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EFFECT OF pH ON H-^{2}H EXCHANGE, H_2 PRODUCTION AND H_2 UPTAKE, CATALYSED BY THE MEMBRANE-BOUND HYDROGENASE OF *PARACOCCUS DENITRIFICANS*

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(1) The kinetics of isotope exchange catalysed by the membrane-bound hydrogenase of *Paracoccus denitrificans* have been studied by measuring H^{2}H, H_2 or $^2H_2$ produced when the enzyme catalyses the exchange between $^2H_2$ and H_2O or H_2 and ^2H_2O. (2) In the $^2H_2$-H_2O system the measured rate of H_2 production was always higher than that of H^{2}H. The H_2/H^{2}H ratio remained constant (about 1.70) in the protein concentration range 0.08–1.32 mg. The very rapid formation of H_2 with respect to H^{2}H is consistent with the hypothesis of a heterolytic cleavage of $^2H_2$ into a deuteron and an enzyme hydride that can exchange with the solvent. (3) In the H_2-^2H_2O system, the exchange rate was much lower than in the $^2H_2$-H_2O system, indicating a marked isotopic effect of ^2H_2O. (4) The H-^{2}H exchange activity, determined from the initial velocity of H^{2}H formation, is optimal at pH 4.5. A second maximum of activity is observed at pH 8.3. The pH value of 4.5 is also the pH optimum for H_2 production while at pH 8.3–8.5 there is a maximum of H_2 oxidation activity. (5) In ordinary H_2O the K_m for hydrogen uptake estimated either from H_2 consumption or from benzyl viologen reduction was 0.06–0.07 μM for both H_2 and $^2H_2$ indicating a strong affinity of the enzyme for hydrogen at pH 8.3–8.5. Shifting from H_2O to ^2H_2O does not affect the K_m of the enzyme for H_2 but lowers the V_{max} value about 10-fold. The K_m for benzyl viologen and methyl viologen was 0.08 and 2 mM, respectively.

Introduction

Hydrogenases are iron-sulfur proteins which catalyse the so-called activation of H_2 according to the reversible reaction:

$$H_2 \rightleftharpoons 2H^+ + 2e^- \quad (1)$$

Soluble hydrogenases, as found in clostridia, are reversible but catalyse mostly H_2 production. Membrane-bound hydrogenases, as found in N_2-fixing bacteria and in hydrogen bacteria, function as uptake hydrogenases [1–3]. These exhibit a high affinity for H_2 (K_m for H_2 in the range of micromolar concentrations) which allows them to use H_2 as an electron donor even in the presence of trace amounts of hydrogen gas.

Paracoccus denitrificans, Strain Stanier 381 (DSM 65) can develop a membrane-bound hydrogenase when grown autotrophically on H_2 as the sole hydrogen donor [4]. The enzyme is inducible [4,5] and functions physiologically as an uptake hydrogenase. It is an intrinsic membrane protein [4] which can transfer electrons from H_2 to the respiratory chain embedded in the cytoplasmic membrane of the bacterium [4,6].

Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Hipip, high-potential iron-sulfur protein.

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This paper shows that although the physiological role of the enzyme is to use H_2 as an electron donor, in vitro the hydrogenase of *P. denitrificans* can evolve H_2 and therefore function reversibly. The optimal pH conditions for both activities have been determined and are discussed in relation to the H-^{2}H exchange reaction catalysed by the hydrogenase.

Materials and Methods

Materials

Bacterial culture and membrane preparation. P. denitrificans (strain Stanier 381 DSM 65, a gift from Professor H.G. Schlegel, Göttingen, F.R.G.) was grown autotrophically in 5-l Erlenmeyer flasks in the medium described by Burnell et al. [7]. At the time of harvesting, the absorbance at 550 nm ranged from 1.2 to 2.3. The cells were collected by centrifugation (12 000 × *g* for 10 min), washed with distilled water and resuspended in a 10 mM Hepes, 2 mM $MgCl_2$ buffer, pH 8.0. Membrane particles were prepared by sonication (five times for 1 min with 1 min intervals), centrifuged at 140 000 × *g* for 2 h, and resuspended in the Hepes-$MgCl_2$ buffer, pH 8.0. They were stable for several weeks at −22°C under an H_2 atmosphere.

Enzymic assays

Isotopic H-^{2}H exchange. The reaction was monitored continuously in the aqueous phase by a direct mass spectrometric method [8] using a reaction vessel fitted with a porous fritted-steel disc covered with a Teflon membrane. A fraction of the dissolved gases diffuses continuously through the Teflon membrane; it is directed via a vacuum line first into a cold trap containing liquid N_2 then into the ion source of a mass spectrometer. To take into account the mass fractionation that occurs during the diffusion of the gases through the membrane and especially into the ion source, the raw data from the mass spectrometer were corrected as described in Ref. 8. The vessel contained 7 ml of 50 mM buffer pregassed with argon; the vessel was sparged with argon until the level of O_2 (mass 32) was lower than 1 μM, then either 30 μM dithionite or a mixture of glucose and glucose oxidase (0.1 mM and 38 units, respectively) was added to make and maintain the medium totally anaerobic.

