Vol. 84, No. 4, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 30, 1978 Pages 1144-1150

ISOLATION AND PROPERTIES OF A UNIDIRECTIONAL H_2-OXIDIZING HYDROGENASE FROM THE STRICTLY ANAEROBIC N_2-FIXING BACTERIUM CLOSTRIDIUM PASTEURIANUM W5

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Received August 28,1978

SUMMARY

A unidirectional H_2-oxidizing hydrogenase (H_2ase-HO) has been isolated from the anaerobic N_2-fixer Clostridium pasteurianum W5. Extensively purified (> 200-fold or > 400-800 μmoles H_2 oxidized/min/mg protein) H_2ase-HO can use ferredoxin (Fd), methyl viologen (MV), benzyl viologen, methylene blue or dichlorophenolindophenol as the sole electron acceptor but does not produce H_2 from reduced Fd or MV. H_2ase-HO has a lower mol. wt., is less negatively charged and less O_2-sensitive than the classical bidirectional H_2ase from the same organism. The level of the H_2-oxidizing enzyme is higher in N_2-fixing cells than in NH_3-grown cells. H_2ase-HO occurs mainly outside the cell membrane and is inhibited by carbon monoxide.

INTRODUCTION

Clostridium pasteurianum W5 is a strictly anaerobic N_2-fixing bacterium that produces abundant amount of H_2 as a normal end-product from catabolism. A classical bidirectional hydrogenase (H_2-producing and -oxidizing; H_2:ferredoxin oxidoreductase EC 1.12.7.1), which is responsible for the catabolic production of H_2, has been purified to homogeneity and extensively characterized (1-5). Although unidirectional H_2-oxidizing hydrogenase is present in many aerobic N_2-fixers, e.g., azotobacter, the presence of a unidirectional H_2-oxidizing hydrogenase in the strictly anaerobic C. pasteurianum was not previously recognized or anticipated.

During the purification of hydrogenase from C. pasteurianum W5, we observed that the ratio of the H_2-oxidizing activity versus the H_2-producing activity was significantly different between the crude extract and the purified bidirectional hydrogenase. Through monitoring this ratio during the purification of hydrogenase, we located fractions which contained predominantly the

0006-291X/78/0844-1144$01.00/0

H_2-oxidizing activity. Further purification of these fractions yielded a unidirectional H_2-oxidizing hydrogenase which did not show any H_2-producing activity under all assay conditions tested.

In this communication, we present the isolation and some characterization of this new enzyme. It is believed that this represents the most extensive purification of a unidirectional H_2-oxidizing hydrogenase. Both the presence of this unidirectional hydrogenase in a H_2-producing anaerobe (and a N_2-fixer) and the localization of this enzyme outside the cell membrane are intriguing and suggest specific physiological function. Research concerning the physiological role of this new hydrogenase is in progress.

MATERIALS AND METHODS

Cells. N_2-fixing and NH_3-grown cells of C. pasteurianum W5 were prepared as described (6). Cell paste was either used fresh or immediately frozen in liquid N_2 and then stored at -20 C.

Enzyme purification. Crude extracts were prepared as described (7). The purification of the bidirectional hydrogenase followed the published procedure (1, 2). For the unidirectional hydrogenase, the crude extract was passed through a DE 52 column (equilibrated with 0.05 M Tris.Cl, pH 8), and the effluent collected during sample charging contained the H_2-oxidizing activity. These fractions were chromatographed on a hydroxyapatite (BioRad HTP) column (eluted with a linear gradient of potassium phosphate between 0.01 and 0.25 M, pH 7.2), and the H_2-oxidizing activity was eluted at about 0.1 M phosphate. The desired fractions were concentrated by ultrafiltration through Amicon Diaflo Ultrafilter PM-30 and then loaded onto a Sephadex G-100 column eluted with 0.05 M Tris.Cl, pH 8, containing 0.1 M KCl. Active fractions were pooled and chromatographed on a second hydroxyapatite column. Peak fractions eluted from the second hydroxyapatite column had specific activities (in the routine H_2-oxidation assay) around 800 units/mg protein (one unit = 1 μmole H_2 oxidized per min) which represented a purification of over 200-fold. All steps of the purification were performed under anaerobic conditions.

Enzyme assays. Details of the routine H_2-oxidation (using 5 mM methylene blue (MB) as the electron acceptor) and H_2-production (using 1 mM dithionite-reduced methyl viologen (MV) as the electron donor) assays were described (1). Bovine serum albumin at 1 mg/ml was included in the assay mixture. The manometric assay was carried out in Warburg flasks with the Gilson Submarine Respirometer. MB or MV was omitted from the assay mixture when other electron carriers were tested. Spectrophotometric assays were performed in anaerobic cuvettes with a Beckman ACTA VI spectrophotometer.

Protein determination. Protein was measured either by the Lowry method (8) or the dye-binding method (BioRad) using dried bovine serum albumin as a standard.

Electrophoresis. Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS) was performed as described (9).

