Biocatalysis

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Control of the Transition between Ni-C and Ni-SI_a States by the Redox State of the Proximal Fe–S Cluster in the Catalytic Cycle of [NiFe] Hydrogenase**

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Abstract: *[NiFe] hydrogenase catalyzes the reversible cleavage of H_2. The electrons produced by the H_2 cleavage pass through three Fe–S clusters in [NiFe] hydrogenase to its redox partner. It has been reported that the Ni-SI_a, Ni-C, and Ni-R states of [NiFe] hydrogenase are involved in the catalytic cycle, although the mechanism and regulation of the transition between the Ni-C and Ni-SI_a states remain unrevealed. In this study, the FT-IR spectra under light irradiation at 138–198 K show that the Ni-L state of [NiFe] hydrogenase is an intermediate between the transition of the Ni-C and Ni-SI_a states. The transition of the Ni-C state to the Ni-SI_a state occurred when the proximal $[Fe_4S_4]_p^{2+/+}$ cluster was oxidized, but not when it was reduced. These results show that the catalytic cycle of [NiFe] hydrogenase is controlled by the redox state of its $[Fe_4S_4]_p^{2+/+}$ cluster, which may function as a gate for the electron flow from the NiFe active site to the redox partner.*

Hydrogenase is a metalloenzyme that catalyzes one of the simplest molecular reactions, that is, reversible oxidation of dihydrogen to two protons and two electrons: $H_2 \rightleftharpoons 2H^+ + 2e^-$.[1] This reaction plays an important role in the energy metabolism of many microorganisms, with three main groups of hydrogenases known: [NiFe], [FeFe], and [Fe] only hydrogenases.[2] [NiFe] hydrogenase from *Desulfovibrio vulgaris* Miyazaki F (*Dv*MF) consists of two subunits (large and small; Figure 1).[3] The catalytic center of [NiFe] hydrogenase from *Dv*MF is located in its large subunit, and contains two metal ions, Ni and Fe, bridged by two cysteinyl thiolates. Another two cysteine residues are bound to the Ni ion, whereas three diatomic ligands (one CO and two CN^-) are coordinated to the Fe ion.[3,4] Three Fe–S clusters are located in the small subunit of [NiFe] hydrogenase (proximal $[Fe_4S_4]_p^{2+/+}$, medial $[Fe_3S_4]_m^{+/0}$, and distal $[Fe_4S_4]_d^{2+/+}$). The electrons obtained by the heterolytic cleavage of H_2 at the catalytic NiFe center are transferred through these Fe–S clusters to the physiological redox partner, cytochrome c_3 (cyt c_3).[5]

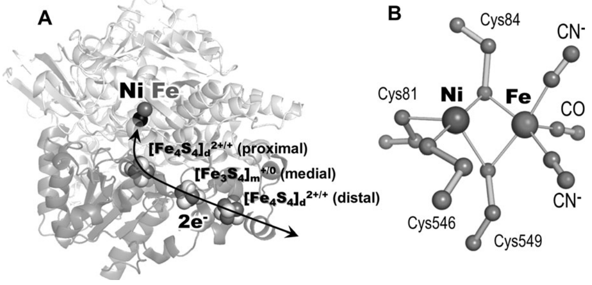

Figure 1. Structure of H_2-activated [NiFe] hydrogenase from *Dv*MF (PDB: 1WUL). A) Overall structure with the NiFe active site and $[Fe_4S_4]_p^{2+/+}$, $[Fe_3S_4]_m^{+/0}$, and $[Fe_4S_4]_d^{2+/+}$ clusters highlighted. B) NiFe active-site structure.

The "as-isolated" oxidized state of [NiFe] hydrogenase is a mixture of two paramagnetic Ni-A (un-ready) and Ni-B (ready) states, where an oxygenic ligand is bridged between the Ni and Fe ions in each state.[6] An EPR-silent state (Ni-SI_a) is produced through the EPR-silent Ni-SU and Ni-SI_r states by activation (reduction) of the Ni-A and Ni-B states, respectively, with H_2, and a paramagnetic state (Ni-C) and fully reduced EPR-silent state (Ni-R) generated by further reduction.[1c–f] The Ni-SI_a, Ni-C, and Ni-R states are reported to be involved in the catalytic cycle of [NiFe] hydrogenase, and these states interconvert among each other by addition or release of protons, electrons, and H_2.[1b–f, 7] Light sensitivity has been observed in various states of [NiFe] hydrogenases.[8] For example, the Ni-C state has been shown to convert into the paramagnetic Ni-L state by light irradiation below 180 K.[8a–c] However, the precise mechanism of the transition between the Ni-C and Ni-SI_a states, and regulation of the catalytic cycle remain unrevealed. In this study, we show, by measuring the Fourier transform infrared (FT-IR) spectra under light irradiation, that the transition between the Ni-C and Ni-SI_a states in the catalytic cycle of [NiFe] hydrogenase is controlled by the redox state of its $[Fe_4S_4]_p^{2+/+}$ cluster.

