ELSEVIER

Journal of Molecular Catalysis A: Chemical 95 (1995) 93–98

JOURNAL OF MOLECULAR CATALYSIS A: CHEMICAL

Purification and properties of intact hydrogenase from *Desulfovibrio vulgaris* (Miyazaki)

Toshiaki Kamachi, Seiichi Uno, Tomohiro Hiraishi, Ichiro Okura *

Department of Bioengineering, Tokyo Institute of Technology, Nagatsuta, Yokohama 227, Japan

Received 15 May 1994; accepted 12 July 1994

Abstract

The intact hydrogenase from *Desulfovibrio vulgaris* (Miyazaki) was purified and characterized. The purified hydrogenase had a molecular weight of 92.0 kDa, with two different subunits (Mw = 62.5 kDa and 29.5 kDa). The molecular weight of the large subunit of the intact hydrogenase was 2 kDa larger than the tryptic digested hydrogenase. The absorption spectrum and the EPR spectrum of the intact hydrogenase were identical to those of tryptic digested hydrogenase. Phase separation study indicated the detergent solubilized hydrogenase was more hydrophobic than tryptic digested hydrogenase.

Keywords: *Desulfovibrio vulgaris*; Enzyme purification; Hydrogenase

1. Introduction

Hydrogenases are the enzymes that catalyze reversible oxidation of molecular hydrogen. Three classes of hydrogenases, the iron-only hydrogenase (Fe hydrogenase), nickel containing hydrogenase (NiFe hydrogenase) and nickel selenium containing hydrogenase (NiFeSe hydrogenase) have been found in sulfate reducing bacteria [1–8]. The NiFe hydrogenase has been isolated from *Desulfovibrio gigas*, *D. fructosovorans* and *D. vulgaris* (Miyazaki) [3–5]. NiFe hydrogenases are generally found to be periplasmic or membrane bound enzymes of molecular weight of 89 kDa (Mw = 63 kDa and 26 kDa for large and small subunit, respectively).

To purify the membrane bound hydrogenases, the detergent and/or the tryptic treatment of cell membranes are needed in advance. *D.vulgaris* (Miyazaki) hydrogenase has been solubilized from membrane with tryptic treatment. In this method, however, the isolated hydrogenase is partially hydrolyzed, and the physicochemical and/or the enzymatic properties are supposed to be different from the intact one. In this study, the intact hydrogenase from *D.vulgaris* (Miyazaki) was purified and characterized.

2. Materials and methods

Chemicals – Triton X-100 was purchased from Boehringer Mannheim GmbH. DEAE Sepharose Fast Flow, Sephacryl S-200 HR and Phenyl Sepharose Fast Flow were from Pharmacia LKB Biotechnology. Other reagents were commercially available.

* Corresponding author.

SSDI 1381-1169(94)00147-2

Cultivation of bacteria – *Desulfovibrio vulgaris* (Miyazaki) was grown in a medium at 30°C as reported previously [9], and stored at −80°C before use.

Hydrogenase assay – The hydrogenase activity was measured by the hydrogen evolution assay in the presence of dithionite-reduced methyl viologen. One unit of activity was defined as 1 μmol H_2 evolved per min.

Protein determination – Protein concentration was determined by modified Lowry protein assay [10] with bovine serum albumin as a standard.

Purification of the hydrogenase – Hydrogenase [H_2:ferricytochrome c_3 oxidoreductase, EC 1.12.2.1] was purified by the following procedure at room temperature under aerobic condition unless otherwise mentioned. The frozen cells of *D.vulgaris* (Miyazaki) were thawed at room temperature and suspended in 25 mM Tris–HCl buffer, pH 7.4. This cell suspension was sonicated on the ice bath (60 W, 0.5 min/g wet cell) followed by centrifugation at 203,000*g* for 1 h at 4°C. The supernatant was discarded. The particulate fraction was resuspended in the same buffer containing 1%(w/v) of Triton X-100. The suspension was stirred gently at room temperature to solubilize hydrogenase. The suspension was centrifuged at 203,000*g* for 1 h at 4°C after solubilization. The supernatant thus obtained was applied on the following chromatographic procedures. The buffer used in the chromatography was 25 mM Tris–HCl buffer, pH 7.4, containing 0.1% (w/v) of Tween 20 unless otherwise mentioned. The solubilized hydrogenase solution was put onto DEAE Sepharose Fast Flow column (2.5×33 cm) which was previously equilibrated with the buffer. The adsorbed hydrogenase was eluted from the column with linear gradient of NaCl concentration in the buffer 0 to 1.0 M. The fractions containing hydrogenase activity were pooled and were concentrated by ultrafiltration using a Diaflo cell (Amicon Corporation) with a PM30 membrane, and chromatographed on a Sephacryl S-200 column with the buffer containing 0.1 M NaCl. The eluent fractions containing the hydrogenase activity were diluted twice with the buffer containing 400 mM ammonium sulfate, and applied onto Phenyl Sepharose Fast Flow column (1.5×25 cm) pre-equilibrated with the buffer containing 200 mM ammonium sulfate. The enzymes were eluted from the column by decreasing the concentration of ammonium sulfate in the buffer from 0.2 to 0 M. The active fraction of the eluent was concentrated by ultrafiltration, and chromatographed again on Sephacryl S-200 column(1.5×90 cm) with the buffer containing 0.1 M NaCl. The fractions containing hydrogenase activity were directly applied onto DEAE Sepharose Fast Flow column(1.5×23 cm). The enzymes were eluted from the column with a linear gradient of NaCl concentration in the buffer. The tryptic digested hydrogenase was purified by the method reported previously [9].