$^2H_2$-H_2O system. The medium was sparged with a mixture of Ar and $^2H_2$ (9:1 or 8:2, v/v). When the concentration of $^2H_2$ (mass 4) reached a constant level, the apparatus was set on mass 3 and the reaction was initiated by adding an aliquot of the membrane suspension (5 μl, 20–35 mg protein/ml). The formation of H^{2}H was followed continuously and recorded. H-^{2}H exchange activity was determined from the initial slope of H^{2}H formation. In some experiments, H_2 production (mass 2) and $^2H_2$ uptake (mass 4) were monitored as well as H^{2}H production (mass 3).

H_2-^2H_2O system. The various buffers and reagents were prepared as indicated by Yagi et al. [9]. The chemicals were first dissolved in ^2H_2O (0.998 ^{2}H) then freeze-dried. The residue was dissolved again in the appropriate volume of ^2H_2O. The final enrichment was over 0.95. In the H_2-^2H_2O system, the sparging gas was a mixture of Ar and H_2 (8:2, v/v) and the molecular species envolved were ^{2}HH and $^2H_2$.

H_2 production. Production of H_2 was measured by gas chromatography. 9-ml serum flasks fitted with rubber serum stoppers and containing 2.5 ml of buffer at appropriate pH were degassed under argon. Then 0.5 ml of 50 mM methyl viologen semiquinone, produced by cathodic reduction of methyl viologen in an electrochemical vessel, was transferred anaerobically by a gas-tight syringe, perflushed with argon, to each flask. The flasks were equilibrated at 30°C in a water bath. The reaction was started by adding 25 μl of the membrane suspension (30–35 mg protein/ml). Samples (200 μl) of the gas phase were withdrawn at intervals and injected into a model IGC 16 gas chromatograph (Intersmat Instrument, Pavillon-sous-Bois, France) equipped with a thermal conductivity detector and provided with a 5 Å molecular-sieve column and argon as carrier gas.

H_2 uptake. H_2-uptake activity was assayed spectrophotometrically [10] by following the appearance of either reduced benzyl viologen (at 555 nm) or reduced methyl viologen (at 602 nm). The increase in absorbance was monitored with a Perkin-Elmer 557 dual-wavelength spectrophotometer using 1 cm light path cuvettes hermetically sealed with gas-tight rubber stoppers (Gallenkamp, London, U.K.). The 3-ml cuvettes containing the membranes and the buffer were flushed first for 10

min with Ar, then for 15 min with H_2 at 30°C. The reaction was started by addition of the oxidized viologen dye saturated with H_2. 100 µl of dye suspended in 50 mM Tris-HCl, pH 8.5, were added to reach a final concentration of 2.5–5 mM for benzyl viologen and 15 mM for methyl viologen. For the determination of K_m values for H_2 or $^2H_2$, the rubber-stoppered cuvette containing 2 ml of 50 mM Tris-HCl, pH 8.45, and 5 mM benzyl viologen was flushed for 15 min with Ar. Then a membrane preparation (5µl, 14 mg protein/ml) stored under H_2 was added; traces of H_2 present in the membrane fraction were immediately consumed as indicated by a slight reduction of benzyl viologen. Once the absorbance at 555 nm remained constant small amounts (from 2.5 to 100 µl) of H_2-saturated buffer were added. The rate of reduction of benzyl viologen was linear during the first minute. This initial velocity was used for plotting a Lineweaver-Burk graph. To evaluate the concentration of hydrogen gas present in the aqueous phase (2 ml), the theoretical partial pressure of H_2 (or $^2H_2$) was calculated assuming that the total amount of added H_2 escaped in the gas phase (1.6 ml). We have checked by gas chromatography that after adequate shaking the H_2 added as H_2-saturated buffer escaped practically instantaneously into the gas phase. Then from the value of the partial pressure using a solubility coefficient of 0.01819 for H_2 [11] and of 0.0203 for $^2H_2$ [12] at 30°C in H_2O, the concentration of dissolved gas was calculated.

Oxygen uptake. Oxygen uptake of membrane particles was measured amperometrically at 22°C using a Clark-type oxygen electrode (Y.S.I. 5331 oxygen probe, Yellow Springs Instruments, OH, U.S.A.) equipped with a standard Teflon membrane (Y.S.I. 5332). The 2 ml reaction chamber was filled with a mixture of air-saturated and H_2-saturated buffer (1:1, v/v); the reaction was initiated by the addition of the membrane particles.

Proteins. Proteins were assayed by the Coomassie brillant blue G-250 method, using bovine serum albumin as a standard [13].

Chemicals

Methyl viologen, benzyl viologen and Coomassie brillant blue G-250 were from Serva, Heidelberg, F.R.G. Mes, Tris, Hepes and glycylglycine were from Sigma Chemical Co, St. Louis, MO, U.S.A. Glycine, carbonate, bicarbonate, sodium phosphate and citrate, all analytical grade, were from Merck, Darmstadt, F.R.G., or Rhône-Poulenc Industries, Paris, France. $^2H_2$ gas and ^2H_2O were from the Commissariat à l'Energic Atomique (CEA), Saclay, France.

Results

The reaction catalysed by hydrogenase involves H^+ as either substrate or product. The catalytic center of hydrogenase involves acid-base groups as well as redox components [2]. It may therefore be expected that the pH can affect the kinetic properties of the enzyme. To avoid artefactual interference of pH-dependent electron-donating systems, the activity of the membrane-bound hydrogenase of *P. denitrificans* was studied with two types of techniques: (a) the H-^{2}H exchange reaction; (b) the oxidoreduction of viologens used as electron donors or acceptors.

