METHODS AND APPLICATIONS

New assay method based on Raman spectroscopy for enzymes reacting with gaseous substrates

Yuka Kawahara-Nakagawa,[1†] Koji Nishikawa,[1,2†] Satoru Nakashima,[3] Shota Inoue,[1] Takehiro Ohta,[4] Takashi Ogura,[1‡] Yasuteru Shigeta,[5] Katsuyuki Fukutani,[6] Tatsuhiko Yagi,[7] and Yoshiki Higuchi[1,2]*

[1]Department of Picobiology, Graduate School of Life Science, University of Hyogo, Hyogo, 678-1297, Japan
[2]Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency (JST), Kawaguchi Center Building, Saitama, 332-0012, Japan
[3]Division for Research Strategy, Institute for Research Initiatives, Nara Institute of Science and Technology, Nara, 630-0192, Japan
[4]Department of Applied Chemistry, Faculty of Engineering, Sanyo-Onoda City University, Yamaguchi, 756-0884, Japan
[5]Division of Life Science, Center for Computational Science, University of Tsukuba, Ibaraki, 305-8571, Japan
[6]Department of Fundamental Engineering, Institute of Industrial Science, The University of Tokyo, Tokyo, 153-8505, Japan
[7]Shizuoka University, Shizuoka, 422-8529, Japan

Received 8 November 2018; Accepted 31 December 2018
DOI: 10.1002/pro.3569
Published online 16 January 2019 proteinscience.org

Abstract: **Enzyme activity is typically assayed by quantitatively measuring the initial and final concentrations of the substrates and/or products over a defined time period. For enzymatic reactions involving gaseous substrates, the substrate concentrations can be estimated either directly by gas chromatography or mass spectrometry, or indirectly by absorption spectroscopy, if the catalytic reactions involve electron transfer with electron mediators that exhibit redox-dependent spectral changes. We have developed a new assay system for measuring the time course of enzymatic reactions involving gaseous substrates based on Raman spectroscopy. This system permits continuous monitoring of the gas composition in the reaction cuvette in a non-invasive manner over a prolonged time period.**

Abbreviations: DvMF, *Desulfovibrio vulgaris* Miyazaki F.

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: Japan Science and Technology Agency CREST, JPMJCR12M4; Grant sponsor: Japan Society for the Promotion of Science 24657077 (Challenging Exploratory Research) 25291038 (Scientific Research (B)) JP18J11420 (JSPS Fellows); Grant sponsor: Ministry of Education, Culture, Sports, Science and Technology 18H05516 (Scientific Research on Innovative Area, Hydrogenomics) 18H05518 (Scientific Research on Innovative Area, Hydrogenomics).

*Correspondence to: Yoshiki Higuchi, Graduate School of Life Science, University of Hyogo, 3-2-1 Koto, Kamigori-cho, Ako-gun, Hyogo, 678-1297, Japan. E-mail: hig@sci.u-hyogo.ac.jp

†Yuka Kawahara-Nakagawa and Koji Nishikawa contributed equally to this work.

‡Deceased July 23, 2017

We have applied this system to the kinetic study of the [NiFe] hydrogenase from *Desulfovibrio vulgaris* Miyazaki F. This enzyme physiologically catalyzes the reversible oxidation of H_2 and also possesses the nonphysiological functions of H/D exchange and nuclear spin isomer conversion reactions. The proposed system has the additional advantage of enabling us to measure all of the hydrogenase-mediated reactions simultaneously. Using the proposed system, we confirmed that H_2 (the fully exchanged product) is concomitantly produced alongside HD by the H/D exchange reaction in the D_2/H_2O system. Based on a kinetic model, the ratio of the rate constants of the H/D exchange reaction (k) at the active site and product release rate (k_{out}) was estimated to be 1.9 $\pm$ 0.2. The proposed assay method based on Raman spectroscopy can be applied to the investigation of other enzymes involving gaseous substrates.

Keywords: Raman spectroscopy; H/D exchange reaction; [NiFe] hydrogenase; kinetic experiment; quantitative analysis of gaseous substrates; non-invasive measurement

