BBA 45660

PURIFICATION AND PROPERTIES OF HYDROGENASES OF DIFFERENT ORIGINS

TATSUHIKO YAGI, MASURA HONYA AND NOBUO TAMIYA

Department of Chemistry, Shizuoka University, Shizuoka (Japan), and Department of Chemistry, Tohoku University, Sendai (Japan)

(Received November 20th, 1967)

SUMMARY

Hydrogenase of *Desulfovibrio desulfuricans* was purified. It was found that highly purified hydrogenase could catalyze the evolution of hydrogen from $Na_2S_2O_4$ in the presence of cytochrome c_3 or methyl viologen and not in the presence of ferredoxin, and the reduction by H_2 of ferricytochrome c_3 or methyl viologen but not of ferredoxin, methylene blue or hexacyanoferrate(III). Hence, H_2:ferricytochrome c_3 oxidoreductase is proposed for the systematic name of Desulfovibrio hydrogenase. It could catalyze the H–^{2}H exchange in the absence of added electron carriers. Hydrogenase of *Clostridium pasteurianum* (H_2:ferredoxin oxidoreductase, EC 1.12.1.1) was purified and found to be specific to ferredoxin and not to cytochrome c_3 or methyl viologen.

The optimum pH of the Desulfovibrio enzyme was 5–6 (cytochrome c_3-dependent H_2 evolution), 8–9 (cytochrome c_3 reduction by H_2) or 6–8 (H–^{2}H exchange reaction). K_m value was $7.5 \cdot 10^{-5}$ M for ferrocytochrome c_3 in the H_2 evolution reaction. H_2 production from $Na_2S_2O_4$ in the presence of cytochrome c_3 was irreversibly inhibited by CO, but that in the presence of methyl viologen was reversibly inhibited by CO.

INTRODUCTION

Hydrogenase is an enzyme which catalyzes the production and consumption of gaseous H_2 by bacteria.

$$H_2 + \text{electron carrier} \rightleftarrows \text{reduced electron carrier}$$

Methyl viologen has been used as an artificial electron carrier in both forward and backward reactions by various hydrogenase preparations. ISHIMOTO, YAGI AND SHIRAKI[1] observed the H_2 evolution from $Na_2S_2O_4$ or formate in the presence of added cytochrome c_3 or methyl viologen with a crude extract of *Desulfovibrio desulfuricans*. MORTENSON, VALENTINE AND CARNAHAN[2] observed the H_2 production from $Na_2S_2O_4$ or pyruvate in the presence of added ferredoxin or methyl viologen with a cell-free extract of *Clostridium pasteurianum*. These results suggest the difference in the electron carrier specificity of hydrogenases of different origins. Specificity studies of purified hydrogenase, however, have not yet been reported.

In this paper, the electron carrier specificity and some properties of hydrogenase are described. A preliminary account of portions of this work has been previously reported[3].

MATERIALS AND METHODS

Bacteria

Desulfovibrio desulfuricans was cultured as described before[4,5] except that the culture was bubbled with N_2 at 38°. From two 40-l cultures, 95 g wet cells were harvested.

Clostridium pasteurianum W5 was cultured in a N-free medium used by CARNAHAN *et al.*[6] except that biotin and *p*-aminobenzoate were replaced by Difco yeast extract. After several transfers from a potato medium, a 200-l culture was grown at 37° in the N-free medium which was bubbled with N_2 and stirred mechanically. The pH of the medium was maintained at 6.5 by the occasional additions of 5 M NaOH. At the end of the 12-h incubation during which 4750 ml of the alkali were added, 1.8 kg wet cells were harvested.

Electron carriers

Cytochrome c_3 was isolated from the sonicate of *D. desulfuricans* by an Amberlite XE 64 (NH_4^+) column[1]. $(NH_4)_2SO_4$ was added to the cytochrome c_3 solution thus obtained (up to 90% saturation) and the contaminants precipitated were removed by centrifugation. The supernatant was dialyzed. The cytochrome was again adsorbed on the above column, eluted with 0.1 M NH_3 and concentrated by partial lyophilization. Concentration of cytochrome c_3 was measured photometrically assuming the molar extinction coefficient of heme of ferrocytochrome c_3 at 553 mμ (ref. 7) to be $2.5 \cdot 10^4$, and expressed in the heme-molar concentrations.

