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THE MEMBRANE-BOUND HYDROGENASE OF *RHODOPSEUDOMONAS CAPSULATA*

STABILITY AND CATALYTIC PROPERTIES

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The hydrogenase of *Rhodopseudomonas capsulata* is an intrinsic membrane protein extractable from the membrane by detergents. Triton X-100 produces stable soluble extracts. Stability of solubilized hydrogenase depends drastically on two factors: temperature and gas-phase. The solubilised hydrogenase is more stable at 20°C than in the cold and is further stabilised under an H_2 atmosphere. The kinetic properties of the membrane-bound and Triton-solubilised forms of the enzyme have been compared. Both forms of the enzyme show a pH optimum for the reduction of benzyl or methyl viologen at 8.5–9.0, for H_2 production with methyl viologen semiquinone at 5.7 and for H^2H exchange at 4.5. In vitro, the hydrogenase functions as a reversible enzyme although at a slower rate for H_2 evolution than for H_2 uptake. The apparent K_m for H_2 (uptake) is 0.25 μM. The artificial electron acceptors having the highest affinity for hydrogenase are methylene blue (K_m = 60 μM) and benzyl viologen (K_m = 100 μM). Methyl viologen has a higher affinity in the semiquinone form (K_m = 450 μM) than in the oxidized dicationic form (K_m = 3.6 mM). The Arrhenius plot of the activity of hydrogenase in the membrane and in the solubilised extract shows a break at 13°C. This transition temperature of 13°C is probably linked to a change of protein conformation. The activation energy is 110 kJ/mol (26.4 kcal/mol) adnd 38 kJ/mol (9.1 kcal/mol) below and above the transition temperature, respectively. While hydrogenase is a cold labile enzyme, it is remarkably resistant to heat inactivation, for example the membrane-bound form can withstand heating at 80°C for 3 h without loss of activity.

Introduction

The phototrophic bacterium, *Rhodopseudomonas capsulata* can use H_2 as an electron donor for autotrophic growth either in the light or dark [1,2]. In the strain B10, growth in the presence of H_2 increases the hydrogenase activity 10–20-fold [2]. The hydrogenase is located in the membrane [2,3] and can transfer electrons from H_2 to the other redox components of the photosynthetic/respiratory chain. In cells grown chemoautotrophically, hydrogenase is the first enzyme involved in the oxyhydrogen reaction, a reaction coupled to ATP synthesis [4]. In cells grown photoautotrophically, hydrogenase activates molecular hydrogen for the photoreduction of CO_2 [5]. In cells grown chemoheterotrophically hydrogenase can catalyse the recycling of some of the H_2 [6] produced by nitrogenase [5,7–9]. In all these reactions the hydrogenase of *R. capsulata* functions physiologically as an H_2 uptake hydrogenase.

Hydrogenases, first described in 1931 [10], are iron-sulphur proteins. Information on the molecular properties of hydrogenases has become available during the last decade; it was first obtained with soluble hydrogenases and more recently with membrane-bound hydrogenases (cf. Refs. 11–13 for review). Hydrogenases catalyse the reversible activation of H_2, $H_2 \rightleftharpoons 2H^+ + 2e^-$, but, depending upon the availability of electron acceptors or donors, they may

Abbreviation: Mops, 4-morpholinepropanesulfonic acid.

function preferentially in one direction or another.

This paper presents data on the effect of the non-ionic detergent Triton X-100 on the enzymic properties of hydrogenase from *R. capsulata* and describes conditions which enhance the stability of the solubilised enzyme.

Materials and Methods

Materials

The electrochemical cell used to reduce methyl viologen to the semiquinone form was from Methrom (Switzerland), the potentiostat from Tacussel, Lyon (France).

FMN, Triton X-100, β-mercaptoethanol, *N*-ethylmaleimide, *p*-chloromercuribenzoate and iodoacetamide were from Sigma Chem. Co, St. Louis, MO (U.S.A.) Dithiothreitol was from Calbiochem, La Jolla, CA (U.S.A.). Methylene blue, Coomassie brillant blue G-250, benzyl viologen and methyl viologen were from Serva, Heidelberg (F.R.G.), deoxycholate and Tris were from Merck, Darmstadt (F.R.G.). All other reagents were from Rhône-Poulenc Industries, Paris (France), emulphogen BC720 was from GAF (France) and lauryldimethylaminooxide from Franconyx, Lyon (France).

Bacterial strain and culture

R. capsulata, strain B10, was a generous gift from the Photosynthetic Bacteria Group, Department of Microbiology, Indiana University, Bloomington, IN 47401 (U.S.A.). The various growth conditions were detailed in a previous paper [2]. The most commonly used medium was a mineral salt medium [9,14] supplemented with 30 mM malate and 7 mM glutamate. The medium was adjusted to pH 7.5. All the cultures were grown at 30°C under illumination (approx. 10 000 lux) during 16 h.

Preparation of membrane and Triton extract

Routinely the cultures were harvested at the late logarithmic phase ($A_{660\,nm}$ about 1.6; 0.7 g dry weight/l), and the cells were sedimented and washed in 20 mM Tris-HCl buffer, pH 8 (20 000 × *g*, 15 min). The cells were resuspended in this buffer to a concentration of about 6–8 mg protein/ml and broken once in a French pressure cell at 20 000 lbs./inch^2 (1 lbs./inch^2 = 6.9 kPa), 4°C, (Ribi Sorvall, Norwalk, CT, U.S.A.). After centrifugation in a Sorvall centrifuge at 20 000 × *g* for 15 min, the pellet (containing less than 10% of unbroken cells) was discarded. The supernatant was centrifuged at 105 000 × *g* for 1 h (Spinco, rotor R40) at 10°C. The membrane pellets were resuspended in 20 mM Tris-HCl buffer, pH 8, and washed as above. The final suspension in 20 mM Tris-HCl, pH 8, was adjusted to 10–12 mg protein/ml, gassed with H_2 and stored at 4°C. Under those conditions hydrogenase activity was stable for several weeks.

A Triton extract was prepared by treating a membrane suspension with Triton X-100 (1 mg Triton/mg protein) at room temperature for 15 min. During the extraction, the mixture was continuously gassed with H_2 and shaken by hand. The mixture was then centrifuged at 105 000 × *g* for 1 h at 15°C. The pellet of remaining membranes was discarded and the supernatant (Triton extract) stored under H_2 in a gas-tight flask, in the dark and at room temperature. Under these conditions hydrogenase activity remained stable for at least 2 weeks.

