Physiological Function of Hydrogen Metabolism During Growth of Sulfdogenic Bacteria on Organic Substrates

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Desulfovibrio vulgaris Madison and Thermodesulfobacterium commune contained functionally distinct hydrogenase activities, one which exchanged \(^3\)H\(_2\) into \(^3\)H\(_2\)O and was inhibited by carbon monoxide and a second activity which produced \(H_2\) in the presence of CO. Cell suspensions of \(D.\ vulgaris\) used either lactate, pyruvate, or CO as the electron donor for \(H_2\) production in the absence of sulfate. Both sulfdogenic species produced and consumed hydrogen as a trace gas during growth on lactate or pyruvate as electron donors and on thiosulfate or sulfate as electron acceptors. Higher initial levels of hydrogen were detected during growth on lactate-sulfate than on pyruvate-sulfate. \(D.\ vulgaris\) but not \(T.\ commune\) also produced and then consumed CO during growth on organic electron donors and sulfate or thiosulfate. High partial pressures of exogenous \(H_2\) inhibited growth and substrate consumption when \(D.\ vulgaris\) was cultured on pyruvate alone but not when it was metabolizing pyruvate plus sulfate or lactate plus sulfate. The data are discussed in relation to supporting two different models for the physiological function of \(H_2\) metabolism during growth of sulfdogenic bacteria on organic electron donors plus sulfate. A trace \(H_2\) transformation model is proposed for control of redox processes during growth on either pyruvate or lactate plus sulfate, and an obligate \(H_2\) cycling model is proposed for chemiosmotic energy coupling during growth on CO plus sulfate.

Molecular hydrogen appears to play a significant role in the energy-generating mechanisms of some species of sulfdogenic bacteria (1–3, 12, 14, 15, 21, 23). Desulfovibrio species can alternately utilize hydrogen as the sole energy source (1–3) or can produce hydrogen when growing fermentatively on pyruvate in the absence of sulfate (16, 26). Desulfovibrio spp. contain lactate dehydrogenase (13) and can grow on lactate in mixed culture with hydrogen-consuming bacteria such as methanogens in the absence of sulfate (6, 20), or alternatively, in the presence of sulfate they act as a hydrogen-utilizing sink in mixed cultures with fermentative bacteria (4). Recently, it has been shown that Desulfovibrio species produce or consume (or both) various amounts of hydrogen when growing on organic electron donors in the presence of sulfate (7, 21, 23). The physiological significance of this phenomenon has been ascribed to (i) control of the redox state of electron transfer components during metabolism (7, 23), (ii) an obligate hydrogen cycle required for chemiosmotic vectorial electron transfer and energy coupling (12, 14, 15), or (iii) the need to produce ATP via fermentation of organic substrates in the initial phase of growth for the activation of sulfate by ATP sulfurylase. Multiple hydrogenase activities in Desulfovibrio species (11, 24, 25, 27) are considered to function in \(H_2\) production and consumption. For example, obligate hydrogen cycling as a mechanism for energy generation by Desulfovibrio species is dependent upon the existence of two functionally distinct hydrogenases, one located in the cytoplasm and involved in hydrogen production and another periplasmic hydrogenase involved in hydrogen consumption (12, 14, 15).

The purpose of the present investigation was to assess the three hypotheses proposed to account for hydrogen transformation during growth of sulfdogenic bacteria on organic energy sources. The experiments analyzed \(H_2\) transformation by Desulfovibrio vulgaris Madison and Thermodesulfobacterium commune; both species grow on \(H_2\), lactate, or pyruvate as the electron donor (3, 29). Recently, we reported that \(D.\ vulgaris\) Madison produced \(H_2\) as one obligate intermediary metabolite during growth and energy conservation with carbon monoxide as the electron donor (F. S. Lupton, R. Conrad, and J. G. Zeikus, FEMS Microbiol. Lett., in press).