Introduction

Understanding the detailed mechanisms of catalytic reactions mediated by enzymes is fundamental for developing new industrial biocatalysts and applying them to biomimetic processes. Enzymatic reaction mechanisms have been studied using biochemical and spectroscopic techniques, and structural information of the active site has been fundamentally important. The experimental data are typically analyzed based on simple kinetic models focusing on the chemical reaction occurring at the active site.[1,2] However, the overall catalytic performance of an enzyme depends on not only the reaction occurring at the active site but also the protein system, which regulates the transfer of the substrates and products as well as that of electrons. Consequently, the protein system contributes to the suppression of side reactions. For enzymatic reactions involving gaseous substrates, the gas channel through which the substrate molecules pass from the enzyme surface to the active site and the release route of the products to the reaction medium are important for high catalytic performance. Hydrogenase, which catalyzes the reversible conversion of molecular hydrogen (H_2) to protons (H^+) and electrons (e^-), is one example of enzymes that react with gaseous substrates.[3] As hydrogenase is a potential candidate for a biofuel cell catalyst, understanding its catalytic mechanism is desirable for developing more active and inexpensive catalysts. In addition to the physiological reaction, namely, the reversible oxidation of H_2, hydrogenases catalyze two reactions *in vitro* without electron transfer to the mediators. These are the H/D exchange reaction between $H^+(H_2O)/D^+(D_2O)$ and D_2/H_2 and the nuclear spin isomer conversion reaction between para-H_2 and ortho-H_2.[4–8] The former reaction consists of the transfer of the gaseous substrate via the gas channel followed by exchange between H^+ and D^+ (which requires the transfer of H^+/D^+ via the proton pathway) after the cleavage of the covalent bond of H_2, and subsequent re-formation of the bond at the active site. The hydrogenase-mediated H/D exchange reaction is conventionally measured by gas chromatography, mass spectrometry, or a combination thereof (GC/MS).[8,9] Although these methods are well established, they require the use of cumbersome procedures to determine the composition of the gas phase in the reaction cuvette. Moreover, sampling the gas from the cuvette at regular intervals, which is necessary to monitor the reaction, changes the pressure of the gas phase after each operation. Alternatively, the gas composition can be measured *in situ* using Raman spectroscopy without the need to remove sample material from the reaction cuvette. This means that Raman spectroscopy enables us to trace the time course of the enzymatic activity without requiring any data corrections owing to the change in gas pressure inside the cuvette. Raman spectroscopy has the additional advantage of permitting the continuous and simultaneous measurement of the changes in the ratios of isotopes and nuclear spin isomers. Despite the great potential of Raman spectroscopy, there have been a few reports of the application of this technique for monitoring enzymatic reactions[10] and no reports involving enzymatic reactions of gaseous substrates to the best of our knowledge. In this study, we developed a new Raman-spectroscopy-based assay system for measuring the enzymatic activity toward gaseous substrates and successfully measured the H/D exchange reaction catalyzed by the [NiFe] hydrogenase from *Desulfovibrio vulgaris* Miyazaki F (DvMF).

Results

Continuous monitoring of the catalytic reaction by Raman spectroscopy

In the system shown in Figure 1(A), only the gas phase in the reaction cuvette is illuminated by the excitation light, such that the enzyme in the solution phase is not damaged or otherwise affected. As there is no need to withdraw aliquots of gas from the reaction cuvette to measure the Raman spectra, the reaction conditions such as pressure and temperature can be kept constant. In fact, Raman intensity of D_2/H_2 (100 kPa) in the reaction cuvette did not change at all for over 1 day. It is

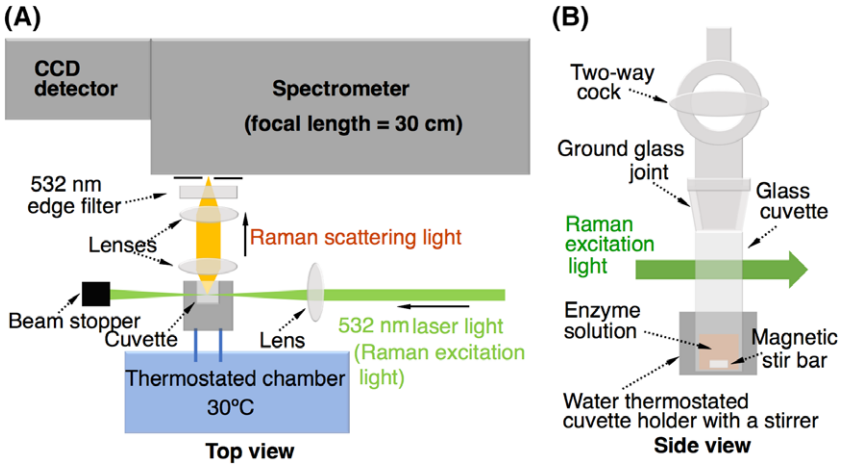

Figure 1. Experimental setup. (A) Schematic illustration of the Raman-spectroscopy-based assay system. (B) Custom-built reaction cuvette for anaerobic measurements of the reaction. The glass cuvette possesses four transparent surfaces for Raman measurements.

