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A proton-deuterium exchange study of three types of *Desulfovibrio* hydrogenases

Guy D. Fauque[a,b], Yves M. Berlier[a], Melvin H. Czechowski[b], Bernard Dimon[a], Paul A. Lespinat[a] and Jean LeGall[a,b]

[a] *A.R.B.S. – Equipe Commune d'Enzymologie CNRS-CEA, C.E.N. Cadarache, Saint-Paul-lez-Durance, France, and* [b] *Department of Biochemistry, School of Chemical Sciences, University of Georgia, Boyd Graduate Studies Research Center, Athens, GA, U.S.A.*

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SUMMARY

Hydrogenases are among the main enzymes involved in bacterial anaerobic corrosion of metals. The study of their mode of action is important for a full comprehension of this phenomenon. The three types of *Desulfovibrio* hydrogenases [(Fe), (NiFe), (NiFeSe)] present different patterns in the pH dependence of their activity. The periplasmic enzyme from *Desulfovibrio salexigens* and the cytoplasmic enzyme from *Desulfovibrio baculatus* both have pH optima at 7.5 for H_2 uptake and 4.0 for H_2 evolution and H^+-D_2 exchange reaction (measured by membrane-inlet mass-spectrometry). The H_2 to HD ratio at pH above 5.0 is higher than 1.0. The periplasmic hydrogenase from *D. gigas* presents the same pH optimum (8.0) for the H^+-D_2 exchange as for H_2 consumption. In contrast, the enzyme from *D. vulgaris* has the highest activity in H_2 production and in the exchange at pH 5.0. Both hydrogenases have a H_2-to-HD ratio below 1.0.

INTRODUCTION

The sulfate-reducing bacteria form a morphologically and physiologically distinctive group of anaerobic microorganisms, their oxidative metabolism being based not on fermentation, but on the utilization of sulfate or other sulfur anions as terminal electron acceptors [31,41,55].

Correspondence: Dr. G.D. Fauque, A.R.B.S. – Equipe Commune d'Enzymologie CNRS-CEA, C.E.N. Cadarache, 13108 Saint-Paul-lez-Durance Cedex, France.

These microorganisms are the principal agent for the anaerobic bacterial corrosion of steel, with important environmental and economic consequences [24]. The sulfate-reducing bacteria also participate in interspecies sulfur transfer with photosynthetic organisms [8,9] and in interspecies hydrogen transfer in the complex fermentation leading to the formation of methane [11].

Hydrogenases and dissimilatory-type bisulfite-reductases from sulfate-reducers are two key enzymes in microbial corrosion. Hydrogenases are implicated in hydrogen consumption from the

metal surface [53] or from the iron sulfide film on the metal [27], and also in hydrogen production, which results in the phenomenon of hydrogen embrittlement of steels [24]. Hydrogen sulfide, the product of dissimilatory sulfate reduction, acts in the corrosion process either as a cathodic or as an anodic reactant [14,54]. It has been implicated in the formation of a reactive volatile phosphorus compound [26].

From a biochemical point of view, hydrogenases constitute a class of enzymes of diverse structure, though, they perform a limited number of functions [1]. They catalyze the oxido-reduction of the dihydrogen molecule:

$$H_2 \rightleftharpoons 2H^+ + 2e^-$$

Although this reaction is reversible, its overall balance can lead, depending on the microorganism and on the enzyme localization in the cell, to either production or uptake of hydrogen [13,44]. In anaerobic bacteria with an oxidative metabolism, such as *Desulfovibrio* sulfate-reducers, the hydrogenase activity can be diverted in either direction, according to the electron transfer system present [40]. In some species of *Desulfovibrio*, such as *D. gigas*, two hydrogenases with different localization could recycle H_2 between the internal and external sides of the membrane during sulfate reduction to sulfide [39].

The localization of hydrogenases in the cell is of particular importance in metal corrosion, since periplasmic hydrogenases can more easily gain access to the metal surface than membrane-bound or cytoplasmic enzymes. Furthermore, in *D. vulgaris* Hildenborough, the periplasmic hydrogenase appears to be two orders of magnitude more active than the membrane-bound enzymes [35a].