MATERIALS AND METHODS

Chemicals. All chemicals used were of reagent grade or better. All gases were obtained from Matheson Scientific, Inc. (Joliet, Ill.). Tritium gas was a gift of Paul DeLuca, University of Wisconsin—Madison.

Bacterial strains and growth conditions. \(D.\ vulgaris\) Madison (3) and \(T.\ commune\) YSRA (29) were grown in the minimal salts medium of Brandis and Thauer (5) with 0.5 g of yeast extract added per liter. Cultures were grown in 158-ml serum vials (Wheaton Scientific, Millville, N.J.) with 50 ml of medium and either sodium lactate or sodium pyruvate sterilized separately as the carbon and energy source. Sulfate was omitted from the medium in experiments in which pyruvate was fermented as the sole energy source. \(D.\ vulgaris\) and \(T.\ commute\) were incubated at 30 and 60°C, respectively.

Analysis of growth and gas production. Growth was measured as bacterial protein, which was determined by the Lowry procedure (10). Lactate and pyruvate concentrations were determined by the gas-liquid chromatographic technique of Holdeman et al. (9). Samples (0.5 ml) of culture were removed by syringe and added to 2-ml plastic centrifuge tubes that contained 0.5 ml of boron trifluoride-methanol (B1252; Sigma Chemical Co., St. Louis, Mo.) and 0.01 ml of concentrated HCl. These tubes were incubated at 60°C for 3 h, and then 0.5 ml of chloroform was added and the tubes were vortexed. The organic phase (4 ml) was analyzed on a Packard 407 chromatograph equipped with a flame ionization detector. The column was packed with 5% FFAP on Chromosorb WHP 80/100 mesh (Supelco). Trace concentrations of hydrogen (<15 nmol/ml) in the headspace concentration were quantified by the procedure of D. Deshpande, R. Conrad, and J. G. Zeikus (unpublished method) via HPLC analysis of lactate and pyruvate conversions with headspace sampling.
was then withdrawn periodically by syringe and transferred to vials that contained 5 ml of Instagel (Packard) as scintillant. Radioactivity was then measured in a Packard Tri-Carb liquid scintillation counter. To determine the effect of carbon monoxide on the in vivo hydrogenase activity, we added 10 ml of 100% carbon monoxide to the headspace of cultures 30 min after the addition of tritium gas. Sampling then continued for an additional 30 min.

RESULTS

Hydrogenase activities in cell suspensions. Experiments were initiated to detect the presence of different functional hydrogenase activities in cells grown to the mid-exponential phase on organic substrates. Figure 1 compares the in vivo hydrogenase activity in cells growing on lactate plus sulfate, as determined by the tritium exchange technique. D. vulgaris and T. commune displayed active hydrogenase activity which was significantly inhibited by CO.

Figure 2 shows that T. commune lacked CO-consuming activity and that the addition of CO enhanced hydrogen production by lactate-SO4\(^{2-}\)-grown cells more than pyruvate-SO4\(^{2-}\)-grown cells. The absence of CO dehydrogenase activity suggests that organic substrates and not CO are the electron donors for H\(_2\) production by T. commune in the presence of CO. CO did not stimulate H\(_2\) production by pyruvate-grown cells of T. commune. On the other hand, CO was consumed by cells of D. vulgaris grown on the analogous substrates, with the highest rates of CO oxidation of growing cultures were determined by removing 1 ml of gas with a gas-tight syringe (Glencoe, Houston, Tex.), followed by analysis in a CO-H\(_2\) analyzer based on the HgO to Hg vapor conversion technique (18, 19). The lower detection limit was 10 ml of H\(_2\) per liter of gas. Gas samples with H\(_2\) mixing ratios higher than 5 \(\mu\)l/liter were diluted before analysis. The gas samples were diluted with air purified of traces of H\(_2\) by passing the air over a metal oxide converter (Hopkalite; Drager-Werke, Lubeck, Federal Republic of Germany). The precision of a 100-fold dilution was greater than 90%. The total amount of H\(_2\) present in the headspace of the culture vials was calculated from the H\(_2\) mixing ratio and the volume and pressure of the headspace. Gas pressure was measured by using a mechanical needle manometer. Concentrations of H\(_2\) and CO in the headspace of culture vials greater than 1.0 \(\mu\)mol/ml were determined by removing 0.4 ml of headspace gas with a gas-tight syringe, followed by analysis on a Packard series 802 gas chromatograph equipped with a thermal conductivity detector for H\(_2\) and a Packard model 417 gas chromatograph with molecular sieve 13 by 40/60 mesh for CO.