common practice to add a small amount of dithionite to remove residual O_2 from the assay system of hydrogenases.[8,11,12] In this study, however, the reaction mixture was anaerobically prepared in the custom-built reaction cuvette shown in Figure 1(B) inside a glovebox and no reductants are required (see Materials and Methods). The highest peaks in the rotational–vibrational Raman spectrum of D_2, HD, and H_2 were located at 2987, 3628, and 4155 cm^{-1}, respectively, as shown in Figure 2.[13] All of the peaks originating from the sample gases were

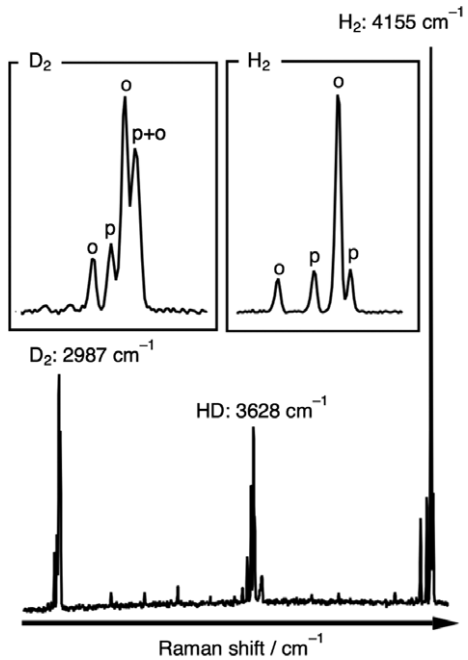

Figure 2. Typical rotational–vibrational spectrum of the gaseous molecular hydrogen species (D_2, HD, and H_2) measured using the Raman-spectroscopy-based assay system. The indicated wavenumbers are for the highest peaks of the individual isotopic hydrogen molecules. The characters p and o indicate the *para* and *ortho* states, respectively. The upper two boxes show the enlarged views of D_2 and H_2.

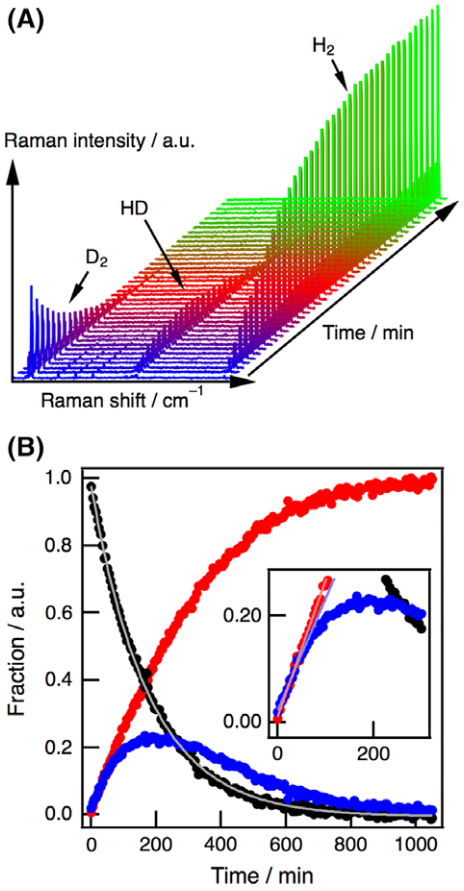

Figure 3. H/D exchange reaction in the D_2/H_2O system catalyzed by [NiFe] hydrogenase from *Desulfovibrio vulgaris* Miyazaki F. (A) Waterfall plot of the Raman spectra obtained at 25-min intervals during the H/D exchange reaction in the D_2/H_2O system. (B) Time course of the isotopic composition of molecular hydrogen. The black, blue, and red markers represent the fractions of D_2, HD, and H_2, respectively. The gray line over the black markers represents the fitting curve for the exponential decay of D_2. The pale blue and pale red lines in the inset represent the fitting curves for the initial reaction rates of HD and H_2 production, respectively.

observed simultaneously in a single spectrum. The resolution of the system was sufficient for discrimination of the rotational states (spin isomers); five peaks corresponding to the possible isotopic/isomeric species (*para*-D_2, *ortho*-D_2, HD, *para*-H_2, and *ortho*-H_2) could be clearly distinguished as labeled in Figure 2.

