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PURIFICATION AND PROPERTIES OF THE H_2-OXIDIZING (UPTAKE) HYDROGENASE OF THE N_2-FIXING ANAEROBE *CLOSTRIDIUM PASTEURIANUM* W5

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SUMMARY: *Clostridium pasteurianum* has two distinct hydrogenases, the bidirectional hydrogenase and the H_2-oxidizing (uptake) hydrogenase. The H_2-oxidizing hydrogenase has been purified (up to 970-fold) to a specific activity of 17,600 μmol H_2 oxidized/min·mg protein (5 mM methylene blue) or 3.5 μmol H_2 produced/min·mg protein (1 mM methyl viologen). The uptake hydrogenase has a M_r of 53,000 (one polypeptide chain). Depending upon how protein was measured, the Fe and $S^=$ contents (gatom/mol) were 4.7 and 5.2 (by the dye-binding assay) or 7.2 and 8.0 (by the Lowry method). Both reduced and oxidized forms of the enzyme gave electron paramagnetic resonance signals. The activation energy for H_2-production and H_2-oxidation by the uptake hydrogenase was 59.1 and 31.2 kJ/mol, respectively. In the exponential phase of growth, the ratio of uptake hydrogenase/bidirectional hydrogenase in NH_3-grown cells was much lower than that in N_2-fixing cells.

In 1978, we reported the discovery of a second hydrogenase, the H_2-oxidizing (uptake) hydrogenase, in the N_2-fixing anaerobe *Clostridium pasteurianum* W5 (1). The presence of this enzyme has since been confirmed by two other laboratories (2; M. W. W. Adams and L. E. Mortenson, personal communication). *C. pasteurianum* has a bidirectional hydrogenase (ferredoxin:H^+ oxidoreductase EC 1.18.3.1) which is involved in H_2 production and it has been extensively studied (3-9). We have purified the uptake H_2ase and compared the properties of the two H_2ases. Based upon their different catalytic and structural properties, we conclude that the two H_2ases are chemically distinct. Although both H_2ases are found in N_2-fixing and NH_3-grown *C. pasteurianum* cells, their relative levels differ under these two growth conditions. The uptake H_2ase perhaps plays a role in regulating the redox state of ferredoxin which is the electron donor for nitrogenase; however the exact role of this enzyme remains to be established.

MATERIALS AND METHODS

All conditions, materials and methods were the same as in reference (1) except for the following:

Growth of organism. For enzyme purification, N_2-fixing cultures were grown in 40-L batches with the pH maintained at 5.5 with KOH. The cultures were harvested when $A_{550\ nm}$ reached 3-4

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Abbreviations: H_2ase, hydrogenase; Fd, ferredoxin; Fld, flavodoxin; MB, methylene blue; MV, methyl viologen; BV, benzyl viologen.

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(Spectronic 20); the cells were in late exponential or between exponential and stationary phases. The cells were stored in liquid N_2 in more recent studies, but were first frozen in liquid N_2 and then stored at -20C under H_2 in earlier studies. The activity of both H_2ases decreased with time at -20C, especially when cells were under aerobic conditions. When studying enzyme levels in N_2-fixing and NH_3-grown cells, the cultures were grown in medium (8L) containing 5 g/L $CaCO_3$ for pH control. The cultures were sampled when $A_{550\ nm}$ was about 1, 2, 3, and 4.

Preparation of crude extract. Frozen cells were thawed at room temperature and under H_2 in Tris·Cl (pH 8.5, 50 mM) containing lysozyme (1mg/mL) and DNase (0.1 mg/mL) for 2 hrs. Enzyme purification started with 0.5 or 1 kg of cell paste which was thawed at 1 g cells per 2 mL of buffer. Crude extract was the supernatant after centrifugation at 35,000 x g for 20 min. All operations were carried out under a H_2 or N_2 atmosphere and were at room temperature unless otherwise specified. All anaerobic buffers contained 1 mM of $Na_2S_2O_4$.

Enzyme assays. The H_2-oxidation assay was carried out with appropriate amounts of hydrogenase so that the rate of H_2 uptake was below 30 μL/min. One unit is the oxidation or production of 1 μmol H_2/min. The H_2 uptake activities reported in (1) were greatly underestimated because too much enzyme was used in the assay. The activation energy was measured in potassium phosphate buffer (pH 8, 50 mM).