H-^{2}H exchange

The use of hydrogen isotope species enables the splitting of the hydrogen molecule by hydrogenase to be detected. Krasna and Rittenberg [14], who investigated the machanism of hydrogenase action, postulated that hydrogenase catalyses a heterolytic splitting of hydrogen with formation of an intermediate enzyme hydride. Further studies indicated that the enzyme intermediate could exchange with hydrogen atoms from the solvent [9,15].

In the $^2H_2$-H_2O system, the reaction can be written:

$$^2H_2 + E \rightleftharpoons E\text{-}^2H^- + {}^2H^+ \quad (2)$$

(where E is hydrogenase). The back reaction, in the presence of excess protons from the solvent, leads to the formation of H^2H:

$$E\text{-}^2H^- + H^+ \rightleftharpoons E + H^2H \quad (3)$$

Formation of H^2H reflects the reversibility of reaction 2. Overall there is no electron transfer. Electron acceptors, if present, compete with H^+ for the hydride intermediate so that the exchange

reaction is lowered and may even be abolished. In particular, the membrane-bound hydrogenase of *P. denitrificans* can transfer electrons from H_2 to O_2 through the respiratory chain [6]. Thus, in the assays described hereafter, dithionite or glucose + glucose oxidase were added to trap traces of oxygen.

In the H_2-^2H_2O system, H^+ from H_2 will be released in a large pool of $^2H^+$ and the back-reaction will also yield H^2H as in reaction 3. [2,16].

Production of H^2H and H_2 by isotope exchange. Fig. 1 shows the rate of production of H_2 and H^2H and the rate of $^2H_2$ consumption, in the $^2H_2$-H_2O system, catalysed by the hydrogenase of *P. denitrificans* at pH 7.0. The peak heights of mass 2, 3 and 4 were converted into H_2, H^2H and $^2H_2$ concentration by using the factors 0.0746, 0.0624 and 0.0502 nmol/ml per mV, respectively. These factors take into account the mass fractionation that occurs during the diffusion of the gases through the Teflon membrane in the reaction vessel and especially in the ion source. The rate of H_2 and $^2H_2$ production was also corrected for simultaneous consumption by the mass spectrometer using the velocity constant ($k = 0.045$ min^{-1}) measured for $^2H_2$ consumption in the absence of enzyme which obeys first-order reaction kinetics.

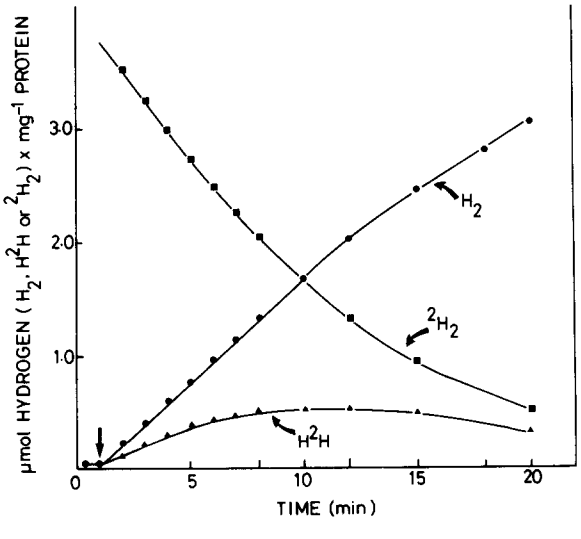

Fig. 1. Time course of H_2 and H^2H production and of $^2H_2$ consumption in the $^2H_2$-H_2O system. Membrane particles from *P. denitrificans* (0.16 mg protein) were incubated in 7 ml of 50 mM phosphate buffer, pH 7.0, as described in Materials and Methods. Measurements were taken at time intervals successively at mass 2 (H_2), mass 3 (H^2H) and mass 4 ($^2H_2$). The membranes were added at the arrow.

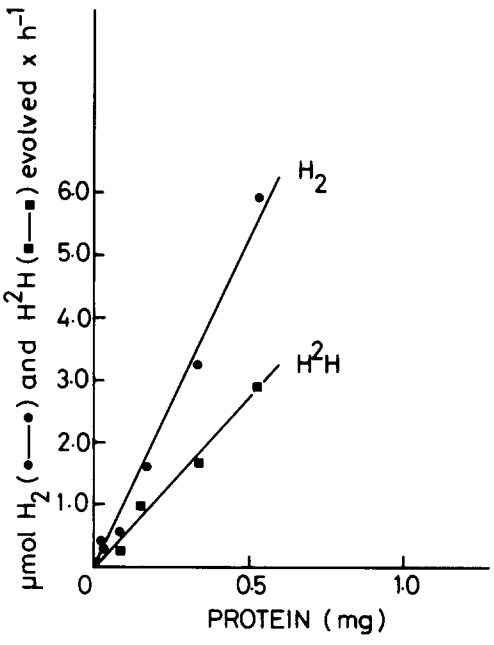

Fig. 2. Rate of H_2 and H^2H evolution as a function of enzyme concentration. Various amounts of membrane vesicles were incubated under the same conditions as in Fig. 1. The initial velocities were determined and were plotted.

It is seen that the production of H^2H is linear during the first 3 min of incubation. The H^2H curve levels off after 4 min due, on the one hand, to the rapid disappearance of $^2H_2$ and, on the other, to further exchange with H^+ of the solvent (H_2 formation). The concentration of H_2 was always higher than that of H^2H right from the beginning of the exchange reaction (indicated by the arrow on Fig. 1).