Hydrogenase assays

H_2 production. This was assayed by gas chromatography with dithionite-reduced dyes as electron donors. The reaction was carried out in 10 ml glass vials fitted with gas-tight rubber stoppers and containing 0.5–1 mg protein (either membranes or solubilised extract) and oxidised dye. The total volume was adjusted to 2 ml with 50 mM citrate-phosphate buffer, pH 6.2. The reaction vessels were gassed for 30 min with argon and incubated in a shaking water bath (140 strokes · min^{-1}, 3 cm/stroke) at 30°C. The reaction was started by adding dithionite, freshly prepared in the buffer and pregassed with argon, to a final concentration of 10 mM, gas samples (50 µl) were withdrawn from the gas phase at time intervals with a gas-tight syringe and injected into an Intersmat IGC 120 Gas chromatograph (Intersmat Instruments, 93320 Pavillons-sous-Bois, France) equipped with a thermal conductivity detector and provided with a Porapak Q column (80–100 mesh, 2 m × 3.2 mm) heated at 40°C.

H_2 uptake. H_2 uptake was measured spectrophotometrically at 25°C in a Zeiss PMQ II spectrophotometer, as previously described in detail [2]. For the various electron acceptors used, the molar extinction coefficients of the reduced form were; 7550 M^{-1} ·

cm^{-1} at 555 nm for benzyl viologen [15], and 13 000 at 600 nm for methyl viologen [16]; they were, for the oxidised form, 16 500 for methylene blue and 8200 at 550 nm for safranine (A. Colbeau, unpublished data). Reduction of NAD was measured at 340 nm and ferricyanide and FMN at 420 nm. Reduction of dichlorophenol indophenol was assayed by measuring H_2 uptake by gas chromatography. When the oxidized viologen dyes were used as electron acceptors the hydrogenase activity was unmasked by adding Triton X-100 directly to the test tube (final concentration 0.2%, w/v). The oxyhydrogen reaction was determined from the consumption of O_2 measured with a Clark-type oxygen electrode.

For the determination of the K_m values for methyl viologen, benzyl viologen and methylene blue, the diluted protein sample was placed in 2 ml anaerobic cuvette and gassed with H_2, then 1.5 ml 20 mM Tris-HCl buffer, pH 8, pregassed with H_2, was added; the reaction was finally initiated by injecting various volumes of anaerobic solution of the electron acceptor pregassed with H_2.

For the determination of the K_m value for H_2 the diluted sample of enzyme (about 50 μg protein) was placed under anaerobiosis in a stoppered 2 ml cuvette and gassed with N_2. The volume was adjusted to 1 ml with 20 mM Tris-HCl buffer, pH 8/5 mM benzyl viologen and pregassed with N_2. The reaction was started by injecting various volumes of H_2-saturated buffer (10–200 μl). The rate of reduction of benzyl viologen was linear during 0.5 to 5 min allowing reproducible measurements of the rate of H_2 uptake.

For the determination of the effect of pH on H_2 uptake or H_2 evolution the pH was rechecked after the assay.

Direct reduction of ferredoxin by hydrogenase was assayed by the method of Chen and Blanchard [17] in which reduction of metronidazole is coupled to reoxidation of the ferredoxin. Purified ferredoxins from *R. capsulata* and *Clostridium pasteurianum* were used. The formation of reduced ferredoxin was also assessed from the reduction of acetylene in a nitrogenase coupled system. In this case the medium saturated with H_2 contained in 1 ml, ferredoxin from *R. capsulata* (3 μM) or from *C. pasteurianum* (5 μM), 0.75 mg nitrogenase from *R. capsulata* (purity checked by protein staining of a gel electrophoretogram) and hydrogenase from *R. capsulata* (0.5 mg protein of membrane or Triton-extract). The gas phase was made 10% with C_2H_2 and the reaction was initiated by injecting 200 μl of an ATP-generating system (10 mM $MgCl_2$/3 mM ATP/25 mM creatine phosphate/1 mg/ml bovine serum albumin/50 μg/ml creatine kinase/2 mM NaOH/25 mM Mops buffer, pH 6.8). Formation of C_2H_4 was determined after 30 min incubation in the light by gas chromatography.

H-^{2}H exchange reaction. Hydrogen-deuterium (H-^{2}H) exchange was determined from the initial rate of H^2H formation. H^2H evolution was followed continuously by mass spectrometry using a reaction vessel connected directly to a mass spectrometer as described earlier [5]. The reaction vessel received 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 30 μl of 30 mM dithionite were added to make and maintain the medium completely anaerobic. The mixture was sparged with a mixture of Ar : ^2H (9 : 1). When the concentration of $^2H_2$ (mass 4) reached a constant level, the apparatus was set on mass 3 (H^2H) and the reaction was started by adding chromatophores or Triton extract. The formation of H^2H was followed continuously and recorded. The rate of H-^{2}H production was proportional to the amount of enzyme used. When the activity was measured as a function of pH, 25 μl chromatophores or Triton-extract (10–15 mg protein · ml^{-1}) were used and the initial velocity of H^2H formation determined.

Protein determination. Protein was measured by the method of Bradford [18] using bovine serum albumin as standard.

Results

A. Solubilisation of the hydrogenase from R. capsulata

1. Intracellular location of hydrogenase. After breaking the cells by passage through a French pressure cell and separation of the membranes by high-speed centrifugation (105 000 × *g*, 2 h) the hydrogenase activity was found to be associated with the membrane fraction [2,3]. Hydrogenase activity was tightly bound to membranes and no extraction of the enzyme from membranes could be obtained by salt treatment at high ionic strength (e.g., NaCl up to

1 M). On the other hand, the hydrogenase is not a periplasmic enzyme since no hydrogenase activity was released after treatment of the entire cells by 50 mM EDTA in alkaline buffer (50 mM Tris-HCl, pH 9) [19]. The hydrogenase of *R. capsulata* is therefore an intrinsic membrane protein, it requires the action of detergent to be extracted from the membrane.

Table I shows that the same distribution of enzyme activity among the subcellular fractions was obtained with either hydrogenase assay (H_2 uptake or H_2 evolution). No hydrogenase activity was found in the soluble fraction indicating that the same enzyme is probably responsible for both H_2 uptake and H_2 evolution in vitro. For comparison the specific activity of Triton-solubilised hydrogenase is given in Table I together with the activity of Triton-extracted membranes.

When membranes were treated with increasing concentrations of Triton X-100 (from 0.05 to 1% (w/v) final concentration) the percent of solubilised hydrogenase increased from 5 to 90%. When the hydrogenase activity was assayed with benzyl viologen as electron acceptor, the total recovery of activity in solubilised fraction and extracted membranes remained fairly constant (90–95% recovery). However with methylene blue the total recovery decreased from 89% (at 0.05% Triton X-100) to 75% (at 0.5 or 1% Triton X-100) reflecting the increase in solubilised hydrogenase.

