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Purification of Hydrogenase from *Chlamydomonas reinhardtii*[1]

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ABSTRACT

A method is described which results in a 2750-fold purification of hydrogenase from *Chlamydomonas reinhardtii*, yielding a preparation which is approximately 40% pure. With a saturating amount of ferredoxin as the electron mediator, the specific activity of pure enzyme was calculated to be 1800 micromoles H_2 produced per milligram protein per minute. The molecular weight was determined to be 4.5×10^4 by gel filtration and 4.75×10^4 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The enzyme has an abundance of acidic side groups, contains iron, and has an activation energy of 55.1 kilojoules per mole for H_2 production; these properties are similar to those of bacterial hydrogenases. The enzyme is less thermally stable than most bacterial hydrogenases, however, losing 50% of its activity in 1 hour at 55°C. The K_m of purified hydrogenase for ferredoxin is 10 micromolar, and the binding of these proteins to each other is enhanced under slightly acidic conditions. Purified hydrogenase also accepts electrons from a variety of artificial electron mediators, including sodium metatungstate, sodium silicotungstate, and several viologen dyes. A lag period is frequently observed before maximal activity is expressed with these artificial electron mediators, although the addition of sodium thiosulfate at least partially overcomes this lag.

Although the biochemical properties of numerous bacterial hydrogenases have been reported, there is a distinct lack of knowledge concerning the properties of hydrogenase from eucaryotic algae. Hydrogenase has been purified from a variety of procaryotes, including *Rhodospirillum* (1, 2), *Alcaligenes* (18), *Desulfovibrio* (10, 20), *Clostridium* (6), *Megasphaera* (21), and *Escherichia* (3). In contrast, attempts to purify the enzyme from a eucaryotic source have been very limited. Erbes *et al.* (8) partially purified hydrogenase from *C. reinhardtii* in order to examine the kinetics of O_2 inactivation, but the authors are unaware of any other reports concerning the purification of a eucaryotic hydrogenase.

Conversely, there have been a substantial number of studies concerning *in vivo* characteristics of algal hydrogenase (4, 11). It is well known that the enzyme is only detectable after cells have undergone a period of anaerobic incubation. Furthermore, the enzyme is completely and irreversibly inactivated by low oxygen concentrations. Both H_2 oxidation and H_2 evolution are catalyzed by algal hydrogenase, with the latter process being linked to PSI via Fd.

In this paper we describe the purification of hydrogenase from the green alga *C. reinhardtii*, along with several biochemical properties of the enzyme.

MATERIALS AND METHODS

Culture and Adaptation Conditions. *C. reinhardtii* 137C (+) was grown phototrophically under fluorescent lights (~40 w/m^2) at 30°C in spinner flasks containing 12 L of minimal medium (16). Cultures were bubbled with 5% CO_2 in air at a flow rate of 235 ml/min. Cells were harvested in the late exponential stage of growth (~15 μg Chl/ml) by microporous filtration (Pellicon Casette System; Millipore Corp.), washed once with 50 mM Tris-Cl buffer (pH 8.0) containing 3 mM $MgCl_2$, and then resuspended in fresh buffer at a concentration of 450 to 550 μg Chl/ml. Cells were anaerobically adapted by bubbling Ar (99.995% purity) through the cell suspension for 4.5 h in darkened 1-L polyethylene bottles. After addition of 10 mM sodium dithionite, the bottles were tightly stoppered and stored at −20°C.

Assays. H_2 production was measured at 25°C in samples diluted into 50 mM Mops[3] buffer (pH 6.8) containing 10 mM dithionite with a hydrogen electrode as described previously (16), using Fd purified from *C. reinhardtii* (16) as the electron mediator. One unit is defined as 1 μmol H_2 produced/min with 10 μM Fd as the electron mediator.

H_2 oxidation was measured spectrophotometrically as described elsewhere (17). An extinction coefficient of 1.57 mM^{-1}·cm^{-1} at 676 nm was used for sodium metatungstate.

