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Photoactivation of the Ni-SI$_r$ state to Ni-SI$_a$ state in [NiFe] hydrogenase: FT-IR study on the light reactivity of the ready Ni-SI$_r$ state and as-isolated enzyme revisited†

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The Ni-SI$_r$ state of [NiFe] hydrogenase from *Desulfovibrio vulgaris* Miyazaki F was photoactivated to its Ni-SI$_a$ state by Ar$^+$ laser irradiation at 514.5 nm, whereas the Ni-SL state was light induced from a newly identified state, which was less active than any other identified state and existed in the "as-isolated" enzyme.

Introduction

Hydrogenase is a metalloenzyme which catalyzes the reversible H_2 oxidation reaction, $H_2 \rightleftharpoons 2H^+ + 2e^-$.[1-4] According to the active site metal composition, hydrogenases are classified into three types: [NiFe], [FeSe], and [Fe].[4] [NiFe] hydrogenase from *Desulfovibrio vulgaris* Miyazaki F (*Dv*MF) is a membrane-attached enzyme comprising two subunits, one large and one small.[5-7] The large subunit contains the Ni-Fe active site, where the Ni and Fe ions are bridged with two cysteinyl thiolates (Fig. 1). Another two cysteine residues are terminally bound to the Ni ion, whereas one CO and two CN^- ligands are coordinated to the Fe ion.[7-10] The small subunit contains three Fe-S clusters which mediate the electron transfer between the Ni−Fe active site and cytochrome c_3.[11]

Aerobically isolated [NiFe] hydrogenase, herein referred to as "as-isolated", is a mixture of mainly two paramagnetic Ni-A (Ni^{3+}) and Ni-B (Ni^{3+}) states with some other EPR-silent states.[10,12,13] The Ni-B state is readily activated in the presence of H_2 or under electrochemically reducing conditions, while the Ni-A state requires longer time for activation.[14,15] A bridging hydroxo (OH^-) ligand between the Ni and Fe ions has been identified for the Ni-B state (Fig.1).[6,16] For the Ni-A state, the nature of an oxygenic bridging ligand remains contentious,[6,16-21] however, bridging OH^- and cysteine-sulfenate ligands between the Ni and Fe ions have been indicated recently.[22] One electron reduction of the Ni-A and Ni-B states produces EPR-silent unready Ni-SU and ready Ni-SI$_r$ states (Ni^{2+}), respectively.[10,16] The Ni-SI$_r$ state is activated into another EPR-silent Ni-SI$_a$ state (Ni^{2+}) by protonation at the Ni−Fe active site through an acid−base equilibrium, where the Ni-SI$_r$ and Ni-SI$_a$ states represent the deprotonated and protonated states, respectively.[10,12,23] Several mechanisms have been proposed to explain the acid−base equilibrium. In one of them, the bridging OH^- ligand is present in the Ni-SI$_r$ state, and a proton is transferred to the OH^- ligand, which then leaves the active site as a H_2O molecule.[2,12,24,25] In the other proposals, a bridging OH^- ligand may be present, absent, or replaced by a hydride (H^-) or a H_2O molecule in the Ni-SI$_r$ state, and the proton is transferred to one of the terminal Ni-coordinating cysteine-thiolate[26,27] or cysteine-sulfenate[28] ligand that acts as a proton accepting base in the Ni-SI$_r$ state. The acid−base equilibrium between the Ni-SI$_r$ and Ni-SI$_a$ states is a common feature among [NiFe] hyrogenases, and thus the Ni-SI$_r$ state has been identified as a key intermediate for the enzyme activation.[4] Further reduction of the Ni-SI$_a$ state produces a paramagnetic state (Ni-C, Ni^{3+}) and a fully reduced EPR-silent state (Ni-R, Ni^{2+}), where the Ni-SI$_a$, Ni-C, and Ni-R states form a catalytic cycle.[3,4,10]

Light sensitivity of [NiFe] hydrogenase has been reported for various states and utilized to elucidate its catalytic reaction.[29-36] For example, we have reported photo-conversion of the Ni-C state to the Ni-L and Ni-SI$_a$ states for *Dv*MF [NiFe] hydrogenase, and proposed the Ni-L state as an intermediate between the transition of the Ni-C and Ni-SI$_a$ states.[31] The Ni-L state has also been shown to be a catalytic intermediate for [NiFe] hydrogenases from *Pyrococcus furiosus* and *Escherichia coli* by chemical potential jump kinetic and direct electrochemical studies.[37-40] We have also simultaneously detected two Ni-L states (Ni-L2 and Ni-L3) by FT-IR, and proposed that Ni-coordinating Cys546 is deprotonated during the conversion from the Ni-L2 to Ni-L3 state.[32] Furthermore, it has been proposed that the Ni-SI$_r$ state is light sensitive, reversibly forming an EPR-