Analytical methods. Iron (3) and inorganic sulfide (10) were measured as described. Core extrusion was by the method of Gillum et al. (6). Electron paramagnetic resonance was measured with a Varian E9 spectrometer. Sodium dodecyl sulfate (SDS)-gel electrophoresis was performed according to (11) and (12). Peptide mapping (13) was performed as described (14). Unless specified otherwise, protein was measured by the dye-binding method (15) as described by BioRad Technical Bulletin No. 1051. For the uptake H_2ase, the dye-binding assay gave a protein concentration 55% higher than that given by the Lowry method (16). Thus, a sample with a protein concentration of 5.51 mg/mL (dye-binding assay) became 3.57 mg/mL (Lowry method).

Enzyme purification. Heat treatment of crude extract was described (3). The first DE 52 column step was as in (1); a column of 5 x 15 cm was used for the extract from 0.5 kg of cells. Using this column/extract ratio, the uptake H_2ase was not adsorbed to DE 52 and the effluent containing the uptake H_2ase was designated the early fraction. The bidirectional H_2ase was eluted at about 0.17 M chloride, which was designated the late fraction. The first hydroxyapatite (BioRad HTP) column was 5 x 12 cm, and was eluted by a linear gradient of potassium phosphate (0.01-0.4 M, pH 7.2, total volume 800 ml, also containing 0.1 M KCl). H_2ase was eluted between 90 and 220 ml, and it was concentrated by ultrafiltration with an Amicon PM-10 membrane before being applied to the next column. The Sephadex G-100 column (5 x 56 cm) was eluted as in (1). H_2ase-containing fractions were diluted with an equal volume of 10 mM Tris·Cl (pH 8) and then loaded onto the second DE 52 column. This column (2.5 x 19.5 cm) was eluted by a linear gradient of KCl (0.01-0.2 M, total volume 600 ml) in Tris·Cl (50 mM, pH 8). H_2ase was eluted between 355 and 395 ml. The final HTP column (2.5 x 12 cm) was eluted by a linear gradient of potassium phosphate (0.04-0.15 M, pH 7.2, total volume 400 ml, also containing 0.1 M KCl). H_2ase was eluted between 117 and 142 ml as a yellow-brown band. Between column steps, H_2ase samples were stored at 5-10C. The purified enzyme was stored in liquid N_2 as frozen pellets.

RESULTS AND DISCUSSION

The two H_2ases of *C. pasteurianum* were easily separated by DEAE-cellulose (DE 52) column chromatography. Fig. 1 shows that the H_2-oxidizing and H_2-producing activities of a crude extract were nearly quantitatively recovered after the heat treatment, with the ratio of H_2-oxidation/H_2-production remaining at 54-56. After the first DE 52 column step, the H_2-oxidizing (uptake) activity was roughly evenly distributed between the early and the late fractions, whereas the H_2-producing activity eluted essentially in the late fraction. The late fraction corresponds to the previously characterized bidirectional H_2ase, and its H_2-oxidation/H_2-production activity ratio was 30 at this point (the ratio for purified bidirectional H_2ase is 25). The early fraction had a H_2-oxidation/H_2-production activity ratio of 3,500, and the H_2ase in this fraction is designated the H_2-oxidizing (uptake) H_2ase. Further purified uptake H_2ase had a H_2-oxidation/H_2-production

