resolved high-frequency intra-ligand modes of the diatomic CO/CN^- ligands at the RH and RH^{E13Q} [NiFe] active sites that allows an unambiguous assignment of the states detected by RR spectroscopy (Fig. S2).[44] This in turn suggests that a large portion of the active sites in RH^{E13Q} resides in the Ni_a–S state, which is diamagnetic and therefore consistent with the EPR analysis. Interestingly, the ν_{CO} IR band at 1952 cm^{-1}, which presumably represents the Ni_a–S intermediate of RH^{E13Q}, is shifted to higher frequencies (*ca.* 9 cm^{-1}, Fig. 2a), while the corresponding RR Fe–CO/CN absorptions are slightly shifted to lower energies (Fig. 2c) compared to the corresponding bands of native RH. A similar observation has been recently made in an nuclear resonance vibrational spectroscopy (NRVS)/IR spectroscopic study of the RH large subunit HoxC.[6] This means that the Fe–CO/CN in the Ni_a–S species of RH^{E13Q} exhibit weaker metal–ligand bonding (red-shifted RR Fe–CO/CN bands, Fig. 2c), which strengthen the diatomic CO/CN bonds as evidenced by the blue-shifted CO/CN stretching vibrations in IR, Fig. 2a). The increased energies of the CO and CN vibrations of the Ni_a–S state, which are clearly observable across all observed redox states (see below), probably result from the particular electrostatic interactions with the surrounding atoms in the catalytic center of RH^{E13Q}.[31] In particular, while the glutamine side chain is electrically neutral under physiological conditions, our recent data indicate that Glu13 is deprotonated in several active-site intermediates of the native RH enzyme and thus, carries a negative charge.[29] The observed shift to higher energies for the CO and CN stretching frequencies in RH^{E13Q} may therefore result from the absence of this negative charge, which likely reduces the electron density around the metal center.

After H_2 incubation in the presence of sodium dithionite (NaDT) to prevent rapid reoxidation (Fig. S3), the IR spectrum of the reduced RH^{E13Q} variant exhibits two major CO bands at 1969 and 1956 cm^{-1} (Fig. 3a, top), which are shifted to higher energies relative to those of the predominant species observed in reduced RH (Fig. 3a, bottom). The corresponding CN absorption frequencies are shown in Fig. 3a and listed in Table S2.

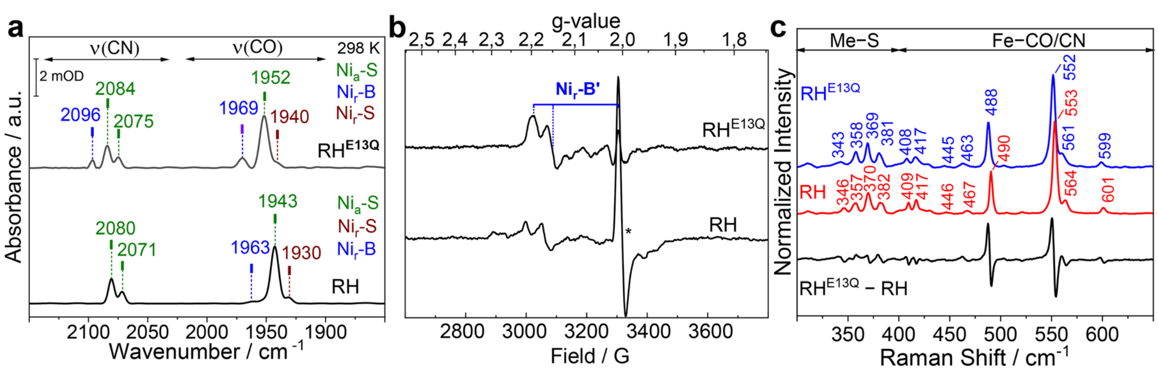

Fig. 2 Spectroscopic characterization of as-isolated RH^{E13Q}. (a) IR spectra of the RH^{E13Q} variant (top) and native RH (bottom) recorded at 298 K in the spectral range where the CO/CN-stretching vibrations of the diatomic ligands occur. The ν_{CO} and ν_{CN} bands are labeled with their corresponding wavenumbers. The IR spectra are dominated by signals attributed to the Ni_a–S (dark green). Minor contributions of the Ni_r–B-like (blue) and Ni_r–S (brown) species were also detected in RH^{E13Q}. (b) EPR spectra of the as-isolated RH^{E13Q} variant and native RH recorded at 80 K. The asterisk marks the weak signal of a [3Fe–4S] cluster most likely resulting from a degraded [4Fe–4S] cluster.[16] (c) Solution-phase RR spectra (80 K, excited at 568 nm) of RH^{E13Q} (blue trace) and native RH (red trace). Spectra are normalized with respect to the integral of the Fe–CO/CN marker band centered at 552/553 cm^{-1}. To highlight spectra changes, a difference spectrum RH^{E13Q}–native RH is shown as well (black trace). Spectral regimes dominated by Ni-centered metal–sulfur (Me–S) modes and Fe-centered Fe–CO/CN stretching and bending modes are indicated. Due to the high excitation wavelength, the data is free of [Fe–S] clusters contributions. The data of native RH are reproduced from ref. 44 © 2024 The Authors. Published by Elsevier Inc.