The CO stretching (ν_{CO}) and CN^- stretching (ν_{CN}) frequencies of the NiFe active site are reliable sensors for

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the changes in the electron density of the Fe ion in [NiFe] hydrogenase.[9] Positive and negative IR bands were detected in the difference (light-minus-dark) IR spectra of H_2-activated [NiFe] hydrogenase, from *Dv*MF, using the spectra with and without irradiation using Ar^+ laser light ($\lambda = 514.5$ nm, 500 mW cm^{-2} at the sample point) under an H_2 atmosphere at 138–198 K (Figure 2 A). Positive bands were observed at 1911, 2048, and 2062 cm^{-1}, and negative bands at 1963, 2077, and 2089 cm^{-1} in the difference spectra, where the positive and negative bands were related to the light-induced products and light-sensitive reactants, respectively.[8c] The negative band at 1963 cm^{-1} corresponded to the ν_{CO} band of the Ni-C state, whereas those at 2077 and 2089 cm^{-1} corresponded to its conjugated ν_{CN} bands.[4b] The frequencies of the positive bands were in agreement with those reported previously for the light-induced Ni-L state of [NiFe] hydrogenase from *Dv*MF[8c] and *Ralstonia eutropha* H16,[8e] thus showing formation of the Ni-L state by light irradiation. Under an N_2 atmosphere, in addition to the 1911 cm^{-1} ν_{CO} band, a positive ν_{CO} band was observed at 1943 cm^{-1} in the light-minus-dark difference spectra (Figure 2 B). The value of 1943 cm^{-1} corresponded well to the frequency of the ν_{CO} band of the Ni-SI$_a$ state.[4b] These results indicate that the Ni-C state also converts into the Ni-SI$_a$ state by light irradiation under an N_2 atmosphere.

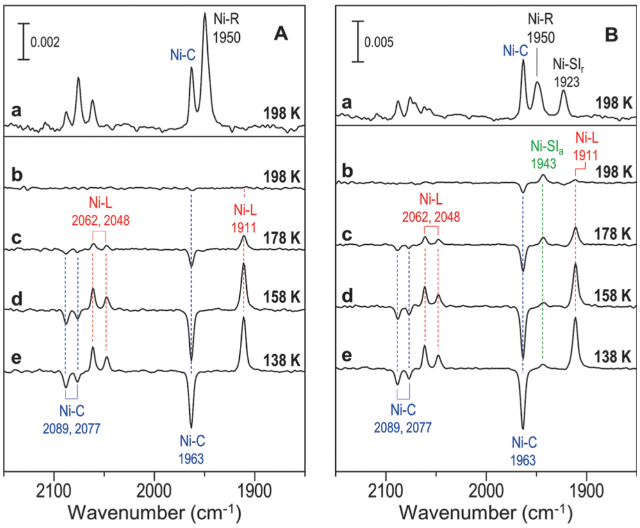


Figure 2. FT-IR spectra of H_2-activated [NiFe] hydrogenase from *Dv*MF under A) H_2 and B) N_2 atmosphere at 138, 158, 178, and 198 K: a) FT-IR spectra before light irradiation. b–e) Difference (light-minus-dark) FT-IR spectra between the spectra with and without light irradiation.

