The Ni-Si state of [NiFe] hydrogenase from Desulfovibrio vulgaris Miyazaki F was photoactivated to its Ni-Si state by Ar⁺ laser irradiation at 514.5 nm, whereas the Ni-Si 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 H₂ oxidation reaction, H₂ ⇌ 2H⁺ + 2e⁻. According to the active site metal composition, hydrogenases are classified into three types: [NiFe], [FeFe], and [Fe]. [NiFe] hydrogenases, and thus the Ni-Si state has been identified as a key intermediate for the enzyme activation. Further reduction of the Ni-Si state produces a paramagnetic state (Ni-C, Ni⁴⁺) and a fully reduced EPR-silent state (Ni-R, Ni⁴⁺), where the Ni-Si, Ni-C, and Ni-R states form a catalytic cycle. Light sensitivity of [NiFe] hydrogenase has been reported for various states and utilized to elucidate its catalytic reaction. For example, we have reported photo-conversion of the Ni-C state to the Ni-L and Ni-Si states for DvMF [NiFe] hydrogenase, and proposed the Ni-L state as an intermediate between the transition of the Ni-C and Ni-Si states. 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.

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 state, and the proton is transferred to the OH⁻ ligand, which then leaves the active site as a H₂O molecule. In the other proposals, a bridging OH⁻ ligand may be present, absent, or replaced by a hydride (H⁻) or a H₂O molecule in the Ni-Si state, and the proton is transferred to one of the terminal Ni-coordinating cysteine-thiolate or cysteine-sulfenate ligand that acts as a proton accepting base in the Ni-Si state. The acid–base equilibrium between the Ni-Si and Ni-Sir states is a common feature among [NiFe] hyrogenses, and thus the Ni-Si state has been identified as a key intermediate for the enzyme activation.

Aerobically isolated [NiFe] hydrogenase, herein referred to as “as-isolated” or “isolated”, is a mixture of mainly two paramagnetic Ni-A (Ni³⁺) and Ni-B (Ni³⁺) states with some other EPR-silent states. The Ni-B state is readily activated in the presence of H₂ or under electrochemically reducing conditions, while the Ni-A state requires longer time for activation. A bridging hydroxide (OH⁻) ligand between the Ni and Fe ions has been identified for the Ni-B state (Fig. 1). For the Ni-A state, the nature of an oxygenic bridging ligand remains contentious, however, bridging OH⁻ and cysteine-sulfenate ligands between the Ni and Fe ions have been indicated recently. One electron reduction of the Ni-A and Ni-B states produces EPR-silent unordered Ni-SU and ready Ni-Si states (Ni²⁺), respectively. The Ni-Si state is activated into another EPR-silent Ni-Sir state (Ni²⁺) by protonation at the Ni-Fe active site through an acid–base equilibrium, where the Ni-Si, and Ni-Sir states represent the deprotonated and protonated states, respectively. 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 state, and a proton is transferred to the OH⁻ ligand, which then leaves the active site as a H₂O molecule. In the other proposals, a bridging OH⁻ ligand may be present, absent, or replaced by a hydride (H⁻) or a H₂O molecule in the Ni-Si state, and the proton is transferred to one of the terminal Ni-coordinating cysteine-thiolate or cysteine-sulfenate ligand that acts as a proton accepting base in the Ni-Si state. The acid–base equilibrium between the Ni-Si and Ni-Sir states is a common feature among [NiFe] hyrogenses, and thus the Ni-Si state has been identified as a key intermediate for the enzyme activation. Further reduction of the Ni-Si state produces a paramagnetic state (Ni-C, Ni⁴⁺) and a fully reduced EPR-silent state (Ni-R, Ni⁴⁺), where the Ni-Si, Ni-C, and Ni-R states form a catalytic cycle.

Light sensitivity of [NiFe] hydrogenase has been reported for various states and utilized to elucidate its catalytic reaction. For example, we have reported photo-conversion of the Ni-C state to the Ni-L and Ni-Si states for DvMF [NiFe] hydrogenase, and proposed the Ni-L state as an intermediate between the transition of the Ni-C and Ni-Si states. 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. 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. Furthermore, it has been proposed that the Ni-Si state is light sensitive, reversibly forming an EPR-

**Fig. 1** Active site structure of DvMF [NiFe] hydrogenase in the Ni-B state (PDB: 1WUJ). One CO and two CN⁻ ligands are assigned as Fe ligands. Carbon, nitrogen, oxygen, sulphur, nickel, and iron atoms are shown in grey, blue, red, yellow, green, and pink spheres, respectively.
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-SIr state, but rather the Ni-SIr state is photo-induced to the Ni-SIr state.