Purification of Hydrogenase. All purification steps were carried out at 8°C in an anaerobic chamber (Forma Scientific, model 1024) under an atmosphere of 90% N_2/10% H_2. All solutions were buffered with 50 mM Tris-Cl (pH 8.5) containing 10 mM dithionite (buffer A), unless otherwise indicated.

(a) Extraction and Batch Adsorption to DEAE. Crude extracts were obtained by subjecting suspensions of anaerobically adapted cells (up to 6 L) to two freeze/thaw cycles, followed by centrifugation at 15,000*g* for 1 h. The supernatant solution, containing >90% of the initial hydrogenase activity, was then divided between two 3-L spinner flasks and the pH was adjusted to 8.3 to 8.5 with NaOH. One volume of DEAE-Sephacel was added to 10 volumes of the extract, and this suspension was stirred for 30 min. After the gel had settled, the supernatant solution (containing no detectable hydrogenase activity) was poured off and the gel was collected in a 5-cm diameter column and washed with one column volume of buffer A. Hydrogenase was then eluted from the column with buffered 0.35 M KCl. The eluate was concentrated by the use of a Millipore CX-30 submersible ultrafilter to about 40 ml and dialyzed overnight.

Due to the space constraints of the anaerobic chamber, it was only possible to process a maximum of 6 L of cell suspension at a time for this first step. Thus, for the purification scheme shown in Table I, this step was repeated three times before additional purification steps were carried out.

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[2] A division of the Midwest Research Institute, Kansas City, MO.

[3] Abbreviations: Mops, morpholinopropanesulfonic acid.

(b) Gradient Elution Ion Exchange Chromatography. The combined sample was loaded onto a second DEAE-Sephacel column (2.5 × 45 cm) and the hydrogenase was eluted with a buffered 50 to 400 mM KCl linear gradient (1400 ml total volume). The fractions containing hydrogenase were combined and concentrated to 8 ml as described above.

(c) Gel Filtration Chromatography. The concentrated sample was then loaded onto a column containing Sephadex G-100 Superfine gel (two 2.5 × 37 cm columns in series) and eluted with buffer A.

(d) Hydroxylapatite Chromatography. The pooled active fractions from the previous step were loaded onto a column (2.5 × 12.5 cm) containing Bio-Rad HTP, which was then washed with 1.5 column volumes of 50 mM K-phosphate/50 mM Tris-Cl, pH 8.0. The hydrogenase was eluted with a 50 to 150 mM K-phosphate linear gradient containing 50 mM Tris-Cl, pH 8.0 (volume = 180 ml). The active fractions were combined, concentrated, and dialyzed overnight. This sample was loaded onto a second HTP column (1.5 × 11.5 cm) and eluted as before (wash volume = 20 ml; gradient volume = 60 ml).

(e) Affinity Chromatography. The affinity gel was formed by combining 70 mg of *C. reinhardtii* Fd (in 4 ml of Mops buffer, pH 7.5) with 7 ml of Affi-gel 10 (Bio-Rad Laboratories), followed by shaking for 3.5 h at 4°C in the presence of 0.4 M $CaCl_2$. The gel that was produced contained 4.3 mg bound Fd/ml gel.

One ml of the combined sample from the second HTP column (containing 30 units, or approximately 33 μg of hydrogenase in the presence of 1130 μg of total protein) was passed through a 4-ml Bio-Rad P-4 desalting column in order to change the buffer to 20 mM Mes (pH 6.0) containing 4 mM dithionite. This sample was then loaded onto the Fd-affinity column (equilibrated with the same Mes buffer). After 1.5 column volumes of additional Mes buffer had passed through the column, the hydrogenase was eluted with buffer A.