Absorption spectra – Optical spectra were measured on a Shimadzu UV-265 spectrophotometer using the cells of 1 cm path length.

Molecular weight – The molecular weight of the enzyme subunit was determined by electrophoresis on SDS–Polyacrylamide gel performed by the method of Laemmli [11].

Hydrophobicity analysis – Phase separation was carried out according to the literature [12].

EPR measurement – EPR spectra were recorded with JEOL FE3XG spectrophotometer equipped with variable temperature cryostat, Air Products Heli-tran LTR-3-110 for controlling sample temperature.

Metal analysis – Analysis of metals (iron and nickel) was performed with Seiko SPR1500VR ICP emission spectrometer.

3. Results

3.1. Solubilization of hydrogenase

In previous studies, the hydrogenase was solubilized from the particulate fraction of *D.vulgaris* by tryptic digestion. In this method, the solubilized enzyme is partially hydrolyzed, so to obtain the intact hydrogenase, the solubilization of the enzyme with non-ionic detergents was tried. To

Table 1
Purification scheme of Triton C-100 solubilized hydrogenase from *D. vulgaris* (Miyazaki)

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (*n*-fold) *n*=
Membrane	3850	4235	1.1	100	1
Crude extract	1153	2882	2.5	68	2
DEAE–Sepharose	188	1240	6.6	29	6
Sephacryl S-200	39	468	12	11	11
Phenyl Sepharose	8.4	252	30	6	27
Sephacryl S-200	2.2	118	54	3	49
DEAE–Sepharose	0.5	68	136	2	124

solubilize the hydrogenase, Triton X-100, *n*-octyl-β-D-glucoside, Brij 58 and Tween 20 with various concentrations were used. Their solubilization ability was compared with that of the tryptic digested hydrogenase.

D.vulgaris (Miyazaki) particulate fraction was suspended with 25 mM Tris–HCl, pH 7.4 containing above detergents and stirred for 1 h at room temperature followed by ultracentrifugation. The hydrogenase activity of the supernatant and the precipitate were examined. Among the detergents examined, Triton X-100 had the highest solubilizing ability. Almost all the hydrogenase activity was found in the supernatant and no hydrogenase activity was found in the precipitate when the particulate was treated with 1% (w/v) Triton X-100, whereas the hydrogenase was solubilized up to 80% of total hydrogenase in the particulate fraction when the particulate was treated with trypsin. This result indicates the solubilization by non-ionic detergent Triton X-100 is more effective than tryptic digestion.

3.2. Purification of hydrogenase

Table 1 shows the summary of the purification of membrane bound hydrogenase from *D.vulgaris* (Miyazaki) as described in the 'Materials and Methods'. In this experiment, 124-fold purification and 2% recovery over the membrane fraction were achieved. When the purified hydrogenase was subjected to native polyacrylamide gel, a single protein band was obtained, indicating that the hydrogenase is homogeneous.