Effect of enzyme concentration. Fig. 2 shows the rate of H_2 and H^2H evolution as a fuction of the enzyme concentration. Initial velocities were determined and plotted in the figure. As the enzyme concentration increased, the linear part of the H^2H production curve shortened and H^2H concentration decayed more rapidly. With 1.32 mg of membrane protein, H^2H production was linear during 30 s only. The H_2/H^2H ratio calculated from the initial velocities remained the same (1.70) in the range of membrane concentration from 0.08 to

0.45 mg protein. All experiments on isotope exchange were performed with an amount of membrane protein lower or equal to 0.3 mg per assay.

As discussed below, formation of H_2 results probably from further interaction of the enzyme hydride with the solvent. To minimize possible isotopic effects from the solvent, measurements of H-^{2}H exchange were restricted to H^{2}H determinations although such determinations give underestimates of the true exchange activity. No exchange reaction was observed with a membrane preparation which had been heated at 100°C for 5 min.

Effect of pH. The H-^{2}H exchange activity of hydrogenase as a function of pH is shown in Fig. 3. The activity is expressed as the initial rate of H^{2}H formation. The pH of maximum activity

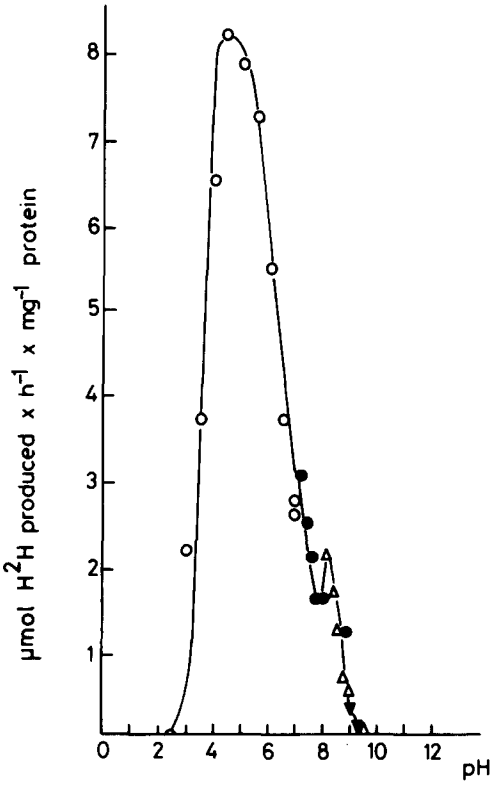

Fig. 3. pH profile of H^{2}H production by the membrane-bound hydrogenase of *P. denitrificans*. H^{2}H formation was recorded continuously for 1 min giving a straight line. The initial velocity was determined from the slope of the line. Protein concentration was 0.16 mg. Buffers (50 mM) were: (○——○) citrate-phosphate; (●——●) phosphate; (△——△) phosphate-glycine-NaOH; (▼——▼) carbonate-bicarbonate-phosphate.

of the enzyme is 4.5. A second peak of activity, much smaller, which in preliminary experiments appeared only as a shoulder, was observed at pH 8.3.

The nature of the buffers influenced the rate of H^{2}H evolution at alkaline pH values; the rate was higher in the presence of Tris and lower in the presence of phosphate. To normalize the test, phosphate was present at the same final concentration at each pH tested (Fig. 3).

The same pH activity curve, with an optimum at pH 4.5, was also obtained when hydrogenase was assayed either with spheroplasts of *P. denitrificans* or with a Triton X-100 extract, instead of membrane particles (not shown).

Comparison of the two systems: $^2H_2$-H_2O and H_2-^2H_2O. The exchange kinetics at the two pH optima (4.5 and 8.3) were determined in the $^2H_2$-H_2O and the H_2-^2H_2O system (Table I). The membranes used for all the determinations reported in Table I were from the same preparation as those used for the experiment of Fig. 1. These membranes had been kept for 18 months at −20°C. At either pH value, the observed rates of H^{2}H and $^2H_2$ (vs. H_2) evolution were much smaller in ^2H_2O than in ordinary H_2O, indicating a negative isotopic effect of ^2H_2O. This effect results in a lower $^2H_2/H^2H$ ratio than the

TABLE I

COMPARISON OF THE EXCHANGE KINETICS WITH THE $^2H_2$-H_2O AND THE H_2-^2H_2O SYSTEMS AT TWO pH VALUES

Conditions as described in Materials and Methods with 0.3 mg protein of membrane particles. The anaerobic conditions were ensured by adding glucose (final concentration 100 µM) and glucose oxidase (38 U). The sparging gas was Ar/$^2H_2$, or Ar/H_2, (8:2, v/v).

Hydrogen species	Initial rates of hydrogen evolution (nmol/min per mg protein)			
	$^2H_2$-H_2O system		H_2-^2H_2O system	
	pH 4.5	pH 8.3	pH 4.5	pH 8.3
H_2	140.0	39.5		
$^2H_2$	–	–	46.4	14.8
H^{2}H	61.0	23.5	26.2	11.6
H_2/H^2H	2.30	1.68	–	–
$^2H_2/H^2H$	–	–	1.77	1.27

H_2/H^2H ratio. Both of these ratios are higher than unity but lower at pH 8.3 than at pH 4.5 (Table I). The isotopic effect observed with the hydrogenase of *P. denitrificans* is, however, not as striking as that reported for the hydrogenase of *Rhodopseudomonas capsulata* chromatophores of which the ratio $^2H/H^2H$ in the H_2-^2H_2O system was lower than unity [8].