Preparation of protoplasts. Cells were suspended, at 1 gm wet cells/25 ml, in 0.05 M Tris.Cl, pH 8/0.58 M sucrose/1 mg per ml of lysozyme and incubated at 30 C anaerobically for 1 hr. Protoplast formation was confirmed by microscopic examination. The protoplasts were collected by centrifugation at 7,000 x g for 10 min and the supernatant, which was further clarified by centrifugation at 27,000 x g for 15 min., was designated as the washing from the protoplasts. The protoplasts were further suspended in 0.05 M Tris.Cl, pH 8.5, for lysis, and cell-free extract was prepared as the crude extract.

Test for oxygen sensitivity. Enzyme solutions (0.5-2 ml) were first evacuated and then gently bubbled with air through a Pasteur pipette for 1 min, and remained exposed to air. Samples were removed after a specified time of exposure and immediately subjected to five cycles of evacuation and equilibration with H_2. H_2-oxidizing activity was measured 12 min. after the termination of O_2-exposure.

RESULTS AND DISCUSSION

In the routine activity assays, the purified bidirectional H_2ase of C. pasteurianum W5 shows a ratio of 2.5 between the H_2-oxidizing activity and the H_2-producing activity (Table I). The crude extracts of N_2-fixing cells show a ratio of 9 (ranging from 8 to 10 in 16 determinations) between the two activities, while the extracts of NH_3-grown cells show a ratio of 4.3 (ranging from 4.0 to 4.6 in 6 determinations). This suggested the presence of an active component(s) that contributed to the higher H_2-oxidizing activity in the crude extracts. As shown in Table I, such H_2-oxidizing activity was located in the effluent that contained the unabsorbed material from the DE 52 column. When the crude extract was fractionated on a Sephadex G-150 column (Fig. 1; Table I), two H_2-oxidizing activity peaks were observed, one coincided with the H_2-production activity peak (containing the bidirectional H_2ase) while the other followed the bidirectional H_2ase. These results suggest that the higher H_2-oxidizing activity in the crude extract is attributable to a second H_2ase which is less negatively charged and has a lower mol. wt. than the bidirectional H_2ase.

Further purification of the predominantly H_2-oxidizing fractions eventually yielded a unidirectional H_2-oxidizing H_2ase which did not show any H_2-producing activity in assays given reduced Fd or MV as the electron donor. Fig. 2 shows an elution profile of the second hydroxyapatite column, and the

Table I. Ratio of H_2-Oxidation Activity[a] versus H_2-Production Activity[a] of Various Fractions from N_2-Fixing Cells of Clostridium pasteurianum W5

(I)	Purified Bidirectional Hydrogenase	2.5
(II)	Crude Extracts	9.0 (8.0 - 10.0)
(III)	Fractions from the Crude Extracts	
	(1) DE 52 Fraction 1[b]	∿ 100
	(2) DE 52 Fraction 2	∿ 5
	(3) Sephadex G-150 Fraction 1[c]	∿ 10
	(4) Sephadex G-150 Fraction 2	∿ 30
(IV)	Fractions from the Protoplast Preparations	
	(1) Washing	∿ 50
	(2) Intracellular	∿ 6
(V)	Extensively Purified Unidirectional Hydrogenase	∞

[a] Hydrogenase activity was measured with the Gilson submarine respirometers at 30 C. The routine assay was carried out in 0.05 M Tris.Cl at pH 8 under H_2. Other conditions were as given in Materials and Methods.

[b] DE 52 Fraction 1 designates the fractions that contain materials not absorbed by the DE 52 column during sample charging. DE 52 Fraction 2 designates the fractions that are eluted with 0.1 - 0.2 M KCl in 0.05 M Tris.Cl at pH 8.

[c] Sephadex G-150 Fraction 1 designates the fractions eluted with a median mol. wt. of 50,000. Sephadex G-150 Fraction 2 designates the fractions eluted with a median mol. wt. of 32,000.

fractions on the back of the activity peak reach a H_2-oxidizing activity around 800 units/mg protein (a purification over 200-fold). SDS-polyacrylamide gel electrophoresis of the most purified unidirectional H_2ase still showed multiple protein bands, and further purification of the new H_2ase is in progress.

It is assumed that the same bidirectional H_2ase exists in both the N_2-fixing and NH_3-grown cells of C. pasteurianum. The level of the H_2-oxidizing H_2ase is thus estimated as the difference between the total H_2-oxidizing activity and the H_2-oxidizing activity attributable to the bidirectional H_2ase (total H_2-oxidizing activity - 2.5 x H_2-producing activity) in both

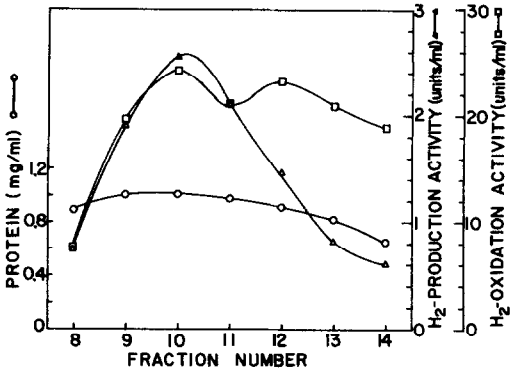

Fig. 1. The elution profile of hydrogenases from the Sephadex G-150 column (2.5 x 39 cm). Crude extract (100 mg protein) of C. pasteurianum was loaded and eluted by 0.05 M Tris.Cl, pH 8; 0.1 M in KCl; 1 mM $Na_2S_2O_4$. Fractions of 5 ml were collected. Protein and hydrogenase assays were performed as described in Materials and Methods.