Approximately 4 and 14 % of the Ni-C state was converted into the Ni-L and Ni-SI$_a$ states, respectively, by light irradiation under an N_2 atmosphere at 198 K, according to the intensities of the ν_{CO} bands in the light-minus-dark difference spectra (Figure 2 B, curve b). The light-induced FT-IR spectrum changed back to the initial spectrum (spectrum before irradiation) when light irradiation was stopped under both H_2 and N_2 atmospheres at 198 K, and no intensity change was observed for the ν_{CO} and ν_{CN} bands of the Ni-L and Ni-SI$_a$ states in the difference (after-minus-before) spectra between the spectra after and before irradiation (all spectra were measured in the dark; see Figures S1 and S2 in the Supporting Information). However, the positive ν_{CO} and ν_{CN} bands of the Ni-L state were observed in the after-minus-before difference spectra under an N_2 atmosphere at 138 and 158 K. These results show that the Ni-L state was trapped at low temperatures. The ν_{CO} band of the Ni-SI$_a$ state was also observed in the after-minus-before difference spectra at 138–178 K (see Figure S2), thus showing that the Ni-SI$_a$ state was also trapped at low temperatures. By increasing the measurement temperature, the intensities of the ν_{CO} bands of the Ni-L and Ni-SI$_a$ states in the after-minus-before difference spectra began to decrease from those of the corresponding bands in the light-minus-dark difference spectra. The intensity of the 1911 cm^{-1} ν_{CO} band of the Ni-L state began to decrease at a lower temperature (ca. 158 K) compared to that of the 1943 cm^{-1} ν_{CO} band of the Ni-SI$_a$ state (ca. 178 K; Figure S2), thus showing that the Ni-L state was less stable to high temperature. The intensity of the ν_{CO} band of the Ni-L state in the light-minus-dark difference spectra under an N_2 atmosphere increased with a decrease in temperature, whereas that of the Ni-SI$_a$ state decreased (Figure 2 B). These results indicate that the Ni-L state was converted efficiently into the Ni-SI$_a$ state under an N_2 atmosphere at higher temperatures, such as 198 K, whereas a certain amount of the Ni-L state was trapped as an intermediate and could not convert into the Ni-SI$_a$ state at lower temperatures, such as 138 K, although there are possibilities that the Ni-C state may have converted directly into the Ni-SI$_a$ state by light irradiation.

It is noteworthy that no transition of the Ni-C state to the Ni-SI$_a$ state was observed with light irradiation under an H_2 atmosphere, whereas the transition was observed under an N_2 atmosphere (Figure 2). According to the intensity change of the ν_{CO} band by light irradiation, the overall percentage of the Ni-C state converted by light irradiation at 198 K under H_2 and N_2 atmosphere was about 3 and 18 %, respectively (Figure 3). The difference in the transition efficiency (ca. 15 %) of the Ni-C state under H_2 and N_2 atmospheres corresponded well to the percentage of the Ni-C state converted into the Ni-SI$_a$ state under an N_2 atmosphere (ca. 14 %). Although the Ni-L state was trapped and observed in the light-minus-dark difference spectra at lower temperatures (138–178 K), the difference in the transition efficiency of the Ni-C state under H_2 and N_2 atmospheres also corresponded

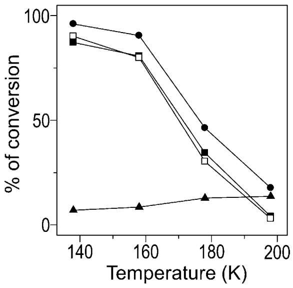


Figure 3. Overall percentages of the Ni-C state converted by light irradiation under H_2 (open square) and N_2 (closed circle) atmospheres, together with the percentages of the Ni-C state converted into the Ni-L state (closed square) and Ni-SI$_a$ state (closed triangle) by light irradiation under an N_2 atmosphere. The Ni-C state only converted into the Ni-L state under an H_2 atmosphere.

well to the percentage of the Ni-C state converted into the Ni-SI$_a$ state under an N_2 atmosphere at lower temperatures (Figure 3). Therefore, the percentage of the conversion of the Ni-C state increased under an N_2 atmosphere compared to that under an H_2 atmosphere.

It has been reported that the proximal $[Fe_4S_4]_p^{2+/+}$ cluster is reduced when the paramagnetic Ni-C state is generated in H_2-activated [NiFe] hydrogenase under an H_2 atmosphere.[1d,10] Therefore, we measured the EPR spectra of [NiFe] hydrogenase from *Dv*MF under H_2 and N_2 atmospheres to characterize the redox state of the proximal $[Fe_4S_4]_p^{2+/+}$ cluster under each set of conditions. The Ni EPR signals of the Ni-C state of H_2-activated [NiFe] hydrogenase were observed at $g_x = 2.197$, $g_y = 2.144$, and $g_z = 2.010$ under an H_2 atmosphere at 50 K (Figure 4). The intensities of these Ni^{3+} signals increased when the atmosphere was changed from H_2 to N_2 at 50 K, owing to the increase in the amount of the Ni-C state (mainly because of the decrease in the amount of the Ni-R state). A broad signal of the reduced $[Fe_4S_4]_{p/d}^{+}$ clusters was also observed at $g = 1.86$ in the EPR spectra under both H_2 and N_2 atmospheres below 10 K.[10a–c] The intensity of the broad $g = 1.86$ signal in the spectra under an N_2 atmosphere was lower than that in the spectra under an H_2 atmosphere, thus suggesting a smaller amount of $[Fe_4S_4]_p^{2+/+}$ clusters were reduced in the Ni-C state under an N_2 atmosphere. Similar characteristics in the EPR signals have been reported for [NiFe] hydrogenase from *D. gigas*.[10d] Changing the atmosphere from H_2 to N_2 may shift the redox potential of the solvent (water) to a less negative redox potential, where the protons from the water act as oxidants.[10d]