H/D exchange reaction in the D_2/H_2O system

Figure 3(A) shows the time course of the Raman peak intensities of D_2, HD, and H_2 during the H/D exchange reaction in the D_2/H_2O system catalyzed by the [NiFe] hydrogenase from DvMF (10 μM) at 30°C. During the course of the reaction, the Raman peak intensities of D_2 and H_2 exhibited a simple decay and rise, respectively. In contrast, the Raman peak intensity of HD increased over the period from 0 to 200 min, began to decay after 200 min, and then finally disappeared at 1000 min. The amounts of D_2, HD, and H_2 were estimated by calculating the area intensities of the corresponding Raman bands, which were normalized using standard gases (see Materials and Methods). As the total number of gas molecules in the reaction cuvette during the H/D exchange reaction was kept unchanged, each of the gaseous components is hereafter represented as a molar fraction. As shown in Figure 3(B), the time course data obtained for the H/D exchange reaction were sufficiently accurate to permit precise evaluation of the kinetics of our system. The time course of the D_2 fraction revealed that this component underwent exponential decay, where the time constant (pseudo–first-order rate constant; τ_D) at the enzyme concentration of 10 μM was 0.0050 ± 0.0004 min^{-1}. The dependency of enzyme concentration was observed in τ_D at the range of 2–20 μM in Figure 4. As no accelerated features were observed on the H/D exchange reaction at the initial stage of the reactions [Fig. 3(B)], the enzyme samples used in this study have a full activity from the beginning of the measurements.

In our developed system, the reaction mixture could not be stirred vigorously (see Materials and Methods). In addition, this measurement setup resulted in the large volume of the gas phase compared to the liquid phase. For these reasons, we had to use the concentrated enzyme solutions (2, 5, 10, and 20 μM) to measure the time course of the H/D exchange reaction in an appropriate period of time. The H/D exchange activities [2–6 mol (D_2) s^{-1} (mol enzyme)$^{-1}$] calculated from the initial rates of D_2 consumption for the concentrated enzyme solutions (Supporting Information Table S1) were considerably lower than those estimated from the previous reports[8,14] [about 120 mol s^{-1} (mol enzyme)$^{-1}$]. As the dependency of the H/D exchange activity on the enzyme concentration was reported before,[8] we performed experiments with the diluted enzyme solutions. The activity estimated from 20 nM enzyme showed comparable value (47 mol s^{-1} [mol enzyme]$^{-1}$) as shown in Supporting Information Table S1, if the large differences of the experimental conditions between two methods are taken into account (see Materials and Methods). To investigate the characteristics of the reaction system, τ_D at various volumes of enzyme solutions were calculated (Supporting Information Table S2).

The initial production rate of HD (v_1, 0.0018 ± 0.0002 min^{-1} at the enzyme concentration of 10 μM) was almost equal to that of H_2 (v_2, 0.0022 ± 0.0003 min^{-1}). These results could not be adequately analyzed using the simple stepwise reaction mechanism shown in Equations 1 and 2, indicating that not only the single exchange reactions (e.g., $D_2 + H^+ \rightarrow HD + D^+$) but also the double exchange reaction (i.e., $D_2 + 2H^+ \rightarrow H_2 + 2D^+$) occurred macroscopically in the reaction cuvette.

$$D_2 + H^+ \rightarrow HD + D^+ \quad (1)$$

$$HD + H^+ \rightarrow H_2 + D^+ \quad (2)$$

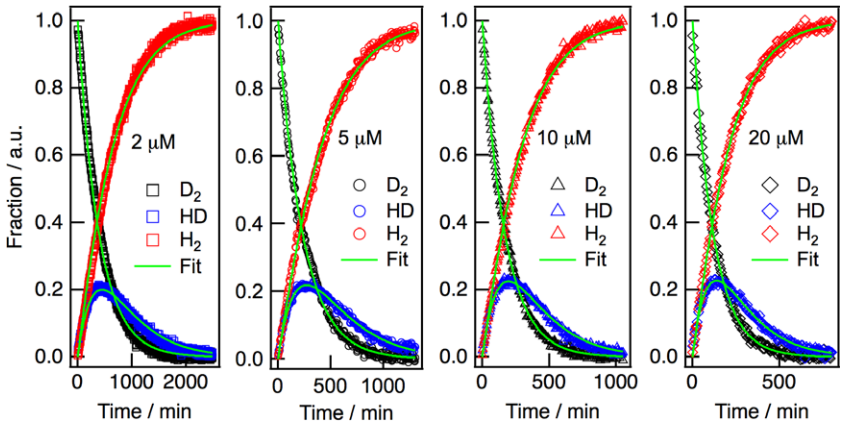

Figure 4. Kinetic analysis of the time course of the H/D exchange reaction catalyzed by [NiFe] hydrogenase. The time course of the reaction was measured at enzyme concentrations of 2, 5, 10, and 20 μM. The black, blue, and red markers represent the molar fractions of D_2, HD, and H_2, respectively. The green lines over the markers represent the fitting curves calculated using the equations based on the model depicted in Figure 5. The interval between each measurement was 5 min.

