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HYDROGENASE ACTIVITY IN THE DRY STATE

ISOTOPE EXCHANGE AND REVERSIBLE OXIDOREDUCTION OF CYTOCHROME c_3

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Summary

Hydrogenase (hydrogen:ferricytochrome c_3 oxidoreductase, EC 1.12.2.1) catalyzes three types of reactions, i.e., (1) conversion between hydrogen modifications, *para*-H_2 and *ortho*-H_2, (2) exchange reaction between hydrogen isotopes, and (3) reversible oxidoreduction of an electron carrier with H_2 and protons. We observed that purified desulfovibrio hydrogenase in the dry state could catalyze not only the conversion and exchange reactions (Yagi, T., Tsuda, M., Mori, Y. and Inokuchi, H. (1969) J. Am. Chem. Soc. 91, 2801) but also the reversible oxidoreduction of the electron carrier, cytochrome c_3 with H_2.

The rate of the conversion was in the range from 0.1 to 0.65 mol H_2 converted per mol hydrogenase per s, and the ratio of the conversion rate to the exchange rate was near 5. The rate of oxidoreduction of cytochrome c_3 in the dry state was 0.015 mol H_2 taken up in the forward reaction and 0.003 mol H_2 released in the reverse reaction per mol hydrogenase per s. The process of these reactions could be explained by the observations that the hydrogenase molecule in the dry state has protons which are directly exchangeable with H_2 during catalytic process. The reversible oxidoreduction of cytochrome c_3 is also explained by inter- and intramolecular electron transfer among cytochrome c_3 molecules.

Introduction

Enzymes act on substrates in aqueous solution, where all the molecules are hydrated. The enzymic activities in the dry state will, therefore, give us infor-

mations on the effect of hydration on enzymic reactions. The enzymic reactions in the dry state can also be considered as model for a membrane-bound enzyme systems such as mitochondrial or chloroplast electron transfer system, where enzymes and carrier molecules are not able to move as in aqueous solution. In this paper, we present experimental results on the enzymic activity of anhydrous hydrogenase preparations.

Desulfovibrio hydrogenase (hydrogen:ferricytochrome c_3 oxidoreductase, EC 1.12.2.1) catalyzes three types of reactions shown in Eqns. 1—3, which are usually observed in aqueous solution:

$$2\ H_2 + \text{ferricytochrome } c_3 \rightleftharpoons 4\ H^+ + \text{ferrocytochrome } c_3 \quad (1)$$

$$H_2 + {}^2H_2O \overset{H^2H + H^2HO}{\rightleftharpoons} {}^2H_2 + H_2O \quad (2)$$

$$\textit{para}\text{-}H_2 \rightleftharpoons \textit{ortho}\text{-}H_2 \quad (3)$$

In 1969, we reported that dry hydrogenase powders could catalyze the conversion of *para*-H_2 to *ortho*-H_2 (Eqn. 3) together with slow isotope exchange reaction (Eqn. 4, which corresponds to Eqn. 2 in the aqueous reaction) [1]. The purity of the enzyme used at that time was found to be not very high, however.

$$H_2 + {}^2H_2 \rightleftharpoons 2\ H^2H \quad (4)$$

In this paper, we present evidence that reversible oxidoreduction of cytochrome c_3 with H_2 (Eqn. 1) as well as conversion (Eqn. 3) and isotope exchange (Eqn. 4) could also be catalyzed by dry hydrogenase of high purity.

Materials and Methods

Hydrogenase and cytochrome c_3. Hydrogenase and cytochrome c_3 were purified from *Desulfovibrio vulgaris*, Miyazaki, as reported previously [2,3], and were thoroughly dialyzed against distilled water to remove salt admixtures for use in the dry state experiments. Activity of hydrogenase in the aqueous state was assayed by the enzymic electric cell method [4], and expressed in units defined in Ref. 5. 1 unit hydrogenase corresponds to 18.4 pmol or 1.64 μg of pure hydrogenase [2]. The concentration of cytochrome c_3 in aqueous solution was estimated from the absorbance of the α-peak of the ferro-form at 552 nm, and was expressed in molar basis of protein instead of heme. The amount of cytochrome c_3 in dry film was also estimated from the absorbance of the α-peak after it had been fully reduced enzymatically under H_2 at atmospheric pressure. In this case, the α-peak position shifted to 553.2 nm. The time-course of the reduction of cytochrome c_3 in dry film was followed spectrophotometrically with a Cary 17 recording spectrophotometer by observing the increase in the absorbance at 553.2 nm. The ratio of the absorbance at 553.2 nm of the ferro-form to that of the ferri-form was 2.65.