2. Solubilisation. Various detergents have been tested for solubilising the enzyme. Solubilisation of hydrogenase could be achieved with Triton X-100, emulphogen BC 720, lauryldimethylamineoxide or deoxycholate. Extraction was carried out at room temperature, no difference in the rate of extraction was found between 20 and 30°C. The specific activity was about the same immediately after extraction of membranes by Triton X-100 or lauryldimethylamine oxide, however the activity was completely lost after one day in the lauryldimethylamine oxide extract, whereas it was stable for more than one week in the Triton X-100 extract. Emulphogen BC 720 and deoxycholate gave intermediate results [3]. Triton X-100 which had the mildest effect on the stability of the enzyme was selected and used in the ratio of 1 mg Triton/mg membrane protein.

3. Stability. The instability of the detergent-extracted hydrogenase made it necessary to study the factors which affect the stability of the solubilised hydrogenase of *R. capsulata*.

TABLE I

INTRACELLULAR LOCATION OF HYDROGENASE

Specific activities are expressed in μmol H_2 consumed or evolved/h per mg protein. H_2 uptake was assayed at pH 8 with 0.2 mM methylene blue or 5 mM benzyl viologen as described in Materials and Methods (0.05–0.1 mg protein per assay) and H_2 evolution was assayed at pH 7 in 20 mM citrate-phosphate buffer (0.5–1 mg protein by assay) using dithionite reduced 5 mM methyl viologen. In Exp. 2 a membrane preparation was extracted with Triton X-100 (1 mg Triton/mg protein, final concentration 1%, w/v). n.d. = not determined.

Expt.	H_2 uptake				H_2 evolution	
	Methylene blue		Benzyl viologen		Specific activity	Total activity (%)
	Specific activity	Total activity (%)	Specific activity	Total activity (%)		
1 Homogenate	25.2	100	21.1	100	0.27	100
20 000 X *g* supernatant	20.9	82	17.0	80	0.21	76
Membranes	41.5	76	29.3	66	0.47	64
100 000 X *g* supernatant	3.1	3.4	2.1	3	0.03	3
2 Membranes	30.2	100	20.4	100	n.d.	n.d.
Triton-extract	26.0	70	24.7	82	n.d.	n.d.
Triton-extracted membrane pellet	3.0	5	1.3	7	n.d.	n.d.

Temperature had a strong influence on the stability of the hydrogenase of *R. capsulata*. Fig. 1A shows that it is a cold labile enzyme and that 80–90% of the activity was lost after one day at 4°C or at −20°C even if the enzyme was stored under H_2. The same inactivation by cold was obtained whether the enzyme was stored at pH 7.8 in either Tris, Hepes or phosphate buffer. When in the membrane-form, the hydrogenase could be stored at 4°C, for several weeks under H_2 and for a few days under air.

The gas phase is the second factor which strongly influenced the stability of solubilised hydrogenase. As shown in Fig. 1B there was no loss of activity in a Triton extract stored at room temperature, under H_2, for 7 days. The enzyme does not seem very sensitive to oxygen since it lost only 25% of its activity after 2 days under air while more than 60% was lost under N_2.

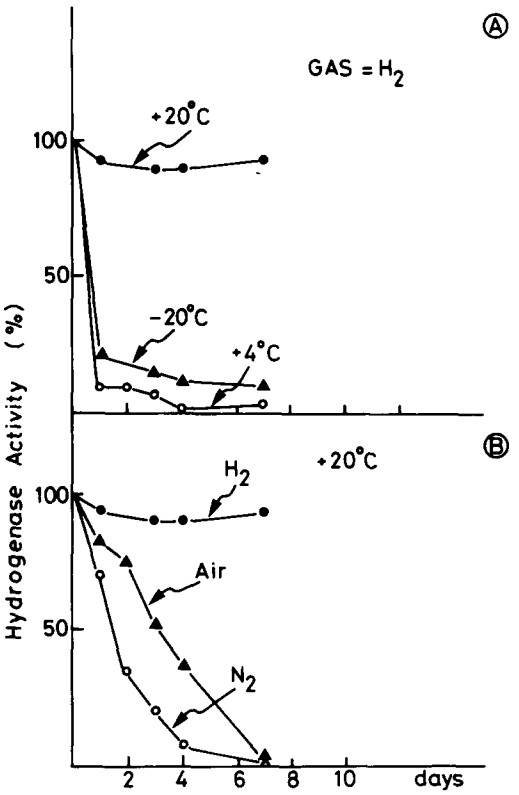

Fig. 1. Effect of temperature and gas phase on the stability of solubilised hydrogenase. A soluble extract was obtained by stirring a suspension of membranes in 20 mM Tris-HCl, pH 8, with 0.5 mg Triton X-100/mg protein for 15 min. at room temperature, under H_2. Tne solubilised proteins were separated from the membranes by centrifugation at 105 000 × *g*/h. The extract (6 mg protein/ml) had a specific activity of 15 μmol H_2 consumed/h per mg protein. A Storage under H_2. For storage at −20°C aliquot fractions were distributed in several test tubes before freezing in order to avoid inactivation by repeated thawing. B. Storage at 20°C, under H_2, air or N_2 as indicated with 2 mM azide.

In short H_2 appears to be necessary for the stability of the solubilised hydrogenase; the solubilised enzyme has been kept under H_2 at room temperature for 3 weeks without loss of activity. The membrane-bound form of hydrogenase can be stored at 4°C even in the absence of H_2.

Various chemicals have been tested as possible stabilizers of hydrogenase activity. Addition of dithionite, dithiothreitol or mercaptoethanol did not bring about any protection of the solubilised hydrogenase stored under N_2 but rather increased the inactivation rate. It was observed that the reducing conditions brough about by dithionite even destabilised the enzyme stored under H_2 [3].

In conclusion Triton X-100 provides soluble extracts which can be stored under appropriate conditions (20°C, under H_2) for several days, without loss of hydrogenase activity. This type of enzyme preparation has then been used for determining the kinetic properties of the solubilised hydrogenase (see below).

4. Hydrogenase activity. Hydrogenases catalyse the following reversible reaction $H_2 \leftrightarrow 2H^+ + 2e^-$. Theoretically it is possible to assess hydrogenase activity by measuring either H_2 consumption or H_2 production. Both types of methods have been applied in this study for the determination of the activity of *R. capsulata* hydrogenase in vitro.