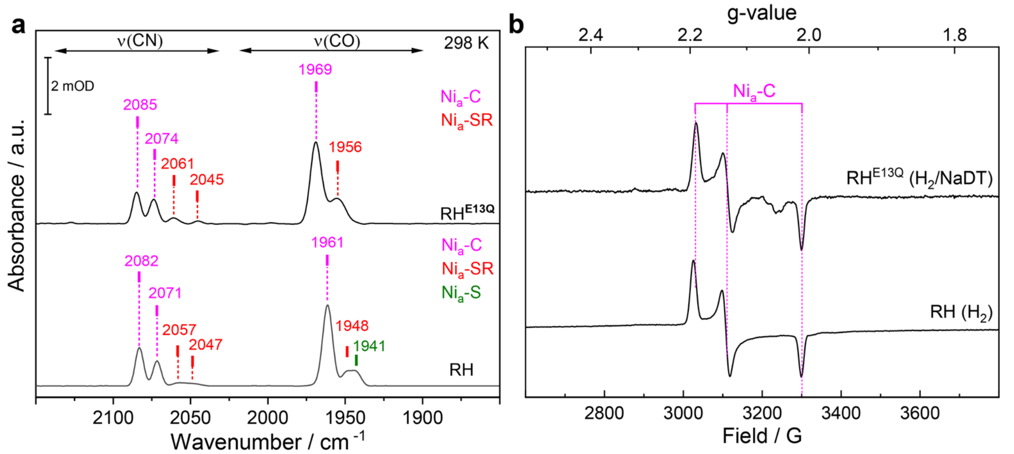

Fig. 3 Spectroscopic characterization of reduced RH^{E13Q}. (a) IR data of the H_2/NaDT-reduced RH^{E13Q} variant (top) and native RH (bottom) at 298 K in the spectral range where the CO/CN-stretching vibrations occur. The ν_{CO} and ν_{CN} bands are labeled with their corresponding wavenumbers. The spectra of both samples are dominated by signals attributed to the Ni$_a$–C (magenta) intermediate. Small bands attributed to Ni$_a$–S (dark green) and Ni$_a$–SR (red) were also detected. (b) EPR spectra of the H_2/NaDT-reduced RH^{E13Q} variant (top) and H_2-reduced native RH (bottom) recorded at 90 K. The EPR spectrum of RH^{E13Q} incubated only with H_2 exhibits *g* values identical to those of the H_2/NaDT-reduced RH^{E13Q} sample (see also Fig. S4).

Complementary EPR data on reduced RH^{E13Q} revealed, regardless of the specific reduction protocol used (Fig. S4a), the typical spectrum of the Ni$_a$–C intermediate observed in native RH (g_x = 2.19, g_y = 2.14, g_z = 2.01, Fig. 3b), which enabled the assignment of the band at 1969 cm^{-1} to Ni$_a$–C (Ni^{3+}–H^-–Fe^{2+}). Finally, the broad CO band at 1956 cm^{-1} likely contains contributions from the diamagnetic bridging-hydride Ni$_a$–SR state (Ni^{2+}–H^-–Fe^{2+}), which typically accumulates in small amounts in reduced native RH (Fig. 3a, bottom; Fig. S3),[27] as well as minor contributions from Ni$_a$–S (1952 cm^{-1}, Fig. 2a). Significantly, the enrichment of Ni$_a$–C in RH^{E13Q} upon reduction is similar to that in native RH, as previously observed for the corresponding variants of *Ec*Hyd1 and *Sf*H_2ase.[30,31] Thus, the conserved glutamate presumably does not act as H^+ acceptor during the Ni$_a$–SR → Ni$_a$–C transition (Fig. 1).

3.2. Infrared spectroscopic analysis of RH^{E13Q} at low temperatures

To further examine the impact of the Glu-to-Gln exchange in RH, the protein variant was analyzed in detail using low-temperature IR spectroscopy, which has been demonstrated to provide valuable details about changes in the outer coordination sphere of the [NiFe] active site.[22,29,45] As-isolated and H_2/NaDT-reduced samples RH^{E13Q} and native RH were rapidly cooled to 90 K using a liquid-nitrogen bath cryostat, and IR spectra were subsequently recorded (Fig. 4). Notably, the IR

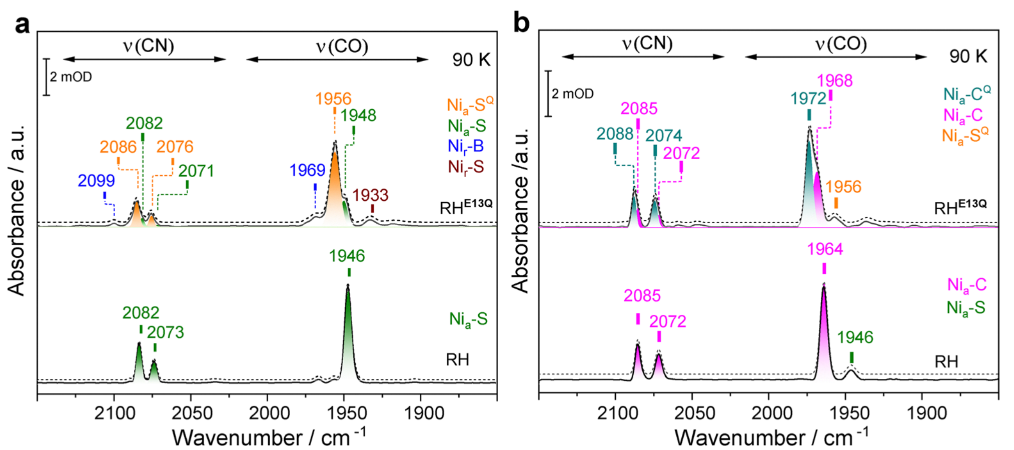

Fig. 4 IR spectra of as-isolated and H_2/NaDT-reduced RH^{E13Q} at 90 K. (a) In contrast to as-isolated native RH (bottom), which shows a single Ni$_a$–S species, Ni$_a$–S state of RH^{E13Q} (top) splits into two distinct species with ν_{CO} bands at 1956 (Ni$_a$–S^Q, orange) and 1948 cm^{-1} (Ni$_a$–S, olive). The RH^{E13Q} spectrum also shows small absorptions attributed presumably to the non-catalytic Ni$_r$–B (blue) and Ni$_r$–S (wine red) state. (b) Low-temperature IR spectrum of H_2/NaDT-reduced RH^{E13Q} (top) exhibits two Ni$_a$–C species, designated Ni$_a$–C (magenta) and Ni$_a$–C^Q (dark cyan) with ν_{CO} bands at 1968 and 1972 cm^{-1}, respectively. Trace signals of the Ni$_a$–S^Q were also observed. The IR spectrum of H_2-reduced RH (bottom) shows predominantly signals of the Ni$_a$–C species. All observed active site states, and their corresponding frequencies are provided in Table S2. Dotted lines indicate the Gaussian fits of the CO/CN bands.