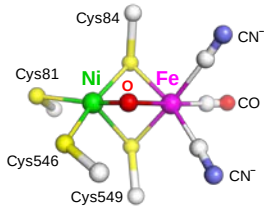

Fig. 1 Active site structure of *Dv*MF [NiFe] hydrogenase in the Ni-B state (PDB: 1WUJ). One CO and two CN^- ligands are assigned as Fe ligands.[7,16,23] Carbon, nitrogen, oxygen, sulphur, nickel, and iron atoms are shown in grey, blue, red, yellow, green, and pink spheres, respectively.

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silent Ni-SL state (Ni^{2+}) at 90−110 K.[36] However, in this work, we found that the Ni-SL state is not light induced from the Ni-SI_r state, but rather the Ni-SI_r state is photo-induced to the Ni-SI_a state.

Experimental

Preparation of [NiFe] hydrogenase

[NiFe] hydrogenase was isolated from sulfate reducing bacterium *Dv*MF, and purified as described previously.[5] The concentration of [NiFe] hydrogenase was adjusted with its absorption at 400 nm using its absorption coefficient ($\varepsilon = 47\ mM^{-1}cm^{-1}$).[11]

FT-IR measurements

[NiFe] hydrogenase (concentration 1.0–2.0 mM) in 25 mM Tris-HCl buffer (pH 7.4 at 298 K) was degassed with a vacuum line, purged with 1 bar of H_2, and incubated at 310 K for 5.5 h (if not mentioned) to obtain the H_2-activated sample. The sample solution was further degassed with the vacuum line and purged with 1 bar of N_2. The Ni-SI_r state was obtained by partial oxidation of the H_2-activated enzyme with an anaerobic addition of 5 equivalents of phenosafranin (Sigma-Aldrich) using a glove box (YSD-800L, UNICO, Tsukuba). The sample solution was transferred anaerobically into an infrared cell with CaF_2 windows in the glove box. FT-IR spectra were measured before, during, and after light irradiation at 103–238 K with a FT-IR spectrometer (FT-IR 6100V, JASCO, Tokyo) equipped with an MCT detector. A cryostat system (CoolSpeK IR USP-203IR-A, Unisoku, Hirakata) was used to control the temperature of the cell. The light irradiation spectra were measured 5–22 min after light-irradiation was started. Light irradiation of the sample was performed at 514.5 nm with an Ar^+ laser (Model 2017, Spectra-Physics, Santa Clara). The laser power was adjusted to 0.5–3.3 W/cm^2 at the sample point. The corresponding buffer spectrum was collected as a reference spectrum and subtracted from the sample spectra. Spectral data were collected at 2-cm^{-1} resolution and averaged from 1024 scans.

Results and discussion

Observation of the light-induced states at low temperatures

It has been reported that the midpoint potential (E_m) for the redox transition between the Ni-B and Ni-SI (Ni-SI_r and Ni-SI_a) states of *Dv*MF [NiFe] hydrogenase is −151 mV at pH 7.4, whereas between the Ni-SI and Ni-C states it is −375 mV.[23] Under N_2 atmosphere, the H_2-activated enzyme contained the Ni-C and Ni-R states for ~70% and ~30%, respectively (See S1, ESI†), with ~90 % of the proximal Fe-S cluster reduced.[29] The Ni-SI_r state was obtained by partial oxidation of the H_2-activated enzyme with an anaerobic addition of 5 equivalents of phenosafranin under N_2 atmosphere, since phenosafranin exhibits its redox potential at $E_m = -252$ mV between −375 and −151 mV.