H_2 uptake. It has been shown earlier [5,6] that, in the cell, the hydrogenase of *R. capsulata* functions as an H_2-uptake hydrogenase. However the physiological electron acceptor of hydrogenase has not yet been identified. NAD, FMN and the ferredoxins from *C. pasteurianum* and *R. capsulata* did not act as a electron acceptor with either the membrane-bound or the Triton-solubilised hydrogenase.

Artificial electron acceptors such as the viologens (methyl or benzyl viologen) and methylene blue were good electron acceptors (Table II). However while benzyl viologen could react directly with the membrane-bound hydrogenase of *P. denitrificans* [15], it

TABLE II

H_2 UPTAKE ACTIVITY WITH VARIOUS ELECTRON ACCEPTORS

H_2 uptake was measured spectrophotometrically as described. 5 mM methyl viologen (pH 9), 5 mM benzyl viologen (pH 8.5), 0.2 mM methylene blue (pH 8), 5 mM FMN, 1 mM NAD, (pH 8.5), and 0.12 mM O_2 (pH 7.6) were used as electron acceptors. Activities are expressed in μmol reduced substrate/h per mg protein. The activity with benzyl viologen (100%) was 85 and 105 μmol BV/h per mg prot for membrane and Triton extract, respectively. n.d. = not determined.

Electron acceptor	Hydrogenase activity in	
	Membrane	Triton extracted
Methyl viologen	9	15
Benzyl viologen	100	100
NAD	0	0
FMN	0	0
Methylene blue	103	76
O_2	11	n.d.

TABLE III

H_2 EVOLUTION ACTIVITY

All substrates were used at pH 6.2 in 50 mM citrate-phosphate buffer. The mediators were reduced by 10 mM dithionite. The substrate concentrations were 5 mM for methyl viologen, 2.5 mM benzyl viologen, 0.2 mM for methylene blue, 2 mM for Safranine and 2 mM for NADH. H_2 produced was measured by gas chromatography. The 100% activity corresponded to 0.30 μmol H_2 produced/h per mg protein. n.d. = not determined.

Reduced electron donor	E_0' (mV)	Hydrogenase activity (%)	
		Membrane bound	detergent solubilised
Methyl viologen	(−446)	81	128
Benzyl viologen	(−360)	100	100
NADH	(−300)	n.d.	7
Safranine	(−289)	n.d.	60
Methylene blue	(+ 39)	n.d.	7

was not the case with the chromatophores of *R. capsulata*. The viologen could not accept electrons from chromatophore-embedded hydrogenase unless small amounts of Triton X-100 (0.2%, w/v) were added to facilitate access of the dye to the enzyme. Another dye widely used for measuring hydrogenase activity is methylene blue [20]. As shown in Table II, methylene blue and benzyl viologen were the best electron acceptors and were reduced at comparative rates by the membrane-bound and the Triton-solubilised hydrogenase of *R. capsulata*. Since methylene blue accepts two electrons and benzyl viologen accepts only one electron, it appears that methylene blue is the best electron acceptor. Furthermore contrary to benzyl viologen, methylene blue does not require Triton X-100 to be reduced by hydrogenase. The rate of reduction of other electron acceptors, dichlorophenol indophenol (10 mM), ferricyanide (10 mM), safranine (5 mM) tested at pH 8.5, was hardly detectable (data not shown). The reduction of O_2, which involves the electron transport chain was low compared to the reduction of artificial electron acceptors (Table II).

H_2 evolution. The hydrogenase of *R. capsulata* either membrane-bound or solubilised by Triton X-100 could evolve H_2 from reduced electron donors (Table III). The reduced viologens were the best electron donors and benzyl viologen was practically as good as methyl viologen. On the other hand practically no H_2 was produced with NADH or reduced methylene blue.

From the results presented in Tables II and III it can be concluded that benzyl viologen is the best substrate to measure hydrogenase activity in either direction.

B. Kinetic properties of the membrane-bound and the Triton-solubilised hydrogenase

1. Effects of pH.

pH dependence of H_2 uptake activity. The effect of pH on the rate of H_2 oxidation by benzyl viologen is shown in Fig. 2A. Optimal activity was found around pH 8.5–9.0 for both the membrane-bound and the Triton-solubilised hydrogenase. A similar pH optimum (8.5–9.0) was observed when methyl viologen was used as electron acceptor (data not shown).

The pH curve for H_2 oxidation with methylene blue is shown on Fig. 2B. A broad optimum (7.5–8.5) is observed for the membrane-bound hydrogenase; on the other hand a more narrow pH activity curve centered at pH 7.7 was obtained for the

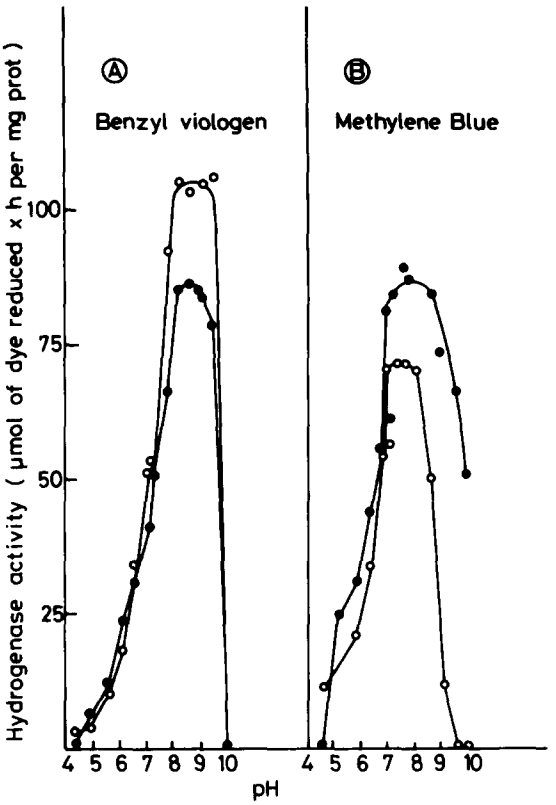

Fig. 2. pH dependence of the rate of H_2 uptake. A. With benzyl viologen as electron acceptor. Hydrogenase activity was assayed in 20 mM buffer, citrate-phosphate (pH 4–7.3), phosphate (pH 7–8), Tris-HCl (pH 7.2–9.2) and bicarbonate (pH 9–10). Membranes (45 µg protein) or Triton extract (40 µg protein) were preincubated in the same buffer under H_2 for 20 min before adding H_2 saturated benzyl viologen (final conc. 5 mM) •———•, membrane-bound hydrogenase; ○———○, Triton-solubilised hydrogenase. B. With methylene blue as electron acceptor. Same conditions as in Fig. 2A. Methylene blue was used at the final concentration of 0.2 mM.