Electrophoresis. SDS-PAGE was performed using the buffer system of Laemmli (13) with a 10% acrylamide/2.7% bis-acrylamide separating slab gel. Nondenaturing polyacrylamide tube gel electrophoresis used the discontinuous buffer system of Chrambach (7) with a 10% acrylamide/5% bis-acrylamide separating gel. The location of hydrogenase in the gel was determined by using the activity staining procedure of Adams and Hall (2). Proteins in the gel were detected by a modification of the silver staining procedures of Merril *et al.* (15) and Wray (22). The modified procedure (personal communication; Dr. G. M. W. Adams, Department of Botany, Louisiana State University) involves (a) prestaining with Coomassie Blue, (b) soaking in a solution of DTT followed by a neutral $AgNO_3$ solution, and (c) stain development with a Na_2CO_3/formaldehyde mixture. Oxidation with $HNO_3/K_2Cr_2O_7$ as recommended in the procedure of Merril *et al.* (15) was omitted.

Iron Analysis. Iron was determined by a modification of the bathophenanthroline method described by Lovenberg *et al.* (14). The primary modifications involved acidification with HCl rather than TCA and the use of sodium acetate rather than ammonium acetate; these substitutes reduced the background level of iron. SDS (0.1%) was also included to solubilize precipitated protein. All reagents except HCl were passed through a Chelex-100 column (Bio-Rad) and glassware was acid-washed in order to remove contaminating iron.

Protein Determination. Protein was quantified by the method of Bradford (5), using bovine-γ-globulin as a standard.

RESULTS AND DISCUSSION

Several precautions must be taken during the purification of hydrogenase from *C. reinhardtii*. Since the enzyme is irreversibly inactivated by low oxygen levels (8), strict anaerobicity must be maintained throughout the entire process. Also, it is necessary to use relatively dilute cell suspensions during the anaerobic adaptation period, due to the production of acidic fermentation products. Furthermore, concentration of adapted cells by centrifugation is not feasible, since over 30% of the hydrogenase is released into the suspending medium. Finally, the amount of hydrogenase produced during the 4.5-h adaptation period is quite low (*e.g.* about 0.5 mg of active hydrogenase/100 g of cells [wet weight] in crude extracts).

The results of the purification scheme described in "Materials and Methods" are shown in Table I. This represents the hydrogenase from cultures totalling 420 L. The total yield of active hydrogenase after the second hydroxylapatite column is approximately 1.3 mg.

Chromatographic Properties of Hydrogenase. The elution profile of a DEAE-Sephacel column utilizing a KCl gradient for fractionation indicates that hydrogenase is more acidic than the bulk of other soluble proteins from *C. reinhardtii* (Fig. 1). Most hydrogenases isolated from procaryotic sources are also quite acidic, often having pI values below 5.0 (2, 3, 9, 18, 19). Binding of hydrogenase to DEAE is greatly reduced when the pH is lowered from 8.5 to 6.8 (*e.g.* elution occurs with 270 and 170 mM KCl, respectively).

Gel filtration chromatography of hydrogenase on a calibrated Sephadex G-100 Superfine column results in a single activity peak, corresponding to a mol wt of 4.5×10^4. This agrees quite well with the value of 4.9×10^4 reported by Erbes *et al.* (8).

The single most powerful step in the purification procedure is that of affinity chromatography with covalently linked Fd as the ligand. A major drawback of this step, however, is the low capacity and short lifetime of the Fd gel. A 2750-fold increase in specific activity over the crude extract is obtained for the peak fraction (Table I). With Fd at a saturating concentration, the specific activity of this fraction would be 720 μmol H_2 produced · mg^{-1} protein · min^{-1}. SDS-PAGE of this fraction (Fig. 2) indicates two major bands; the densest of these corresponds to a mol wt of 4.75×10^4, and was deduced to be hydrogenase. This band was determined by scanning densitometry to contain approximately 40% of the total protein. Therefore, the specific activity of pure hydrogenase from *C. reinhardtii* can be calculated to be about 1800 μmol H_2 produced · mg^{-1} protein · min^{-1}. Native polyacrylamide gels show a single band when stained for hydrogenase activity, and this band corresponds to the major band of a Coomassie Blue-stained gel (Fig. 2).

In contrast to the effects of pH on hydrogenase-DEAE interaction, it was observed that the binding of hydrogenase to Fd is much stronger at lower pH. Hydrogenase is fully retained by the Fd-affinity column at pH 6.0, but passes through in the void volume at pH 8.5 (Fig. 3). There are several possible reasons for this effect, including: (a) a change in an ionizable functional group; (b) enhanced affinity for Fd due to increased binding of the other substrate (*i.e.* protons); and (c) higher affinity of hydrogenase for oxidized Fd (which would increase with decreasing pH due to the lessened reducing power of dithionite).