absorptions of the CO and CN^- ligands displayed significantly narrower line shapes at 90 K than at 298 K. This improved resolution facilitated the identification of additional overlapping active-site species that would otherwise be masked by line broadening at room temperature. For example, the broad CO band at 1952 cm^{-1} (298 K) observed for as-isolated RH^{E13Q} and assigned to the Ni_a–S state (Fig. 2a) was resolved more clearly at 90 K, revealing two adjacent ν_{CO} bands at 1948 and 1956 cm^{-1}. Gaussian fitting of the CO/CN bands suggests the presence of two distinct subforms of the Ni_a–S state (Fig. 4a). Furthermore, the ν_{CO} band at 1969 cm^{-1} of the reduced RH^{E13Q} sample, assigned to the Ni_a–C state (Fig. 3a), was also resolved into two distinct bands at 1972 and 1968 cm^{-1} (Fig. 4b). Interestingly, complementary EPR measurements on the H_2/NaDT-reduced protein variant reveal the presence of a single Ni_a–C species (Fig. 3b). This suggests that the structural differences between the two Ni_a–C states detected by IR spectroscopy are unlikely to be accompanied by a significant change in the local environment or coordination geometry of the Ni center, resulting in EPR spectra that are indistinguishable for the two species. Our results therefore emphasize an important point: relying on a single spectroscopic technique may not be sufficient to unambiguously assign and distinguish hydrogenase active-site species. Additionally, EPR measurements at 10 K, together with power-dependent saturation analyses at 20 K (Fig. S4b–d), are consistent with the presence of a reduced proximal [4Fe–4S] cluster in H_2/NaDT-reduced RH^{E13Q}. This behavior is in agreement with previous observations made for native RH upon incubation with chemical reductants.[27] Thus, unlike the *Pf*SH hydrogenase, where distinct Ni_a–C species have been attributed to differences in the oxidation state of the proximal [4Fe–4S] cluster,[14] the two Ni_a–C species detected in RH^{E13Q} are unlikely to originate from such redox variations. Notably, low-temperature IR spectrum of reduced RH^{E13Q} revealed an almost complete disappearance of the Ni_a–SR-associated ν_{CO} band at 1956 cm^{-1} (Fig. 3a *vs.* Fig. 4b). We attribute this behavior to a temperature-dependent change in the redox equilibrium of the Ni_a–SR and the Ni_a–C states, as recently described for membrane-bound [NiFe]-hydrogenase from *C. necator*[45] and *Ht*SH.[46]

The IR spectra of the RH^{E13Q} variant differ from those of the native enzyme in two significant ways. First, the dominant IR bands recorded at room temperature appear broader, which is due to the presence of two subforms that can be disentangled at low temperatures. We assume that these subforms differ in some structural element(s) at or near the active site. In support of this interpretation, crystallographic analyses of the analogous E28Q variant of the O_2-tolerant *Ec*Hyd1 showed that a subset of the active site molecules accommodate an additional H_2O or OH^- adjacent to the amide headgroup of the glutamine residue.[31] Notably, the presence of a sub-population with an anionic species electrostatically equivalent to the deprotonated carboxylate of E13 could account for the IR observations of a minor fraction in RH^{E13Q} characterized by CO/CN stretching signals at lower energies relative to the main fraction. In particular, the bands at 1948 and 1968 cm^{-1} (Fig. 4a and b) closely match those of the Ni_a–S and Ni_a–C states in oxidized and reduced samples of native RH, detected at 1946 and 1964 cm^{-1}, respectively. This strong similarity suggests that the active site sub-states represented by CO bands at 1948 and 1968 cm^{-1} might reflect a sub-population that bears a hydroxide anion in proximity of the [NiFe] site. This would reinstate the electrostatic effect of the missing negatively charged carboxylate group, thereby, lowering the CO stretching frequencies. These Ni_a–S and Ni_a–C subforms account for ~20–30% of the total absorption integral and are hereafter referred to as the prototypical states of RH^{E13Q}. The second major difference between RH^{E13Q} and RH is that the IR bands belonging to the dominant subforms are blue-shifted by about 6–9 cm^{-1} both in the as-isolated and reduced RH^{E13Q} samples. Similarly to the IR data recorded at 298 K (Fig. 2a and 3a), the absence of a negative charge near the bimetal center (*e.g.*, deprotonated glutamate or OH^-) may strengthen the bonds of the diatomic Fe-ligands, leading to the observed shift of the CO bands to higher energies. The superscript "Q" was incorporated into designation of the more dominant subforms of RH^{E13Q} (1956 and 1972 cm^{-1}), and we refer to them as Ni_a–S^Q and Ni_a–C^Q in the caption of Fig. 4 and the text below. Table S2 summarizes all observed redox states of the active site and their corresponding CO/CN absorptions.