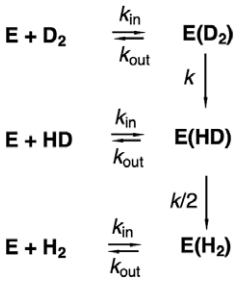

Figure 5. Scheme of the H/D exchange reaction used for the kinetic analysis.[15] E represents the free enzyme, and E(D_2), E(HD), and E(H_2) represent the enzyme bound to D_2, HD, and H_2, respectively.

Effect of the product release rate from the hydrogenase in the H/D exchange reaction using a kinetic model

To analyze the time course of the H/D exchange reaction in the D_2/H_2O system quantitatively, we adopted the reaction model proposed by Leroux et al., as depicted in Figure 5.[15] In this model, k is the rate constant of the H/D exchange reaction at the active site and k_{in} and k_{out} are the rate constants for the trapping and release, respectively, of molecular hydrogen species by the hydrogenase molecule (including the binding or dissociation of them at the Ni–Fe active site and the transfer through the gas channel). To obtain the ratios of k to k_{out}, the time courses of D_2, HD, and H_2 at several enzyme concentrations were fitted against the analytical solutions derived from the model equations presented in Figure 5. As indicated by the green lines in Figure 4, the fitted curves were in good agreement with the experimental data. The values of k/k_{out} (1.8–2.1) were approximately constant for enzyme concentrations in the range of 2–20 μM (Table I), and similar to those calculated by the method where the maximum points of HD are used.[15]

Discussion

Until the 1990s, H/D exchange reactions by hydrogenases were studied and discussed with respect to the initial reaction rates of HD and H_2 production, where the main focus was the mechanism of the double exchange reaction.[4,5,8] Recently, the technique of online mass spectrometry, which allows monitoring of the time course of the variation of the gas composition of the reaction, has been used to obtain more detailed information regarding the mechanism of the H/D exchange reaction.[15,16] However, it is difficult to survey the entire time course of the reaction by mass spectrometry owing to the continuous consumption of the gas in the reaction cuvette during sampling. Using the newly developed system based on Raman spectroscopy, we confirmed that H_2 (i.e., the fully exchanged product) is concomitantly produced alongside HD during the H/D exchange reaction in the D_2/H_2O system. As shown in Figure 3(B), the initial production rates of HD and H_2 were almost equal. It is generally accepted that D_2 is heterolytically cleaved to produce D^- and D^+ following binding to the Ni–Fe active site. The D^- bridges the Ni and Fe and the D^+ is proposed to bind to the cysteine (Cys546) sulfur atom that coordinates the Ni (the Ni-R state in Fig. 6).[3,7,14,17] In the D_2/H_2O system, the formation of HD and H_2 at the Ni–Fe active site is supposed to proceed according to Equations 3–6:

$$A + D_2 \rightarrow A\text{-}D^- + D^+ \quad (3)$$

$$A\text{-}D^- + H^+ \rightarrow A + HD \quad (4)$$

$$A + HD \rightarrow A\text{-}H^- + D^+ \quad (5)$$

$$A\text{-}H^- + H^+ \rightarrow A + H_2 \quad (6)$$

where "A" and "A-H^- + H^+" represent the Ni–Fe active site and the Ni-R state, respectively, as shown in Figure 6. According to Equations 3–6, H_2 should appear after HD production. In the D_2/H_2O system, the fully exchanged product (H_2) could be produced by two successive single H/D exchange reactions (1) in different active sites or (2) at the same active site (macroscopically double exchange reaction).[6,8,18] Since the ratio of v_2/v_1 depended little on the enzyme concentrations (Table I), the former is denied. Besides, τ_D depended on the enzyme concentration and did not depend on the volume of the enzyme solution. The results indicate that the reaction probably occurred near the gas–liquid interface and not controlled by the diffusion of the substrate. Hence, the entire time courses of individual components in the reaction were quantitatively analyzed and well fitted to the equations derived from the previously described reaction model (Fig. 5).[15] From the fitting results in Figure 4, k/k_{out} was estimated to be 1.9 ± 0.2 (Table I). This obtained value indicates that the rates of the H/D exchange reaction at the active

Table I. τ_D, v_2/v_1, k_a, *and* k_b for Several Enzyme Concentrations

Enzyme/μM	τ_D /min^{-1}	v_2/v_1	k_a	k_b
2	0.0022 ± 0.0003	1.34 ± 0.08	0.007 ± 0.001	2.14 ± 0.18
5	0.0038 ± 0.0003	1.20 ± 0.05	0.011 ± 0.001	1.86 ± 0.02
10	0.0050 ± 0.0004	1.22 ± 0.07	0.015 ± 0.001	1.83 ± 0.03
20	0.0074 ± 0.0010	1.18 ± 0.06	0.016 ± 0.003	1.83 ± 0.04

All data are the average results obtained from three independently purified enzyme solutions.
$k_a = (k \times k_{in} \times [E]_0)/(k_{out} + k_{in} \times [D_2]_0)$.
$k_b = k/k_{out}$.