Iron Content. Every procaryotic hydrogenase that has been examined in detail has been shown to be an iron-sulfur protein. Thus, it was of interest to determine whether hydrogenase from *C. reinhardtii* also exhibits this characteristic. The iron concentrations of fractions eluting from the second hydroxylapatite column are shown in Figure 4. The hydrogenase activity profile corresponds quite well with that of iron concentration. This strongly suggests that algal hydrogenase is indeed an iron-containing protein. Apparently there is a small amount of an additional iron-containing contaminant that elutes slightly after the hydrogenase peak, however. By comparing the hydrogenase activity of fractions 17, 19–21, and 23 with the calculated specific activity of pure hydrogenase, it was possible to estimate the concentration of hydrogenase for each of these fractions, and

Table I. *Purification of Hydrogenase from C. reinhardtii*

All activity measurements were made with 10 μM Fd as the electron mediator (1 unit = 1 μmol H_2 produced·min^{-1}). The values expressed refer to pooled active fractions, except for the affinity chromatography step, in which case only the peak fraction (vol = 1 ml) was measured.

Step	Total Volume	Total Activity	Total Protein	Yield	Specific Activity	Purification
	ml	*units*	*mg*	*%*	*units/mg protein*	*fold*
Crude extract	12600	4380	33500	100	0.131	1.00
First DEAE column	282	2720	11300	62.1	0.241	1.84
DEAE column with KCl gradient	172	2300	1380	52.5	1.67	12.8
Sephadex G-100 column	42.5	1700	376	38.8	4.52	34.5
First hydroxylapatite column	117	1700	92.6	38.8	18.4	140
Second hydroxylapatite column	27.0	1190	44.9	27.2	26.5	202
Fd-affinity chromatography[a]	(27.0)	(48.6)	(0.135)	(1.1)	360	2750

[a] Due to the limited capacity of the affinity column, the entire sample was not processed. The values in parentheses expressed for this step were calculated as if the entire sample had been utilized.

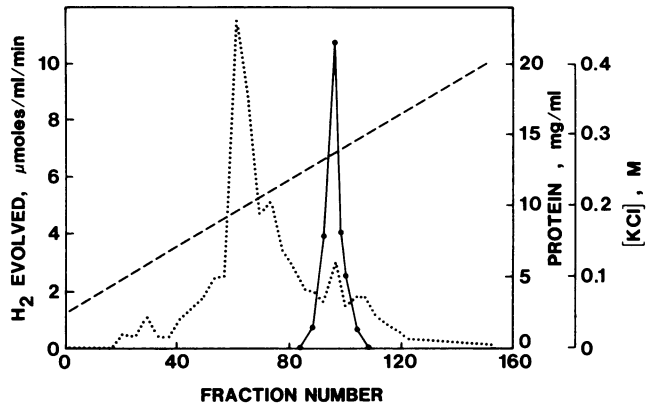

FIG. 1. DEAE-Sephacel chromatography of *C. reinhardtii* hydrogenase. The [KCl] gradient was buffered throughout with 50 mM Tris-Cl, pH 8.5. Protein (····); hydrogenase activity (●——●); [KCl] (– – –).

thus also the number of iron atoms per hydrogenase molecule. Such calculations indicate that there are 25.1 ± 2.8 (SD) mol iron/mol hydrogenase. The large (2.05×10^5 mol wt) hydrogenase of *Proteus mirabilis* was reported to contain 24 Fe/mol (19), while smaller ($5.0–6.0 \times 10^4$ mol wt) hydrogenases from *Clostridium pasteurianum* (6), *Desulfovibrio vulgaris* (20), and *Megasphaera elsdenii* (21) were all reported to contain 12 Fe/mol. Due to the uncertainty involved in the determination of hydrogenase content in these fractions, our estimate for *C. reinhardtii* hydrogenase iron content could differ from the actual value by a factor of two or more.