H_2 modification and H_2 isotopes. Enriched *para*-H_2 was prepared as reported before [6]. ${}^2H_2O$ (99.75%) was a product of Merck. ${}^2H_2$ was a product of Showa Denko Co. Ratio of dihydrogen isotopes, H_2, H^2H, and ${}^2H_2$, was measured with a mass spectrometer, Type 21-620A (Consolidated Electrody-

namics Corp.). Ratio of H_2 modifications, *para*-H_2 and *ortho*-H_2, was determined gas chromatographically using a heat-treated alumina column [6], with corrections for the content of H^2H, because ortho-H_2 and H^2H were not separated in the gas chromatographic system as reported previously [7].

Rates of conversion between H_2 modifications and isotope exchange. A salt-free solution containing purified hydrogenase (1.9 nmol) and cytochrome c_3 (5.3 nmol) in 1 mM NH_3 was placed in a reaction vessel (volume, 61 ml) and freeze-dried. The vessel was evacuated at 0.13 Pa for 6 h at 26°C, and then for additional 8 h at 60°C to remove humidity from the mixture as possible. Then a 1 : 1 mixture of *para*-H_2 and $^2H_2$ was introduced to the vessel at a pressure of 15.5 kPa. Therefore, 380 μmol of total dihydrogen was introduced into the vessel. The vessel was then incubated at 30°C. At intervals, the gas samples were withdrawn, and analyzed for H_2 modifications and isotopes.

Deuterated hydrogenase. Purified hydrogenase dissolved in ^2H_2O was lyophilyzed, and was again dissolved in ^2H_2O (0.96 mg, or 10.8 nmol per ml). A 1.0 ml portion of this solution was transferred to a reaction vessel (volume, 11.3 ml) and freeze-dried. The vessel was then evacuated for additional 24 h at 0.4 Pa at room temperature. This sample was used for the estimation of exchangeable protons in dry hydrogenase.

Anhydrous film of cytochrome c_3 containing hydrogenase. This was prepared by a film-casting method. A salt-free solution containing 370 pmol of hydrogenase and 150 nmol of cytochrome c_3 in 5.0 ml distilled water was transferred to a Pyrex glass apparatus shown in Fig. 1. The gas phase of the apparatus was pumped off with stirring for 30 min in order to remove dissolved gas. Upon gradual evaporation of the solution, a thin film was deposited on a quartz plate (30 × 15 mm, 1 mm thick). The quartz plate coated with a thin film of the hydrogenase-cytochrome c_3 mixture in both surfaces was taken out from the apparatus, and kept in a drying box for 2 h to remove humidity at atmospheric pressure. The thickness of the film thus prepared was measured

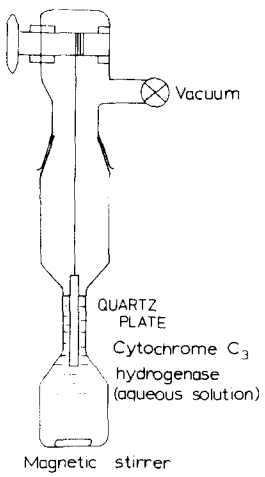


Fig. 1. Apparatus for preparing film of the reaction mixture. The procedure of preparing the film is described in the text.

spectrophotometrically, and was found to be 230 nm. The direct measurement of the film thickness with a surface roughness tester gave a value of about 210—250 nm. This film was used for the assay of the rate of reversible oxido-reduction of cytochrome c_3 with H_2 as described in the text.

Measurement of film thickness. Thickness of the anhydrous film of cytochrome c_3 described in the preceding section was measured by two methods. In spectrophotometric measurement, the film thickness (δ) is given by Eqn. 5:

$$\delta = AM\rho^{-1} \cdot \epsilon^{-1} \tag{5}$$

where A is the absorbance at 553.2 nm after full reduction of cytochrome c_3, M, the molecular weight of cytochrome c_3, i.e. 14 000 [3], ρ, the density of the cytochrome, and ϵ, the molar absorbance of the ferrocytochrome c_3 at 553.2 nm. We assumed that ϵ was not different from that in aqueous solution, i.e. $1.1 \cdot 10^5$ M^{-1} [3]. ρ was estimated crystallographically. The molecular volume of cytochrome c_3 crystals calculated from crystallographic data was $2.33 \cdot 10^{-30}$ m^3 per atomic mass unit (Bando, S., Matsuura, Y., Tanaka, N., Kakudo, M., Yagi, T. and Inokuchi, H., unpublished observation). From this, the density of the cytochrome c_3 crystal was calculated to be 0.713. We assumed that the density of amorphous cytochrome c_3 solid was not different from that of cytochrome c_3 crystals.