Ferredoxin was prepared as described by MORTENSON[8].

Assay of hydrogenase

The rate of H_2 evolution from a reduced electron carrier[9]. A reaction mixture contained hydrogenase preparation and $6 \cdot 10^{-4}$ M methyl viologen (or another electron carrier to be tested) in 0.020 M phosphate buffer (pH 7.0) in 3.0 ml, and was placed in the main compartment of a Warburg vessel. The center well contained alkaline pyrogallol (0.2 ml). The gas phase was N_2. Anhydrous $Na_2S_2O_4$ (2.4 mg) was added from the side arm and the rate of H_2 production determined at 30°. Activity was expressed in units (μmoles H_2 produced per min).

Reduction of electron carriers with H_2. The reaction mixture contained enzyme preparation, $2 \cdot 10^{-5}$ M electron carrier, $3.3 \cdot 10^{-4}$ M thioglycolate and a buffer solution of the desired pH in 6.0 ml, and was placed in a Thunberg tube. The tube was evacuated, filled with H_2 (500 mm Hg) and incubated at 30°. The reduction of the electron carrier was measured photometrically, and the activity in units (μmoles H_2 consumed per min) was calculated.

Hydrogen–deuterium exchange. The reaction mixture contained enzyme preparation and a buffer solution of desired pH in 3.0 ml, and was placed in a 170-ml vessel. The content of the vessel was lyophilized, dissolved in 2.0 ml ^2H_2O (99.8 atom % ^2H), lyophilized again and then dissolved in 3.0 ml ^2H_2O. The vessel was

chilled with solid CO_2–ethanol, evacuated and filled with H_2. The contents of the vessel were thawed and anhydrous $Na_2S_2O_4$ (2.4 mg) was added from the side arm. Then the vessel was immediately chilled with solid CO_2–ethanol, evacuated and filled with H_2 (500 mm Hg).

The reaction was carried out by shaking the vessel at 100 rev./min at 30°. After 3 h the gas was analyzed for H_2, H^2H and $^2H_2$ by a mass spectrometer, Type RMU-3B (Hitachi), or Type 21-620A (Consolidated Electrodynamics Corporation). Activity was expressed in units (μmoles H^2H formed per min).

RESULTS

Purification of hydrogenase of D. desulfuricans

The bacterial sonicate[5] was centrifuged at 80000 × *g* for 60 min. The supernatant was passed through an Amberlite XE 64 column to remove cytochrome c_3 (see MATERIALS AND METHODS) and used as the crude enzyme. Streptomycin sulfate (1.2 g) was added to the crude enzyme (140 ml) and centrifuged. From the supernatant fluid (Streptomycin supernatant, 140 ml) the enzyme was precipitated with $(NH_4)_2SO_4$ (between 30 and 75 % saturation) and dissolved in 10 ml distilled water. After centrifugation at 26000 × *g* for 20 min, a dark green solution ($(NH_4)_2SO_4$ fraction, 15 ml) was obtained. The solution was applied in 5 portions to a Sephadex G-200 column (2.7 cm × 65 cm) which had been equilibrated with 0.05 M Tris–HCl buffer (pH 7.3) containing 0.2 M NaCl at 4°. By chromatography with the same buffer, the activity was eluted in 2 peaks (the first peak, Sephadex Fraction A, from 128 to 168 ml; the second peak, Sephadex Fraction B, from 208 to 272 ml; see Fig. 1). In earlier experiments, the enzyme in the Sephadex Fraction B was precipitated with $(NH_4)_2SO_4$, dissolved in distilled water and rechromatographed on the same column. The active fraction was collected, and the enzyme was precipitated with $(NH_4)_2SO_4$, dissolved in distilled water and dialyzed overnight against distilled water. The supernatant of the dialyzed solution was designated as the second Sephadex fraction. The process resulted in a 7-fold purification with 2 % recovery of the activity[3]. In later experiments, the Sephadex Fraction B was dialyzed overnight against distilled water (10 l)