The CO stretching (ν_{CO}) and CN^- stretching (ν_{CN}) frequencies of the Fe site are reliable sensors for the changes in the electron density of the Fe ion in [NiFe] hydrogenase.[41] Negative IR bands at 1924, 2056, and 2071 cm^{-1} and positive bands at 1943, 2077, and 2089 cm^{-1} were observed in the difference (light-minus-before) FT-IR spectra between the spectra during and before light irradiation by Ar^+ laser (514.5 nm) for phenosafranin-oxidized [NiFe] hydrogenase at 178–238 K under N_2 atmosphere at pH 8.0 (Fig. 2A). The negative and

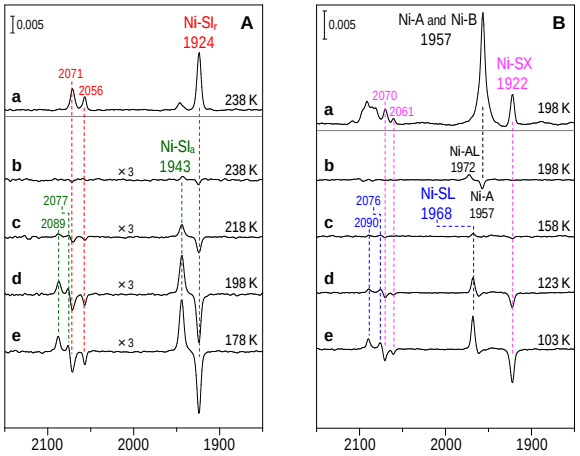

Fig. 2 FT-IR spectra of (A) phenosafranin-oxidized and (B) as-isolated *Dv*MF [NiFe] hydrogenase at 178–238 and 103–198 K, respectively, under N_2 atmosphere at pH 8.0. (a) FT-IR spectra before light irradiation and (b–e) light-minus-before difference spectra between the spectra during and before light irradiation are shown. Phenosafranin-oxidized enzyme was obtained by partial oxidation of the H_2-activated enzyme with an anaerobic addition of 5 equivalents of phenosafranin. The difference spectra of phenosafranin-oxidized enzyme are expanded by three. The laser power was adjusted to 2.5 W/cm^2 at the sample point. The pH value was measured at 274 K.

positive bands were related to the light-sensitive reactant and light-induced product, respectively. The frequency of the negative band at 1924 cm^{-1} corresponded to that of the ν_{CO} band of the Ni-SI_r state of *Dv*MF [NiFe] hydrogenase, whereas 2056 and 2071 cm^{-1} corresponded well to the frequencies of its conjugated ν_{CN} bands.[23] The positive frequencies at 1943, 2077, and 2089 cm^{-1} corresponded well to those of the ν_{CO} and two conjugated ν_{CN} bands of the Ni-SI_a state of the H_2-activated enzyme.[23,31,32] These results showed that the Ni-SI_r state was converted to the Ni-SI_a state by the light irradiation (Fig. 3). Ciaccafava *et al.* have reported that electrochemical activation of an O_2-tolerant [NiFe] hydrogenase from *Aquifex aeolicus* is promoted by UV-vis light irradiation, but the detailed activation mechanism was unspecified.[30] Although the Ni-SI_r state has not been observed by electrochemical FT-IR measurements for O_2-tolerant [NiFe] hydrogenases, the Ni-SI_r state may be highly reactive leading to the fast transition of the Ni-B state to Ni-SI_a state by the light irradiation.[42] Judging from the intensities of the ν_{CO} bands of the Ni-SI_r state in the light-minus-before difference FT-IR spectra, approximately 3% of the Ni-SI_r state was converted to the Ni-SI_a state by the light irradiation at 238 K. The intensities of the ν_{CO} bands of the Ni-SI_r and Ni-SI_a states increased in the light-minus-before difference spectra with a decrease in the temperature, and approximately 34% of the Ni-SI_r state was converted to the Ni-SI_a state at 178 K. Notably, the light-induced conversion of the Ni-SI_r state decreased significantly at pH 9.6 (See S2, ESI†), indicating that protonation occurred in the photo-activation process. The reported photochemical reactions in various [NiFe] hydrogenases are usually associated with dissociation of non-protein ligands bound to the metal ions at the Ni−Fe active site.[13,29-36,43] Stronger laser power was required for photo-activation of the Ni-SI_r state to the Ni-SI_a state compared to photo-activation of the Ni-C state to the NI-L state associated with dissociation of the bridging H^-.[31] Considering these results, we propose that the protonation of the Ni-SI_r state is related to dissociation of the putative bridging OH^- ligand as a H_2O molecule by the light irradiation, although the possibility of the Ni-