Film thickness was also measured with a surface roughness tester, DEKTAK, obtained from Sloan Co. Calibration was made with a calibration flat of Au supplied from the same company.

Results

Conversion between H_2 modifications and isotope exchange

Conversion of *para*-H_2 to *ortho*-H_2 and the exchange between H_2 and $^2H_2$ to produce H^2H took place simultaneously as shown in Fig. 2. In this figure, the extent of conversion was expressed in open circles; c_0 and c_∞ being the percentages of *para*-H_2 in H_2 modifications at time zero and at equilibrium, respectively, and was 83% (measured) and 25% (theoretical), respectively. The percentage of *para*-H_2 at time t is expressed by c_t. The extent of isotope exchange was also illustrated in this figure in solid circles; c_0 and c_∞ in this case

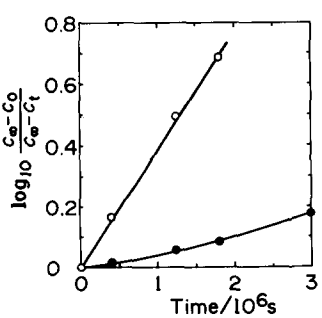


Fig. 2. Time-course curves for the conversion and exchange reactions. The reaction conditions are described in the text. ○, conversion; ●, exchange reactions.

being the percentages of H^2H in total isotopic H_2, and was 0.45% (measured) and 47.45% (calculated from the equilibrium constant of reaction 4), respectively.

The conversion proceeded without lag period, whereas the exchange proceeded steadily only after the induction period. From the slope of curves in steady state in Fig. 2, the rate of conversion was calculated to be 0.180 mol H_2 converted and 0.036 mol H_2 mutually exchanged per mol hydrogenase per s. The ratio of conversion rate to exchange rate in this particular experiment was 5.0. Omission of cytochrome c_3 from the reaction mixture did not affect the rates of both reactions appreciably, but the omission of hydrogenase completely nullified both of the reactions. The rates of both reactions were influenced by the conditions of drying, and were not easily controlled. In several experiments, the rates of conversion were 0.1—0.65 mol H_2 converted per mol hydrogenase per s, and the ratio of conversion rate to exchange rate was constantly near 5. The rate of conversion of H_2 modifications was, thus, at best only 0.22% of that of the conversion catalyzed by aqueous hydrogenase solution (calculated from Refs. 2 and 6).

Estimation of exchangeable protons in the dry hydrogenase

The ability of dry hydrogenase to catalyze not only conversion of H_2 modifications but also exchange between H_2 isotopes indicated that the covalent bond in an H_2 molecule is actually split on the hydrogenase molecule, and, at least, one hydrogen atom in H_2 was exchanged with protons on the enzyme molecule. The presence of the exchangeable protons in the dry hydrogenase was, therefore, tested.

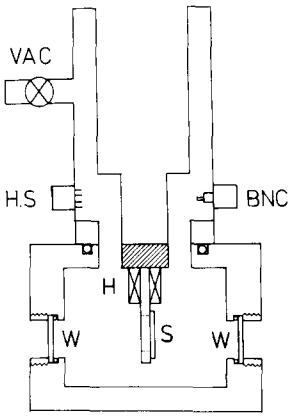
Normal H_2 (2.64 kPa at 20°C) was introduced into a vessel (volume 11.3 ml) containing deuterated hydrogenase powders (10.8 nmol) which had been kept in vacuo for 24 h. Therefore, 12.3 μmol of H_2 was introduced. After the vessel was left standing for 35 days, the gas was analyzed for H_2 isotopes. The molar fractions of H^2H and $^2H_2$ were found to be 6.9 and 0.2%, respectively. This means that 0.90 μmol ^2H atoms were liberated into the gas phase. After the analysis, the gas was withdrawn, and fresh H_2 was again introduced, and the gas analyzed for isotopes after a month. This procedure was repeated again. Total amount deuterium atoms liberated to the gas phase was calculated by summing up the amount of ^2H atoms liberated into the gas phase in each exchange reaction, and was found to be 1.53 μmol. This value corresponds to 140 ^2H atoms liberated per molecule hydrogenase (M_r 89 000 [2]) in the dry state.