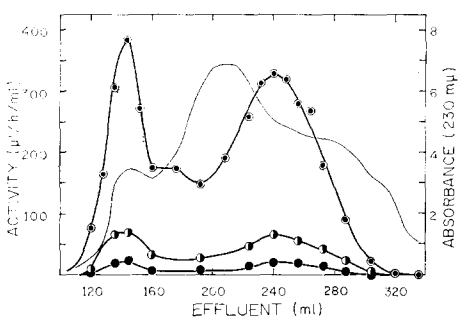


Fig. 1. Elution pattern of Desulfovibrio hydrogenase from a Sephadex G-200 column (2.7 cm × 65 cm). Conditions of chromatography are described in the text. ⊙—⊙, the rate of H_2 evolution from $Na_2S_2O_4$ in the presence of $6 \cdot 10^{-4}$ M methyl viologen; ◑—◑, the rate of H^2H formation in the exchange reaction; ●—●, the rate of $^2H_2$ formation; and ———, absorbance at 230 mμ* of the effluent.

* Because of sensitivity, $A_{230\,m\mu}$ in preference to $A_{280\,m\mu}$ was plotted.

and applied to a DEAE-cellulose column (1.7 cm × 24 cm) in 3 portions. Prior to being packed in the column, the DEAE-cellulose was stored overnight in 0.05 M Tris–HCl (pH 7.3), and after being packed in the column, was washed with 10 vol. of distilled water. The activity of the hydrogenase was eluted by linear concentration gradient technique after the column was washed with 50 ml distilled water. The initial solvent in the mixing chamber was 250 ml 0.01 M Tris–HCl (pH 7.3), and that in the reservoir 250 ml 0.04 M Tris–HCl (pH 7.3) containing 0.075 M NaCl. The activity of hydrogenase eluted in a sharp peak between 288 and 352 ml was collected (DEAE Fraction B1). It was dialyzed overnight against distilled water (10 l), and rechromatographed on a DEAE-cellulose column under the same conditions. The active fraction from the second chromatography was collected, dialyzed overnight against distilled water (10 l), lyophilized and dissolved in 2.3 ml water (DEAE Fraction B2). The preparation had no absorption in the visible region. This procedure apparently resulted in 80-fold purification, but as the hydrogenase activity was separated into 2 fractions by the Sephadex column chromatography, the actual purification was 125-fold. The results are summarized in Table I. The activity of the purified preparation gradually deteriorated upon repeated freezing and thawings.

TABLE I

SUMMARY OF THE PURIFICATION PROCESS OF THE DESULFOVIBRIO HYDROGENASE

	Volume (*ml*)	*Total* $A_{280\,m\mu}$	*Exchange reaction* (*units/ml enzyme*)		*Activity* (*units/ml enzyme*) (*b*)	*Ratio* (*a/b*)	*Total activity* (*units*)	*Recovery* (%)	*Specific activity* (*units/* $A_{280\,m\mu}$)
			H^2H (*a*)	$^2H_2$					
Crude enzyme	140	6440	—	—	3.77	—	530	100	0.08
Streptomycin supern.	140	1410	0.26	0.096	2.00	0.13	280	53	0.20
$(NH_4)_2SO_4$ fraction	15	760	2.22	0.66	12.6	0.18	190	36	0.25
Sephadex Fraction A	200	115	0.037	0.013	0.193	0.19	39	7.3	0.34
Sephadex Fraction B	320	285	0.039	0.013	0.220	0.18	71	13	0.25
DEAE Fraction B1	192	45	—	—	0.147	—	28	5.3	0.61
DEAE Fraction B2	2.3	1.6	0.66	0.15	4.44	0.15	10	1.9	6.3

