SEPARATION OF HYDROGENASE FROM INTACT CELLS OF *DESULFOVIBRIO VULGARIS*

Purification and properties

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1. Introduction

Purified preparations of hydrogenase have been reported from two strains of *Desulfovibrio vulgaris* (Hildenborough [1–3] and Miyazaki [4,5]). In most of the purification procedures that have been described the bacteria were broken mechanically, and in the case of the Miyazaki strain, a subsequent treatment with pancreatin or trypsin was found to be required to solubilize the enzyme [4,5]. The recoveries of activity after purification were low (0.4–2.8%), and purified preparations from a single strain differed in a number of properties, including molecular weight, subunit composition and iron content [2,3]. Part of the hydrogenase activity in a related organism, *Desulfovibrio gigas*, is rather easily removed from the cell and it was concluded [6] that the enzyme is located outside the cytoplasmic membrane. This paper reports that all of the hydrogenase activity in *D. vulgaris* Hildenborough is removed from intact cells by a simple washing procedure with Tris–HCl buffer plus EDTA. The enzyme extracted has been obtained pure in high yield, and shown to consist of a single polypeptide chain of approx. mol. wt. 50 000 with 12 atoms of iron and 12 atoms of acid-labile sulphur. In contrast to certain other hydrogenases, it is not rapidly inactivated in air.

2. Materials and methods

Desulfovibrio vulgaris strain Hildenborough, NCIB 8303, was grown at 35°C in stirred 1.5 liter or 20 liter cultures with Saunders medium N [7]. At the end of the active phase of growth (0.8–1 g wet cells/liter) the cultures were cooled to 4°C and the bacteria harvested.

Protein was measured by the method [8] after it had been precipitated with 5% trichloroacetic acid.

Hydrogenase was assayed at 30°C by manometric measurement of either hydrogen evolution from dithionite with 1 mM methyl viologen as electron carrier [9], or hydrogen consumption with benzyl viologen as electron acceptor. One unit of hydrogenase is defined as the amount to catalyze the production or consumption of 1 μmol H_2/min. In the gas evolution assay, Warburg manometers contained in final vol. 2 ml, in the main compartment: Tris–HCl buffer, pH 8, 100 μmol; $Na_2S_2O_4$, 30 μmol; methyl viologen, 2 μmol; and bovine serum albumin, 1 mg; the side arm contained enzyme (0.1–1.5 units) that had been diluted in 50 mM Tris–HCl buffer, pH 8, plus bovine serum albumin, 0.1 mg/ml, to minimize adsorption of the enzyme to glass during dilution; the centre well contained 0.2 ml 10% (w/v) NaOH; the gas phase was N_2. Hydrogen production was linear with time. In the gas consumption assay, manometers contained in final vol. 2 ml, in the main compartment: Tris–HCl buffer, pH 8, 200 μmol; benzyl viologen, 20 μmol; EDTA, 2 μmol; bovine serum albumin, 1 mg; the side arm contained enzyme (0.5–1.5 units) diluted as above; the centre well contained 0.2 ml 10% NaOH. The gas phase was H_2 that had been purified by passage over BASF catalyst at about 120°C. Manometers were run separately and read at 30 s intervals because the uptake reaction was linear for only about 3 min.

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3. Results and discussion

3.1. *Extraction and purification of hydrogenase*

Conditions for the removal of proteins from the external layers of Gram-negative bacteria are described [10]; these include the use of a slightly alkaline pH and a chelating agent, such as EDTA, to remove divalent metal ions that normally stabilize the outer membrane of the bacterial envelope. When freshly harvested cells of *D. vulgaris* were treated with 50 mM Tris–HCl buffer, pH 9, and 50 mM EDTA, according to the procedure in the table 1 legend, much hydrogenase and cytochrome (A_{410}) was released into the extraction medium. Microscopic examination, and the failure to detect absorbance at 630 nm due to desulfoviridin in the extract [6], showed that very little breakage of the bacteria occurred during this treatment.

The amount and specific activity of the hydrogenase extracted depended on the ratio mol EDTA/g bacteria, on the pH, and, as shown in table 1, on the temperature and time of extraction. An incubation at 30°C for 15 min under the conditions in the legend to table 1 was found to extract most of the hydrogenase at a high specific activity (385 U/mg in the hydrogen production assay). Experiment A shows that cytochrome was released at 0°C, but not hydrogenase; the removal of hydrogenase was also incomplete when the concentrations of buffer and EDTA were decreased to 25 mM, or when Tris–HCl buffer, pH 9, was replaced by phosphate buffer, pH 7. The specific