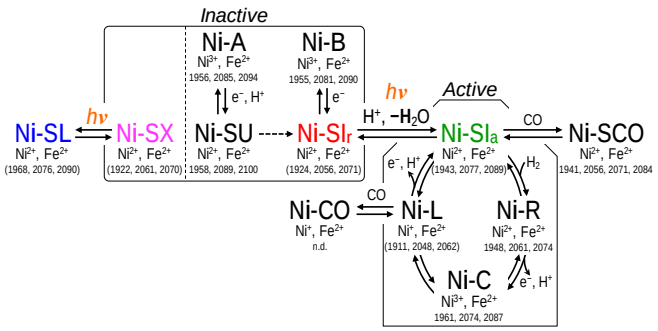

Fig. 3 Reaction states of [NiFe] hydrogenase. The ν_{CO} and ν_{CN} frequencies for each state of *Dv*MF [NiFe] hydrogenase are depicted.[13] The frequencies at 198 and 298 K are shown with and without parentheses, respectively.

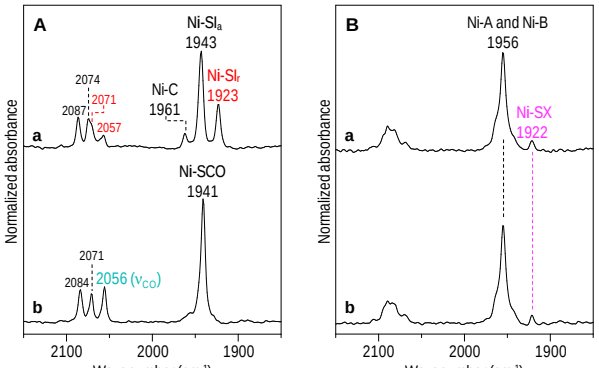

Fig. 4 FT-IR spectra of (A) phenosafranin-oxidized and (B) as-isolated *Dv*MF [NiFe] hydrogenase under (a) N_2 and (b) CO atmospheres at pH 7.4 and 298 K.

coordinating cysteine-thiolate or cysteine-sulfenate ligand being protonated after the light irradiation cannot be excluded.[26-28]

The light-induced FT-IR spectrum converted back immediately to the initial spectrum when the light irradiation was stopped at 218 and 238 K, and no ν_{CO} or ν_{CN} band was observed in the difference (after-minus-before) FT-IR spectra between the spectra after and before light irradiation (See S3, ESI†). However, the ν_{CO} and ν_{CN} bands of the Ni-SI_a state were observed in the after-minus-before difference spectrum at 198 K and the intensities in the difference spectrum increased at 178 K, indicating that the light-induced Ni-SI_a state was trapped at low temperatures. Interestingly, the relative intensities of the ν_{CO} bands of the Ni-SI_r and Ni-SI_a states in the light-minus-before difference spectra at 103 K depended on the irradiation time and light intensity (See S4, ESI†). The Ni-SI_r state photo-converted to the Ni-SI_a state for only ~50 % after ~40 min of light irradiation with the laser power of 3.3 W/cm^2 at 103 K and pH 8.0, indicating that the photo-conversion process of the Ni-SI_r state to the Ni-SI_a state was relatively slow. However, the photo-conversion rate of the Ni-SI_r state did not change significantly at pH 7.0 compared to that at pH 8.0, although the relative intensity of the Ni-SI_r ν_{CO} band before light irradiation decreased.

A negative IR band at 1957 cm^{-1} and a positive band at 1972 cm^{-1} were observed in the light-minus-before difference FT-IR spectrum of as-isolated *Dv*MF [NiFe] hydrogenase under N_2 atmosphere at pH 8.0 and 198 K (Fig. 2B). The negative and positive bands corresponded well to the ν_{CO} bands of the Ni-A and light-induced Ni-AL states, respectively.[29,36] Additionally, negative IR bands at 1922, 2061, and 2070 cm^{-1} and positive bands at 1968, 2076, and 2090 cm^{-1} were observed in the light-minus-before difference spectra at 103–158 K. The frequencies of the positive bands were similar to the ν_{CO} and ν_{CN} frequencies of the light-induced Ni-SL state obtained from as-isolated *Dv*MF [NiFe] hydrogenase,[36] indicating formation of the Ni-SL state. However, the frequencies of the negative bands (ν_{CO}: 1922 cm^{-1}, ν_{CN}: 2061 and 2070 cm^{-1}) were 1–5 cm^{-1} shifted from those of the ν_{CO} and two conjugated ν_{CN} bands of the Ni-SI_r state (ν_{CO}: 1924 cm^{-1}, ν_{CN}: 2056 and 2071 cm^{-1}) (Fig. 2).[23] These frequency differences indicate that the light-reacted state other than the Ni-SI_r state converted to the Ni-SL state. We term this newly identified light-reacted state, Ni-SX (Fig. 3). Previously, spectroscopic studies on the as-isolated *Dv*MF [NiFe] hydrogenase have determined a third bridging ligand between the Ni and Fe ions, but misassigned the state to the Ni-SI_r state.[36] The Ni-SX state corresponds to this previously studied state. The intensities of the ν_{CO} and ν_{CN} bands of the Ni-SX and Ni-SL states in the difference (light-minus-after) spectra between the spectra during and after light irradiation increased with decrease in the temperature from 158 K to 103 K, indicating reversibility for the formation of the Ni-SL state by the light irradiation (See S5, ESI†).