Similar experiments were carried out with a mixture of 10.8 nmol hydrogenase and 26.0 nmol cytochrome c_3. In this case, the number of exchangeable protons per molecule hydrogenase was 360.

Reversible oxidoreduction of cytochrome c_3 with H_2

Dried hydrogenase, as used in the above-mentioned experiments prepared by lyophilyzing frozen hydrogenase solution, was not suitable for spectrophotometric measurements, which are essential for measuring cytochrome c_3 reduction. Therefore, dry films of cytochrome c_3 containing hydrogenase were used for this purpose.

A quartz plate coated with dry films of cytochrome c_3 containing hydrogen-



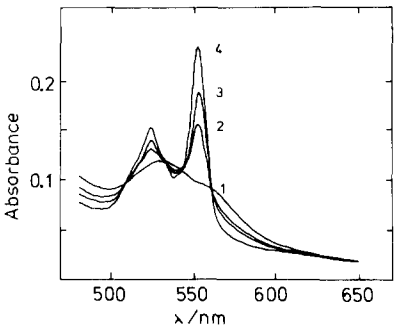


Fig. 3. Measuring cell for spectra recordings. The compartment of the cell was made of aluminium and the flange was made of stainless steel. H, heater for temperature control; VAC, vacuum valve, also used as gas inlet valve; W, quartz window; S, quartz plate coated with sample film; BNC and HS, high frequency connector and hermetic seal for conductivity measurement.

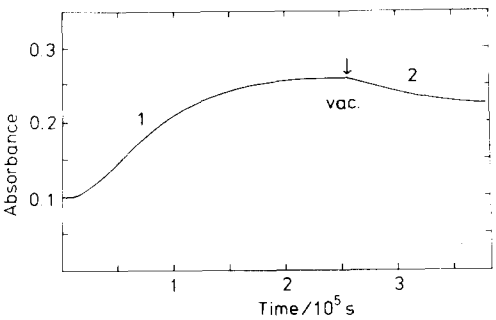
Fig. 4. Absorption spectra of cytochrome c_3 in dry film at intervals. 1, fully oxidized; 2, at $5.85 \cdot 10^4$ s; 3, at $7.98 \cdot 10^4$ s; and 4, at $1.35 \cdot 10^5$ s.

ase on both surfaces was placed in a measuring cell (Fig. 3). After evacuation of the cell at 13 mPa, 102 kPa of H_2 was introduced to the cell, and the time-course of the reduction of cytochrome c_3 was successively monitored at 25°C. After 3 days ($2.55 \cdot 10^5$ s), the cell was evacuated at about 13 mPa for 60 min, and the spectral change for the reverse reaction, i.e., the reoxidation of cytochrome c_3 by releasing H_2 was monitored.

Fig. 4 shows the time course of visible spectra recorded at various intervals. The observed isosbestic points were 510.0, 533.7, 543.0, and 561.5 nm, in contrast to those in solution, 508, 532, 542, and 560 nm [3]. Fig. 5 shows the absorbance of α-peak as a function of time. This figure shows that there was an induction period at the initial state of the reaction. In the reverse reaction, however, no induction period was observed. In this case, the reaction did not completely proceed to ferri-form, but ceased attaining equilibrium. Applying first-order rate kinetics to the curve, one can express the extent of the reaction as $\log_e [(a_\infty - a_0)/(a_\infty - a_t)]$ versus time t, as shown in Fig. 6. In this plot, a_0 and a_∞ were molar fractions of the ferro-form of cytochrome c_3 in the total cytochrome at time 0 and at equilibrium, respectively. In this particular case, they were 0.10 and 0.26, respectively. a_t is the molar fraction of ferrocytochrome c_3 at time t. In the reverse reaction, a_0 was 0.26 (equals to a_∞ in the forward reaction), but a_∞ was 0.21 instead of 0.10 (see Fig. 4). Since the plots of reactions were linear in both directions except at the induction period of the forward reaction, we have concluded that the redox reactions of cytochrome c_3 on dry film follows first-order rate kinetic as in Eqn. 6.

$$kt = \log_e [(a_\infty - a_0)/(a_\infty - a_t)] \quad (6)$$

The rate constant, k, was calculated to be $1.9 \cdot 10^{-5}$ s^{-1} in the forward direction, and $1.1 \cdot 10^{-5}$ s^{-1} in the backward direction.