Table 1

Exp.	Changes to extraction conditions	Hydrogenase extracted		A_{410} nm
		Units (% total)	Spec. act. (units/mg)	(% total)
A	0°C, –EDTA	2		2
	0°C	3		103
	39°C, –EDTA	2		1
	39°C	98		100
	39°C, –EDTA, + 20% sucrose	8		47
	39°C, –EDTA, + 0.2 M $MgCl_2$	1		4
B	10°C	25	73	91
	20°C	97	126	95
	30°C	98	138	94
	39°C	92	120	98
C	0 min	3	18	
	5 min	50	385	
	15 min	93	385	
	25 min	93	306	
	35 min	90	289	
	45 min	98	228	

Freshly-harvested cell paste from cultures of 1.5 liter was suspended in 10 ml extraction medium/g wet cells. The standard extraction medium for the experiments was 50 mM Tris–HCl buffer, pH 9, and 50 mM EDTA; the mixtures were stirred gently for 45 min at 30°C, then centrifuged at 7000 × *g* for 10 min at 4°C. Changes to the extraction medium (exp. A) and to the temperature (exp. A,B) or time of extraction (exp. C), were made as indicated. The hydrogen production activity and the A_{410} due to cytochrome in the supernatant are given as percentages of the values measured in a suspension of broken cells. The hydrogenase content of different cultures varied between 1000 and 1400 production units/g wet cells. The total A_{410} due to cytochrome varied between 1.0 and 1.3 in suspensions of 1 g wet cells/10 ml.

Table 2
Purification of hydrogenase from *D. vulgaris*

Purification step	Vol. (ml)	Total protein (mg)	Total units		Spec. act. (units/mg)	
			P	C	P	C
Cell suspension	237	3310	28 600	345 000	8.6	104
Extract	210	189	24 600	303 000	130	1600
DEAE-cellulose	64	15.9	15 300	158 000	960	9900
Sephadex G-150	42	4.3	10 300	112 000	2400	26 000
Hydroxylapatite	23	2.1	7700	79 900	3800	38 000

The starting material was 23 g wet cell paste. Catalytic activities were measured in the hydrogen production (P) and hydrogen consumption (C) assays. All manipulations during the purification procedure were performed at 4°C in air

activity of the hydrogenase extracted decreased with increasing time of extraction (exp. C, table 1), indicating that additional protein was more slowly released. The use of anaerobic conditions during the extraction did not affect the amount of hydrogenase activity extracted.

In contrast with the high yields of soluble enzyme that were obtained with the mild extraction method, only 20–40% hydrogenase was obtained in solution after sonication or treatment of bacterial suspensions in a French press. The major part of the activity in mechanically disrupted suspensions was found to be associated with particulate material, as reported for *Desulfovibrio* hydrogenase [4,11,12]. The reason for this association of enzyme with particles has not been investigated.

In the larger scale extraction that was used for the purification of hydrogenase (table 2), freshly-harvested bacteria cell paste was suspended in H_2O at 0°C (5 ml/g), and the temperature of the mixture then raised quickly to 30°C before slowly adding equal vol. 0.1 M Tris–HCl buffer, pH 9, plus 0.1 M EDTA. The mixture was gently stirred at 30°C for 15 min and then centrifuged (7000 × *g*, 10 min, 4°C). The red–brown supernatant was adjusted from pH 9 to pH 8 by addition of 1 M Tris–HCl, pH 6. It contained some black colloidal material that interfered with the subsequent chromatography step. The black material, which was probably due to metal sulphides, was removed by stirring the mixture at 30°C for 30 min followed when necessary by centrifugation (15 000 × *g*, 10 min, 4°C). The resulting clear solution was dialyzed for 16 h versus 10 mM Tris–HCl buffer, pH 8, plus 20 mM NaCl and 5 mM EDTA. The specific activity of the enzyme obtained in this extraction was lower than the best values in small scale experiments.

The dialyzed solution was treated on a column of DEAE-cellulose (Whatman DE 32, 20 × 2.5 cm). A red fraction that contained cytochrome passed directly through the column; the column was eluted with a linear gradient of NaCl to remove the hydrogenase activity in a golden–brown band at 10 mM Tris–HCl, pH 8, plus 90 mM NaCl. The most active fractions were combined and concentrated to 5 ml by adsorption to and elution from a small column of DEAE-cellulose (4 × 1.5 cm), then treated on a column of Sephadex G-150 (100 × 2.5 cm) which had been equilibrated with 25 mM phosphate buffer, pH 7.5, plus 20 mM NaCl. The hydrogenase activity was eluted in a symmetrical band, from which the most active fractions were pooled and treated on a column of hydroxylapatite (Bio-Rad biogel HTP; 10 × 1.5 cm). This column was eluted with a linear phosphate gradient; the hydrogenase was removed at about 0.15 M phosphate, pH 7.5.