Temperature Effects. H_2 production catalyzed by partially purified hydrogenase with Fd as the electron mediator increases exponentially from 2.0 to 32.0°C. An Arrhenius plot of the data thus produces a straight line from which an activation energy of 55.1 kJ·mol^{-1} can be calculated. Similarly, activation energies of 57, 59, and 61 kJ·mol^{-1} have been calculated for hydrogenase reactions in *Rhodospirillum* (1), *Alcaligenes* (18), and *Clostridium* (12), respectively. The rate of the reaction continues to increase above 32°C to at least 56°C, indicating that algal hydrogenase has a high optimum temperature, which is characteristic of many other hydrogenases.

Hydrogenase from *C. reinhardtii* loses less than 10% of its activity when incubated for 1 h at 40°C or lower. Indeed, the half-life of a post-Sephadex preparation of hydrogenase maintained at 25°C was determined to be 14 d. Incubation at 55°C for 1 h results in a 50% reduction in activity, however, and activity is almost completely lost after 5 min at 70°C. This is in

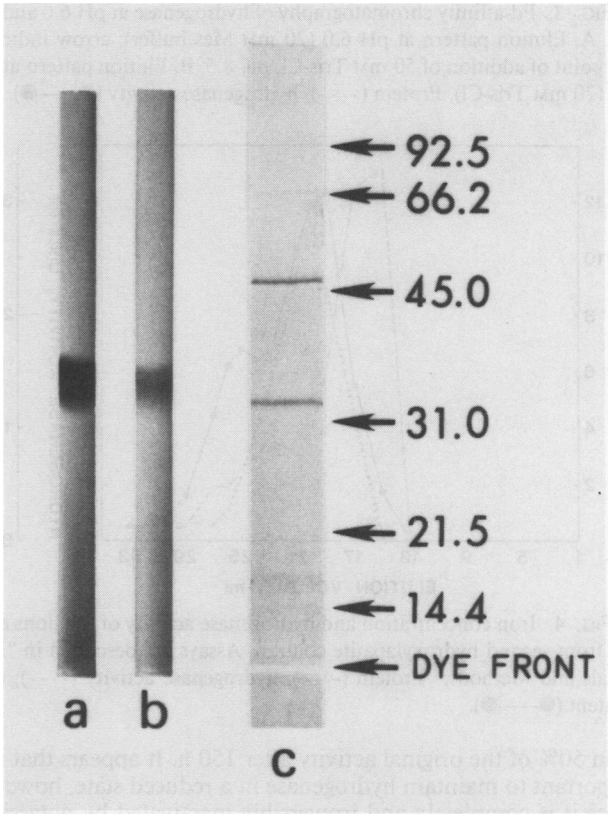

FIG. 2. Native and SDS-PAGE of a 2750-fold purified preparation of hydrogenase. Left, Native gels stained for activity (a) and protein (b). Right, SDS-containing gel (c). Arrows indicate migration distances of mol wt marker proteins (expressed in kD). The band migrating slightly slower than the 45 kD mol wt marker (ovalbumin) was deduced to be hydrogenase.

contrast to the hydrogenases of *Thiocapsa* (9) and *Rhodospirillum* (1, 2), which show no loss of activity after incubation for 10 min at 70°C. Samples can be frozen for several months at −20°C with little loss in activity, as long as the sample remains anaerobic.

Although dithionite helps to preserve hydrogenase by scavenging oxygen, its presence, *per se*, is not required as long as strict anaerobicity is maintained. This is known because hydrogenase (4 units/ml) oxidizes 10 mM dithionite to undetectable levels (<3 μM) within 30 h at 25°C, while hydrogenase activity is still greater