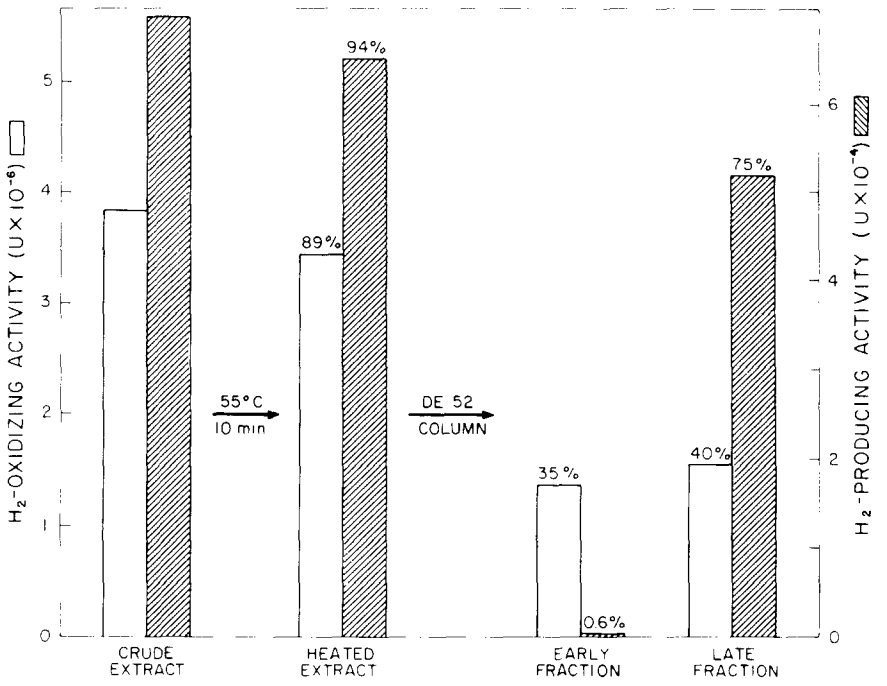


Figure 1. Separation of the H_2-oxidizing H_2ase (the early fraction) and the bidirectional H_2ase (the late fraction) of *C. pasteurianum* by a DEAE-cellulose (DE 52) column. An extract from 1 kg of cells was used, and conditions were given in Materials and Methods. Percentages denote the recovery of activities by taking the total activity in the crude extract as 100%.

activity ratio of 5,000; the H_2-producing activity of the uptake H_2ase is negligible for practical purposes. Using the H_2-oxidation/H_2-production activity ratio of the bidirectional H_2ase and the H_2-oxidizing and H_2-producing activities of a crude extract, we can deduce the amount of the two H_2ases, in terms of H_2-uptake activities, in crude extracts. In crude extracts of N_2-fixing cells (with the H_2-oxidation/H_2-production activity ratio near 55), about 50% of the H_2-uptake activity was from the uptake H_2ase.

Table 1 shows the results of one preparation of the uptake H_2ase from 0.5 kg of cells. The peak fraction had an activity of 22,300 units/mg. Another preparation from 1 kg of cells yielded a final activity of 15,540 units/mg. We also tried to replace the first HTP column step with $(NH_4)_2SO_4$ fractionation (H_2ase was in the supernatant of 60% saturated $(NH_4)_2SO_4$) and an Octyl-Sepharose column step (eluted by a gradient of 60-0% saturated $(NH_4)_2SO_4$ in 50 mM Tris-Cl, pH 8, at 10C). The latter procedure gave a final activity of 15,900 units/mg (peak = 17,600 units/mg) with a yield of 6%. Because the starting activity varied from 17.6 to 25 units/mg, the fold of purification was between 700 and 970. The purity of the enzyme was examined by SDS-gel electrophoresis followed by densitometry. The intensity of the H_2ase band (M_r = 53,000) was proportional to activity in

TABLE 1. Purification of the H_2-Oxidizing (Uptake) Hydrogenase from *C. pasteurianum*

Step	Volume (ml)	Total protein (mg)	Spec. Act. (units/ mg protein)	Fold of purification	Total activity (units)	Recovery of Activity (%)
Crude extract	900	27,540	17.6[a]	1	484,700	100
Heat treatment	850	10,900	45.2[a]	2.6	492,680	102
First DE 52 column	700	2,933	128	7.3	375,420	77
First HTP column	131	704	443 (peak = 687)	25	311,870	64
Sephadex G-100 column	180	274	923 (peak = 1,374)	52	252,900	52
Second DE 52 column	40	45	4,716 (peak = 6,294)	268	212,220	44
Second HTP column	25	4.05	17,020 (peak = 22,300)	967	68,930	14

[a]The total H_2-uptake activities for these samples were 30.6 and 75.2 units/mg, respectively, whereas the H_2-producing activities were 0.52 and 1.2 units/mg, respectively. The contribution of H_2-uptake activity by the bidirectional H_2ase was subtracted by using a H_2-uptake/H_2-production activity ratio of 25 for the enzyme. There was negligible H_2-producing activity after the fourth step.

the sample. For a sample with an activity of 15,900 units/mg, the H_2ase band represented 84.6% of the Coomassie blue-stained material.