3.3. Molecular weight determination

The Triton X-100 solubilized hydrogenase showed two bands on SDS polyacrylamide gel (Fig. 1). The molecular weight was estimated to be 62.5 kDa and 29.5 kDa for large and small subunit, respectively. The molecular weight of large and small subunit of tryptic digested hydrogenase was estimated to be 60.5 kDa and 29.5 kDa, respectively. From the amino acid sequence of *D.vulgaris* (Miyazaki) hydrogenase, the molecular weight of large and small subunit of hydrogenase were reported as 62.495 kDa and 28.763 kDa, respectively [13]. The molecular weight of Triton X-100 solubilized hydrogenase estimated

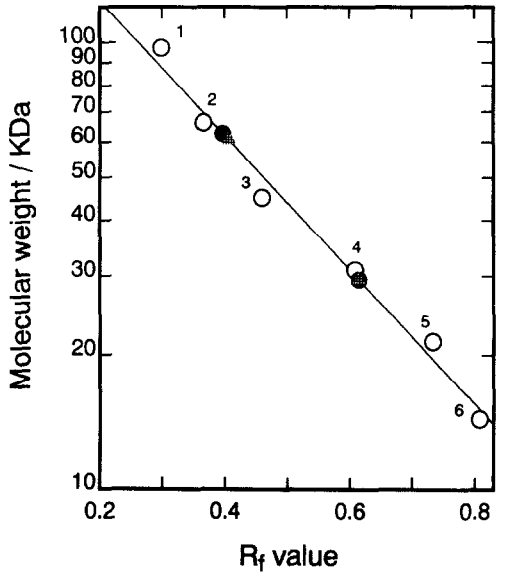

Fig. 1. Determination of the subunit molecular weight of *D. vulgaris* (Miyazaki) by SDS–polyacrylamide gel electrophoresis on 10–20% polyacrylamide gel. (●) Triton X-100 solubilized hydrogenase; (▲) tryptic digested hydrogenase. Marker proteins: 1, rabbit muscle phosphorylase (97 kDa); 2, bovine serum albumin (66.2 kDa); 3, hen egg white ovalbumin (45.0 kDa); 4, bovine carbonic anhydrase (31.0 kDa); 5, soybean trypsin inhibitor (21.5 kDa); hen egg white lysozyme (14.4 kDa).

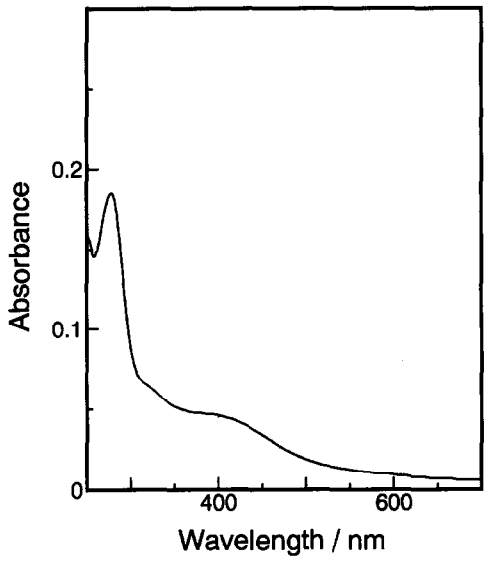

Fig. 2. Absorption spectrum of *D. vulgaris* (Miyazaki) hydrogenase solubilized with Triton X-100 in 25 mM Tris–HCl buffer, pH 7.4.

by SDS polyacrylamide gel electrophoresis was in good agreement with the molecular weight deduced from amino acid sequence. These data indicate that the Triton X-100 solubilized hydrogenase was intact enzyme as in the membrane, and the large subunit of the tryptic digested hydrogenase was partially decomposed by about 2 kDa.

3.4. Absorption spectrum

The absorption spectrum of the Triton X-100 solubilized hydrogenase is presented in Fig. 2. The Triton X-100 solubilized hydrogenase showed typical ultraviolet–visible spectrum of iron–sulfur proteins with a broad absorption band at around 400 nm, which was very similar to those given by tryptic digested hydrogenase [9]. The extinction coefficient ($42\ mM^{-1}cm^{-1}$ at 400 nm) for Triton X-100 solubilized hydrogenase was smaller than that for tryptic digested hydrogenase ($47\ mM^{-1}cm^{-1}$) [9]. The smaller value of the extinction coefficients of Triton X-100 solubilized hydrogenase is due to the larger molecular weight of Triton X-100 solubilized hydrogenase and/or partial loss of iron–sulfur cluster(s) of hydrogenase during the purification. The maximum ratio, A_{400}/A_{280}, was 0.25, which is smaller than that reported for tryptic digested hydrogenase ($A_{400}/A_{280}=0.29$).

