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Regulation of Hydrogenase Activity in Enterobacteria

ALVIN I. KRASNA

Department of Biochemistry and the Cancer Center/Institute of Cancer Research, College of Physicians and Surgeons, Columbia University, New York, New York 10032

Proteus vulgaris, *Escherichia coli*, and *Citrobacter freundii* cells were devoid of hydrogenase activity when grown on complex medium or minimal medium plus glucose in the presence of saturating levels of dissolved oxygen. Anaerobically grown cells had appreciable hydrogenase activity. Cells grown anaerobically in the presence of CO (an inhibitor of hydrogenase) or nitrate (an electron acceptor) lacked hydrogenase activity. To make hydrogenase essential for anaerobic growth, cells were grown on fumarate, a nonfermentable carbon source. *P. vulgaris* and *C. freundii* evolved H_2 gas under these conditions, and the hydrogenase-specific activity was 8 to 10 times greater than that in cells grown on glucose. Cell growth was inhibited by CO, and the cells grew but lacked hydrogenase activity when grown in the presence of nitrate. *E. coli* grew on fumarate plus H_2, and the specific activity was five times greater than that in cells grown on glucose. Thus, hydrogenase activity is inducible and is expressed maximally when the enzyme is essential for cellular growth. Under conditions of growth where the enzyme would not be catalytically active, cells contain little active hydrogenase. Under anaerobic conditions where the enzyme is not essential for growth, the level of hydrogenase activity is intermediate.

Hydrogenase is found in a variety of enterobacteria, and the role of the enzyme in normal cellular metabolism and growth is not clear. Both obligate and facultative anaerobes have the ability to produce H_2 gas when grown anaerobically, and in these cases protons are used as terminal electron acceptors. Although hydrogenase is synthesized by cells, it is not always required for normal growth and metabolism. Hydrogenase is inactive in the presence of oxygen and could not play any obvious role when cells are grown aerobically. The properties of hydrogenase have recently been reviewed (6).

There are conflicting reports as to whether hydrogenase is a constitutive or inducible enzyme. *Escherichia coli*, *Proteus vulgaris*, and *Aerobacter aerogenes* cells were reported (1, 4, 14) to have hydrogenase activity when grown aerobically on complex medium. When grown aerobically on minimal medium plus glucose, the organisms were reported (1-3, 10, 15) to be completely devoid of hydrogenase activity, suggesting that the enzyme is repressed.

The present study shows that in the presence of saturating levels of dissolved oxygen, cells are devoid of hydrogenase activity under all growth conditions. The enzyme is inducible, and by selecting conditions in which hydrogenase is essential for growth, the level of enzyme activity can be greatly increased.

MATERIALS AND METHODS

The organisms studied were *P. vulgaris*, *Citrobacter freundii* and *E. coli* K-12W6 *met bio*. The former two organisms were long-standing laboratory isolates. Cells were grown at 37°C in complex or minimal medium. The complex medium contained, per liter: nutrient broth, 10 g; Casamino Acids, 5 g; glucose, 5 g; Na_2HPO_4, 7.93 g; KH_2PO_4, 1.47 g; NaCl, 5 g; $FeSO_4 \cdot 7H_2O$, 0.005 g. The minimal medium had the following composition (in grams per liter): Na_2HPO_4, 6; KH_2PO_4, 3; NH_4Cl, 1; NaCl, 0.5; $MgSO_4$, 0.1; $CaCl_2$, 0.01; carbon source, 5. For growth of *E. coli*, the medium was supplemented with 0.02 g of L-methionine and 0.012 mg of biotin per liter. For growth of *P. vulgaris*, the medium was supplemented with 0.01% nutrient broth. Growth rates were determined by measuring the absorbance of cultures at 660 nm. Cells were harvested at mid-log phase and assayed immediately. The activity of the cells did not change by storage for a number of days at 4°C.

Cells were grown in pure oxygen by bubbling the purified gas into the growth medium, in air by shaking shallow cultures at 350 rpm, and anaerobically in sealed bottles filled to the top. When cells were grown in the presence of H_2 or CO, the appropriate purified gas was constantly bubbled into the growth medium.