In vitro, these hydrogenases are reversible and catalyze either hydrogen evolution or consumption in the presence of appropriate electron mediators. They also mediate an exchange reaction between protons and different hydrogen isotopes [6,28]. The proton-deuterium exchange reaction, in which D_2 disappears and is quantitatively replaced by HD and H_2, was used for the first time [57] to determine the intrinsic activity of the membrane-bound purified hydrogenase from *D. vulgaris* strain Miyazaki in long-term kinetic experiments with electron mediators such as sodium dithionite or low redox potential tetraheme cytochrome c_3. Recently, the use of a membrane-inlet to the mass-spectrometer ion source allowed determination of the initial stages of reaction and permitted the distinction between the single (HD) and the double (H_2) products of the H^+-D_2 exchange reaction [6].

The pH controls the concentration of protons which are either products or substrates of the reversible oxido-reduction of H_2 and is a critical hydrogenase parameter [34].

In this paper we describe the pH profiles obtained for the proton-deuterium exchange activities of the iron-enzyme from *D. vulgaris* strain Hildenborough, the nickel-iron protein from *D. gigas* and the nickel-iron-selenium hydrogenases from *D. salexigens* British Guiana and *D. baculatus* 9974.

MATERIALS AND METHODS

Organisms and growth conditions

D. vulgaris strain Hildenborough (NCIB 8303), *D. gigas* holotype strain (NCIB 9332), *D. salexigens* strain British Guiana (NCIB 8403) and *D. baculatus* strain 9974 (DSM 1743), an isolate from the mixed culture '*Chloropseudomonas ethylica*' strain N_2 [8], were grown at 37°C on a standard lactate-sulfate medium [45] supplemented with 3.0% sodium chloride for the halophilic *D. salexigens*. Mass cultures of the four strains were obtained as described previously [20].

Analyses

Protein concentrations were determined according to the method of Lowry et al. [36].

Chemicals and gases

All reagents and chemicals were analytical reagent grades and were used without further purification. Gases were from l' Air Liquide (Paris, France), grade N 30. Deuterium gas (99.8% D_2) was purchased from Oris Saclay (Gif-sur-Yvette, France). Oxygen was removed from gas mixtures

by passing them through a solution of photo-reduced methylviologen [46].

Purification of hydrogenases

The cytoplasmic hydrogenase from *D. baculatus* and the periplasmic enzymes from *D. gigas*, *D. vulgaris* and *D. salexigens* were purified to homogeneity as previously reported [16,17,25,38,47,50,51]. Enzyme purity was established by polyacrylamide gel electrophoresis under non-denaturing conditions [10].

Assays of hydrogenase activities

Hydrogen uptake, hydrogen evolution and the proton-deuterium exchange were followed under strict anaerobic conditions by mass-spectrometry of the dissolved gases in a reaction vessel connected by a membrane-inlet to the ion source of the mass-spectrometer (VG 8–80 equipped with an Apple II data acquisition system). The mass-peaks of interest [2 (H_2), 3 (HD), 4 (D_2) and 32 (O_2)] were automatically scanned by a peak jumping system.

Hydrogen uptake was determined by following the disappearance of this gas from buffers saturated with a mixture of 80% N_2 and 20% H_2 in the presence of 5 mM oxidized benzylviologen [4]. Hydrogen production was followed in the presence of 1 mM methylviologen, semi-quinone form, prepared by cathodic reduction in an electrochemical vessel [52]. The proton-deuterium exchange reaction was performed after saturating the buffered medium with a mixture of 20% D_2 in N_2 [6]. The three reactions were initiated by injecting in the vessel 1–5 μl of a hydrogenase solution containing 0.5–4 μg of protein. Hydrogen consumption and evolution were followed without preactivation of the enzyme, whereas for the H^+–D_2 exchange reaction the hydrogenases were pre-incubated for at least 1 h under D_2 or H_2, except for the *D. gigas* periplasmic enzyme, which was too unstable under these conditions [7,34]. Buffers which varied by half pH values between pH 10.5 and 2.5 were prepared from standard solutions: glycine-sodium hydroxide, disodium phosphate-monosodium phosphate and citrate-phosphate. The different buffers present similar kinetics for the same pH value.