3.5. Effect of mercurial ion on hydrogenase activity

Triton X-100 solubilized and tryptic digested hydrogenase were incubated with Hg(II) at 10 mM, and their hydrogen evolution activities were measured. Effect of incubation time is shown in Fig. 3. Both Triton X-100 solubilized hydrogenase and tryptic digested hydrogenase were inhibited with Hg(II). The residual activities of Triton X-100 solubilized and tryptic digested hydrogenase after Hg(II) treatment for 10 min were ca. 75% and 40%, respectively. This indicates the tryptic digested hydrogenase is more sensitive to Hg(II) treatment than Triton X-100 solubilized hydrogenase. The polypeptide of 2 kDa in Triton X-100

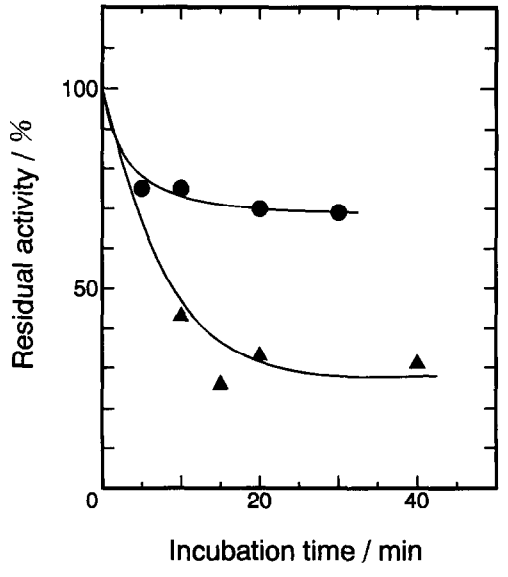

Fig. 3. Effect of Hg(II) ion on hydrogen evolution activity. The Triton X-100 solubilized hydrogenase(– ● –) and tryptic digested hydrogenase(– ▲ –) were incubated with 10 mM Hg(II) in 25 mM Tris–HCl buffer, pH 7.4 and their relative activities were measured at the time indicated.

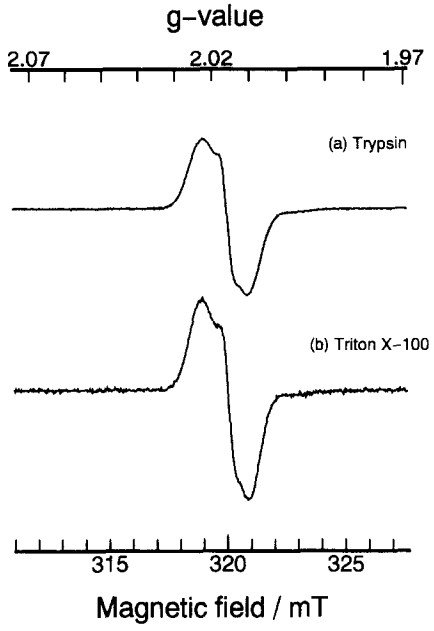

Fig. 4. EPR spectrum of *D. vulgaris* (Miyazaki) hydrogenase. (a) Tryptic digested hydrogenase, (b) Triton X-100 solubilized hydrogenase. Temperature 9 K; microwave frequency 9.03 GHz; microwave power 100 μW; modulation frequency 100 kHz; modulation amplitude 0.1 mT.

solubilized hydrogenase may play an important role to disturb the attack of Hg(II) against the active site of hydrogenase.

3.6. Phase separation

A phase separation is a technique for separating hydrophobic proteins from hydrophilic ones based on the low solubility of Triton X-114 above its cloud point. Hydrophilic proteins are found exclusively in an aqueous phase and integral membrane proteins with amphiphilic nature are recovered in a detergent phase. When this technique was applied for the Triton X-100 solubilized and the tryptic digested hydrogenases, Triton X-100 solubilized hydrogenase was almost found in detergent phase and tryptic digested hydrogenase was recovered in aqueous phase. These results show that the hydrogenase solubilized with trypsin is partially hydrolyzed.

3.7. Metal content

The Triton X-100 solubilized hydrogenase contained 12.4 mol iron and 0.63 mol nickel per molecule (Mw = 92 kDa) by ICP emission spectrometry, while the tryptic digested hydrogenase contained 12.3 mol iron and 0.68 mol nickel per molecule.

3.8. EPR spectrum

Fig. 4 shows EPR spectra from *D.vulgaris* (Miyazaki) hydrogenase at low temperature. EPR spectra of the Triton X-100 solubilized hydrogenase shows a nearly isotropic signal at $g = 2.015$. This spectrum is attributed to an oxidized [3Fe–4S] cluster and is identical to that of tryptic digested hydrogenase.