Hydrogen production with methyl viologen semiquinone as electron donor

Usually dithionite is used to reduce the colorless, oxidized form of methyl viologen (MV^{2+}), as the semiquinone form ($MV^{+\cdot}$), a dark-violet radical cation absorbing maximally at 602 nm. However, as also reported by Erbes and Burris [17], we observed that below pH 7.0, excess dithionite brought about decoloration of colored $MV^{+\cdot}$ solutions. Furthermore, Mayhew [18] has shown that the redox potential of dithionite depends upon the pH and that dithionite may dismutate and become a less efficient reductant at pH values lower than 7.0 so that the production of H_2 by hydrogenases has not been observed to be linear with time below pH 7.0 when dithionite-reduced methyl viologen was the electron donor [2].

To avoid uncertainties as to the degree of reduction of methyl viologen by dithionite, the dye was reduced instead electrochemically by cathodic reduction in an anaerobic cell flushed with argon (see Materials and Methods). Using this technique it was possible to obtain stable solutions of $MV^{+\cdot}$ and therefore to measure hydrogenase-catalyzed H_2 evolution, even at acidic pH values. Fig. 4 shows that at a pH as low as 4.0, precise linear kinetics for H_2 evolution could be determined even over periods as long as 30 min. The use of electrochemically reduced methyl viologen enabled an accurate pH-dependence curve for H_2 production in *P. denitrificans* to be produced over a large range of pH (pH 2–10). As indicated in Fig. 5, the H_2-producing activity of the hydrogenase displays a pH optimum at 4.5, a result already obtained using the H-^{2}H exchange raction (Fig. 3).

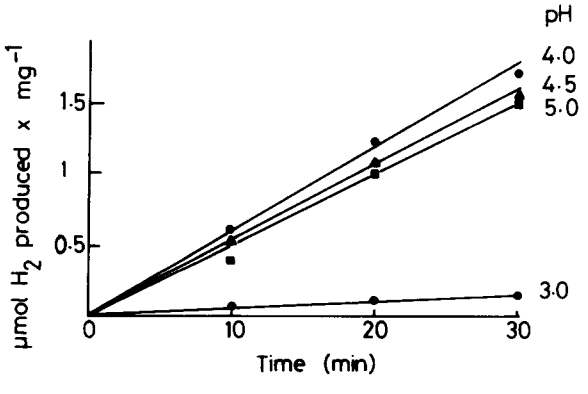

Fig. 4. Kinetics of H_2 production with methyl viologen semiquinone. Membrane vesicles (0.66 mg protein) were incubated anaerobically with reduced methyl viologen as described in Materials and Methods. At various intervals of time samples from the gas phase were withdrawn and assayed for H_2 by gas chromatography. The buffer used was citrate-phosphate.

Hydrogen uptake

Hydrogen uptake with oxygen as electron acceptor. In the presence of electron acceptors, hydro-

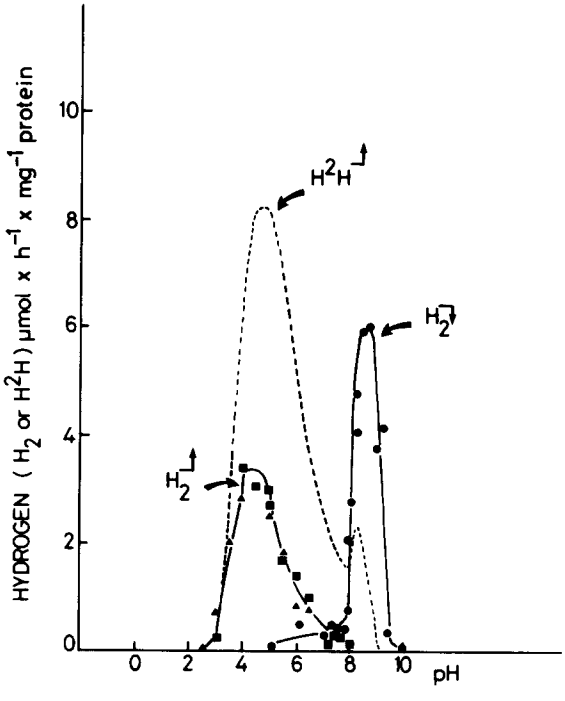

Fig. 5. Effect of pH on H_2 and H^2H production, and on H_2 uptake. H_2 uptake (●———●) in the presence of 0.5 mM benzyl viologen and 0.24 membrane protein was measured by mass spectrometry as described in text. H_2 produced with methyl viologen semiquinone (▲, ■) was measured by gas chromatography as in Fig. 4. The curve of H^2H production of Fig. 3 is reproduced in the dotted line. Buffers were the same as in Fig. 3. The same batch of membrane preparation was used for the three sets of experiments.

genase can catalyse H_2 uptake. Membrane particles of *P. denitrificans* have been shown to transfer electrons from hydrogen to ferricyanide or O_2 [4]. However, in this process redox components of the respiratory chain other than hydrogenase are also involved [4,6]. The pH-dependence of the oxyhydrogen reaction exhibited a profile rather similar to that observed when benzyl or methyl viologen served as electron acceptors (see below).