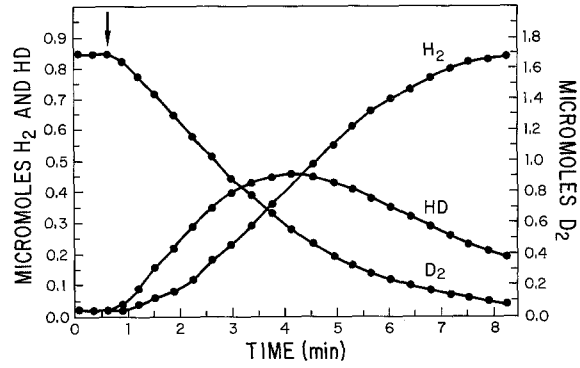


Fig. 1. H^+-D_2 exchange kinetics catalyzed by the periplasmic hydrogenase from *Desulfovibrio gigas*. The Tris-HCl buffer (50 mM, pH 7.6) was sparged till equilibrium with a mixture of 20% D_2 in N_2. At the time indicated by the arrow, 1.5 μg of enzyme were injected. Peaks 4 (D_2), 3 (HD) and 2 (H_2) were recorded.

RESULTS

The respective exchange kinetics obtained with the purified hydrogenases from *D. gigas* (periplasmic) and *D. baculatus* (cytoplasmic) show differences in the ratio between the initial HD and H_2 productions (Figs. 1 and 2). With the *D. gigas* hydrogenase the H_2 to HD ratio was much lower than 1.0 (Fig. 1) (H_2/HD + H_2 around 0.35), whereas the *D. baculatus* enzyme presented a H_2 to HD ratio higher than 1.0 (Fig. 2) (H_2/HD + H_2 around 0.60).

The effect of pH on the H^+-D_2 exchange reaction catalyzed by the *D. salexigens* periplasmic hydrogenase was compared to the results obtained

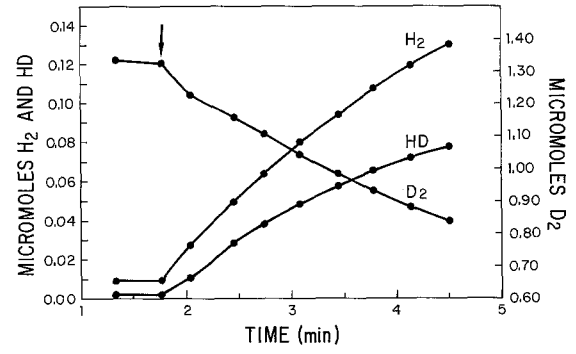


Fig. 2. H^+-D_2 exchange kinetics catalyzed by the cytoplasmic hydrogenase from *Desulfovibrio baculatus* strain 9974. Same conditions as in Fig. 1, except pH 6.0 and 0.5 μg of enzyme.

with the other three enzymes [34]. Different pH profiles were also obtained with the hydrogenase from *D. salexigens* for the HD and H_2 components (Fig. 3). Both rose as the pH decreased from 7.6 to 5.0 (with the ratio $H_2/HD + H_2$ around 0.6), but below pH 5.0 the rate of H_2 production slowed down while the HD evolution continued increasing until pH 3.5. The overall exchange activity reached a maximal value at pH 3.5–4.0 (900 μmol HD + H_2 evolved/min/mg protein).