The distance between the Ni atom and the nearest Fe atom of the proximal $[Fe_4S_4]_p^{2+/+}$ cluster is 10.9 Å for [NiFe] hydrogenase from *Dv*MF.[3] At a relatively high temperature (50 K), the spin relaxation of the $[Fe_4S_4]_p^{+}$ cluster was fast and averaged out, thus resulting in relatively sharp Ni signals in the EPR spectra. The spin–spin interaction between the paramagnetic Ni^{3+} site ($S = 1/2$) and paramagnetic reduced proximal $[Fe_4S_4]_p^{+}$ cluster ($S = 1/2$) in the Ni-C state causes broadening and splitting in the Ni signals at low temperatures.[1d,10] The Ni signals at $g_y = 2.144$ and $g_z = 2.010$ of the Ni-C state under an H_2 atmosphere at 50 K became broader at 20 K (Figure 4; see Figure S3). The Ni signals at $g_x = 2.197$ and $g_y = 2.144$ of the Ni-C state under an H_2 atmosphere at 50 K split into two components at $g_x = 2.176$ and 2.218 and $g_y = 2.106$ and 2.182, respectively, at 10 K, and their intensities increased at 4 K. The Ni signal at $g_z = 2.010$ at 50 K also split into four components at $g_z = 1.978$, 1.994, 2.034, and 2.057, at 10 K, and their intensities also increased at 4 K. No un-split Ni signal was observed at $g_y = 2.144$ and $g_z = 2.010$ in the spectra under an H_2 atmosphere at 4 and 10 K. These results strongly indicate that almost all the proximal $[Fe_4S_4]_p^{2+/+}$ clusters in the Ni-C state were reduced under an H_2 atmosphere, where the $[Fe_4S_4]_p^{+}$ cluster spin–spin coupled with the Ni^{3+} center.

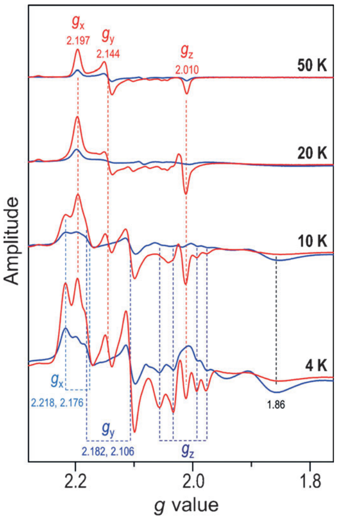
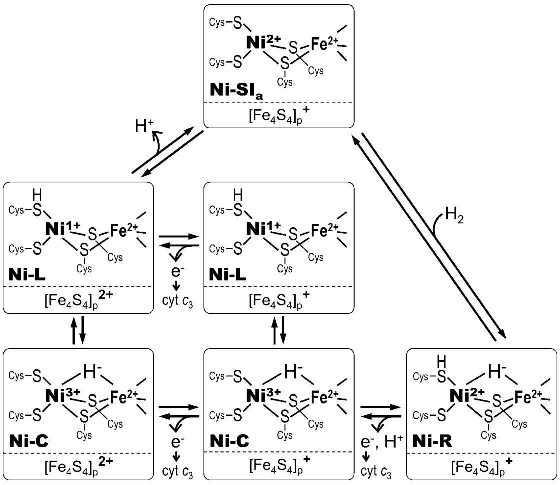


Figure 4. EPR spectra of reduced [NiFe] hydrogenase from *Dv*MF under H_2 (blue) and N_2 (red) atmospheres at 4, 10, 20, and 50 K. The g values of the $[Fe_4S_4]_{p/d}^{+}$ cluster and un-split Ni-C signals are shown in black and red broken lines, respectively. The g values of the split Ni-C signals are shown in cyan (g_x), blue (g_y), and dark blue (g_z) broken lines. Measuring conditions: microwave frequency = 9.645 GHz; microwave power = 2 mT; modulation power = 100 KHz.