**Experimental**

**Preparation of [NiFe] hydrogenase**

[NiFe] hydrogenase was isolated from sulfate reducing bacterium *DvMF*, and purified as described previously.\(^5\) The concentration of [NiFe] hydrogenase was adjusted with its absorption at 400 nm using its absorption coefficient (\(\varepsilon = 47 \text{ mM}^{-1} \text{cm}^{-1}\)).\(^{31}\)

**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, 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 - the cell. The light irradiation spectra were measured 5–22 min after 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-SIr and Ni-SL) states of *DvMF* [NiFe] hydrogenase is ~151 mV at pH 7.4, whereas between the Ni-SI and Ni-C states it is ~375 mV.\(^{31}\) 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 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 −375 and −151 mV, respectively (See S1, ESI†), with ~90 % of the proximal 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 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 v\(_{\text{CO}}\) band of the Ni-SIr state of *DvMF* [NiFe] hydrogenase, whereas 2056 and 2071 cm\(^{-1}\) corresponded well to the frequencies of its conjugated v\(_{\text{CN}}\) bands.\(^{23}\) The positive frequencies at 1943, 2077, and 2089 cm\(^{-1}\) corresponded well to those of the v\(_{\text{CO}}\) and two conjugated v\(_{\text{CN}}\) bands of the Ni-SL state of the H\(_2\)-activated enzyme.\(^{23,31,32}\) These results reveal that the Ni-SI, state sonnets to the Ni-SIr state by the light irradiation (Fig. 3). Ciaccafa 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-SL state has not been observed by electrochemical FT-IR measurements for O\(_2\)-tolerant [NiFe] hydrogenases, the Ni-SI state may be highly reactive leading to the fast transition of the Ni-B state to Ni-SI state by the light irradiation.\(^{42}\) Judging from the intensities of the v\(_{\text{CO}}\) bands of the Ni-SI state in the light-minus-before difference FT-IR spectra, approximately 3% of the Ni-SI state was converted to the Ni-SL state by the light irradiation at 238 K. The intensities of the v\(_{\text{CO}}\) bands of the Ni-SI, and Ni-SL states increased in the light-minus-before difference spectra with a decrease in the temperature, and approximately 34% of the Ni-SL state was converted to the Ni-SI state at 178 K. Notably, the light-induced conversion of the Ni-SL state decreased significantly at pH 9.6 (See S2, ESI†), indicating that protonation occurred in the photoactivation 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 photoactivation of the Ni-SL state to the Ni-SI state compared to photoactivation 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-SL state is related to dissociation of the putative bridging OH\(^-\) ligand as a H\(_2\)O molecule by the light irradiation, although the possibility of the Ni
The light-induced FT-IR spectrum converted back immediately to the initial spectrum when the light irradiation was stopped at 218 and 298 K, indicating reversibility for the formation of the Ni-SL state by the light irradiation (See S5, ESI†).

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

The acid–base equilibrium between the Ni-SL, and Ni-SX states is important for the activation of [NiFe] hydrogenase (Fig. 3). In the FT-IR spectrum of phenosafranin-oxidized [NiFe] hydrogenase under N₂ atmosphere at pH 7.4 and 298 K, the υCO bands of the Ni-SL (1923 cm⁻¹) and Ni-SX (1941 cm⁻¹) states were major υCO bands, whereas a weak υCO 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-SL, and Ni-SX υCO bands intensities revealed that the Ni-SL, and Ni-SX states form an acid–base equilibrium. In the spectrum of as-isolated [NiFe] hydrogenase, the υCO bands corresponding to the Ni-A (1956 cm⁻¹), Ni-B (1955 cm⁻¹), and Ni-SX (1922 cm⁻¹) states were observed, 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-SL 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. X-ray crystallographic experiments have demonstrated that exogenous CO coordinates to the active site Ni ion of DvMF [NiFe] hydrogenase. 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 υCO and two conjugated υCN 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. Previous spectroscopic studies have showed that CO reacts selectively with the Ni-SL and Ni-L...
states. These results indicate that the Ni-SIr state was converted into the Ni-SO 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 was inactive state (Ni-SOx') similar to the Ni-SX state has been proposed by X-ray crystallographic analysis to possess a state (Ni-SIr) and 2069 cm\(^{-1}\)) were 1–5 cm\(^{-1}\) shifted from those of its Ni-SX state (ν\(_{\text{CO}}\): 1913 cm\(^{-1}\); ν\(_{\text{CN}}\): 2059 and 2069 cm\(^{-1}\)). 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-SIr state of DyMF [NiFe] hydrogenase is converted to the active Ni-SIr state by laser light irradiation at 514.5 nm (Fig. 3). From the pH-dependent light-reactivity of the Ni-SIr state, we propose that the bridging OH ligand dissociates as a H\(_2\)O molecule from the Ni-Fe active site by light irradiation at low pH. We have identified a light-sensitive Ni-SX state (ν\(_{\text{CO}}\): 1922 cm\(^{-1}\); ν\(_{\text{CN}}\): 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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References
Supplementary Information

Photoactivation of the Ni-SIr state to Ni-SIa state in [NiFe] hydrogenase: FT-IR study on the light reactivity of the ready Ni-SIr state and as-isolated enzyme revisited

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**Fig. S9** $\text{H}_2$ activation kinetics of the Ni-SX state at 310 K. Intensities of the Ni-SX $\nu_{\text{CO}}$ band were calculated from Figures S7 and S8. The intensity of the $\nu_{\text{CO}}$ band decreased exponentially with a time constant of ~50 min.