detergent-solubilised hydrogenase. This apparent shift of pH optimum upon solubilisation of membranes by Triton and the loss of activity at high pH values had already been reported for extracts of *R. capsulata* grown on lactate + glutamate [3] and for extracts of the membrane-bound hydrogenase of *Alcaligenes eutrophus* [20].

pH dependence of H_2 production activity. The effect of pH on the rate of H_2 production in the presence of methyl viologen semiquinone is shown in Fig. 3. It has been shown by Mayhew [21] that at acidic pH values, dithionite is a less efficient reducing agent. To avoid such complications with dithionite and in order to have the same concentration of semiquinone at all pH values, methyl viologen was reduced electrochemically under argon and transferred anaerobically to closed vials pregassed with argon and containing the hydrogenase preparation. H_2 released in the gas phase was analysed by gas chromatography. H_2 evolution was monitored for 60 min to check that it was linear with time. The optimal pH for H_2 production was 5.7, that is in a more acidic range than that for H_2 uptake.

pH dependence of the H-^2H exchange reaction.

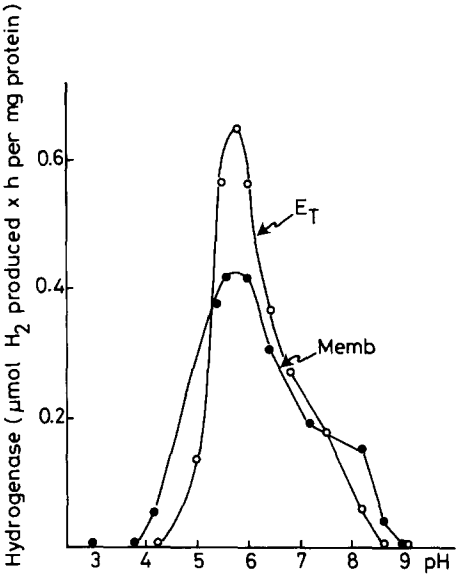

Fig. 3. pH dependence of the rate of H_2 production from electrochemically-reduced methyl viologen. Methyl viologen (30 mM) in 20 mM phosphate buffer, pH 7.0, was reduced electrochemically under argon by cathodic reduction on a platinum cathode (2.5 × 9 cm) operating at a potential of −800 mV relative to the calomel electrode; in a separate compartment, linked with a 0.1 M KCl bridge to the cathodic compartment, a platinum wire served as anode. When the methyl viologen was totally reduced to the semiquinone form, aliquots of 500 µl were transferred with gas-tight Hamilton syringes to rubber-stoppered vials filled with 1 ml buffer containing 0.8 mg protein and pregassed with argon. The vials were incubated in a shaking water-bath at 30°C for 60 min. The H_2 content of gas samples withdrawn from the gas phase at time intervals was analysed by gas chromatography.

When splitting molecular hydrogen, hydrogenase (E) has been postulated to form an intermediate enzyme hydride ($E \cdot H^-$) [22]. In the presence of deuterium gas the enzyme hydride will be $E : {}^2H^-$ which by exchange with the protons of light water will produce H^2H [22] as illustrated in Eqn 1.

$$E : {}^2H^- + H^+ \rightarrow H\text{-}{}^2H + E \qquad (1)$$

The hydrogenase in chromatophores of *R. capsulata* has already been shown to release H^2H and H_2 when incubated with ${}^2H_2$ gas and H_2O in the absence of electron acceptors [5]. The production of H^2H, reflecting the $H\text{-}{}^2H$ exchange activity of hydrogenase [22], has been chosen here to determine the pH optimum for the enzyme activity.

The production of H^2H was recorded continuously as described earlier [5], and was linear during the first minutes. The initial rate of H^2H production has been measured at various pH values. The pH activity curve shows a marked optimum of $H\text{-}{}^2H$ exchange at pH 4.5 and a shoulder around pH 7, (Fig. 4). The same curve as in Fig. 4, with an optimum at pH 4.5, was obtained when hydrogenase was embedded in chromatophore membranes (data not shown).

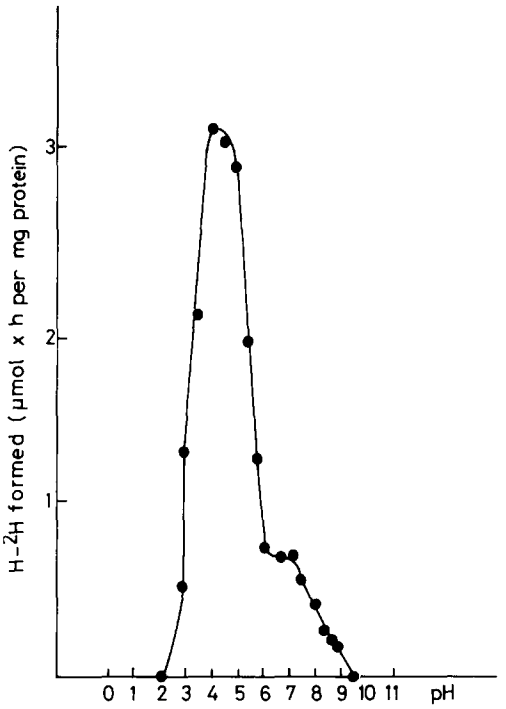

Fig. 4. pH dependence of the $H\text{-}{}^2H$ exchange reaction catalysed by the Triton-solubilised hydrogenase. Conditions as described. 0.36 mg protein (in 25 µl) of Triton-extract was used per assay.

In conclusion H_2 uptake was maximal at alkaline pH (8–9) while H_2 production and $H\text{-}{}^2H$ exchange were optimal in the acidic pH range (4.5–5.7).

2. Kinetic parameters. The affinity of hydrogenase for methylene blue, benzyl viologen, methyl viologen and H_2 was determined (Table IV). It has been observed with membranes of *Escherichia coli* [23] that oxidised viologens do not penetrate the membranes while reduced viologens can. In the case of *R. capsulata* small amounts of Triton X-100 had to be added to whole cells to elicit maximal hydrogenase activity with viologens as electron acceptors. Practically the same K_m values were obtained with the membrane-bound and the solubilised enzyme, only the latter are reported in Table IV.

The K_m for H_2, with benzyl viologen as electron acceptor, was the lowest (0.25 µM). This very high affinity of hydrogenase for H_2 enables hydrogenase to recycle H_2 even when present in trace amounts.