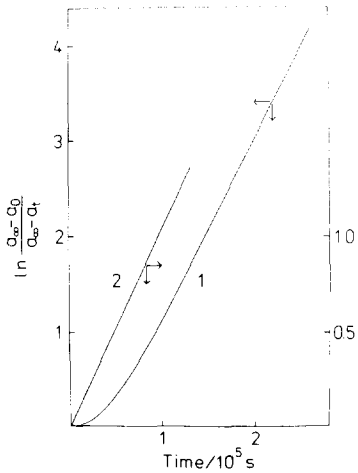


Fig. 5. Absorbance of the α-peak as a function of time. Stage 1 stands for the forward reaction, i.e., the reduction of ferricytochrome c_3, and stage 2, for the backward reaction after evacuation of the cell to 13 mPa.

Fig. 6. First-order rate plot of stages 1 and 2 in Fig. 5.

From the molar ratio of cytochrome c_3 to hydrogenase, i.e., 400 : 1, and the rate constant, the rate of cytochrome c_3 reduction was calculated to be 0.0077 (400 k) mol cytochrome c_3 reduced per mol hydrogenase per s. To reduce 1 mol tetrahemoprotein, cytochrome c_3, 2 mol H_2 are required (Eqn. 1). Therefore, the rate of H_2 uptake with dry cytochrome c_3 with dry hydrogenase was 0.0154 mol H_2 uptaken per mol hydrogenase per s. In the reverse reaction, only 128 out of 400 mol cytochrome c_3 were reoxidized by a single hydrogenase. This means that 0.0028 mol H_2 was liberated per mol hydrogenase per s. These reaction rates were only 0.0003% of those in the reactions catalyzed by aqueous hydrogenase.

Control experiments of cytochrome c_3 reduction were made on films with denatured hydrogenase boiled at 100°C for 2 min, and without hydrogenase. No change was observed for both samples after $2 \cdot 10^5$ s. This means that native hydrogenase is essential for cytochrome c_3 reduction.

Discussion

It is noteworthy that hydrogenase in the dry state could catalyze not only the conversion between H_2 modifications and the exchange between H_2 isotopes, but also reversible oxidoreduction of cytochrome c_3 with H_2. In our previous paper [1], we observed that hydrogenase could catalyze the conversion reaction in the dry state only when the enzyme was reduced with $Na_2S_2O_4$ prior to drying. At that time, 1969, the purification technique for hydrogenase was not sufficient. In the present experiments with highly purified hydrogenase, we observed the occurrence of the conversion and exchange reactions without preliminary reduction of the enzyme. It reminds us that Couper et al. [8] were not able to observe the conversion reaction using dried cells of *Escherichia*

coli or *Proteus vulgaris* even though these cells had strong hydrogenase activity in aqueous conditions. Probably the purity of the enzyme is one of the most important factors for the expression of the catalytic activities under rather unusual conditions.

The ability of dry hydrogenase to catalyze isotope exchange (Eqn. 4) suggests the presence of exchangeable protons on the dry enzyme as shown below:

$$EnzH + {}^2H_2 = Enz{}^2H + H{}^2H \quad (7)$$

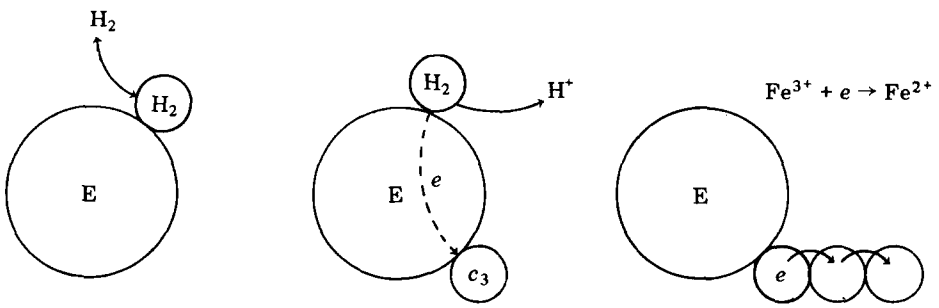
$$Enz{}^2H + H_2 = EnzH + H{}^2H \quad (8)$$

From amino acid analysis [2], there are 1200—1300 protons which are expected to exchange with water when in the aqueous medium, if conformational restrictions are disregarded. Our experiment showed that 140 protons thereof were actually exchangeable with H_2 molecules. The increase of the exchangeable protons in the presence of cytochrome c_3 suggested the presence of this kind of protons also in the cytochrome molecule.