The enzyme from the hydroxylapatite column gave a single band of protein after electrophoresis in polyacrylamide gel [13,14] and the position of the band coincided with that of the single violet band which was formed when a duplicate gel was incubated under H_2 in the presence of benzyl viologen. The preparation was therefore judged to be pure. The data of table 2 indicate that hydrogenase forms about 0.2% of the cellular protein in *D. vulgaris*. The total units of activity in the extract, the recoveries of activity in the pure preparation (27% and 23% in the

hydrogen production and uptake assays, respectively), and the final specific activities are high by comparison with values reported previously for hydrogenase from this organism [1–5]. The ratio of specific activity in the H_2-consumption assay to that in the H_2-production assay was constant after the DEAE-cellulose treatment, indicating that a single enzyme catalyzes both gas production and consumption.

3.2. *Properties of purified hydrogenase*

The molecular weight of the protein was estimated to be 52 000 from its elution volume during gel filtration in a calibrated column of Sephadex G-150 [15]. A similar value (49 000) was determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate and 2-mercaptoethanol [16], indicating that the molecule consists of a single polypeptide chain. As isolated, the protein contained up to 20 g atoms of iron/molecule, but a large part of this iron could be removed by dialysis of the protein versus 1 mM Tiron plus 1 mM *o*-phenanthroline for 24 h. Only 7% of the catalytic activity of the protein was lost during the dialysis, and it is therefore clear that the iron removed was not essential for activity. After dialysis, the protein contained 12.5 atoms non-haem iron [17] and 11.5 atoms acid-labile sulphur [18,19] per 50 000 mol. wt molecule. These measurements suggest that the iron and acid-labile sulphur content of the enzyme preparation is higher than that of earlier preparations of *D. vulgaris* hydrogenase [2–5], but similar to that reported [9] for hydrogenase from *Clostridium pasteurianum*.

The preparation is brown, absorbs light throughout the visible region of the spectrum, and has an A_{280} max and a broad shoulder around 400 nm, as is typical of other proteins that contain Fe–S centres as their only redox chromophores. The ratio A_{400}/A_{280} is 0.36. The extinction coefficient at 400 nm (46 mM^{-1} cm^{-1}) is 3.7 mM^{-1} cm^{-1} per Fe, and therefore similar to that of the 8 Fe ferredoxin from *Clostridium acidi urici* (3.8 mM^{-1} cm^{-1} per Fe at 390 nm [20]). The visible absorbance of the protein was not affected by the dialysis against iron chelating agents above, indicating that the iron removed by this treatment was not associated with the visible chromophore. The brown colour was lost, however, when the enzyme was denatured with acid. The A_{400} decreased by 23% when the protein was treated with excess sodium dithionite.

The K_m value for methyl viologen in the H_2-production assay with sodium dithionite was 0.20 mM and V_m was 4600 U/mg. The K_m value for benzyl

Table 3
Effect of storage conditions on the stability of *D. vulgaris* hydrogenase

Exp.	Storage conditions	Storage time (weeks)	Remaining act. (% initial)		
			−20°C	4°C	22°C
A	Air	2	94	44	13
	Nitrogen	2	92	69	54
	Air + BSA	2	98	93	42
	Nitrogen + BSA	2	93	89	62
B	Air	1		69	
		2		45	
		5		29	
		8		24	
		23		16	

Samples of purified hydrogenase in 50 mM phosphate buffer, pH 7.5, and 0.01% sodium azide were stored as indicated. Activities were measured in the hydrogen production assay. The protein concentration in experiments A and B was 21 μg/ml and 42 μg/ml, respectively. Where indicated, bovine serum albumin (BSA) was added to give a concentration of 0.1 mg/ml

viologen in the hydrogen consumption assay was 3.0 mM and V_m was 50 000 U/mg. Comparison of these data with values in the literature for previous preparations of hydrogenase from *Desulfovibrio* is not straight-forward because the assays were not all the same. We estimate, however, that the activity of our preparation in the hydrogen uptake assay is about 400 times higher than that of the preparation [2], but only about 3 times higher than [1,3]. In view of the high activity of their preparation, it is surprising that only approx. 1 iron atom and 1 acid-labile sulphur atom/protein molecule was found [3]. We also estimate that the present preparation is about 50-times more active in the hydrogen production assay than the hydrogenase isolated [5] from the Miyazaki strain of *D. vulgaris*. Its molecular and catalytic properties most closely resemble those of hydrogenase from *C. pasteurianum*, as isolated [9,21], which contained 12 iron atoms and 12 acid-labile sulphur atoms/60 000 mol. wt molecule, and had a V_m of 4500 U/mg in the hydrogen production assay. In contrast to *C. pasteurianum* hydrogenase, however, and also in contrast to an earlier preparation of the *D. vulgaris* enzyme [2], our preparation is not particularly sensitive to oxygen either during the purification, which was carried out in air, or during subsequent storage (table 3). It could be stored frozen in air for several weeks with only small losses in activity. Roughly half of the activity was lost in 2 weeks during storage of a dilute solution in air at 4°C, but the preparation still retained 16% of its original activity after a further 21 weeks. A smaller loss in activity occurred when the hydrogenase was stored anaerobically, but at 4°C the addition of bovine serum albumin conferred even greater stability than the use of a nitrogen gas phase.

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