3.3. Photoconversion of active site states in reduced RH^{E13Q} at cryogenic temperature

The Ni_a–C intermediate,[47–49] and, more recently, one Ni_a–SR subform,[46] have been shown to convert into Ni_a–L species under cryogenic conditions upon exposure to blue light. This approach allows these active-site species to be trapped kinetically and characterized spectroscopically. However, Ni_a–L species have also been observed at ambient temperature in IR spectroscopic studies on *Pf*SH,[14,40] *Ec*Hyd1,[25,50,51] and isolated catalytic subunits of RH and MBH from *C. necator*.[3,42] Photolysis of the hydride ligand leads to proton translocation to one of the cysteines (Cys479 in *C. necator* RH) that terminally ligate the Ni ion.[22] The two electrons originating from the hydride are transferred to the Ni ion and one of the adjacent [Fe–S] clusters in case of the Ni_a–SR to Ni_a–L conversion. During the Ni_a–C to Ni_a–L conversion, however, neither protons nor electrons are released from the first coordination sphere, *i.e.*, Ni_a–C and Ni_a–L are tautomers. As outlined in the introduction, we have recently elucidated the details of the latter photo-transformation for native RH.[29] After illumination of RH in the Ni_a–C state, photolysis results in the formation of the Ni_a–L1 subform, which then (in the dark) thermally converts to the thermodynamically more stable Ni_a–L2 state $\left(\mathrm{Ni_a - C} \xrightarrow{h\nu} \mathrm{Ni_a - L1} \rightarrow \mathrm{Ni_a - L2}\right)$.

When the same experiment was performed with H_2/NaDT-reduced RH^{E13Q}, illumination with blue LED light (460 nm) at 90 K led to the conversion of the two distinct Ni_a–C states into two Ni_a–L states characterized by ν_{CO} bands at 1921 and 1915 cm^{-1} (Fig. 5a).

Their relative intensities were similar to those observed for the Ni_a–C/Ni_a–C^Q subforms. Kinetic analysis of the light-induced reaction indicates that the two Ni_a–L subforms can be traced back (rate constants $K_1 \approx K_2$ and $K_1' \approx K_2'$) to the

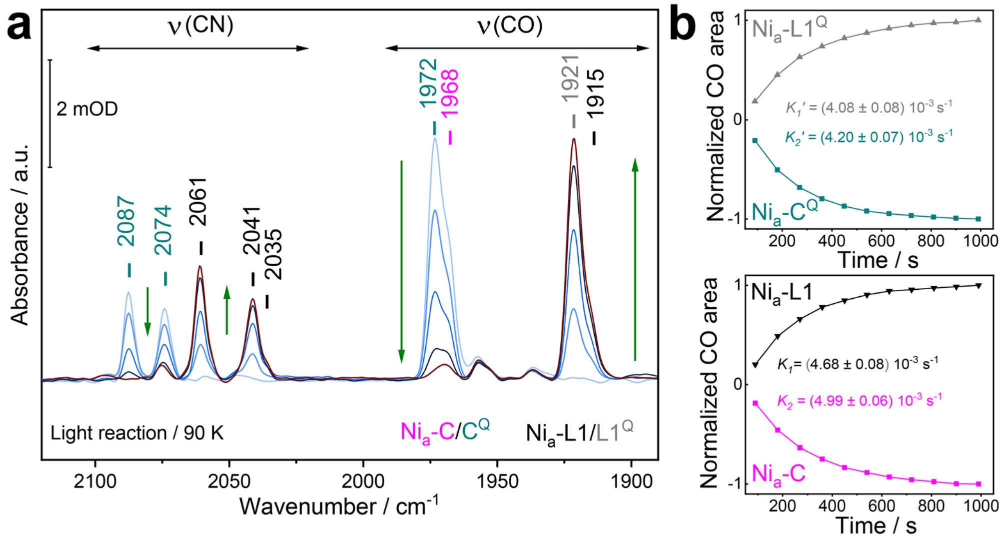

Fig. 5 IR spectra of as-isolated and H_2/NaDT-reduced RH^{E13Q} at 90 K before, during and after illumination. (a) Representative IR absorption spectra of reduced RH^{E13Q} before, during, and after LED-illumination at 460 nm. Early and late spectra are colored light blue and brown, respectively. CO/CN bands of the Ni$_a$–C (magenta), Ni$_a$–C^Q (dark cyan), Ni$_a$–L1 (black) and Ni$_a$–L1^Q (grey) are labeled with corresponding wavenumbers (b) kinetic profiles of the Ni$_a$–C/C^Q depletion and Ni$_a$–L1/L1^Q formation at 90 K. The normalized CO areas of Ni$_a$–L1 and Ni$_a$–C (ν_{CO} bands at 1915 and 1968 cm^{-1}) and Ni$_a$–L1^Q and Ni$_a$–C^Q (ν_{CO} bands at 1921 and 1972 cm^{-1}) were plotted against time. The fitted curves exhibit monoexponential kinetics, indicating two distinct conversions, *i.e.*, Ni$_a$–C^Q (1972 cm^{-1}) → Ni$_a$–L1^Q (1921 cm^{-1}) and Ni$_a$–C (1968 cm^{-1}) → Ni$_a$–L (1915 cm^{-1}). Color code as in (a). The fit parameters are summarized in Table S3. It should be noted that the kinetic rates associated with Ni$_a$–C depletion (K_2) and Ni$_a$–L1 formation (K_1) appear to be faster than those estimated for $\mathrm{Ni_a-C^Q}$ (K_2') and $\mathrm{Ni_a-L^Q}$ (K_1'). These differences likely originate from partial back conversion of Ni$_a$–L1^Q → Ni$_a$–C^Q, which seems to proceed slightly faster than the corresponding Ni$_a$–L1 → Ni$_a$–C process. This interpretation is supported by the IR difference spectra shown in Fig. 6a, which indicate enrichment of the Ni$_a$–C^Q species in RH^{E13Q} after thermal transformation. In this sense, the values for the forward reaction represent apparent rate constants.

corresponding Ni$_a$–C subforms present prior to illumination (Fig. 5b and Table S3). Accordingly, we designated the more dominant subform at 1921 cm^{-1} as Ni$_a$–L1^Q.