Under an N_2 atmosphere, relatively sharp signals at $g_x = 2.197$, $g_y = 2.144$, and $g_z = 2.010$ were detected for the Ni-C state at 20 K, thus indicating the presence of the oxidized $[Fe_4S_4]_p^{2+}$ clusters (Figure 4). At lower temperatures ($T = 4$ and 10 K), both un-split ($g_x = 2.197$, $g_y = 2.144$, and $g_z = 2.010$) and split ($g_x = 2.176$ and 2.218, $g_y = 2.106$ and 2.182, and $g_z = 1.978$, 1.994, 2.034, and 2.057) Ni signals were observed. We confirmed the presence of oxidized $[Fe_4S_4]_p^{2+}$ clusters in the Ni-C state under an N_2 atmosphere by the observation of the un-split signals. According to the intensity of the Ni signals at 4 K, about 15 % of the $[Fe_4S_4]_p^{2+/+}$ clusters in the Ni-C state were oxidized under an N_2 atmosphere. The amount of the oxidized $[Fe_4S_4]_p^{2+}$ clusters corresponded well to the percentage (ca. 14 %) of the Ni-C state converted into the Ni-SI$_a$ state by light irradiation under an N_2 atmosphere at 198 K obtained by IR measurements (Figure 3). At 198 K, the Ni-L state is not trapped and can covert into the Ni-SI$_a$ state (Figure S2). These results indicate that the transition of the Ni-C state to the Ni-SI$_a$ state is related to the redox state of the $[Fe_4S_4]_p^{2+/+}$ cluster. Therefore, the redox state of the proximal $[Fe_4S_4]_p^{2+/+}$ cluster may control the transition of the Ni-C state to the Ni-SI$_a$ state. The transition of the Ni-C state to the Ni-SI$_a$ state produces a proton and an electron (Scheme 1).[1] The produced electron may be transferred to its redox partner cyt c_3 through the proximal $[Fe_4S_4]_p^{2+/+}$ cluster. When the $[Fe_4S_4]_p^{2+/+}$ cluster is reduced ($[Fe_4S_4]_p^{+}$), it may not be able to receive an electron, and thus the transition of the Ni-C state to the Ni-SI$_a$ state is inhibited.

In [NiFe] hydrogenase, H_2 is cleaved heterolytically by transition of the Ni-SI$_a$ state to the fully reduced Ni-R state (Ni^{2+}) with a bridged H^- and reduced $[Fe_4S_4]_p^{+}$ cluster (Scheme 1).[1d,11] By one-electron oxidation of the Ni-R state, the Ni-C state (Ni^{3+}) with a bridged H^- and reduced $[Fe_4S_4]_p^{+}$ cluster is produced,[12] where the electron is transferred to cyt c_3 through the $[Fe_4S_4]_p^{2+/+}$ cluster. A proton is generated by oxidation of H^- at the NiFe active site, thus resulting in the formation of the Ni-L state (Ni^{1+}).[12] The produced proton is transferred to one of the Ni-coordinating, terminal cysteines (Cys546), as proposed by theoretical and



Scheme 1. Proposed catalytic cycle for [NiFe] hydrogenase.

Raman studies.[8e,13] The reaction is terminated at the Ni-L state when the $[Fe_4S_4]_p^{2+/+}$ cluster is reduced ($[Fe_4S_4]_p^{+}$). When the $[Fe_4S_4]_p^{+}$ cluster is oxidized to $[Fe_4S_4]_p^{2+}$, the Ni-L state may be converted into the Ni-SI_a state by electron transfer from the Ni site to the $[Fe_4S_4]_p^{2+}$ cluster with release of the proton from Cys546, thus completing the H_2 cleavage catalytic cycle. In the absence of H_2, the Ni-SI_a state is converted into the Ni-C state through the Ni-L state, when the $[Fe_4S_4]_p^{2+/+}$ cluster is reduced. Kampa et al. proposed that formation of the Ni–Fe bond is essential for generation of the Ni-L state, where the Ni–Fe bond increases the basicity of the active site.[13a]

In conclusion, the Ni-L state of [NiFe] hydrogenase from *Dv*MF is presumably an intermediate between the transition of Ni-C and Ni-SI_a states. The transition occurred only when the $[Fe_4S_4]_p^{2+/+}$ cluster was oxidized and could accept an electron, thus showing that the redox state of the $[Fe_4S_4]_p^{2+/+}$ cluster may control the transition of the Ni-C state to the Ni-SI_a state. These findings provide new insights into the mechanisms of the catalytic cycle of [NiFe] hydrogenases.

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