For determination of hydrogenase activity, cells were harvested, washed with water, and suspended in 0.075 M sodium phosphate buffer, pH 6.8. Hydrogenase activity was measured by the deuterium exchange method (5), and specific activity is expressed as units per milligram of protein. Protein was determined in whole cells or sonicated cells by the Lowry method

(8). A unit of activity is defined as the appearance of 0.05% deuterium per h (with 10% D_2O) and is equivalent to the activation of 0.282 μmol of H_2 per min. Dithionite was added to all assays to deoxygenate the enzyme (6). The activities determined in whole cells were the same as that found in sonicated cells.

RESULTS AND DISCUSSION

Effect of aerobic and anaerobic growth on hydrogenase activity. Table 1 summarizes the effect of aerobic versus anaerobic growth on the hydrogenase activity of cells grown in complex medium or minimal medium plus glucose. In pure O_2, the cells are devoid of activity even in complex medium. Since the enzyme is inactive in oxygen, it is not surprising that the activity is not expressed under these conditions. When grown in air (as is true from all previous experiments cited), cells had little activity in minimal medium plus glucose and appreciable activity when grown in complex medium (Table 1). This is the same observation made in earlier reports (1–4, 10, 14, 15) and is undoubtedly due to the different levels of dissolved oxygen left in the different growth media. However, the data in the table clearly show that in the presence of saturating levels of dissolved oxygen, cells are devoid of hydrogenase activity. The effect of oxygen may be directly on the activity of hydrogenase per se or indirectly by its oxidation of essential metabolites in the cell or growth medium which influence hydrogenase expression.

Anaerobically, on complex or minimal medium, cells had appreciable hydrogenase activity although the enzyme is not essential for growth because the cells can ferment glucose to lactate. The data in Table 1 were obtained when aerobic cells were used as inoculum for anaerobic growth. Identical specific activities were obtained when anaerobically grown cells were used as an inoculum for further anaerobic growth. The data presented do not establish the exact threshold levels of oxygen which inhibit expression of hydrogenase activity. Information relating to this question has been previously reported (15).

TABLE 1. *Effect of aerobic and anaerobic growth on hydrogenase activity*[a]

Medium	Organism	Hydrogenase sp act		
		Pure O_2	Air	Anaerobic
Complex	*P. vulgaris*	0	0.52	1.10
	C. freundii	0.05	1.20	1.59
	E. coli	0	0.23	0.37
Minimal plus glucose	*P. vulgaris*	0	0.02	1.14
	C. freundii	0	0.18	0.76
	E. coli	0	0.08	0.77

[a] The conditions of growth and assay of enzyme activity are described in the text.

Experiments were then undertaken to grow cells anaerobically under conditions in which hydrogenase is inactive to determine whether the enzyme is essential for growth. CO inhibits hydrogenase (6, 11–13), and in the presence of saturating quantities of CO, cells grew anaerobically on minimal medium plus glucose, although at a lower rate than in its absence. These cells were completely devoid of hydrogenase activity, indicating that the enzyme is not required for anaerobic growth on glucose.

Anaerobically on minimal medium plus glucose, cells have active hydrogenase and use protons as terminal electron acceptors, evolving hydrogen (even though the enzyme is not essential for growth). In the presence of 0.01 M nitrate as electron acceptor, the cells had very little hydrogenase activity. (Specific activities were as follows: *P. vulgaris*, 0.13; *C. freundii*, 0.13; *E. coli*, 0.) This effect of nitrate has been reported previously (2, 3, 15) and shows clearly that cells produce active hydrogenase only under conditions in which its presence would be advantageous (although not necessarily essential) to cellular growth. With nitrate as electron acceptor, evolution of H_2 offers no advantage to the cells.

Effect of carbon sources during growth on hydrogenase activity of cells. The above data suggest that hydrogenase activity is not constitutive but is induced under anaerobic conditions when it can play a role in cellular metabolism. The level of activity during anaerobic growth on glucose may not be maximal, since hydrogenase is not essential for growth and cells grow even if hydrogenase activity is inhibited by CO. To determine whether the level of enzyme activity can be further increased, conditions were sought in which hydrogenase would be required for cellular growth. Such conditions should be achieved if cells are grown on a nonfermentable carbon source in the absence of terminal electron acceptors, in which case protons would serve as terminal electron acceptors only if cells contained active hydrogenase. In the absence of hydrogenase, the cells should not grow, and in the presence of other electron acceptors cells would grow but not require hydrogenase.