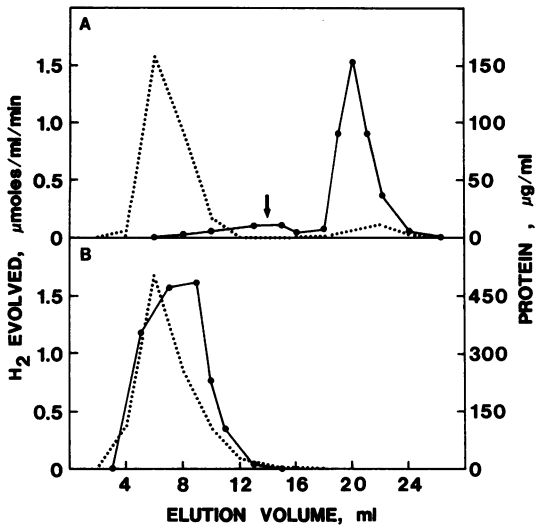

FIG. 3. Fd-affinity chromatography of hydrogenase at pH 6.0 and pH 8.5. A, Elution pattern at pH 6.0 (20 mM Mes buffer); arrow indicates the point of addition of 50 mM Tris-Cl, pH 8.5. B, Elution pattern at pH 8.5 (20 mM Tris-Cl). Protein (····); hydrogenase activity (●——●).

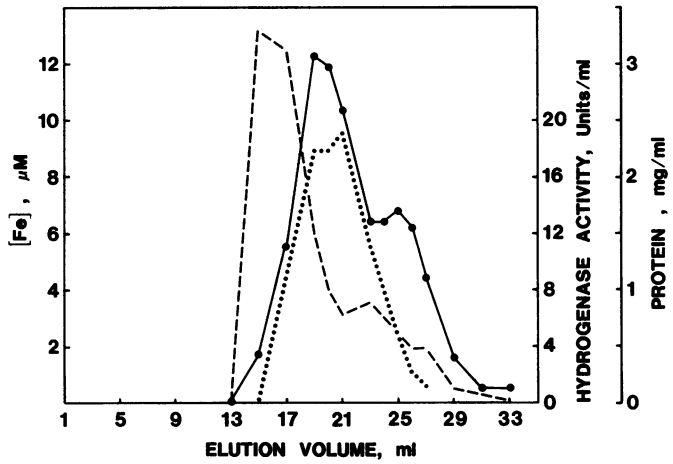

FIG. 4. Iron concentration and hydrogenase activity of fractions eluting from second hydroxylapatite column. Assays are described in "Materials and Methods." Protein (---); hydrogenase activity (····); iron content (●——●).

than 50% of the original activity after 150 h. It appears that it is important to maintain hydrogenase in a reduced state, however, since it is completely and irreversibly inactivated by potassium ferricyanide (2 mM in excess of dithionite). Therefore, concentrated samples should be frozen as soon as possible in order to prevent dithionite depletion. The use of a high pH buffer (*e.g.* pH 8.5) affects the equilibrium of the reaction such that dithionite oxidation, coupled to H_2 production, is reduced. H_2 gas bubble formation in chromatographic columns is also minimized by the use of a high pH buffer.

Interactions with Electron Mediators. Purified hydrogenase reacts with Fd, the physiological electron mediator, as well as a variety of artificial electron mediators. The K_m for dithionite-reduced Fd is 10 μM, which is the same value obtained with crude, cell-free extracts (16). Hydrogen evolution also occurs with reduced methyl viologen, benzyl viologen, sulfonatopropyl viologen, sodium metatungstate, and sodium silicotungstate, but frequently maximal enzyme activity is only observed after a prolonged lag period. This lag period is extremely variable in occurrence and extent. We have not been able to determine the reason for this lag, but the observation that low concentrations of oxidized dithionite (<10 mM) partially eliminated the lag period suggested that sulfur compounds might be involved. Further investigations showed that preincubation with 50 mM sodium sulfite extends the lag period while sodium thiosulfate eliminates the lag (Fig. 5). The extended lag caused by Na_2SO_3 is more than a simple inhibition, since post-lag hydrogenase activity with 2.5 mM methyl viologen is only inhibited by 26% in the presence of 50 mM Na_2SO_3 (apparently due to a lowered redox potential for the reaction mixture, decreasing the amount of reduced dye by 42%). Preincubation with 50 mM Na_2SO_4, $NaNO_3$, NaCl, or NaBr has no effect on the lag period. Furthermore, this lag is rarely observed in crude extracts. The implications of these findings are presently unknown. H_2 oxidation (with no lag period) is observed when methyl viologen, sulfonatopropyl viologen, and metatungstate are included as electron acceptors.