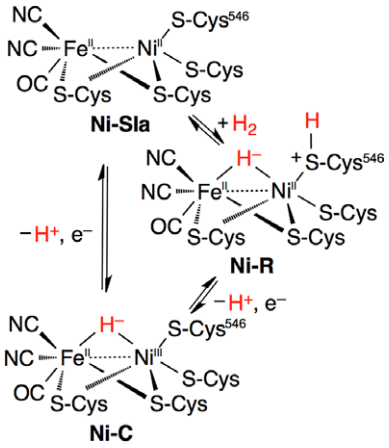

Figure 6. Proposed catalytic mechanism for H_2 at the active site of [NiFe] hydrogenase. The catalytic cycle consists of Ni-SIa, Ni-C, and Ni-R states. Ni-R state harbors a hydride bridge between the Ni and Fe, and S of Cys546 is protonated.

site and the release of the products from the enzyme were comparable. Hamdan et al., reported that k and k_{out} can be separately calculated under the condition of largely excess concentration of substrate over Michaelis constant, K_m.[19] Although k/k_{out} was successfully estimated in this study, it is difficult to apply this approximation because there is insufficient substrate concentration in the solution (760 μM) against K_m of DvMF (130 μM for D_2 in the H/D exchange reaction)[8] and to discuss the relationship between k_{in} and k_{out} based on the present reaction model. In addition, the assumption that all molecular hydrogen species share the same kinetics in this model is not valid, because the masses of D_2 and HD are twofold and 1.5-fold that of H_2, respectively, while the kinetic isotope effects may be as high as 17-fold and fourfold that of H_2, respectively. To clarify these issues, the following reactions must be investigated: (1) the H/D exchange reaction in the H_2/D_2O system; and (2) the nuclear spin isomer conversion reaction, that is, the conversion between *para*-H_2 and *ortho*-H_2 (or between *ortho*-D_2 and *para*-D_2) in H_2O or D_2O. These measurements can be performed using exactly the same assay system proposed in this study.

In the experimental setup of our Raman system using concentrated enzyme solution, the enzymatic reaction mainly takes place at the interface between the gas and liquid phases. This is one of the reasons that the activities of the H/D exchange reaction showed the dependency of the enzyme concentrations (Supporting Information Table S1). Nevertheless, τ_D of D_2 decay with the concentrated enzyme in this study was dependent on the enzyme concentrations (probably on the enzyme density at the interface) and k/k_{out} was successfully estimated from the entire time course of the H/D exchange reaction. These results indicate that the data obtained in this study were not controlled by the diffusion limit of the substrate at the liquid–gas interface, but demonstrated the typical feature of the enzymatic reaction, even though the improvement of the measurement system is required for further assay of the H/D exchange activity of the enzyme more accurately. We believe that our newly developed Raman method is potentially useful and is applicable to assay the enzymes reacting with gaseous substrates.

Materials and Methods

Materials and sample preparation

[NiFe] hydrogenase from DvMF was purified as described in the previous report.[17] Enzyme purification was performed under strict anoxic conditions. The concentration of the hydrogenase was determined using the absorption coefficient at 400 nm (47 mM^{-1} cm^{-1}). H_2-uptake activity of the anaerobically purified enzyme assayed by the conventional spectroscopic method with methyl viologen was 5620 mol s^{-1} (mol enzyme)$^{-1}$, which was a sufficiently high value compared to that [840 mol s^{-1} (mol enzyme)$^{-1}$] prepared aerobically.[14] The H/D exchange activities of [NiFe] hydrogenases were ranging from 45 to 450,[20,21] whereas that obtained using our system was 47 mol s^{-1} (mol enzyme)$^{-1}$ at 20 nM. Although it is difficult to compare them directly due to the large differences of the experimental conditions, including the reaction cuvettes, bubbling conditions, additives (electron transfer media), the obtained value in this study was reasonable. The D_2/H^+ exchange assay was performed in 25 mM Tris–HCl buffer (pH = 7.4). Dissolved oxygen in the buffer solution was removed by bubbling with argon gas prior to use in a glovebox. The glovebox (Coy Laboratory Products Inc.) was maintained under anoxic conditions with H_2 (1.5 ± 0.5%) and N_2 (98.5 ± 0.5%). H_2 gas (99.99%) was purchased from Taiyo Nippon Sanso Corporation. D_2 (99.8%) and HD (D, 97%) were purchased from Cambridge Isotope Laboratories Inc.