After complete photolysis of the Ni$_a$–C/Ni$_a$–C^Q states and enrichment of the Ni$_a$–L1/Ni$_a$–L1^Q subforms, the illumination was switched off. In native RH, the thermal conversion of the Ni$_a$–L1 state to the Ni$_a$–L2 state proceeds very slowly at 90 K, and we have previously observed that slightly higher temperatures can accelerate Ni$_a$–L2 formation.[29] Therefore, the temperature of the RH^{E13Q} sample was first increased to approximately 130 K to accelerate the enrichment of Ni$_a$–L2 by thermal transformation and subsequently lowered back to 90 K prior to record the spectral features of the Ni$_a$–L2/L2^Q states.

Fig. 6a shows an overlay of the IR difference spectra of "after light-minus-dark" (black traces), where the positive bands highlight enrichment of Ni$_a$–L1 before thermal transformation, and "after thermal transformation-minus-dark" (dark yellow and red traces), where the positive bands reflect the enrichment of Ni$_a$–L2 at high temperatures. By comparing both RH samples in H_2/H_2O and D_2/D_2O, we identified clear spectral differences between native RH and RH^{E13Q}. Both the CO and the CN bands of the Ni$_a$–L1 state in native RH (Fig. 6a, upper black and grey traces) undergo a net blue shift in the Ni$_a$–L2 state (upper dark yellow and red traces). In particular, the ν_{CO} shifts from 1911 to 1914 cm^{-1} and ν_{CN} move from 2037/2056 to 2040/2060 cm^{-1}. These changes were proposed to originate from conformational changes around the [NiFe] site including a new hydrogen bond (H-B) between the deprotonated glutamate and the protonated thiolate of Cys479.[29] In contrast, analysis of the CO/CN spectral region of RH^{E13Q} revealed a pronounced similarity between the Ni$_a$–L species populated immediately after photolysis (Fig. 6a, lower black trace) and that obtained following thermal transformation (Fig. 6a, lower dark yellow trace). The main CO band exhibits only a slight red shift from 1921 to 1919 cm^{-1} (marked in wine red), and only the asymmetric CN stretching shows a modest upshift of approximately 2 cm^{-1} (marked in wine red). These changes in RH^{E13Q} are virtually absent in D_2/D_2O (Fig. 6a, lower grey and red traces). Complementary EPR measurements on irradiated RH^{E13Q} (90 K, Fig. 6b) yielded signals with g values characteristic of Ni$_a$–L1 (predominant, g_x = 2.247, g_y = 2.090, g_z = 2.044) and Ni$_a$–L2 (minor, g_x = 2.309, g_y = 2.076, g_z = 2.052) species, closely matching those observed for native RH, similar to the correspondence seen for the parent Ni$_a$–C states (Fig. 3b).[20,29]

To obtain detailed information about differences in the second/outer coordination sphere between the active sites of RH^{E13Q} and native RH, we analyzed and compared the IR difference spectra in the ranges from 1450 to 1800 cm^{-1} (Fig. 7a and b). The IR difference spectra "after light-minus-dark" (Ni$_a$–L1/L1^Q-minus-Ni$_a$–C/C^Q) revealed major positive bands at 1705, 1686 and 1622 cm^{-1} related to the Ni$_a$–L1/1^Q states, and clear negative bands at 1700, 1675 and 1630 cm^{-1} deriving from the Ni$_a$–C/C^Q species. These signals appear in a spectral range that is characteristic for signals from Arg (symmetric, ν_s, and asymmetric stretching vibrations, ν_{as}, of $CN_3H_5^+$) and Gln residues ($\nu_{C=O}$) as well as amide I/II bands