The relative effectiveness of various electron mediators for both H_2 production (measured in the presence of 50 mM thiosulfate) and H_2 oxidation are indicated in Table II. The mediator

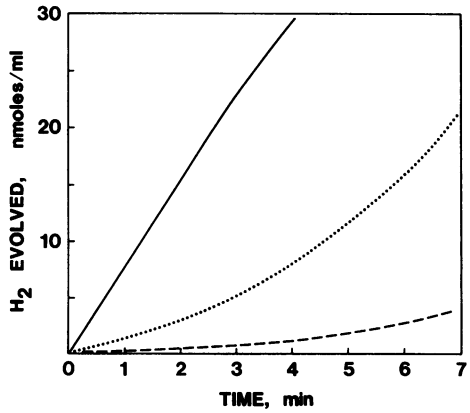

FIG. 5. Effects of sulfite and thiosulfate on lag period observed when using artificial electron mediators. Methyl viologen (2.5 mM) was injected into the reaction mixture (which contained 10 mM sodium dithionite) at time zero. No additons (····); preincubation with 50 mM Na_2SO_3 (---); preincubation with 50 mM $Na_2S_2O_3$ (——).

Table II. *Hydrogenase Activity with Various Electron Mediators*

The initial rates (<5 min duration) expressed are relative to the rates obtained with 2.5 mM methyl viologen as the electron mediator; actual rates are shown in parenthesis as μmol $H_2 \cdot mg^{-1}$ protein $\cdot min^{-1}$. Measurements of H_2 production and H_2 oxidation rates utilized 35- and 80-fold purified post-Sephadex preparations, respectively. For H_2 evolution studies, all mediators were reduced with 10 mM sodium dithionite.

Electron Mediator	50 mM Thiosulfate	Relative Activity
H_2 evolution		
2.5 mM methyl viologen	+	100 (4.53)
2.5 mM benzyl viologen	+	7.7
2.5 mM sulfonatopropyl viologen	+	62.9
90 μM sodium silicotungstate	+	34.7
50 μM sodium metatungstate	+	82.6
30 μM Fd	+	63.4
30 μM Fd	−	155
10 mM sodium dithionite	−	1.8
H_2 uptake		
2.5 mM methyl viologen	−	100 (0.85)
40 mM methyl viologen	−	1090
2.5 mM sulfonatopropyl viologen	−	847
0.83 mM sodium metatungstate	−	3610

concentrations used for the H_2 production studies are approximately 3 times the K_m values obtained for hydrogenase in crude extracts (16, 17). The inhibition of Fd-mediated hydrogenase activity by thiosulfate is expected, based on earlier studies involving the effects of anions on hydrogenase-mediator interactions (16, 17). We have not obtained accurate K_m values for these electron mediators for the process of H_2 oxidation, but the K_m for methyl viologen is approximately 50 to 80 mM. This high K_m for oxidized methyl viologen can be attributed to the fact that it is a divalent cation, and therefore may be repulsed from the positively charged region near the active site of hydrogenase as reported earlier (17). Lower K_m values would be expected for neutral or anionic electron mediators. The high rates of H_2 oxidation obtained when using low concentrations of sulfonatopropyl viologen (with a net neutral charge) and polyanionic sodium metatungstate (Table II) support this view.

The results of these investigations do not suggest any fundamental differences between the hydrogenases purified from various procaryotic sources and that from the eucaryote, *C. reinhardtii*. More detailed analysis of the active site of *C. reinhardtii* hydrogenase by the use of electron paramagnetic resonance spectroscopy would be highly desirable for comparative purposes, but the low quantity of hydrogenase present in this organism makes this a difficult task. This problem could be alleviated if another strain or mutant of *C. reinhardtii* is discovered which contains greatly elevated levels of hydrogenase.

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