Table 2 lists the catalytic and structural properties of the uptake H_2ase and a comparison of the two H_2ases of *C. pasteurianum*. For the uptake H_2ase, a M_r of 53,000 was obtained with both electrophoretic methods (11, 12), but gel filtration on a Sephacryl S-200 column gave a M_r = 48,500. We used the M_r of 53,000 for calculations. Depending upon the methods of protein determination (see MATERIALS AND METHODS), the activity (units/mg) and the Fe,$S^=$ contents (gatom/mol) of a sample can be 15,540, 4.7, and 5.2, respectively (using the dye-binding protein assay), or 23,990, 7.2, and 8.0 (using the Lowry protein assay). We do not yet know which protein assay is more accurate for this enzyme. Core extrusion of a sample (15,540 units/mg) quantitatively yielded FeS clusters of the 4Fe-4S type. The H_2/dithionite-reduced enzyme (15,900 units/mg) had a yellow-brown color and had broad absorption in the 400 nm region, with an ϵ (400 nm) = 12.8 mM^{-1} cm^{-1} (dye-binding assay) or 19.8 mM^{-1} cm^{-1} (Lowry assay). More concentrated samples (5.5 mg/ml) showed a greenish brown color under N_2. Fig. 2 shows the electron paramagnetic resonance spectra of the enzyme. Judging from the intensity of the signals near g=2 in (b) and the ease to generate the oxidized form, we speculate that the minor signal near g=2.0 in (a) was from the oxidized enzyme. Additional redox titrations will be needed to clarify this point and to allow interpretation of these signals.

The significantly higher Ea for H_2-production by the uptake H_2ase (Table 2) may explain the enzyme's low capacity to produce H_2. Fig. 3 shows the temperature dependence of H_2 production

TABLE 2. Properties of H_2-Oxidizing H_2ase and Bidirectional H_2ase of *C. pasteurianum*

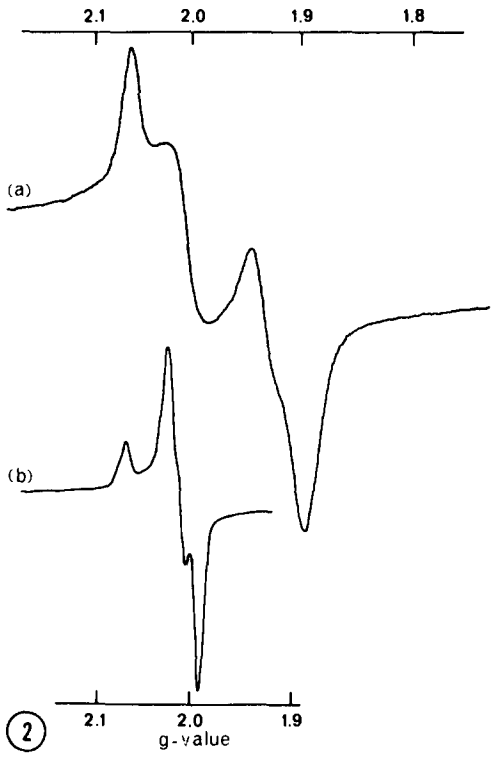
	H_2-Oxidizing H_2ase	Bidirectional H_2ase[a]
Molecular Weight	53,000	60,000
Polypeptide	1	1
Iron (gatom/mol)	4.7[b] or 7.2[c]	12[c]
Acid-lable sulfide (gatom/mol)	5.2[b] or 8.0[c]	12[c]
Structure of FeS center	n x (4 Fe-4S)	3 x (4 Fe-4S)
Catalytic activities (μmol H_2/min·mg)		
H_2-oxidation		
Spec. Act. (5 mM MB)	15,000[b]	13,700[c]
Km for MB (mM)	0.42	ND
Km for Fd (μM)	42.2	ND
Km for H_2 (atm)	0.19	0.57[d]
H_2-production		
Spec. Act. (1 mM MV)	3[b]	550[c]
Km for MV (mM)	0.36	6.2
Km for Fd (μM)	ND	51
H_2-oxidation/H_2-production	5,000	25
Activation energy (kJ/mol)		
H_2-oxidation	31.2	22.9[d]
H_2-production	59.1	22.7[d]
Electron carriers	Fd, Fld, MV BV, MB, FAD, FMN, riboflavin.	Fd, Fld, MV BV, MB, FAD[d], FMN[d], riboflavin[d].