Hydrogen uptake with viologens as electron acceptors. Oxidized viologens can also accept electrons from H_2. This property has been exploited to assay hydrogenase activity spectrophotometrically [10] and to localize the hydrogenase form *P. denitrificans* on polyacrylamide gels [4] which indicates that viologens bind directly to hydrogenase. Viologens were used here to study the catalytic properties of hydrogenase in its membrane-bound form, either in isolated spheroplasts or in membrane particles. The rate of benzyl viologen and methyl viologen reduction by the membrane-bound hydrogenase of *P. denitrificans* was studied as a function of time. At a low protein concentration (0.28 mg/ml) the reaction was linear with time for more than 10 min. The linear regression calculated over a period of 14 min, in the case of benzyl viologen reduction, indicated a regression coefficient (r^2) of 0.997.

The rate of H_2 uptake by spheroplasts was also proportional to the protein concentration. A linear relationship for benzyl viologen reduction as a function of time was observed in a range of protein concentration from 0.2 to 1 mg/ml ($r^2 = 0.998$). Assays at higher protein concentrations were not attempted to avoid turbidity problems which might have hampered the spectrophotometric determinations.

The pH dependence of the H_2 uptake activity was determined by the spectrophotometric determination of viologen reduction or by the measure of H_2 consumption by mass spectrometry (Fig. 5). For both methyl and benzyl viologen, the pH optimum is at 8.5. Accurate spectrophotometric determinations could not be performed below pH 5.0 due to protein precipitation. At pH 8.5, a double-reciprocal plot of the kinetic data for benzyl and methyl viologen was linear and yielded a K_m value of 0.08 mM for benzyl viologen and of 2 mM for methyl viologen.

Affinity of hydrogenase for H_2 and $^2H_2$: (a) Spectrophotometric determination. Using membrane vesicles, the affinity of hydrogenase for hydrogen (H_2 or $^2H_2$) was determined spectrophotometrically by following the reduction of 5 mM benzyl viologen in 50 mM Tris-HCl, pH 8.5. The Michaelis constants calculated from Lineweaver-Burk double-reciprocal plots yielded a K_m for $^2H_2$ of 0.066 μM ($r^2 = 0.95$) and a K_m for H_2 of 0.060 μM ($r^2 = 0.97$). Under optimal conditions, V_{max} values were 3.5 μmol hydrogen consumed/h per mg protein for the oxidation of H_2 and $^2H_2$, respectively. In other words, hydrogenase shows the same affinity (0.06 μM) and has the same V_{max} for either H_2 or $^2H_2$ in ordinary H_2O.

However, when the affinity of hydrogenase for H_2 was measured in ^2H_2O instead of H_2O, the K_m was not significantly affected ($K_m \approx 0.06$ μM) whereas the V_{max} value decreased about 10-fold, showing a marked isotopic effect of ^2H_2O.

It appeared that the nature of the buffer used to determine the pH dependence of the H_2 uptake greatly affected the rate and extent of H_2 uptake without displacement of the pH optimum. At similar pH values, hydrogenase activity increased 2-fold when Tris buffer was used instead of phosphate. Carbonate was slightly inhibitory. All buffers were used at a final concentration of 50 mM. Salts such as sodium carbonate, ammonium, manganese or magnesium sulfate, potassium nitrate or nitrite, calcium, magnesium, potassium or sodium chloride were individually assayed with membrane vesicles from *P. denitrificans* for H_2 uptake activity with benzyl viologen as electron acceptor. No striking effect of these salts was observed at concentrations up to 0.1–0.2 M when the reaction medium was adequately buffered; however, at 0.5 M almost complete inhibition of H_2 uptake was observed.

(b) Determination by mass spectrometry. H_2 and $^2H_2$ uptake was also directly measured in the reaction vessel linked to a mass spectrometer (cf. Materials and Methods). The vessel filled with 7.6 ml of 50 mM glycine-phosphate buffer, pH 8.3, and containing 100 μM glucose and glucose oxidase (38 units), was sparged with a mixture of Ar and H_2 (or Ar and ^2H) (8:2, v/v). Then 0.5 ml of 2.5 mM benzyl viologen was added. At time zero, 10 μl of the membrane suspension (0.3 mg protein)

were injected by a gas-tight syringe into the medium. A K_m for H_2 of 0.09–0.1 μM was obtained, a value in the same range as that obtained with spectrophotometric measurements of benzyl viologen reduction (see above).

Discussion

This paper demonstrates that like other hydrogenases, the membrane-bound hydrogenase of *P. denitrificans* is able to catalyze an isotope exchange gas reaction between ^2H_2O and H_2 or between H_2O and $^2H_2$.

Isotope exchange reactions catalyzed by hydrogenase was first reported by Farkas et al. [19] with whole cells of *Escherichia coli* (*B. coli*). Later, the reaction was studied in more detail with whole cells and cell-free extracts from *Proteus vulgaris* [14,20]. The mechanism of H-^{2}H exchange has also been investigated with the hydrogenase isolated from *Desulfovibrio desulfuricans* [21], *Clostridium pasteurianum* [15], *Desulfovibrio vulgaris* [9] and *Chromatium* [22].

The method used in this study to determine the isotope exchange reaction proved very sensitive, accurate and specific. The technique is based on the continuous determination of changes in concentration of gases dissolved in the incubation medium [8]. At any time during the assay, the sum of $^2H_2$, H^2H and H_2 remained constant. In other words, as illustrated in Fig. 1, the amount of H^2H and H_2 evolved always equalled the amount of $^2H_2$ consumed. The response of the apparatus was so rapid and so sensitive that it was possible to measure an exchange reaction within the first few seconds following the mixing of hydrogen and membrane preparation, while long incubation periods (hours) had been used previously by other authors [9,14,15,23] who measured hydrogen isotopes in the gas phase instead of the incubation medium.