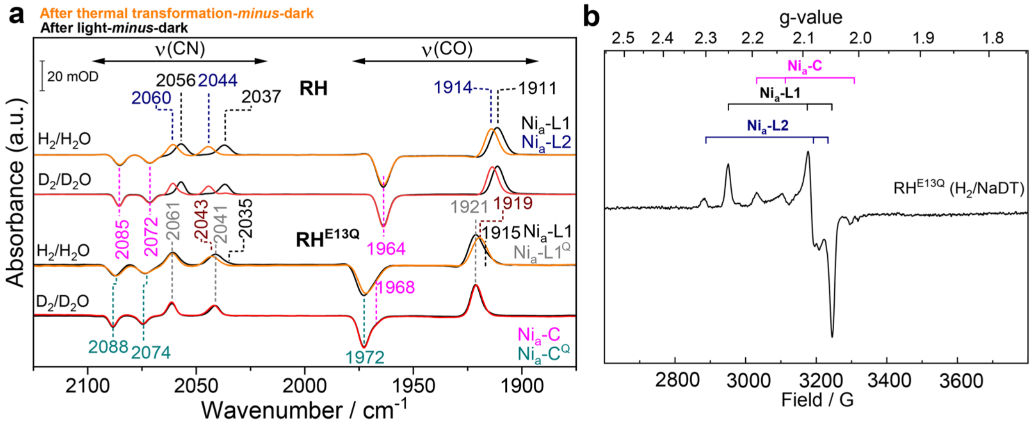

Fig. 6 IR and EPR characterization of the Ni_a–L states of RH^{E13Q} and RH. (a) IR difference spectra at 90 K in the 2150–1850 cm^{-1} regime for H_2/NaDT-reduced native RH (upper traces) and RH^{E13Q} (lower traces) prepared with H_2/H_2O and D_2/D_2O. The figure shows two sets of IR difference spectra: the "after light-minus-dark" spectra (black, H_2/H_2O; grey, D_2/D_2O) and the "after thermal transformation-minus-dark" spectra (orange, H_2/H_2O; red, D_2/D_2O). The spectra of RH^{E13Q} after light exposure show prominent positive absorptions of the active site CO/CN ligands related to the Ni_a–$L1^Q$ at 1921, 2041, and 2061 cm^{-1} (marked in grey), while negative Ni_a–C^Q-related bands were detected at 1972, 2074, and 2088 cm^{-1} (dark cyan, Table S2). Minorly populated Ni_a–L1 (black) and Ni_a–C (magenta) species are also visible (see also Fig. 5). The "after light-minus-dark" spectra of RH exhibit Ni_a–L1-related positive $\nu_{CO/CN}$ bands at 1911, 2037, and 2056 cm^{-1} (black) while Ni_a–C-related signals (magenta) were observed at 1964, 2072 and 2085 cm^{-1}. After thermal transformation, native RH is stoichiometrically enriched in the Ni_a–L2 ($\nu_{CO/CN}$ bands at 1914, 2044 and 2060 cm^{-1}), while RH^{E13Q} still exhibits predominantly Ni_a–$L1^Q$-related signals. Minor changes of the CO (1919 cm^{-1}) absorption and the band related to the antisymmetric CN stretching vibration (2043 cm^{-1}) in RH^{E13Q}, which arise from overlapping Ni_a–$L1^Q$ and Ni_a–L2 CO/CN bands, are marked in wine red (see also Fig. S6). (b) EPR spectrum of the H_2/NaDT-reduced RH^{E13Q} recorded at 90 K upon LED-illumination (455 nm) resulting in the formation of the Ni_a–L1 (g_x = 2.247, g_y = 2.090, g_z = 2.044) and Ni_a–L2 (g_x = 2.309, g_y = 2.076, g_z = 2.052) species. Trace signals of Ni_a–C species are also observed.

and water contributions.[52,53] Notably, the positive and negative absorption features of the RH^{E13Q} variant closely match those of native RH (Fig. 7a, black traces), suggesting that Glu13 is "dispensable" for the Ni_a–C → Ni_a–L1 transition in native RH. In contrast, the analogous glutamate in *Dv*MF hydrogenase has been proposed to undergo significant structural changes during the Ni_a–C → Ni_a–L transition (possibly Ni_a–L2), forming one hydrogen bond (with a cysteine terminally coordinating the Ni) in the Ni_a–C intermediate and two bonds in the Ni_a–L(2) state.[22] These contrasting results imply that the mechanism of H_2 activation in RH and *Dv*MF hydrogenases proceeds with distinct dynamics of outer-sphere residues.

Importantly, the "after thermal transformation-minus-dark" difference spectrum of the RH^{E13Q} variant displays features that are nearly identical to those of the "after light-minus-dark" spectrum (Fig. 7a, top). These observations suggest that the outer coordination sphere of the active site of RH^{E13Q} does not undergo the conformational changes observed in native RH (see, for example, the new set of negative/positive bands at 1641 and 1659 cm^{-1} in Fig. 7a). Importantly, only weak absorptions are observed in the spectral region associated with deprotonated aspartate/glutamate residues between 1530 and 1570 cm^{-1}. This is particularly evident in experiments conducted in D_2/D_2O (Fig. S5), where RH^{E13Q} is characterized by nearly stoichiometric Ni_a–C^Q and Ni_a–$L1^Q$ species, consistent with the absence of the conserved glutamate and possibly reflecting an impaired proton-transfer (PT) pathway involving deprotonated carboxylates interacting with E13.[29] Based on our previous findings for native RH, where the deprotonated glutamate was shown to form a hydrogen bond with the protonated Ni-bound Cys479 in the Ni_a–L2 state, we propose that the thermal conversion of Ni_a–L1/$L1^Q$ in RH^{E13Q} (H_2/H_2O) yields only a minor population of Ni_a–L2 species. In this variant, the role of the deprotonated glutamate would be mimicked by an electrostatically equivalent hydroxide ligand capable of hydrogen bonding to protonated Cys479 (Fig. 7d), while the predominant Ni_a–$L1^Q$ state remains essentially unchanged.

Guided by these observations, we performed Gaussian fitting of the CO and CN bands of H_2/NaDT reduced RH^{E13Q} in H_2/H_2O (Fig. S6), assuming enrichment of the Ni_a–L2 state while retaining the Ni_a–$L1^Q$ population. The resulting fit accurately reproduces the experimental CO/CN spectral features of RH^{E13Q}. Accordingly, the bands corresponding to the predominant Ni_a–$L1^Q$ species remain largely unaffected after thermal transformation, and only a small fraction of molecules—represented by the Ni_a–L1 ν_{CO} band at 1915 cm^{-1}—appears to convert into the Ni_a–L2 state, characterized by a ν_{CO} band near 1917 cm^{-1}, partially overlapping with the ν_{CO} of the Ni_a–$L1^Q$ at 1921 cm^{-1}. These results suggest that the [NiFe] site in RH^{E13Q} predominantly remains in the outer-sphere Ni_a–$L1^Q$ conformation (Fig. 7a). The only exception is the broad negative band at 1654 cm^{-1} (associated with Ni_a–C), which falls within the amide $\nu_{C=O}$ range and may originate in part from the introduced glutamine residue. Accordingly, this band shows a significantly increased intensity compared to native RH (Fig. 7a, bottom) and exhibits a redshift when the sample is prepared in D_2/D_2O (Fig. S5, 1651 cm^{-1}), consistent with previously reported solvent isotope effects.[53] Furthermore, the observed $\nu_{C=O}$ feature vanishes during thermal transformation (Fig. 7a, middle trace), suggesting that the corresponding residue—most likely the