There is a striking difference between the Michaelis constants for benzyl viologen and for methyl viologen, the former being 36-times lower

TABLE IV

K_m VALUES FOR DIFFERENT SUBSTRATES USED BY THE SOLUBILISED HYDROGENASE OF *R. CAPSULATA*

For H_2 uptake assays, hydrogenase activity was measured at the optimum pH in 2 ml anaerobic cuvettes as described in Materials and Methods (50 µg protein with methylene blue and benzyl viologen and 300 µg protein for methyl viologen). H_2 evolution was determined at pH 6.2 using 1 mg protein in 10 ml flasks (total aqueous phase = 1 ml), as described. V was expressed in µmol H_2/h per mg protein.

Activity	Electron acceptor or donor	K_m (µM)	V
H_2 uptake	Methyleneblue	60	67
	Benzyl viologen	100	50
	Methyl viologen	3600	4.5
	H_2 (with benzyl viologen as acceptor)	0.25	40
H_2 evolution	Dithionite-reduced methyl viologen	450	0.7

than the latter. This may be linked to the shape of the molecules; the flat benzyl rings are expected to be more easily inserted in the hydrophobic regions of the membrane, the Triton micelles or the enzyme than the methyl groups. The positive charge at both ends of the methyl viologen molecule also seems to be involved. Reduction of methyl viologen by dithionite produced the semiquinone form with a positive charge at one end only of the molecule; the affinity of the enzyme for the semiquinone is 8-times higher than that for the oxidised form (Table IV).

The Michaelis constants for the dyes routinely used as electron acceptors, benzyl viologen and methylene blue, are low (100 and 60 μM, respectively). Methylene blue is able to accept electrons from electron carriers of the chromatophore membrane other than hydrogenase. This interaction of methylene blue at various points of the membrane could account for the broadening of the observed activity vs. pH curve (Fig. 2B) and may explain the shift in the pH optimum which has been observed upon extraction of hydrogenase from the membrane (Fig. 2B) [3,20]. However the same K_m value (60 μM) was found for the membrane-bound and the solubilised hydrogenase (at pH 6.2 and 8.0) (data not shown) indicating that methylene blue is able to react directly with the enzyme.

The Michaelis constant for methyl viologen semiquinone obtained by reduction with dithionite was initially found to vary with pH; a K_m = 0.6 mM was found at pH 6.2 (with either the membrane-bound or the solubilised hydrogenase) and of 0.45 mM at pH 7.5. However the reducing potential of dithionite was then shown by Mayhew [21] to decrease with decreasing pH. While full reduction of methyl viologen to the semiquinone form can be attained at pH 8.0, at lower pH values reduction is incomplete and there is a difference of about 30% in the degree of reduction of methyl viologen at pH 7.0 and 6.0. After correcting for the pH effect on viologen semiquinone concentration, the same K_m (0.45 mM) was obtained at both pH values (6.2 and 7.5) (Table IV).

3. Effect of temperature on hydrogenase activity. Activation energy. The Arrhenius plot of the reduction of benzyl viologen by H_2 catalysed by either the membrane-bound or the solubilised hydrogenase of *R. capsulata* gives a line with a break at 13°C (Fig. 5). There is no change in the form of the Arrhenius plot upon solubilisation. The break at 13°C therefore appears to be linked to a protein transition rather than to a transition in the membrane lipids.

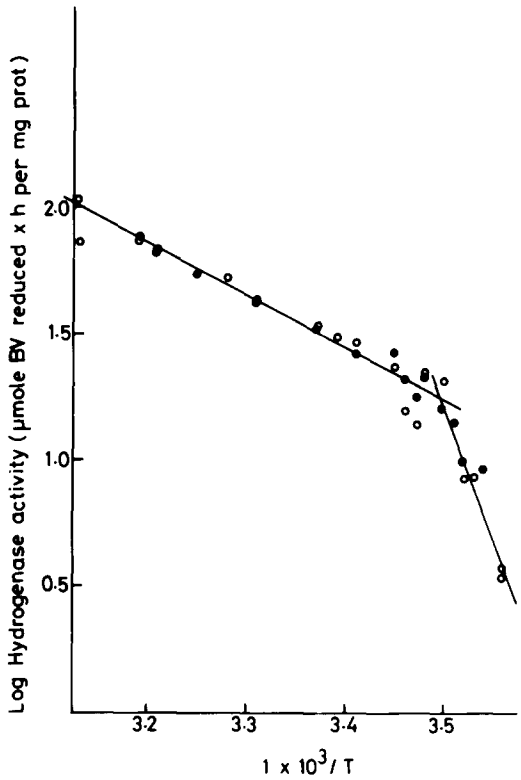

Fig. 5. Arrhenius plot of hydrogenase activity. Assays were made in a thermostated Zeiss PMQ II spectrophotometer. Samples containing the fraction (about 50 μg protein) and the buffer (1.5 ml of 20 mM Tris-HCl, pH 8) were saturated with H_2 and equilibrated in the spectrophotometer at the desired temperature for 15 min. The reaction was then initiated by 0.1 ml of 50 mM benzyl viologen saturated with N_2. Each point is the average of triplicate assays.

Below the transition temperature of 13°C the Q_{10} for the reduction of benzyl viologen was 5 while above 13°C the Q_{10} was 1.6 corresponding to an activation energy of 110.5 kJ/mol (26.4 kcal/mol) and 38.1 kJ/mol (9.1 kcal/mol), respectively, for both the membrane-bound and solubilised form of hydrogenase. The preliminary stability studies ([3] and Section A, 3) had indeed indicated that hydrogenase activity was lost in the cold and it had been found empirically that the isolation steps should be carried out above 15°C to isolate active hydrogenase with reasonable yields.

Resistance to heat inactivation. The hydrogenase of *R. capsulata* is a cold labile enzyme (Fig. 1A). On the other hand, like most hydrogenases, the hydrogenase of *R. capsulata* showed a high stability at elevated temperatures. Both the membrane-bound an the solubilised form could withstand incubation at 70 or 80°C (over 5 min) without loss of activity (Fig. 6A). The membrane-bound form was more resistant than the solubilised enzyme against heat denaturation; even after 5 min of heating at 100°C a small percentage of the activity was still retained.

The kinetics of inactivation of the membrane-bound and solubilised hydrogenase were studied at 60 and 80°C (Fig. 6B). The solubilised form could withstand heating at 60°C for several hours without loss of activity; at 80°C it was 50% inactivated after 15 min. The membrane-bound form was particularly resistant since the activity of hydrogenase remained the same even after heating at 80°C for 3 h (Fig. 6B).