Partial purification of hydrogenase of Cl. pasteurianum

The precipitate obtained by the addition of acetone to the extract of *Cl. pasteurianum*[8] was lyophilized. The dried precipitate (2.5 g) was extracted with 50 ml 0.2 M phosphate buffer (pH 7.0) and centrifuged at 26000 × *g* for 20 min. The hydrogenase was precipitated from the yellowish supernatant (crude enzyme, 43 ml) by $(NH_4)_2SO_4$ (between 30 and 90 % saturation), dissolved in 4.0 ml distilled water, and chromatographed on a Sephadex G-100 column (3 cm × 45 cm) with 0.05 M Tris–HCl containing 0.2 M NaCl. The enzyme was further purified by rechromatography on the same column as in the case of the Desulfovibrio enzyme. The preparation obtained was designated as the second Sephadex fraction. This process resulted in a 6-fold purification with 32 % recovery of the activity[3].

Specificity

The crude hydrogenase preparation from *D. desulfuricans* catalyzed the production of H_2 in the presence of methyl viologen, cytochrome c_3 or ferredoxin[3], whereas the partially purified preparation (second Sephadex fraction) catalyzed the H_2 production in the presence of cytochrome c_3 or methyl viologen and not in the presence of ferredoxin (Fig. 2). Possibly, there are two kinds of hydrogenases in *D. desulfuricans*, one specific to cytochrome c_3 or methyl viologen, and the other specific to ferredoxin. Therefore, the $(NH_4)_2SO_4$ fraction was chromatographed on Sephadex G-100 column (3.0 cm × 45 cm). The effluent was collected in 8-ml fractions and each fraction assayed for activity with $6 \cdot 10^{-4}$ M methyl viologen or $2 \cdot 10^{-5}$ M ferredoxin. Fig. 3 shows that there is no ferredoxin-specific hydrogenase in *D. desulfuricans*.

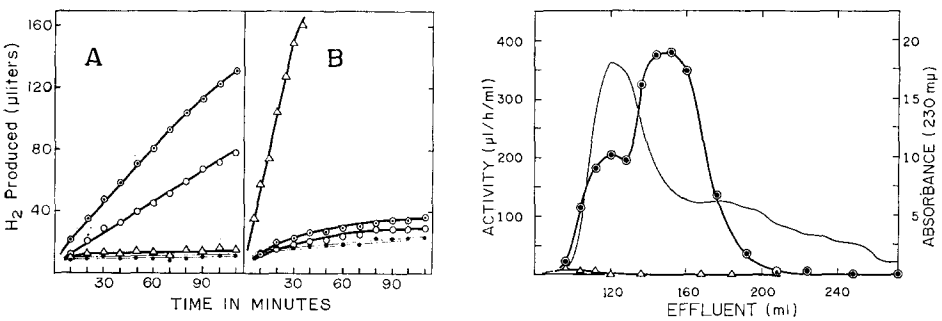


Fig. 2. Evolution of H_2 by the purified hydrogenase preparations. The main compartment of a Warburg vessel contained the enzyme, 2nd Sephadex fraction (A: 3.3 $A_{230\,m\mu}$ units Desulfovibrio enzyme; B: 5.6 $A_{230\,m\mu}$ units Clostridial enzyme), $2 \cdot 10^{-5}$ M each electron carrier (○—○, cytochrome c_3; ⊙—⊙, methyl viologen; △—△, ferredoxin or ●=●, control without addition) and 0.020 M phosphate buffer (pH 7.0) in 3.0 ml. The gas phase was N_2. The center well contained 0.2 ml alkaline pyrogallol. Anhydrous $Na_2S_2O_4$ (2.4 mg) was added and the rate of H_2 evolution was followed at 30°.

Fig. 3. Elution pattern of Desulfovibrio hydrogenase from a Sephadex G-100 column (3.0 cm × 45 cm). Conditions of chromatography are described in the text. ⊙—⊙, the rate of H_2 evolution in the presence of $6 \cdot 10^{-4}$ M methyl viologen; △—△, in the presence of $2 \cdot 10^{-5}$ M ferredoxin; and ———, absorbance at 230 mμ of the effluent.