Table 1 compares the catalytic activities in the H_2 production, H_2 uptake and H^+-D_2 exchange reaction of the three types of *Desulfovibrio* hydrogenases. With the periplasmic hydrogenase from *D. vulgaris*, the highest H^+-D_2 exchange activity was found at pH 4.5–5.0, and the maximum specific exchange activity was equal to 2700 μmol of HD + H_2 evolved/min/mg protein. The pH optima for the HD and H_2 components were 4.75 and 5.25, respectively, and the H_2 to HD ratio was below unity

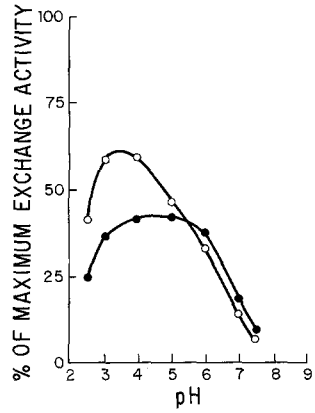


Fig. 3. pH effect on the H^+-D_2 exchange activity of the periplasmic hydrogenase from *Desulfovibrio salexigens* strain British Guiana. 10 ml of the different buffers were sparged until saturation with N_2/D_2, 80:20 (v/v). The reaction was initiated by injecting 2 μg of hydrogenase previously activated under H_2. The appearance of HD (○) and of H_2 (●), as well as the disappearance of D_2 (not shown) together with the level of O_2 (not shown) were monitored. The results are expressed as percentage of the maximum total exchange activity (HD + H_2).

Table 1

Catalytic activities of iron, iron-nickel and iron-nickel-selenium hydrogenases isolated from *Desulfovibrio*

Activity	*D. vulgaris* Hildenborough	*D. gigas*	*D. baculatus*[a] strain 9974	*D. salexigens* British Guiana
Hydrogen production				
S.A.[b]	4 800	440	466	1 830
pH optimum	5.6–6.0	4.0–4.5	4.0	4.5–5.0
Hydrogen uptake				
S.A.	50 000	1 500	120	1 300
pH optimum	8.4	8.0	7.5	7.5–8.0
H^+-D_2-exchange reaction[c]				
S.A.	2 700	267	350	900
pH optimum	4.5–5.0	7.5–8.0	3.0 (HD) 5.0 (H_2)	3.5 (HD) 5.0 (H_2)
$H_2/HD + H_2$	≃0.3[d]	≃0.3[d]	≃0.6 (pH 9 → 4.5) ≃0.2 (pH 3.0)	≃0.6 (pH 7.6 → 5) ≃0.4 (pH 3.5)
Refs.	25,34,42,51	22,34,38	34,50	17,this work

[a] Cytoplasmic enzyme.

[b] Specific activity expressed in μmol H_2 (produced or consumed) or (HD + H_2) evolved per minute and per mg of protein.

[c] At pH 7.6, ratios of $H_2/HD + H_2$ of 0.30 and 0.95 were obtained with ruthenium chloride and platinum oxide, respectively.

[d] At pH optimum for the H^+-D_2 exchange reaction.

(H_2/HD + H_2 around 0.3) [34]. The latter protein had the highest specific activities in all three hydrogen reactions and exhibited pH optima at 5.6–6.0 in H_2 evolution and 8.4 in H_2 consumption (Table 1).

The *D. gigas* periplasmic hydrogenase presented pH optima at 4.0–4.5 in H_2 production and 8.0 in H_2 uptake. The pH optimum in the H^+-D_2 exchange reaction (around 7.5–8.0) was in the range of H_2 consumption even though the specific activity (267 μmol of HD + H_2 evolved/min/mg protein) was of the same order as found in H_2 evolution (440 μmol H_2 produced/min/mg protein) [22,34] (Table 1).

The cytoplasmic hydrogenase from *D. baculatus* exhibited pH optima in H_2 uptake and evolution at 7.5 and 4.0, respectively, with maximum H_2 production higher than maximum H_2 consumption (466 and 120 μmol/min/mg protein, respectively). The H^+-D_2 exchange activity reached a maximal value (350 μmol HD + H_2 evolved/min/mg protein) at about the same pH as H_2 production (Table 1) [34].

DISCUSSION

Hydrogenase is responsible for the reversible oxido-reduction of molecular hydrogen and plays a central metabolic feature in many anaerobic microorganisms, resulting in extensive study of several *Desulfovibrio* species [31,33,40].