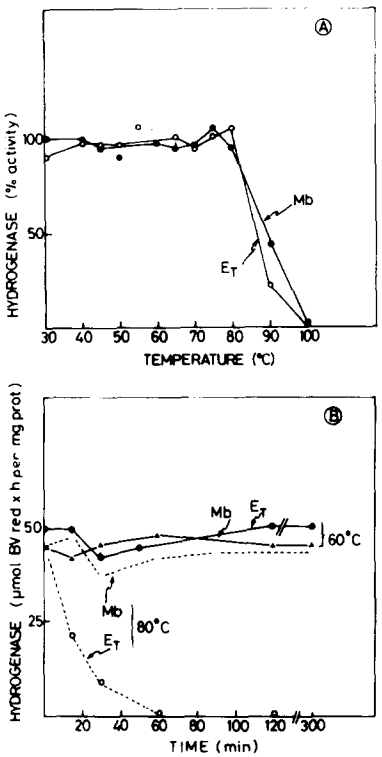

Fig. 6. Thermal stability of *R. capsulata* hydrogenase. Membrane (●——●), (▲——▲) or Triton-extract (○——○), (△——△) preparations (2 mg protein/ml) were incubated under H_2 in a water-bath then cooled at room temperature and hydrogenase uptake was assayed on an aliquot fraction by benzyl viologen reduction. A. Samples were incubated for 5 min at the indicated temperatures. Results are expressed as percentage of residual activity. The 100% activity of non-incubated fractions was 80 µmol benzyl viologen reduced/h per mg protein. B. Samples were incubated at 60°C (full lines) or 80°C (dotted lines) for the indicated time.

4. Effect of inhibitors. Some potential inhibitors have been tested on the hydrogenase of *R. capsulata*. Table V shows that transition metals at 1 mM totally inhibited the hydrogenase in the membrane form. Ferrous sulfate (1 mM) which has been used to stabilise soluble hydrogenases from other sources [24] inhibited by 70% the activity of the solubilised hydrogenase from *R. capsulata*. Sulphydryl reagents are weak inhibitors (10–20% inhibition).

It has already been observed [5] that H-^{2}H exchange catalysed by the hydrogenase of whole cells or chromatophores of *R. capsulata* is unaffected by

TABLE V

EFFECT OF INHIBITORS ON H_2 CONSUMPTION BY THE HYDROGENASE OF *R. CAPSULATA*

The inhibitors, at the indicated final concentration were added to diluted membrane fractions (100 µg protein in 200 µl 20 mM Tris-HCl buffer, pH 8) in 2 ml closed cuvettes. The cuvettes were preincubated for 15 min at room temperature under a stream of H_2. Then a 5 mM solution of benzyl viologen saturated with H_2 was added to a final volume of 2 ml and the reduction of benzyl viologen was followed at 555 nm. pCMB, *p*-chloromercuribenzoate.

Inhibitor	Final concentration (mM)	Inhibition of hydrogenase activity (%)
Sulfhydryl reagents		
pCMB	2	13
Iodoacetamide	10	22
Dithiothreitol	1	10
N-ethylmaleiimide	5	13
β-mercaptoethanol	(2%)	0
Transition metals		
$FeSO_4$	1	62
	5	86
$CuSO_4$	1	100
$HgCl_2$	1	100
SDS	(0.37%)	81
Urea	400	60

CO (10.4 kPa) or C_2H_2 (10.4 kPa). In those experiments, H-^{2}H exchange was measured in a reaction vessel completely filled with liquid, i.e., with no gas phase, CO was added in the form of CO-saturated buffer. For the experiments described hereafter the cuvettes used to measure the reduction of benzyl viologen were only half filled with the buffer and enzyme preparation and the gas phase was enriched in CO as described in the legend of Fig. 7. For the assay of H_2 production the volumes of the gas phase and aqueous phase were, respectively, 8 and 1 ml.

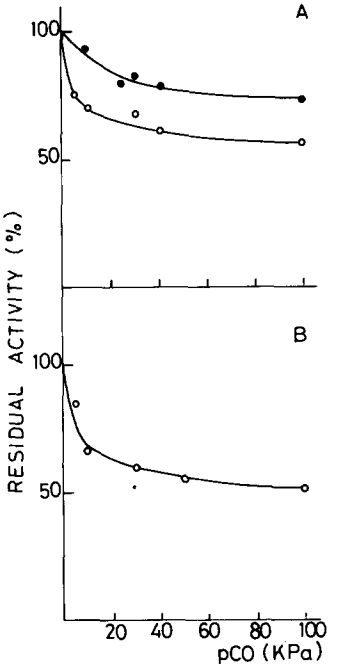

Fig. 7. Inhibition of hydrogenase by CO. A. H_2 uptake assay. Membrane (•———•) or Triton extract (○———○) (50 μg protein) were added to 1 ml 20 mM Tris-HCl buffer, pH 8, saturated with H_2 in 2 ml cuvettes. Various volumes of the gas phase were then replaced by identical volumes of CO gas so that the concentration of CO in the gas varied from 0 to 40%. For 100% CO, the cuvettes with the enzyme fraction were directly saturated with CO for 15 min. The cuvettes were left for 15 min at 20°C and hydrogenase activity was assayed by injecting 100 μl 50 mM benzyl viologen saturated with H_2. B. H_2 evolution assay. Triton extract (0.9 mg protein) was added to 1 ml 20 mM Tris-HCl buffer, pH 7.4/10 mM methyl viologen and saturated with argon in 9 ml flasks. The concentration of CO was adjusted as in A. The reaction was initiated by adding 20 mM dithionite. H_2 production was measured by analysis of aliquots of the gas phase at time intervals during 1 h.

The hydrogenase of *R. capsulata* showed little sensitivity to CO inhibition (Fig. 7). Even under an atmosphere of CO, the activity of either H_2 uptake (Fig. 7A) or H_2 production (Fig. 7B) was less than 50% inhibited.

Discussion

As in many N_2 fixers, e.g., *Rhizobium* [25], *Azotobacter* [26], *blue-green algae* [27] the hydrogenase of *R. capsulata* is membrane-bound and has a very high affinity for H_2. The K_m value for H_2 for the hydrogenase of *R. capsulata* was found in this study to be 0.25 μM; it is 1.4 μM for the hydrogenase of *Rhizobium japonicum* [28]. Such a high affinity of the enzyme for H_2 allows it to remain saturated and therefore function maximally even at very low concentrations of H_2. In other words in bacteria where H_2 is produced by nitrogenase, hydrogenase may very efficiently compete with diffusion and recycle H_2. This K_m for H_2 is the lowest reported value for any known hydrogenase. Aerobic hydrogen bacteria in which the membrane-bound hydrogenase catalyses the oxyhydrogen reaction for ATP synthesis and CO_2 reduction also have a strong affinity for H_2, but the K_m values are more than one order of magnitude higher e.g., the hydrogenase from *P. denitrificans* [29] the membrane-bound hydrogenase of *A. eutrophus* [30] and of *E. coli* [31] have an apparent K_m for H_2 of 20, 32 and 26 μM, respectively. On the other hand the V values are also low even for H_2 uptake. The highest V values are found in aerobic nitrogen-fixing bacteria such as *Azotobacter* where O_2 depletion by respiration is a critical need for the bacteria to maintain its O_2-labile nitrogenase active.