Raman spectroscopy

Raman scattering was measured using a 532.0 nm Nd:YVO_4 laser (Spectra-Physics, Millennia X) as the excitation source with a power of 800 mW at the sample point. The detector was a liquid-nitrogen-cooled CCD (Roper Scientific Inc., Spec-10:400B) attached to a 30-cm spectrometer (Princeton Instruments Inc., Acton SpectraPro 300i) with a 500-nm blazed grating with 1200 grooves/mm. This setup enabled us to carry out the simultaneous measurement of a wide Raman spectral range including the D_2, HD, and H_2 signals. The exposure time was 60 s and five scans were accumulated for each spectrum to provide a sufficiently high signal-to-noise ratio to allow quantitative discussion. The measurements were performed in a custom-built reaction cuvette as shown in Figure 1(B). All of the optical components were installed on an optical table and arranged as depicted in Figure 1(A).

Assay of H/D exchange reaction

The reaction vessel consisted of a glass reaction cuvette and a two-way cock unit attached to the top of the cuvette via a ground glass joint. The hydrogenase solution was gently placed in the bottom of the reaction cuvette. After connecting the two units at the ground glass joint with high-vacuum sealant, the gas inside the cuvette was replaced with D_2 gas at 100 kPa via a vacuum line connected to the inlet of the two-way cock unit. The two-way cock was closed prior to removal of the vacuum line. Finally, the reaction cuvette was placed in the cuvette holder in front of the spectrometer to initiate the Raman measurements. The reaction mixture was continuously stirred (1000 rpm) using a magnetic stirrer and maintained at 30°C in a thermostated chamber. Unless otherwise noted, the volumes of the reaction mixture and gas phase were 1 and 10 mL, respectively. As the droplets attached on the wall of the inside of the cuvette by the splashed solution of the reaction mixture disturb the transmission of the laser light, resulting in the inaccurate measurement of the Raman peak intensity, the enzyme solution was not able to be stirred vigorously in the present measurement system. This could be solved by improving the reaction system in future work. All of the procedures except the Raman measurements were performed in the glovebox.

Calculation of the gas composition

We measured the area intensities of the Raman bands for each pure isotope individually. The D_2:HD:H_2 intensity ratio was 0.71:0.88:1. The observed area intensities of the Raman bands of the individual isotopic species were corrected by dividing by these values to obtain the molar fraction of each isotopic species.

Data analysis

The fractions of the isotopic species in the period from the start of the measurement to half of the half-life of D_2 were used to calculate the initial rates of HD and H_2 production. The data were least-squares fitted with a straight line to obtain the slopes corresponding to v_1 and v_2 for HD and H_2, respectively.

The time courses of the D_2/H^+ exchange reactions were fitted using the analytical solution of the reaction model depicted in Figure 5. The calculations were reported by Leroux et al.[15] The initial conditions were $[D_2]_0 = 1$, $[HD]_0 = 0$, and $[H_2]_0 = 0$. The equations used for the fitting were as follows:

$$[D_2] = [D_2]_0 e^{-[ka/(1+kb)]t} \quad (7)$$

$$[HD] = 2[D_2]_0 \left\{ e^{-[ka/(2+kb)]t} - e^{-[ka/(1+kb)]t} \right\} \quad (8)$$

$$[H_2] = [D_2]_0 \left\{ 1 - 2e^{-[ka/(2+kb)]t} + e^{-[ka/(1+kb)]t} \right\} \quad (9)$$

where $k_a = (k \times k_{in} \times [E]_0)/(k_{out} + k_{in} \times [D_2]_0)$, $k_b = k/k_{out}$, and $[E]_0$ is the enzyme concentration. The other terms are explained in the Results section and Figure 5.

ACKNOWLEDGMENTS

This work was supported by the Japan Science and Technology Agency CREST grant JPMJCR12M4 (Y.H.), a Japan Society for Promotion of Science Grants-in Aid for Scientific Research (B), Grant No. 25291038 (Y.H.), for Challenging Exploratory Research, No. 24657077 (Y.H.) and for JSPS Fellows JP18J11420 (Y.K.), and a Ministry of Education, Culture, Sports, Science and Technology Grant-in-Aid for Scientific Research on Innovative Area (Hydrogenomics, 18H05518 (K.F.) and 18H05516 (Y.H.)). We thank Dr. N. Shibata, Dr. T. Hiromoto, Dr. S. Yanagisawa, and Dr. M. Kubo for helpful discussions, Ms. S. Inomata and Ms. A. Kakimoto for experimental assistance, and Ms. K. Hataguchi and Ms K. Matsumoto for bacterial cultures.

CONFLICTS OF INTEREST

There are no conflicts of interest to declare.