Most often, the *Desulfovibrio* hydrogenase activity is located in the periplasm: *D. vulgaris* strains Hildenborough [25,51], Marburg [3] and Miyazaki K [2], *D. baculatus* 9974 [50], *D. gigas* [5], *D. salexigens* British Guiana [17,47] and *D. desulfuricans* NRC 49001 [37]. Three types of purified periplasmic hydrogenases from *Desulfovibrio* have been characterized to date: the first from *D. vulgaris* Hildenborough contains exclusively non-heme iron [(Fe) hydrogenase] [25,42,51], the second from *D. gigas* contains non-heme iron and redox-active nickel [(NiFe) hydrogenase] [12,23,48], while the third from *D. baculatus* 9974 contains nickel, non-heme iron and selenium [(NiFeSe) hydrogenase] [50]. In contrast to the (Fe) hydrogenase which has only been isolated in *D. vulgaris* Hildenborough, (NiFe) and (NiFeSe) hydrogenases have been purified and characterized in several species of *Desulfovibrio*, including *D. desulfuricans* (ATCC 27774) [29], *D. multispirans* n. sp. (cytoplasmic) [18] for the (NiFe) hydrogenases and *D. salexigens* British Guiana (periplasmic) [16,17,47], *D. desulfuricans* Norway 4 (soluble and membrane-bound) [30,43] and *D. baculatus* 9974 (cytoplasmic and membrane-bound) [50] for the (NiFeSe] hydrogenases.

The existence of more than one hydrogenase has also been recently reported in *D. vulgaris* strains Miyazaki F [56] and Hildenborough [23,35a]. Indeed, there are indications that *D. vulgaris* Hildenborough contains all three types of hydrogenases which could be differentially expressed during the growth cycle or under different growth conditions [35a].

Table 2 summarizes the data on the activity, molecular weight, localization and metal center composition of the four hydrogenases used in this study. All these enzymes contain two subunits and are active both in H_2 uptake and production. The periplasmic hydrogenase from *D. salexigens* British Guiana possesses the highest specific activity in H_2 evolution and in H^+-D_2 exchange reaction presently known for (NiFe) or (NiFeSe) *Desulfovibrio* enzymes [35]. The pH optima found in hydrogen uptake (7.5–8.0) and in H_2 evolution (4–4.5) were very similar for the cytoplasmic hydrogenase from *D. baculatus* and for the periplasmic enzyme from *D. gigas*, even though the major activity for the former is in H_2 evolution while for the latter it is in hydrogen consumption. With the *D. vulgaris* hydrogenase the pH optimum in H_2 production is more compatible with physiological conditions (5.6–6.0) but the major activity is nevertheless in H_2 consumption. In vivo, the hydrogenases seem not always to function at their pH optimum, mainly in the H_2 evolution.

The exchange reaction does not involve any electron transfer step except in the initial activating process of the enzyme in which the hydrogenase becomes reduced by electrons from its substrate, D_2 or H_2 [7]. The periplasmic *D. gigas* hydrogenase has

Table 2

Physico-chemical properties of iron, nickel-iron and nickel-iron-selenium hydrogenases from four *Desulfovibrio* species

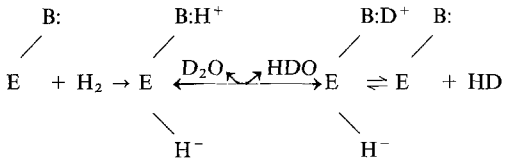
Property	*D. vulgaris* Hildenborough	*D. gigas*	*D. baculatus* strain 9974	*D. salexigens* British Guiana
Molecular weight (kDa)	56	89.5	100	98
Subunits	2	2	2	2
Localization	periplasm	periplasm	periplasm	periplasm[a]
Metal content				
non-heme iron	12	11	14	12
nickel	0	1	1	1
selenium	NR[b]	0	1	1
Specific activity[c]				
uptake	50 000	1 500	120	1 300
production	4 800	440	466	1 830
References	23,42,51	22,38	47	17,47

[a] At least 50% of the hydrogenase was located in the periplasm [17].

[b] Not reported.