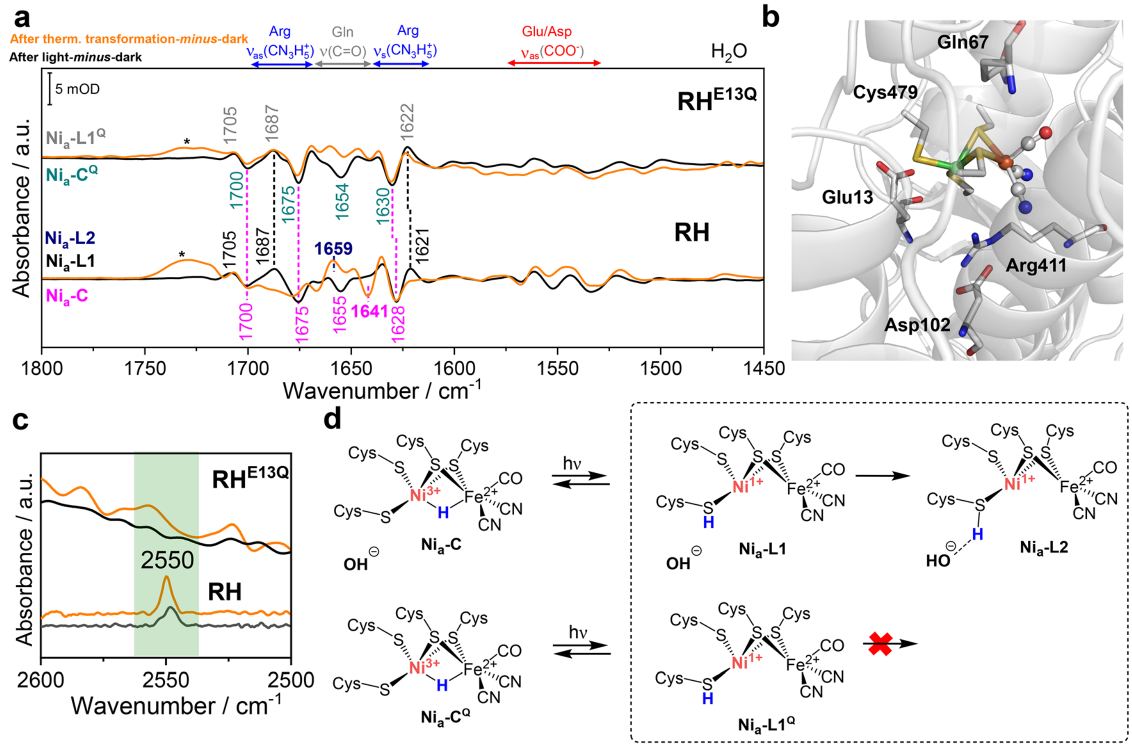

Fig. 7 IR difference spectra of RH^{E13Q} and native RH revealing important elements of the first and outer coordination sphere of the corresponding [NiFe] sites. (a) "After light-minus-dark" (black traces) and "after thermal transformation-minus-dark" (orange traces) difference spectra of native RH (bottom) and RH^{E13Q} (top) prepared with H_2/H_2O. The spectra in the range between 1800 and 1450 cm^{-1} show potential contributions from individual amino acid residues. The spectral regimes characteristic for the main bands of arginine ($CN_3H_5^+$, ν_s and ν_{as}, blue arrow), glutamine ($\nu_{C=O}$, grey arrow) and deprotonated aspartate/glutamate (ν_{as} COO^-, red arrow), are highlighted. Amide I and amide II absorptions, which occur between 1600–1700 cm^{-1} and 1510–1580 cm^{-1}, respectively, as well as water absorptions ($\delta_{(OH)}$ bending, 1635–1670 cm^{-1}) might also contribute to the observed absorptions. We assign the broad band around 1700–1730 cm^{-1} (marked with *) to an artifact due to slight temperature fluctuations over time. (b) AlphaFill-predicted model of the RH large subunit HoxC including the $NiFe(CN)_2CO$ cofactor and selected outer-sphere residues.[29] The coordinating cysteines (Cys60, 63, 479, and 482), together with Glu13, Gln67, Arg411, and Asp102, residues are shown in stick representation. Color code: C, grey; O, red; N, blue; S, yellow; Ni, green; Fe, brown. The protein backbone is shown in cartoon representation (grey). (c) Spectral region typical for S–H stretching vibrations for native RH and RH^{E13Q} after light reaction and after thermal transformation. (d) Schematic representation of the [NiFe] active site in RH^{E13Q} variant depicting the likely involved structural elements characterizing Ni_a–C/C^Q, Ni_a–L1/$L1^Q$ and Ni_a–L2 active site states. For the minorly populated Ni_a–C, Ni_a–L1 and Ni_a–L2 in RH^{E13Q} we propose the presence of an OH^- anion in proximity of the [NiFe] site, which can partially compensate the absence of the negatively charged carboxylate side group of the conserved glutamate (see Fig. 1).

newly introduced Gln13—is stabilized after thermal transformation in a conformation resembling that of the Ni_a–C/C^Q states, while all other nearby vibrational features remain unchanged. Finally, whereas native RH displays clear ν_{SH} bands for both the Ni_a–L1 and Ni_a–L2 states ($\nu_{SH} \approx 2550$ cm^{-1}; Fig. 7c), with the intensity being significantly higher for Ni_a–L2, where the protonated Cys479 is proposed to form an hydrogen bond with Glu13 (Fig. 1),[29] the corresponding IR difference spectra of the RH^{E13Q} variant exhibit no detectable ν_{SH} absorptions (Fig. 7c).