Unlike other photosynthetic bacteria such as *Thiocapsa roseopersicina* [32], *Rhodospirillum rubrum* [33], no soluble hydrogenase activity could be detected either in the culture medium or after cell fractionation. It was possible to demonstrate that in vitro the hydrogenase of *R. capsulata* can evolve H_2 when assayed using methyl viologen and dithionite, but this activity was 50–100-times lower than that of H_2 uptake. In conclusion, in vitro as well as in vivo, the hydrogenase of *R. capsulata* functions preferentially as an H_2-uptake hydrogenase although it can function reversibly and evolve H_2 in vitro with dithionite-reduced methyl viologen.

The catalytic properties of the hydrogenase of *R. capsulata* reflect the function of the enzyme in the cell. The kinetic parameters (Table IV) are different from those of hydrogenases found in obligate anaerobes such as the *Clostridia*, or the sulfate-reducing bacteria where the main function of hydrogenase is to evolve H_2. In such bacteria the H_2-evolving hydrogenase is soluble, has a higher K_m for H_2 and a much higher H_2-producing activity; for example a K_m of 225 μM at pH 7 [34] and a rate of H_2 production of 3960 μmol H_2 $min^{-1} \cdot mg^{-1}$ [35] have been reported for the hydrogenase of *C. pasteurianum*. The same specific activity (3800 μmol $H_2 \cdot min^{-1} \cdot mg^{-1}$) was also obtained with the hydrogenase from *Desulfovibrio vulgaris* [19].

The physiological electron acceptor of the hydrogenase of *R. capsulata* is not identified yet, as in the case for most of the membrane-bound hydrogenases (cf. Refs. 12, 13, for review). Artificial electrons acceptors, in particular methylene blue (K_m = 60 μM) and benzyl viologen (K_m = 100 μM) (Table IV), have strong affinity for the enzyme which exhibited a good activity with these dyes and none with bacterial ferredoxins (isolated either from *R. capsulata* strain B10 or from *C. pasteurianum*). There is a striking difference in affinity between oxidised benzyl viologen (K_m = 100 μM) and oxidised methyl viologen (K_m = 3600 μM); this may result from the flat structure of the benzyl ring which can allow better (hydrophobic) interactions with the enzyme. It is noteworthy that the reduced semiquinone form of methyl viologen has a higher affinity (K_m = 450 μM) for hydrogenase than the oxidised form (Table IV).

Very different pH optima were observed for H_2 uptake (pH 8.5–9.0) (Fig. 2) and for H_2 evolution (pH 5.7) (Fig. 3). Similar differences have been reported for the membrane-bound hydrogenase of *E. coli* [31] or of *R. rubrum* [36]. A change in pH optimum for deuterium exchange by the hydrogenase of *Desulfovibrio desulfuricans* was observed when the enzyme was activated by dithionite instead of cysteine or 2,3-mercaptoethanol [37]; the pH optimum was shifted from 8 to 5.6. In the case of the hydrogenase of *R. capsulata* the acidic pH optimum observed for H_2 evolution is not due to dithionite since methyl viologen was reduced electrochemically (Fig. 3) and not by dithionite.

The H-^{2}H exchange catalysed by the hydrogenase of *R. capsulata* between $^2H_2$ and H_2O which reflects the activity of the enzyme at the hydrogen binding site had still a more acidic pH optimum (pH 4.5) (Fig. 4). As mentioned above a pH optimum in the acidic range for the H-^{2}H exchange reaction has also been observed with the soluble hydrogenase of *D. desulfuricans* (pH 5.5) [37,38] and the membrane-bound hydrogenase of *Chromatium* (pH 6) [39].

The fact that hydrogenase can optimally activate hydrogen at acidic pH known to destabilise synthetic iron-sulphur clusters [40] and ferredoxins [41] indicates that the hydrogenase protein affords a good shielding for the cluster(s) as observed by Maskiewicz and Bruice [41] with *Chromatium vinosum* high potential iron protein. This protection by the polypeptide chain may also explain the relative insensitivity of the hydrogenase of *R. capsulata* to inhibition by sulphydryl reagents (Table V) and to heat denaturation; even the solubilised form of hydrogenase was completely stable for 3 h at 60°C (Fig. 5). Comparable thermal stability has also been observed with the hydrogenase of other photosynthetic bacteria, e.g., *T. roseopersicina* [32] and *R. rubrum* [36].

CO is a competitive inhibitor of hydrogenase with respect to H_2 and it has been shown by EPR with the *C. pasteurianum* hydrogenase and synthetic iron-sulphur analog clusters that both CO and H_2 bind directly to the iron-sulphur cluster of the enzyme [42,43]. Therefore, as expected, practically all hydrogenases, except the soluble hydrogenase of *A. eutrophus* [44], are inhibited by CO (cf. Ref. 12 for review). The hydrogenase of *R. capsulata* shows a relative insensitivity to CO. The maximum inhibition is the same (about 50%) for the H_2 uptake activity and the H_2 production activity. Light has no influence on the inhibition by CO.

The hydrogenase of *R. capsulata* is not particularly sensitive to O_2 since it loses its activity more rapidly upon storage under N_2 gas than under air (Fig. 1). Other hydrogenases have been found to be stable when stored in the presence of O_2 i.e., in the oxidised state, e.g., the hydrogenase from *T. roseopersicina* [32], *Chromatium vinosum* strain D [39,45,46], *R. rubrum* [36], *A. eutrophus* [30], *Proteus mirabilis* [47], *D. vulgaris* (strain Hildenborough) [48], *Desulfovibrio gigas* [49]. However all isolated hydrogenases are very quickly inhibited by O_2 in the

reduced form or when the active site is functioning (cf. Ref. 12 for review). Indeed addition of dithionite (0.3 mM) to a solubilised preparation of hydrogenase from *R. capsulata* not only did not increase the protection but even destabilised the enzyme stored under N_2 or H_2 gas [3].

By using electron donors (H_2, $^2H_2$, MV^+) or acceptor (BV^{2+}) able to react directly with the enzyme it has been possible to study the kinetic properties of the hydrogenase of *R. capsulata* in the membrane-form or in crude extracts. The purification of hydrogenase, hampered by the instability and the high degree of hydrophobicity of the enzyme, is in progress.

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