Differences between the Ni-SI_r and Ni-SX states in pH sensitivity and carbon monoxide reactivity

The acid−base equilibrium between the Ni-SI_r and Ni-SI_a states is important for the activation of [NiFe] hydorogenase (Fig. 3).[4] In the FT-IR spectrum of phenosafranin-oxidized [NiFe] hydrogenase under N_2 atmosphere at pH 7.4 and 298 K, the ν_{CO} bands of the Ni-SI_r (1923 cm^{-1}; corresponding to the 1924-cm^{-1} band at 178–238 K in Fig. 2A) and Ni-SI_a (1943 cm^{-1}) states were the major ν_{CO} bands, whereas a weak ν_{CO} band (1961 cm^{-1}) corresponding to the Ni-C state was also detectable (See S6A, ESI†). The pH-dependence of the ratio between the Ni-SI_a and Ni-SI_r ν_{CO} band intensities revealed that the Ni-SI_a and Ni-SI_r states form an acid−base equilibrium.[10,12,23] In the spectrum of as-isolated [NiFe] hydrogenase, the ν_{CO} bands corresponding to the Ni-A (1956 cm^{-1}), Ni-B (1955 cm^{-1}), and Ni-SX (1922 cm^{-1}) states were observed,[13,23,36] but the intensities of these bands did not change with a change in pH (See S6B, ESI†), showing that no acid−base equilibrium existed for the Ni-SX state. These results support the hypothesis that the Ni-SX state is different from the Ni-SI_r state and indicate that the Ni-SX state is not a ready state.

Carbon monoxide (CO) is known as a reversible inhibitor for [NiFe] hydrogenases from earlier enzymatic studies.[44] X-ray crystallographic experiments have demonstrated that exogenous CO coordinates to the active site Ni ion of *Dv*MF [NiFe] hydrogenase.[43] For the FT-IR spectrum of phenosafranin-oxidized [NiFe] hydrogenase in CO-saturated buffer, IR bands were observed mainly at 1941, 2056, 2071, and 2084 cm^{-1} (Fig. 4A). The bands at 1941, 2071 and 2084 cm^{-1} correspond well to the ν_{CO} and two conjugated ν_{CN} bands of the Ni-SCO state (exogenous CO-bound state), whereas the band at 2056 cm^{-1} corresponds well to that of the exogenous CO bound to the Ni ion, revealing that most of the enzyme molecules were in the Ni-SCO state.[13] Previous spectroscopic studies have showed that CO reacts selectively with the Ni-SI_a and Ni-L states.[13,36,43-45] These results indicate that the Ni-SI_r state was converted into the Ni-SCO state under CO atmosphere apparently through the acid−base equilibrium (Fig. 3). On the other hand, no change was observed in the FT-IR spectrum of as-isolated [NiFe] hydrogenase by introduction of CO, indicating that the Ni-SX state

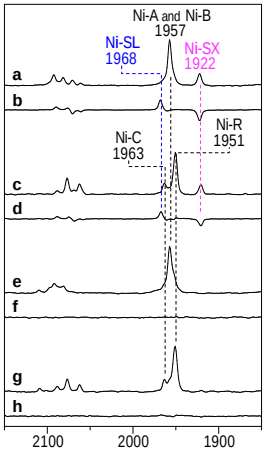

Fig. 5 FT-IR spectra of *Dv*MF [NiFe] hydrogenase under N_2 atmosphere at pH 8.0 and 103 K: (a,c,e,g) Spectra before light irradiation and (b,d,f,h) light-minus-after difference spectra. The spectra of the as-isolated enzyme in the (a,b) absence and (c,d) presence of 10 equivalents of dithionite and the spectra of the air-oxidized enzyme (obtained by exposure of the H_2-activated enzyme to air) in the (e,f) absence and (g,h) presence of 10 equivalents of dithionite are shown. The "after" spectra were measured 30–47 min after light irradiation was stopped. The laser power was adjusted to 2.5 W/cm^2 at the sample point. The pH value was measured at 274 K.

was unreactive toward CO (Fig. 4B), in agreement with the results that an acid−base equilibrium did not exist for the Ni-SX state (See S6B, ESI†).