[c] Expressed in μmol H_2/min/mg protein.

its maximal H^+-D_2 exchange and H_2 uptake activities around pH 8.0, indicating that the limiting step with this protein is dihydrogen activation. In contrast, the cytoplasmic hydrogenase from *D. baculatus* and the periplasmic proteins from *D. vulgaris* and *D. salexigens* have a pH optimum in H^+-D_2 exchange similar to that of H_2 production. The four hydrogenases assayed here differ in their respective rates of HD and H_2 evolution in the H^+-D_2 exchange reaction. The most generally postulated mechanism for the exchange reaction consists in a heterolytic cleavage of the D_2 or H_2 molecule [28] with the formation of one hydride molecule and one proton according to the following scheme [6]:



in which E is the enzyme and B the proton acceptor site. The back reaction leads to the production of the hybrid molecule HD:

$$D_2 + H^+ \rightleftharpoons HD + D^+$$

H_2 is also formed according to:

$$HD + H^+ \rightleftharpoons H_2 + D^+$$

The process finally results in H_2 accumulation because of D^+ dilution in H_2O.

The *Desulfovibrio* hydrogenases can be divided into two types according to the ratio found for the initial HD and H_2 production in the H^+-D_2 exchange reaction. The first type includes hydrogenases which have a H_2/HD ratio higher than 1 [H_2/(HD + H_2) around 0.6], e.g. *D. salexigens* and *D. baculatus*. The second type is represented by the hydrogenases from *D. vulgaris*, *D. gigas* and *D. multispirans* (Y. Berlier, G. Fauque, M. Czechowski, B. Dimon, P.A. Lespinat and J. LeGall, unpublished results) which have a H_2/HD ratio lower than 1 [H_2/(HD + H_2) around 0.3]. The soluble (NiFe) hydrogenase isolated from the methanogenic bacterium *Methanosarcina barkeri* (DSM 800) grown on methanol [21,22] belongs to this second type of proteins, with a H_2/HD ratio of 0.42 at pH 7.6 [19].

The H_2/HD ratios are generally utilized to differentiate between a homolytic versus a heterolytic cleavage of the dihydrogen molecule. In similar

H^+-D_2 exchange experiments with metal salts, $H_2/HD + H_2$ ratios of 0.30 with ruthenium chloride and of 0.95 with platinum oxide were found (Table 1). $RuCl_3$ and PtO_2 could serve respectively as analogs for the heterolytic and homolytic cleavages of the dihydrogen molecule. The different functional patterns of the three types of *Desulfovibrio* hydrogenases are probably related to differences in the composition and structure of their active centers (Table 2). Nickel(III) and one (4Fe-4S] cluster have been proposed respectively as the binding sites of the hydride and of the proton in the *D. gigas* periplasmic hydrogenase [49]. The differences observed in the H^+-D_2 exchange kinetics of (NiFe) and (NiFeSe) *Desulfovibrio* hydrogenases could reflect different ligation to the nickel, because it is not known whether selenium is present at a new catalytic site. The EPR studies of hydrogen-reduced states of (NiFe) and (NiFeSe) hydrogenases show very similar redox patterns of the EPR spectra, suggesting a common mechanism for the activation and oxido-reduction of the dihydrogen molecule [47].

This study of three types of *Desulfovibrio* hydrogenases as explored by the proton-deuterium exchange reaction shows that, in complex anaerobic ecosystems which favor metal corrosion, sulfate-reducing bacteria are able to activate hydrogen. Depolarization of metal surfaces can possibly occur via a displacement of the equilibrium between hydrogen adsorbed at the metal surface and free molecular hydrogen [24]. Then, such a depolarization is possible over a wide range of pHs, with maximal activities ranging from 8 for (NiFe) hydrogenases to 3 for (NiFeSe) hydrogenases (Table 1). Such a phenomenon is not revealed by classical methods used for the detection of hydrogenases, which are hampered by the presence of artificial dyes often used in the detection of bacterial-induced cathodic depolarization [15,24]. The proton-deuterium exchange reaction, as measured by membrane-inlet mass-spectrometry, has also recently allowed the detection of high hydrogenase activity in cells of *Desulfotomaculum orientis* [35]. This result shows that, in contrast to previous conclusions [15], these sporogenic sulfate-reducing organisms can also be involved in anaerobic corrosion of metals via hydrogen depolarization.

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