Attempts were made to grow cells anaerobically on minimal medium with acetate, formate, citrate, lactate, or glycerol as the sole carbon source. The inoculum was taken from cells grown anaerobically on glucose, which had ac-

tive hydrogenase. None of the cells grew on these carbon sources even if the medium was supplemented with 0.1% glutamate or Casamino Acids. Addition of 0.01 M nitrate did not stimulate cell growth, suggesting that anaerobically the cells could not utilize these carbon sources. Aerobically, all cell types grew on lactate or glycerol, *P. vulgaris* and *C. freundii* grew on citrate, and *C. freundii* and *E. coli* also grew on acetate. The cells from aerobic growth on these carbon sources had the same low level of hydrogenase activity as cells grown aerobically on glucose.

P. vulgaris and *C. freundii* grew anaerobically with fumarate as a carbon source. Growth was slow, and hydrogen gas was evolved during growth. These cells had very high hydrogenase activity (Table 2) compared with cells grown on glucose (8- to 10-fold increase). CO completely inhibited cell growth, demonstrating that growth on fumarate is dependent on hydrogenase. These data also demonstrate that the level of hydrogenase activity in cells grown anaerobically on glucose is decreased and becomes maximal when cells are completely dependent on hydrogenase for growth. When cells were grown anaerobically on fumarate in the presence of nitrate, they were devoid of hydrogenase activity (Table 2), presumably because nitrate was serving as terminal electron acceptor instead of protons. CO had no effect on cell growth anaerobically on fumarate plus nitrate. Cells grown aerobically on fumarate had the same low hydrogenase activity as when grown aerobically on glucose.

The effect of azide on growth with fumarate as the carbon source is consistent with the suggested role of hydrogenase. Anaerobically, 10^{-3} M azide had little effect on the growth rate or the hydrogenase activity. Aerobically, cells did not grow in the presence of 10^{-3} M azide. Azide inhibits respiration, and the cells could grow only in the presence of active hydrogenase. Aerobically, hydrogenase is inactive, so this pathway is unavailable and the cells do not grow.

E. coli did not grow on fumarate anaerobically but did grow if the culture was bubbled with H_2 gas. In this case, H_2 is being used as a source of electrons and fumarate is converted to succinate (9, 16). These cells had five times more hydrogenase activity (Table 2) than cells grown anaerobically on glucose. CO inhibited growth of cells on fumarate plus H_2 demonstrating that hydrogenase is essential for cellular growth. *E. coli* grown aerobically on fumarate had little hydrogenase activity.

These experiments suggest that hydrogenase activity in the enterobacteria is inducible and is expressed maximally when the enzyme is required for cellular growth. Under conditions of growth in which the enzyme would not be catalytically active, cells contain little active enzyme. Under anaerobic conditions in which it is not essential for growth, the level of enzyme activity is intermediate. From the data presented, it cannot be ascertained whether the different levels of hydrogenase activity observed reflect actual levels of de novo enzyme synthesis or whether inactive forms of the enzyme are synthesized under some conditions. The relationship of hydrogenase activity to growth conditions is not limited to the enterobacteria. *Hydrogenomonas facilis* grown autotrophically on H_2, O_2, and CO_2 had hydrogenase activity but lacked this activity when grown heterotrophically (7). When heterotrophic cells were incubated in 95% H_2–5% air, hydrogenase activity developed in the absence of cell growth. This induced synthesis was inhibited by 2,4-dinitrophenol or chloramphenicol.

TABLE 2. *Hydrogenase activity of cells grown anaerobically on minimal media plus fumarate*[a]

Organism	Hydrogenase sp act	
	Growth on fumarate	Growth on fumarate + nitrate
P. vulgaris	11.0	0
C. freundii	6.64	0
E. coli	4.05 (+H_2)	

[a] Organisms were grown on minimal medium plus 0.5% fumarate as carbon source. For *E. coli*, H_2 was required for growth. Cells were inoculated from cultures grown anaerobically on 0.5% glucose. *P. vulgaris* and *C. freundii* were also grown in the presence of 0.01 M sodium nitrate. This last experiment was not carried out with *E. coli* because growth on H_2 required hydrogenase regardless of the final electron acceptor.

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