References

1. Lippard SJ, Berg JM (1994) *Principles of bioinorganic chemistry*. Sausalito, United States: University Science Books.
2. Rehder D (2014) *Bioinorganic chemistry*. Oxford, England, UK: Oxford University Press.
3. Lubitz W, Ogata H, Rüdiger O, Reijerse E (2014) Hydrogenases. Chem Rev 114:4081–4148.
4. Krasna AI, Rittenberg D (1954) The mechanism of action of the enzyme hydrogenase. J Am Chem Soc 76: 3015–3020.
5. Berlier Y, Fauque GD, LeGall J, Choi ES, Peck HD, Lespinat PA (1987) Inhibition studies of three classes of Desulfovibrio hydrogenase: application to the further characterization of the multiple hydrogenases found in Desulfovibrio vulgaris hildenborough. Biochem Biophys Res Commun 146:147–153.
6. Vignais PM (2005) H/D exchange reactions and mechanistic aspects of the hydrogenases. Coord Chem Rev 249:1677–1690.
7. Yagi T, Higuchi Y (2013) Studies on hydrogenase. Proc Japan Acad Ser B 89:16–33.
8. Yagi T, Tsuda M, Inokuchi H (1973) Kinetic studies on hydrogenase: parahydrogen-orthohydrogen conversion and hydrogen-deuterium exchange reactions. J Biochem 73:1069–1081.
9. Berlier Y, Lespinat PA, Dimon B (1990) A gas chromatographic-mass spectrometric technique for studying simultaneous hydrogen-deuteron exchange and Para-orthohydrogen conversion in hydrogenases of Desulfovibrio vulgaris Hildenborough. Anal Biochem 188:427–431.
10. Westley C, Fisk H, Xu Y, Hollywood KA, Carnell AJ, Micklefield J, Turner NJ, Goodacre R (2017) Real-time monitoring of enzyme-catalysed reactions using deep UV resonance Raman spectroscopy. Chemistry 23: 6983–6987.
11. Vignais PM, Henry M-F, Berlier Y, Lespinat PA (1982) Effect of pH on H-^{2}H exchange, H_2 production and H_2 uptake, catalysed by the membrane-bound hydrogenase of Paracoccus denitrificans. Biochim Biophys Acta Bioenergetics 681:519–529.

12. McTavish H, Sayavedra-Soto LA, Arp DJ (1996) Comparison of isotope exchange, H_2 evolution, and H_2 oxidation activities of *Azotobacter vinelandii* hydrogenase. Biochim Biophys Acta Protein Struct Mol Enzymol 1294:183–190.
13. Stoicheff BP (1957) High resolution Raman spectroscopy of gases: IX. Spectra of H_2, HD, and D_2. Can J Phys 35: 730–741.
14. Yagi T, Ogo S, Higuchi Y (2014) Catalytic cycle of cytochrome-c_3 hydrogenase, a [NiFe]-enzyme, deduced from the structures of the enzyme and the enzyme mimic. Int J Hydrogen Energy 39:18543–18550.
15. Leroux F, Dementin S, Burlat B, Cournac L, Volbeda A, Champ S, Martin L, Guigliarelli B, Bertrand P, Fontecilla-Camps J, Marc R, Christophe L (2008) Experimental approaches to kinetics of gas diffusion in hydrogenase. Proc Natl Acad Sci USA 105:11188–11193.
16. Cournac L, Guedeney G, Peltier G, Vignais PM (2004) Sustained photoevolution of molecular hydrogen in a mutant of Synechocystis sp. strain PCC 6803 deficient in the type I NADPH-dehydrogenase complex. J Bacteriol 186: 1737–1746.
17. Ogata H, Nishikawa K, Lubitz W (2015) Hydrogens detected by subatomic resolution protein crystallography in a [NiFe] hydrogenase. Nature 520:571–574.
18. Krasna AI (1979) Hydrogenase: properties and applications. Enzyme Microb Technol 1:165–172.
19. Abou Hamdan A, Dementin S, Liebgott PP, Gutierrez-Sanz O, Richaud P, De Lacey AL, Roussett M, Bertrand P, Cournac L, Léger C (2012) Understanding and tuning the catalytic bias of hydrogenase. J Am Chem Soc 134:8368–8371.
20. Bertrand P, Dole F, Asso M, Guigliarelli B (2000) Is there a rate-limiting step in the catalytic cycle of [Ni-Fe] hydrogenases? J Biol Inorg Chem 5:682–691.
21. Fauque GD, Berlier YM, Czechowski MH, Dimon B, Lespinat PA, LeGall J (1987) A proton-deuterium exchange study of three types of Desulfovibrio hydrogenases. J Ind Microbiol 2:15–23.