The direct determination of dissolved gases allows the use of $^2H_2$ gas in ordinary H_2O equally well as H_2 in ^2H_2O, contrary to what was reported by Arp and Burris [23] for the study of isotope exchange by the hydrogenase from soybean root nodules. The system $^2H_2$-H_2O is indeed easier to handle than the system H_2-^2H_2O which requires lyophilization of the membrane vesicles and buffers to eliminate all H_2O molecules before resuspension in ^2H_2O.

The H-^{2}H exchange activity has been mostly studied with soluble hydrogenase and maximal activities were generally observed at acidic pH values: 6.2 with whole cells of *P. vulgaris* [20], 6.0 and 5.5 with the hydrogenase from *Chromatium* [22] and *D. vulgaris* (formerly *desulfuricans*) [21] respectively. In contrast, a pH optimum of 8.3 was reported for the exchange activity mediated by the bidirectional hydrogenase of *C. pasteurianum* [15].

The H-^{2}H exchange mediated by the membrane-bound hydrogenase of *P. denitrificans* had the same pH optimum (4.5) (Fig. 3) as that observed with the hydrogenase embedded in *Rps. capsulata* chromatophores [24]. As protons are substrate in the exchange reaction it may be expected that the isotope exchange will be favored at low pH although iron-sulfur clusters are more labile in that pH range [25]. It is striking that synthetic [4Fe-4S] cluster complexes which exist in solution as acid-base pairs have also a pK_a of 4 [25].

The pH-activity curve for H_2 production which is strikingly similar to that of the H-^{2}H exchange reaction below pH 8.0 (Fig. 5) confirmed that hydrogenase was indeed functioning maximally at pH 4–4.5. It is the first time that hydrogenase-catalysed H_2 production is reported to be optimal at pH 4.5. Technical difficulties in obtaining linear rates of H_2 production at acidic pH [2] have prevented other authors testing the activity of hydrogenase at such low pH values. Since the hydrogenase of *P. denitrificans* was used in its membrane form, it is probable that the Fe-S cluster is protected from the bulk medium. Resistance to acid hydrolysis has also been observed with the Hipip protein from *Chromatium* [26].

The second pH maximum observed for H-^{2}H exchange occurred at pH 8.3, very close to the optimum pH for H_2 uptake (Fig. 5).

The experiments reported in Fig. 5, carried out with the same membrane preparation, enables a comparison to be made of the rates of isotope exchange, H_2 production and H_2 uptake. The rate of isotope exchange is by far the highest, in spite of the fact that the rate of H^2H formation is an underestimate of the isotope exchange rate. At the optimal pH (4.5), the exchange activity is at least twice as great as the H_2-production activity (8.2

μmol H^2H produced/h per mg protein compared to 3.8 μmol H_2 evolved/h per mg protein with $Mv^{+\cdot}$ as electron donor). On the other hand, in the alkaline pH range, H_2-uptake activity is apparently greater than the isotope exchange (but they would be about the same if both the amounts of H^2H and H_2 formed were taken into account). When measured at their respective optimal pH, the H_2-uptake activity was about twice as high as the H_2-production activity (Fig. 5).

As with hydrogenase, activation of hydrogen by metal ions and complexes is assumed to involve a heterolytic splitting of H_2 (or $^2H_2$) [14,16,27]. The involvement of the metal of hydrogenase has not yet been directly demonstrated. Erbes et al. [28] showed by EPR measurements that CO, a competitive inhibitor of H_2, modifies the EPR signal of an iron-sulfur cluster of the *C. pasteurianum* hydrogenase and concluded that the cluster is the active site which serves as site of binding of H_2 and is able to accept one or two electrons from and donate either one or two electrons to substrates. However, Van Heerikhuizen et al. [29] were unable to detect any change of the iron EPR signal of *Chromatium* hydrogenase and concluded that H_2 does not bind to an iron cluster.

The kinetics of the $H\text{-}^2H$ exchange reaction determined with the hydrogenase of *P. denitrificans* present the following features: (a) both the single-exchange product (H^2H) and the double-exchange product (H^2), in the $^2H_2\text{-}H_2O$ reaction, appear from the start of the reaction (Fig. 1); (b) the formation of H_2 is faster than that of H^2H (Figs. 1 and 2); (c) the ratio of the initial rates of formation of H_2 to H^2H is constant and independent of enzyme concentrations. This H_2/H^2H ratio was 1.70 in the range of protein concentration from 0.08 to 0.45 mg protein; (d) the initial rates of H_2 and of H^2H formation increase with enzyme concentration (Fig. 2).

The mechanism proposed by Halpern and James [30] for the $^2H_2\text{-}H_2O$ exchange catalysed by ruthenium chloride is a useful model for the exchange reaction catalysed by the hydrogenase of *P. denitrificans*. In this model:

$$\begin{aligned} &Ru(III)Cl^- + {}^2H_2 \rightarrow {}^2H^+ + Ru(III)^2H^- \\ &\quad \xrightarrow{H^+ + Cl^-} Ru(III)Cl^- + H^2H \\ &\quad \xrightarrow{+H_2O} Ru(III)H^- + H^2HO \\ &\quad \rightarrow Ru(III)Cl^- + 2Fe(II) + {}^2H^+ + 2Fe(III) + Cl^- \end{aligned}$$

The rate-determining step involves the heterolytic splitting of $^2H_2$ and the isotopic exchange occurs through reversal of that reaction. The hydride ion ($^2H^-$) which is transferred to Ru(III) occupies one of the normal ligand positions in the coordination shell of Ru(III). According to that mechanism, both hydrogen atoms of the hydrogen molecule can exchange with the solvent but the atom forming the metal hydride exchanges more slowly than the atom released immediately as H^+. In this model, H^2H is not the necessary intermediate in the formation of H_2 (or $^2H_2$); both H^2H and H_2 (or $^2H_2$) are primary exchange products.