The purified hydrogenase (DEAE Fraction B2) catalyzed the reduction with H_2 of ferricytochrome c_3 and methyl viologen, but not of ferredoxin, methylene blue or hexacyanoferrate (III) ion.

The crude hydrogenase of *Cl. pasteurianum* catalyzed the H_2 evolution from $Na_2S_2O_4$ in the presence of ferredoxin, methyl viologen and cytochrome c_3 (ref. 3), but the purified preparation (second Sephadex fraction) could catalyze the reaction only in the presence of ferredoxin as shown in Fig. 2. The presence of another hydrogenase specific to methyl viologen in *Cl. pasteurianum* was denied by experiments similar to those illustrated in Fig. 3.

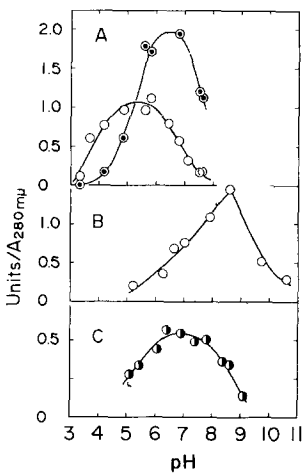
Properties of hydrogenase of D. desulfuricans

The hydrogenase activities measured by H_2 production in the presence of re-

duced methyl viologen and those measured by H–^{2}H exchange reaction were compared. The results are shown in Table I and Fig. 1.

The optimum pH of the cytochrome c_3-dependent H_2 evolution was 5–6, that of the methyl viologen-dependent H_2 evolution, 6–7 (Fig. 4A), that of the cytochrome c_3 reduction by H_2, 8–9 (Fig. 4B) and that of H–^{2}H exchange reaction in the absence of electron carrier, 6–8 (Fig. 4C).

Lineweaver–Burk plots for the rate of H_2 evolution and cytochrome c_3 or methyl viologen concentration are illustrated in Fig. 5. The K_m values for ferrocytochrome c_3 and for reduced methyl viologen were calculated to be $7.5 \cdot 10^{-5}$ M (for heme concentration) and $1.2 \cdot 10^{-4}$ M, respectively.



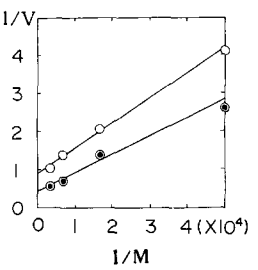


Fig. 4. Effects of pH on the rate of hydrogenase reactions. A. H_2 evolution from $Na_2S_2O_4$ in the presence of ○—○, $6 \cdot 10^{-5}$ M cytochrome c_3 or ⊙—⊙, $6 \cdot 10^{-4}$ M methyl viologen. B. Reduction by H_2 of $2 \cdot 10^{-5}$ M ferricytochrome c_3. C. H^{2}H formation in the exchange reaction between H_2 and ^2H_2O. The enzyme was the DEAE Fraction B2. The buffers were: McIlvaine's Na_2HPO_4–citric acid (diluted 10-fold) below pH 5.0; 0.020 M phosphate between pH 5.0 and 7.7; 0.020 M Tris–HCl between pH 7.7 and 9.0; and Sørensen's NaOH–glycine (containing 0.020 M Na^+) over pH 9.0.

Fig. 5. Lineweaver–Burk plots at pH 6.9. ○—○, for cytochrome c_3 and ⊙—⊙, for methyl viologen. The enzyme was the DEAE Fraction B2. V is specific activity (units/$A_{280\,m\mu}$ unit).

Molecular weight of the hydrogenase was determined to be 60000 from the elution behavior[10] on Sephadex G-200 column at 4°. In this determination dextran blue (mol. wt. 2000000) and $(NH_4)_2SO_4$ had been used as external standards and yeast cytochrome c (mol. wt. 12700) and bovine serum albumin (mol. wt. 67000) as internal standards.

H_2 production in the presence of cytochrome c_3 was completely inhibited by CO and could not be reversed upon replacement of CO by flushing with N_2. H_2 evolution in the presence of methyl viologen also inhibited by CO (97% inhibition). When CO was replaced by N_2, 55% of the original activity was recovered.