[a]From references (3, 6).
[b]Protein determined by the dye-binding assay.
[c]Protein determined by the Lowry method.
[d]This study.
ND, not determined.

by the uptake H_2ase. Fig. 4 shows the effect of pH on activities of the uptake H_2ase. At pH 8, Tris and phosphate buffers gave similar activities, but the bidirectional H_2ase is twice as active in phosphate than in Tris buffer at pH 8 (5). Between pH 8 and 9, there was an abrupt increase in H_2-producing activity by the uptake H_2ase; however, the intracellular pH is not likely to be in this range (17). The effects of temperature and pH may provide a way of probing the mechanism of H_2-formation by this enzyme.

We also compared the primary structure of the two H_2ases of *C. pasteurianum* by the peptide mapping method (13, 14). Fig. 5 shows that the two H_2ases do not share a common polypeptide. Therefore, we conclude that the two H_2ases are chemically distinct.

We studied extensively the levels of the two H_2ases in N_2-fixing, NH_3-grown, $NH_3 \rightleftharpoons N_2$ transitional, and energetically perturbed cultures of *C. pasteurianum*. When we compared the level of the two H_2ases in cells grown under different conditions and harvested at different stages of growth we observed much variation in the absolute level of the two H_2ases (e.g., Table 3). However, when exponentially growing cells were compared at similar culture densities ($A_{550\ nm}$ = 1-2), the N_2-



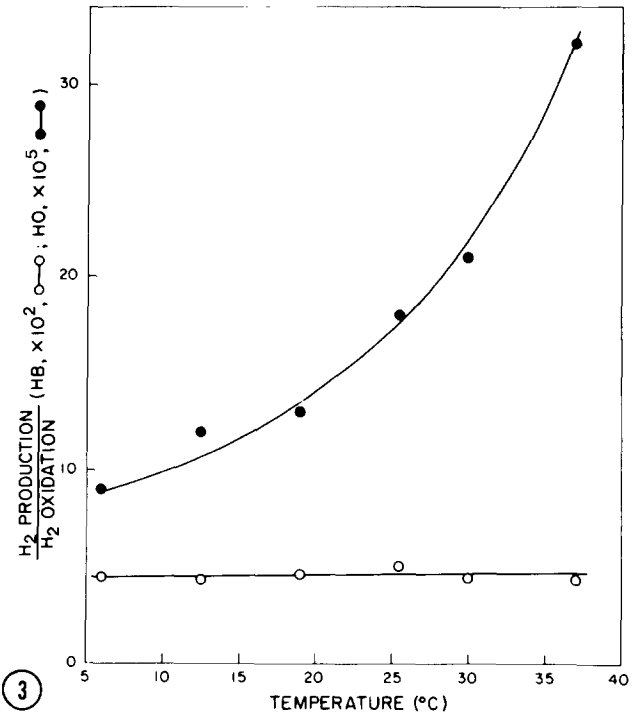
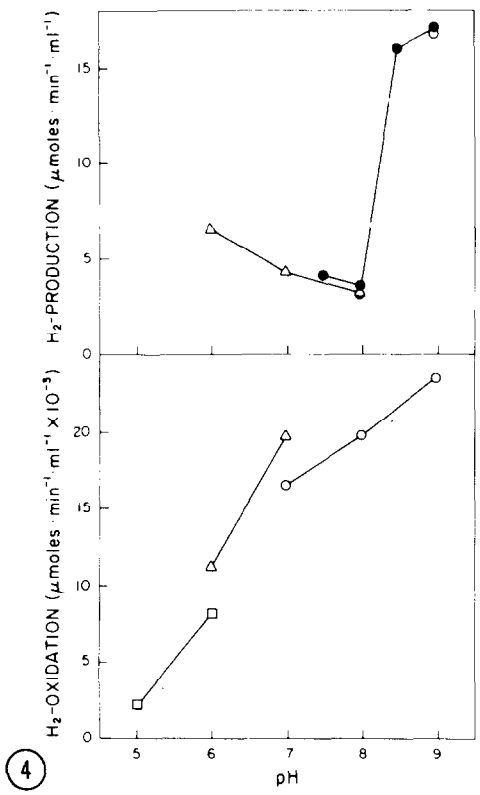


Figure 2. Electron paramagnetic resonance spectra of the uptake H_2ase of *C. pasteurianum*. (a) The H_2ase sample (2.3 mg/ml, 15,750 units/mg) was as isolated in potassium phosphate buffer (pH 7.2, about 0.1 M; with 0.1 M KCl). EPR conditions were: Microwave frequency, 9.260 GHz; temperature, 13 K; power, 50 mW; modulation, 4 mT; gain, $2x10^3$. (b) The H_2ase sample (15,540 units/mg) was thawed and diluted with half volume of dist. H_20 under N_2 to give a protein concentration of 3.67 mg/ml. EPR conditions were: microwave frequency, 9.255 GHz; temperature, 16 K; power, 20 mW; modulation 2.5 mT; gain, 1.6×10^3. (The signal height in (b) was reduced by 50% during graph preparation to bring the g-values to scale.)