However, these observations do not exclude protonation of Cys479 in this variant. We propose that the intrinsic heterogeneity of RH^{E13Q}—specifically, enrichment of Ni_a–L1/$L1^Q$ after light exposure and of Ni_a–$L1^Q$/L2 after thermal transformation (Fig. 6a and S6)—produces a population of Cys-SH conformers that largely lack the hydrogen bond stabilizing the protonated thiolate in native Ni_a–L2 (Fig. 7d). Consequently, the associated ν_{SH} bands are expected to be substantially broadened, less polarized and therefore exhibit substantially lower intensity, rendering them undetectable in the experimental spectra. Nevertheless, protonation of Cys479 in RH^{E13Q} is supported by the close similarities between the IR (difference) spectra and the EPR signatures of the Ni_a–L species of RH^{E13Q} and native RH (Fig. 6, 7a, and S6), indicating that the first-sphere and most second-sphere interactions of the [NiFe] active site are largely preserved in the variant.

4. Conclusion

The present study elucidated the dynamics of residues surrounding the [NiFe] active site in the catalytic cycle of hydrogenases by using a variant of the *C. necator* regulatory hydrogenase in which the conserved proton-accepting glutamate (Glu13) located in the second coordination sphere of the metal center was replaced by glutamine. We performed comprehensive biochemical and spectroscopic analyses, including low-temperature IR, RR, and EPR, and compared the results with those of native RH. According to IR and RR spectroscopy, RH^{E13Q} protein remained in the Ni_a–S state, as observed for RH.[17] Nevertheless, the E13Q variant exhibited only 0.1% of the catalytic activity of native RH, which is consistent with previous studies on other [NiFe]-hydrogenases.[30,31,38,39] Exposure of RH^{E13Q} to H_2 led to the accumulation of the reduced active site species Ni_a–C and Ni_a–SR. Subsequent illumination of the hydrogenase at 90 K induced the photolysis of the bridging hydride of the Ni_a–C state, resulting in the Ni_a–L1 state. The corresponding light-minus-dark IR difference spectra for this photoreaction were almost identical for RH^{E13Q} and native RH, indicating very similar structural and conformational changes in both proteins during the Ni_a–C to Ni_a–L1 transition. These results suggest that the sequence Ni_a–S → Ni_a–SR → Ni_a–C → Ni_a–L1, including the first proton release during the Ni_a–SR to Ni_a–C transition, proceeds in the same way in the E13Q variant as in native RH. This, in turn, indicates that Glu13 does not play a role in the catalytic cycle from Ni_a–S to Ni_a–L1 (Fig. 1).

Comparative low-temperature IR spectroscopy of CO and CN^- ligands revealed two notable differences: (1) compared to native RH, the variant exhibited subforms for all major catalytic states, and (2) these subforms showed IR shifts of $\sim$6–8 cm^{-1} to higher frequencies, consistent with the loss of the negative charge of Glu13. Resonance Raman data supported this with red-shifted Fe–CN/CO vibrations. Interestingly, minor subforms of Ni_a–S, Ni_a–C, and Ni_a–L1 in the variant closely resembled native RH spectra, suggesting the presence of a compensatory hydroxide ligand near the active site, as observed in the corresponding E/Q variant of *Ec*Hyd-1.[31] This entity could partially substitute for Glu13 in proton transfer, albeit inefficiently. However, the conserved glutamate is necessary for the Ni_a–L1 to Ni_a–L2 transition, which is supported by the observation that RH^{E13Q} largely remained in a non-reactive subform of Ni_a–L1 (termed Ni_a–$L1^Q$) after thermal transformation, while native RH formed the Ni_a–L2 state that is characterized by defined outer-sphere interactions. These findings differ from previous studies by the Dyer group on *Pf*SH.[40] In fact, while the Glu-to-Gln exchange in *Pf*SH has been reported to perturb the Ni_a–L1/L2 → Ni_a–S transition, our data on RH suggest that the same exchange disrupts outer-sphere dynamics at an earlier stage, namely at Ni_a–L1. Based on our results, we assign a new mechanistic role to the conserved glutamate residue. It triggers critical outer-sphere rearrangements, such as the formation of a hydrogen bond with the protonated Ni-bound cysteine, which are essential for an efficient proton-transfer (PT) and thus, for the progression of the catalytic cycle.

An even more comprehensive picture emerges when our findings are combined with the recent preprint by Carr, Ash, Vincent and co-workers, which describes the mobility of the conserved glutamate side chain during the conversion of two Ni_a–L subforms of *Ec*Hyd-1 and *Ec*Hyd-2 based on X-ray structural analyses.[32] The results emphasize the relevance of the Ni_a–L2 state and its participation into the hydrogenase catalytic cycle, as shown in Fig. 1. Ultimately, the detailed insights presented here underscore the crucial role of the protein environment in modulating the reactivity of transition metal catalysts—a key factor in the development of synthetic catalysts and biomimetic systems for efficient proton and electron transfer.[54–57] This is particularly relevant for (semi)artificial systems such as Ni-substituted rubredoxins and short Ni-binding peptides, which have been proposed as promising hydrogenase mimetics. In several cases, these systems have been suggested to operate *via* a protonated cysteine thiolate,[55,57] although direct experimental evidence remains limited. Our findings highlight the importance of a precisely engineered outer coordination sphere for stabilizing and controlling reactive intermediates. We therefore propose that the deliberate introduction of a tailor-made outer-sphere, increasingly enabled by modern machine-learning-based protein design approaches (*e.g.*, AlphaFold 2/3,[58,59] RoseTTAFold,[60] RFdiffusion,[61] DeepPredict,[62] ProteinMPNN,[63] and Boltz-2[64]) can significantly enhance catalytic performance and facilitate the stabilization and characterization of key reaction intermediates.

Conflicts of interest

There are no conflicts of interest to declare.

Data availability

The authors declare that the data supporting the findings of this study are available within the article and the supplementary information (SI). See DOI: https://doi.org/10.1039/d6ey00004e.

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