Reduction of ferricytochrome c_3 in the dry film indicated that H_2 was dissociated to provide electrons which are transferred to cytochrome c_3. The protons produced with electrons from H_2 must have diffused into the film in order to keep electric neutrality of the film. This diffusion process could be accounted for by the presence of exchangeable protons in the cytochrome. Since almost all of cytochrome c_3 were reduced as revealed spectroscopically, it is concluded that cytochrome c_3 molecules not in contact with hydrogenase molecules were also reduced, because the molar ratio of hydrogenase to cytochrome c_3 was 1 : 400.

The reduction of 400 mol cytochrome c_3 by a single hydrogenase molecule in solid state strongly implied the possibility of electron propagation among cytochrome c_3 molecules. The electrons may be transferred from the hydrogenase molecule to the nearest neighboring cytochrome c_3 molecules, and then propagating further to other cytochrome molecules as shown in Fig. 7. Since the reduction of cytochrome c_3 with H_2 was reversible, the electrons must be transferred to either directions.

Cytochrome $c_3(Fe^{3+}) + H_2 \rightarrow$ cytochrome $c_3(Fe^{2+}) + 2\ H^+$



H_2 adsorption H_2 dissociation Electron transfer

Fig. 7. Schematic representation of the electron transfer during the dry state reaction. E, enzyme (i.e. hydrogenase); and c_3, cytochrome c_3.

One might suspect the degree of dryness in the experimental conditions, since traces of humidity are known to be not easily removed from proteinaceous material. In a series of dry state experiments, we have measured resistances in Ω of cytochrome *c* and cytochrome c_3 in the ferri- and ferro-forms together with those of simple proteins [9,10]. It has been found that the current-voltage relation strongly depends on the degree of dryness. Ohm's law holds only for well-dried samples. The raising time of the transient currents for the step change of the input voltage was more than 10 min if the proteinaceous sample had been partially dried, whereas the time was only a few seconds if the sample had been as carefully dried as in the present experiments. Therefore, the degree of dryness in our present experiments was high enough to eliminate any free water in the samples. The slow enzymic reactions observed in the present experiments could be considered as a model for slow biological activities naturally occurring in the dry state as seeds.

The observed reaction rate in the dry state was much slower than that in aqueous solution. One possibility is that the reaction was controlled by diffusion of H_2. In the case of reduction of cytochrome c_3 with H_2 where the reaction was measured in dry films of definite thickness, the numerical evaluation of the reaction rate is possible. Suppose the reduction was controlled by diffusion process of H_2, Fick's first law (Eqn. 9) will hold:

$$\phi = -D\,\partial c/\partial x \tag{9}$$

where ϕ is the flux, D the diffusion coefficient, c the concentration of H_2, and x, the distance from the surface of the film. Upon assuming a linear gradient approximation in diffusion layer, Eqn. 9 can be rewritten:

$$\phi = -D(c - c_s)/\delta \tag{10}$$

where δ is the thickness of the film, and c_s the concentration of H_2 at the surface of the film. By applying first-order rate kinetics, the flux is proportional to the change of the average concentration. Eqn. 10, therefore, becomes Eqn. 11:

$$(dc/dt)\delta = -D(c - c_s)/\delta \tag{11}$$

Solution of this differential equation, expressed as Eqn. 12, is in the same form as Eqn. 6:

$$-(D/\delta^2)t = \log_e[(c - c_s)/(c_0 - c_s)] \tag{12}$$

where c_0 is the initial concentration of H_2, and (D/δ^2) corresponds to k in Eqn. 6. Giving the values of $\delta = 2.3 \cdot 10^{-7}$ m and $k = 1.9 \cdot 10^{-5}$ s^{-1}, D was calculated to be $1.0 \cdot 10^{-18}$ $m^2 \cdot s^{-1}$. The calculated value of the diffusion coefficient, assuming the diffusion process to be the rate-limiting step, is too small compared to known diffusion coefficients of gas in dense media [11–13]. This rules out the possibility that the reaction was diffusion-controlled, and leads us to conclude that the enzymic activity itself is lower in the dry state.

Our present experiments clearly show that hydrogenase in the dry state retained all catalytic functions observed in the aqueous media, but its activity was lower than in aqueous media. Detailed kinetic analysis will be published in future.

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