The same authors [30] observed that Ru(III) can also catalyse the oxidation of $^2H_2$ by Fe(III) and postulated that this oxidation of hydrogen involves the same hydride intermediate ($Ru(III)^2H^-$) as the exchange reaction, the formation of the hydride being the rate-limiting step in both the isotope exchange and the hydrogen oxidation. Similarly, an enzyme hydride had been postulated [14,16] to be the active reducing agent for electron acceptors of hydrogenase as well as the intermediate in the evolution of H_2 from reduced redox components or for H^2H formation. A similar pH profile for the exchange reaction and H_2 evolution on the one hand, and the exchange reaction and H_2 uptake on the other (Fig. 5) indicate that a common intermediate is involved in the three reactions catalysed by the hydrogenase of *P. denitrificans*. It further suggests that a very acidic (pK_{app} 4.5) and a basic group (pK_{app} 8.3–8.5) of the hydrogenase protein are involved. In the dry state, the hydrogenase form *D. vulgaris* was shown to have protons which are directly exchangeable with H_2 during the catalytic process [31]. Therefore, amino acid residues of hydrogenase, with apparent pK values of 4.5 and 8.3–8.5, might be expected to participate in the catalytic reaction.

Our experimental conditions (no gas phase, direct, rapid (in the second range) and continuous monitoring of the concentrations of dissolved gases, hydrogenase constantly saturated with hydrogen) allowed us to determine the initial rates of direct product formation. These conditions minimized the possible interference of a multiple exchange process whereby a dissolved H^2H molecule produced by the primary exchange reaction between $^2H_2$ and H_2O undergoes a second exchange

reaction with H_2O to produce H_2. It was shown that the double-exchange product is formed initially and apparently faster than the single-exchange product (Figs. 1 and 2), that its rate of formation is dependent on the pH (Table I) and that the ratio of the products (H_2/H^2H) is independent of enzyme concentration. Such results are predicted by the following mechanism. The enzyme has two distinct H-binding sites; each H atom can exchange with the solvent independently and at a different exchange rate [9]. The ratio of the products (H_2 and H^2H in the $^2H_2$-H_2O system) is independent of enzyme concentration but varies with pH, indicating that the exchange rates of each H atom are affected differently by pH. Work underway aims at the identification of the chemical nature of the two H-binding sites.

The membrane-bound hydrogenase of *P. denitrificans* has a very great affinity for hydrogen with a K_m of 0.06–0.07 μM for H_2 or $^2H_2$ in ordinary H_2O. The hydrogenase of *P. denitrificans* presents, therefore, no difference in affinity for H_2 or $^2H_2$, contrary to what was reported for the hydrogenase of either *D. vulgaris* [9] or *C. pasteurianum* [32]. On the other hand, there was a marked isotopic effect in ^2H_2O, where the V_{max} for hydrogen uptake in the presence of benzyl viologen was 10-times lower than in ordinary H_2O. The rate of H^2H formation was also reduced (by half) in ^2H_2O (Table I).

The unidirectionality of the so-called uptake hydrogenases which are generally membrane-bound appears therefore to result from different factors favoring H_2 oxidation. These factors are; (a) a microenvironment of the active site maintaining an alkaline pH, and perhaps an oxidoreduction state of the iron clusters at high potential; (b) a strong affinity of the enzyme for H_2; (c) the presence of electron acceptors readily available to hydrogenase in the membrane. However, as shown in this study, under appropriate conditions the membrane-bound hydrogenase functions reversibly as the so-called reversible hydrogenases.

Up to now the physiological role of hydrogenase has been linked essentially to its ability to transfer electrons. In fermentative bacteria the physiological role of hydrogenase is to reduce H^+ as a means of oxidizing the carriers reduced during fermentation. In anaerobic bacteria where sulfate, nitrate, CO_2 or fumarate can serve as terminal electron acceptors and in aerobes where O_2 is the final acceptor, hydrogenase enables the cell to utilize H_2 as a source of energy and catalyses the transfer of electrons from hydrogen to appropriate acceptors.

The capacity of the membrane-bound hydrogenase of *P. denitrificans* to produce hydrogen at acidic pH values may reflect another possible physiological function of the enzyme. Spheroplasts of *P. denitrificans* were shown to release H^+ inside the cell when the hydrognase embedded in the cytoplasmic membrane was oxidizing hydrogen gas with benzyl viologen as electron acceptor [33]. The functioning of hydrogenase in the reverse way (at low pH and with a reductant) should lead to the consumption of intracytoplasmic H^+ for the formation of H_2 with external nonpermeant reductant(s) as electron donor(s). This role in the regulation of cytoplasmic pH may be one of the most primitive physiological functions of (ancestral) hydrogenase in *P. denitrificans* [34]. However, the appropriate, naturally occurring, electron donor(s) have not been identified.

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