Reaction of the Ni-SX state with dithionite

To gain information on the activation of the Ni-SX state, light-minus-after difference FT-IR spectra were measured for as-isolated and dithionite-reduced *Dv*MF [NiFe] hydrogenase at 103 K (Fig. 5). The photo-conversion of the Ni-SX state to the Ni-SL state was successfully observed in the light-minus-after difference spectra, because the Ni-SL state converted back to the Ni-SX state (See S5, ESI†) but the light-induced Ni-SI_a and Ni-L states were trapped at 103 K when the light irradiation was stopped (See S7, ESI†).[31] By assuming equal absorption coefficients for the ν_{CO} bands, approximately 16% was in the Ni-SX state for the as-isolated enzyme (Fig. 5, curve a), and almost (~95%) all the enzymes in the Ni-SX state converted to the Ni-SL state by the light irradiation at 103K (Fig. 5, curve b). However, approximately 10% of the enzyme was still in the Ni-SX state after reduction with dithionite (Fig. 5, curves c and d), indicating that the Ni-SX state was very inactive. For [NiFe] hydrogenases from the sulphur-metabolizing bacterium *Allochromatium vinosum* and *Desulfovibrio fructosorans* (*Df*), an inactive state (Ni-'S_{ox}') similar to the Ni-SX state has been reported.[12,22] The ν_{CO} and ν_{CN} frequencies of the Ni-'S_{ox}' state for *Df* [NiFe] hydrogenase (ν_{CO}: 1911, ν_{CN}: 2059 and 2068 cm^{-1}) were 1–5 cm^{-1} shifted from those of its Ni-SI_r state (ν_{CO}: 1913 cm^{-1}, ν_{CN}: 2054 and 2069 cm^{-1}),[22,46] which was very similar to those of the Ni-SX state detected in the present study (Fig. 2B). The Ni-'S_{ox}' state has been proposed by X-ray crystallographic analysis to possess a cysteine-persulfide terminal Ni-coordinating ligand at the active site.[22] The required reduction of the persulfide bond may explain the observed slow activation of the Ni-SX state.

As-isolated *Dv*MF [NiFe] hydrogenase contained the Ni-SX state exhibiting a ν_{CO} band at 1922 cm^{-1} and two ν_{CN} bands at 2061 and 2070 cm^{-1}.[13,36] The intensities of these bands decreased exponentially with a time constant of ~50 min by incubation under H_2 atmosphere at 310 K (See Figs. S8 and S9, ESI†), revealing that the Ni-SX state was activated very slowly under H_2 atmosphere. However, the Ni-SX state was not observed for the air-oxidized enzyme, where the H_2-activated enzyme was exposed to air (Fig. 5, curves e and f). Additionally, the Ni-SX state was not observed after further dithionite reduction of the air-oxidized enzyme (Fig. 5, curves g and h). These results reveal that although the as-isolated enzyme contained the Ni-SX state, the Ni-SX state was not formed during the generation or activation of the Ni-A and Ni-B states in vitro.

Conclusions

We have shown for the first time that the ready Ni-SI_r state of *Dv*MF [NiFe] hydrogenase is converted to the active Ni-SI_a state by laser light irradiation at 514.5 nm (Fig. 3). From the pH-dependent light-reactivity of the Ni-SI_r state, we propose that the bridging OH^- ligand dissociates as a H_2O molecule from the Ni−Fe active site by light irradiation at low pH. We have identified a light-sensitive Ni-SX state (ν_{CO}, 1922 cm^{-1}; ν_{CN}, 2061 and 2070 cm^{-1}), which was photo-converted to the Ni-SL state. A certain amount of the enzyme was still in the Ni-SX state after treatment of the as-isolated enzyme with dithionite, although the enzyme was activated slowly by H_2, revealing that the Ni-SX state was highly inactive. These findings provide new insights into the activation mechanism of [NiFe] hydrogenase.

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