Figure 3. Effect of temperature on the relative rates of H_2-production and H_2-oxidation by the uptake H_2ase (●) and the bidirectional H_2ase (○) of *C. pasteurianum*. The assays were performed in 50 mM potassium phosphate buffer, pH 8, using manometry. The volume of gas was corrected for each temperature.

fixing cells always had a lower level of the bidirectional H_2ase and a higher ratio of uptake H_2ase/bidirectional H_2ase than NH_3-grown cells (Table 3). Because the bidirectional H_2ase can lower the level of reduced Fd in the cell whereas the uptake H_2ase may elevate it, the relative level of the two H_2ases may have an effect on the level of reduced Fd or the electron-donating potential of Fd in the cell. Since reduced Fd is the electron donor for nitrogenase (reduced Fd:dinitrogen oxidoreductase EC 1.18.2.1), the bidirectional H_2ase and nitrogenase compete against each other for reduced Fd, and the bidirectional H_2ase may also lower the ratio of reduced Fd/oxidizied Fd to hamper nitrogenase activity. The redox potential required for half-maximal nitrogenase activity has been reported to be between -460 and -470 mV (18-20); Braaksma et al. (22) reported that nitrogenase activity begins to decrease when the potential is above -440 mV. The bidirectional H_2ase of



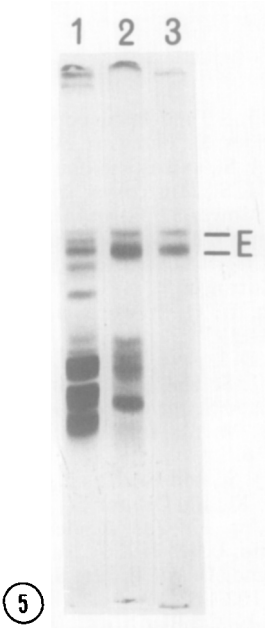


Figure 4. Effect of pH on the activities of the uptake H_2ase of *C. pasteurianum*. The assays were carried out in 50 mM of sodium acetate (□ , pH 5-6), potassium phosphate (△ , pH 6-8), and Tris-Cl (○ , ● , pH 7-9) buffers. Methylene blue (5 mM) and methyl viologen (1 mM) were used as the electron acceptor and donor.

Figure 5. Peptide mapping following limited proteolysis of the uptake H_2ase (1) and the bidirectional H_2ase (2) of *C. pasteurianum*. Gel 3 contained proteinase only, with E marking the bands of the proteinase (*S. aureus* V8 proteinase). The activities of the samples were: uptake H_2ase, 15,540 units/mg; bidirectional H_2ase, 420 units/mg (H_2-production).

C. pasteurianum can produce H_2 when the potential is more positive than these values (8). We speculate that the uptake H_2ase facilitates N_2-fixation in *C. pasteurianum* by maintaining a more favorable level of reduced Fd and/or a more favorable ratio of reduced Fd/oxidized Fd for nitrogenase. The speculation remains to be tested.

TABLE 3. Levels of H_2-Oxidizing H_2ase and Bidirectional H_2ase in Crude Extracts of N_2-Fixing and NH_3-Grown Cells of *C. pasteurianum*.

Growth Phase	Growth Conditions	H_2ase Level (units/mg protein)[a] H_2-oxidizing (HO)	Bidirectional (HB)	HO/HB
Exponential ($A_{550\,nm} \cong 2$)	N_2-fixing	39	35	1.1
	NH_3-grown	30	75	0.4
Stationary ($A_{550\,nm} \cong 4$)	N_2-fixing	15	14	1.1
	NH_3-grown	46	36	1.3

[a]The H_2ase level is based upon the H_2-uptake activity of each enzyme. See footnote to Table 1 for methods of calculation.

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