The activity measured by H_2 evolution in the presence of methyl viologen was not affected by treatment of the enzyme at 50° for 10 min. Treatment at 60°, 70°, 80°, 90° and 100° for 10 min caused 30, 37, 69, 99 and 100% decrease in activity, respectively.

DISCUSSION

The enzyme, hydrogenase, is believed to catalyze the following reactions.

H_2 + electron carrier $\rightleftarrows$ reduced electron carrier

$H_2 + {}^2H_2O \rightleftarrows H^2H + HO^2H$

A discrepancy exists between the relative rates of these activities in different hydrogenases of different organisms[11,12], and the electron carrier specificity has never been determined with the purified preparations. In this study, Desulfovibrio hydrogenase was extensively purified. The purified Desulfovibrio hydrogenase catalyzed the H_2 evolution from ferrocytochrome c_3 or reduced methyl viologen, the reduction of ferricytochrome c_3 or methyl viologen by H_2, and the H–^2H exchange reaction in the absence of added electron carriers; but it did not catalyze the H_2 evolution from reduced ferredoxin, nor the reduction of ferredoxin, methylene blue or hexacyanoferrate(III) by H_2. The results shown in Table I also support the theory that H_2 evolution from a reduced electron carrier and the H–^2H exchange reaction were catalyzed by the same enzyme. The hydrogenase of *Desulfovibrio desulfuricans* is, therefore, specific to cytochrome c_3 as natural electron carrier. Hence H_2:ferricytochrome c_3 oxidoreductase is proposed as the systematic name of Desulfovibrio hydrogenase.

Clostridial hydrogenase (H_2:ferredoxin oxidoreductase, EC 1.12.1.1) on the other hand, is specific to ferredoxin as an electron carrier, and not to cytochrome c_3.

ACKNOWLEDGEMENTS

The authors wish to thank Professor B. MARUO of the University of Tokyo for mass spectrometric analyses, Dr. J. ABE and Dr. A. NAGATA of Toyo Jozo Co., Ltd., Ohito, Shizuoka for large-scale bacterial cultivations and Mr. K. SHIMOMURA for his assistance. We also thank Toyo Rayon Science Foundation for providing us with a Hitachi Mass Spectrometer and Sankyo Co., Ltd., Tokyo for providing us with yeast cytochrome *c*. This study was supported in part by a grant from the Ministry of Education.

REFERENCES

1 M. ISHIMOTO, T. YAGI AND M. SHIRAKI, *J. Biochem. Tokyo*, 44 (1957) 707.
2 L. E. MORTENSON, R. C. VALENTINE AND J. E. CARNAHAN, *Biochem. Biophys. Res. Commun.*, 7 (1962) 448.
3 N. TAMIYA, Y. YAMAGUCHI, M. HONYA AND T. YAGI, *Biochem. Biophys. Res. Commun.*, 22 (1966) 43.
4 M. ISHIMOTO, J. KOYAMA, T. OMURA AND Y. NAGAI, *J. Biochem. Tokyo*, 41 (1954) 537.
5 T. YAGI, *J. Biochem. Tokyo*, 46 (1959) 949.
6 J. E. CARNAHAN, L. E. MORTENSON, H. F. MOWER AND J. E. CASTLE, *Biochim. Biophys. Acta*, 44 (1960) 520.
7 J. R. POSTGATE, *J. Gen. Microbiol.*, 14 (1956) 545.
8 L. E. MORTENSON, *Biochim. Biophys. Acta*, 81 (1964) 71.
9 N. TAMIYA, Y. KONDO, T. KAMEYAMA AND S. AKABORI, *J. Biochem. Tokyo*, 42 (1955) 613.
10 P. ANDREWS, *Biochem. J.*, 96 (1965) 595.
11 H. D. PECK, A. SAN PIETRO AND H. GEST, *Proc. Natl. Acad. Sci. U.S.*, 42 (1956) 13.
12 A. I. KRASNA AND D. RITTENBERG, *Proc. Natl. Acad. Sci. U.S.*, 42 (1956) 180.