At high temperature (> 100 K), the Triton X-100 solubilized and tryptic digested hydrogenases exhibited identical spectrum due to nickel(III) as observed in some other nickel-containing hydrogenases. These spectra consist of two superimposed signals Ni-A ($g = 2.32$, 2.24, 2.01) and Ni-B ($g = 2.34$, 2.10, 2.01) [5].

The dithionite reduced enzyme showed complex EPR signal at low temperature (< 10 K) as shown in the EPR spectrum of the tryptic digested hydrogenase. This signal has been assigned to the reduced [4Fe–4S] clusters [5]. These EPR data indicate that the Ni, the [3Fe–4S] and [4Fe–4S] centers in the Triton X-100 solubilized hydrogenase are almost identical to those in tryptic digested hydrogenase.

4. Discussion

The purification and properties of intact hydrogenase from *Desulfovibrio vulgaris* (Miyazaki) were studied. The molecular weights of Triton X-100 solubilized hydrogenase was 62.5 kDa and 29.5 kDa as determined by SDS polyacrylamide gel electrophoresis. These values are in good agreement with the molecular weight deduced from the amino acid sequences [13]. The large

subunit of tryptic digested hydrogenase was partially hydrolyzed by 2 kDa. These data indicate the tryptic digested hydrogenase is a hydrolyzed artifact enzyme and the Triton X-100 solubilized hydrogenase is a native one. Triton X-100 had a higher efficiency for solubilizing hydrogenase than trypsin treatment, though the over all recovery is slightly low.

The metal contents of Triton X-100 and tryptic digested hydrogenase were almost the same. These data and EPR spectra indicate the hydrogenase contained one [3Fe–4S], two [4Fe–4S] and one nickel per molecule, which are similar to some other NiFe hydrogenases. The inhibition study of hydrogenase with Hg(II) indicates that the Triton X-100 solubilized hydrogenase was more stable than the tryptic digested hydrogenase on Hg(II) treatment.

Acknowledgements

This work was partially supported by a Grant-in-Aid for Scientific Research on Priority Area 'Organic Electrochemistry' (No. 05234104) from the Ministry of Education, Science and Culture, Japan.

References

[1] B.H. Huynh, M.H. Czechowski, H. Kruger, D.V. Der Vartanian, H.D. Peck, Jr. and J. LeGall, Proc. Natl. Acad. Sci. USA, 81 (1984) 3728–3732.
[2] E.C. Hatchikian, N. Forget, V.M. Fernandez, R. Williams and R. Cammack, Eur. J. Biochem., 209 (1992) 357–365.
[3] R. Cammack, D.S. Patil, E.C. Hatchikian, V.M. Fernandez, Biochim. Biophys. Acta, 912 (1987) 98–109.
[4] E.C. Hatchikian, A.S. Traore, V.M. Fernandez and R. Cammack, Eur. J. Biochem., 187 (1990) 635–643.
[5] M. Asso, B. Guigliarelli, T. Yagi and P. Bertrand, Biochim. Biophys. Acta, 1122 (1992) 50–56.
[6] M. Teixeira, G, Fauque, I. Moura, P.A. Lespinat, Y. Berlier, B. Prickril, H.D. Peck, Jr, A.V. Xavier, J. LeGall and J.J.G. Moura, Eur. J. Biochem., 167 (1987) 47–58.
[7] M. Teixeira, I. Moura, G. Fauque, M. Czechowski, Y. Berlier, P.A. Lespinat, J. LeGall, A.V. Xavier and J.J.G. Moura, Biochimie, 68 (1986) 75–84.
[8] G. Fauque, H.D. Peck, Jr, J.J.G. Moura, B.H. Huynh, Y. Berlier, D.V. Der Vartanian, M. Teixeira, A.E. Przybyla, P.A. Lespinat, I. Moura, J. LeGall, FEMS Microbiol. Rev., 54 (1988) 299–344.
[9] T. Yagi, K. Kimura, H. Daidoji, F. Sakai, S. Tamura, and H. Inokuchi, J. Biochem., 79 (1976) 661–671.
[10] A. Bensadoun and D. Weinstein, Anal. Biochem., 70 (1976) 241–250.
[11] U.K. Laemmli, Nature, 227 (1970) 680–685.
[12] C. Bordier, J. Biol. Chem., 256(4) (1981) 1604–1607.
[13] H.M. Deckers, F.R. Wilson and G. Voordouw, J. Gen. Microbiol., 136 (1990) 2021–2028.