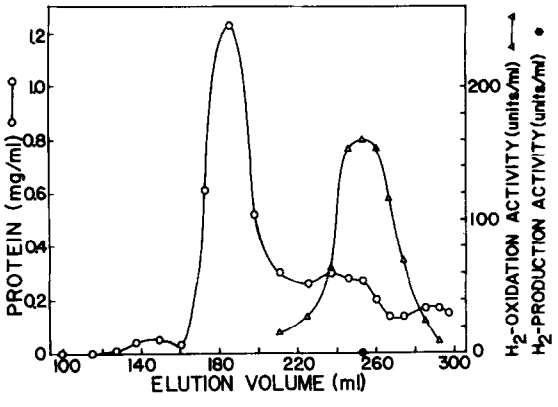

Fig. 2. The elution profile of the unidirectional H_2-oxidizing hydrogenase from the second hydroxyapatite column (2.5 x 15 cm). 60 mg of protein (active fractions from the Sephadex G-100 column) was loaded and eluted by a linear gradient of phosphate (0.01-0.25 M potassium phosphate, pH 7.2; 0.1 M in KCl; 1 mM $Na_2S_2O_4$; total volume 600 ml). Fractions of about 6-8 ml were collected when hydrogenase activity was detected in the effluent. Protein and hydrogenase were assayed as described in Materials and Methods.

cell types. The N_2-fixing and NH_3-grown cells of C. pasteurianum W5 were found to contain, respectively, 370 and 205 units of the unidirectional H_2ase per gm of wet cells. In the N_2-fixing cells, the unidirectional H_2ase accounts for over two thirds of the total H_2-oxidizing activity while the corresponding value is about 40% in NH_3-grown cells. The significance of these differences is not presently clear.

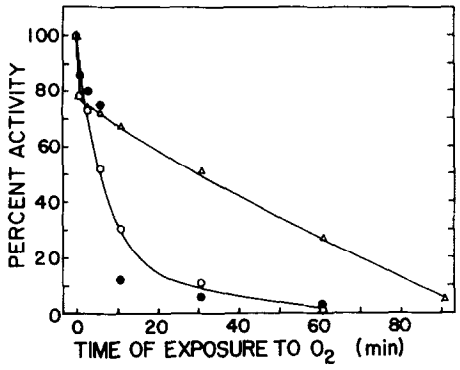

Fig. 3. Inactivation of the unidirectional and the bidirectional hydrogenases by oxygen. The unidirectional hydrogenase (in Tris.Cl, pH 8, with 0.58 M sucrose, Δ - Δ) was from the washings of the protoplast preparation. The bidirectional hydrogenase (in Tris.Cl, pH 8, with (0 - 0) or without (● - ●) 0.58 M sucrose) was partially purified through the Sephadex G-100 column step (1). Other details were described in Materials and Methods.

The extensively purified unidirectional H_2ase can use Fd, MB, MV, benzyl viologen or dichlorophenolindophenol as the sole electron acceptor, and the measured activity decreases when an electron acceptor with a more negative midpoint redox potential is used (data not shown). The H_2-oxidizing activity in the heterocysts of Anabaena 7120 was reported to use O_2 as the electron acceptor (10, 11), and the activity was not significantly enhanced when Fd or MV was added to the assay mixture (10); however, the tests were performed with intact heterocysts.

Significant difference in O_2-sensitivity was found between the unidirectional and the bidirectional H_2ases (Fig. 3). Fifty percent inactivation occurred in approximately 30 and 5 min., respectively, after the unidirectional and the bidirectional H_2ases were exposed to air.

Like the other classical H_2ases (12), the unidirectional H_2ase from C. pasteurianum is also inhibited by carbon monoxide (98% inhibition at 0.5 atm CO). This suggests that the new H_2ase is likely a metalloenzyme, perhaps an iron-sulfur protein, too. Direct metal analysis must await further purification of the enzyme.

Interestingly, the unidirectional H_2ase seems to exist outside the cell membrane because the washings from the protoplasts contained most of the H_2-oxidizing activity (Table I). The bidirectional H_2ase was released only when the protoplasts were lysed. The separate cellular location and the different levels in N_2-fixing and NH_3-grown cells certainly suggest that the unidirectional H_2ase has some specific physiological function, perhaps related to N_2-fixation and/or energy metabolism. Dixon (13) proposed functions for the H_2-oxidizing H_2ase in N_2-fixation; however, direct evidence is still lacking to substantiate them.

"Isozymes" of H_2ase were reported in C. pasteurianum when the enzyme activity was demonstrated using polyacrylamide gel electrophoresis followed by the reduction of MV under H_2 (14-16). It is possible that the unidirectional H_2ase corresponds to one of those "isozymes". Characterization of the unidirectional H_2ase from C. pasteurianum W5 is in progress.

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