Isotopic radioassay for hydrogenase activity. In vivo hydrogenase activity was determined by the isotopic tracer assay based on the hydrogenase-dependent formation of tritiated water from tritium gas as described by Schink et al. (17). A tritium-hydrogen gas mixture (250 \(\mu\)l; specific activity, 10 mCi/liter at 1 atm and 25°C) was added by gas-tight syringe to the headspace of culture vials. The vials were incubated with shaking at 30 and 60°C for D. vulgaris and T. commune, respectively. Samples of culture liquid were withdrawn periodically by syringe and transferred to vials that contained 5 ml of Instagel (Packard) as scintillant. Radioactivity was then measured in a Packard Tri-Carb liquid scintillation counter. To determine the effect of carbon monoxide on the in vivo hydrogenase activity, we added 10 ml of 100% carbon monoxide to the headspace of cultures 30 min after the addition of tritium gas. Sampling then continued for an additional 30 min.

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FIG. 1. Comparison of in vivo hydrogenase activity by cell suspensions of D. vulgaris and T. commune. Cultures were grown on lactate-SO4\(^{2-}\). The analysis was performed at 30°C with shaken mid-exponential-phase cells. CO, when present, was added (10% of culture headspace) after 30 min of incubation.

FIG. 2. Influence of CO on hydrogen evolution by T. commune. Cultures were grown with shaking for 24 h on organic electron donors in serum vials at 60°C. CO, when present, was added to 5% of the headspace. The cultures were shaken, and then gas transformation was measured.
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FIG. 3. Influence of CO on hydrogen evolution by *D. vulgaris*. Cultures were grown in serum vials on the substrates indicated at 30°C with shaking, and CO was added after 2 days.

FIG. 4. Relationship of electron donor to hydrogen evolution by cell suspensions of *D. vulgaris*. Cells were grown in serum vials on lactate-SO$_4^{2-}$ at 30°C, harvested, and washed with phosphate buffer. Reaction conditions: 50 ml of phosphate buffer (50 mM, pH 7.0), 200 µg of cell protein, 50 µM lactate or 1% CO headspace or both. 30°C. nm. Nanomoles.

FIG. 5. H$_2$ and CO transformations during growth of *D. vulgaris* and *T. commune* on organic electron donors and sulfate. Cultures were grown in serum vials with 50 ml of medium at 30 or 60°C for *D. vulgaris* or *T. commune*, respectively. Note that the trace levels of CO in (C) and (D) are due to chemical CO generation by the medium.
absence of sulfate. Controls without an electron donor added displayed low levels of endogenous H₂ evolution. The addition of lactate or CO significantly stimulated the rate of H₂ evolution, and the effects of these electron donors were additive, indicating that organic substrates or CO are electron donors for H₂ evolution by Desulfovibrio spp.

**Hydrogen transformation during growth.** Hydrogen was formed as a trace gas during the initial growth phases of both *D. vulgaris* and *T. commune* cultured on organic electron donors plus sulfate (Fig. 5). Significantly, higher levels of H₂ were formed during growth of these sulfidogenic species with lactate than were formed with pyruvate as the electron donor. Under the growth conditions used (excess sulfate), hydrogen was consumed during growth and was not detectable at the end of these experiments. Lower trace levels of CO were also produced and consumed during growth of *D. vulgaris*, as expected, but CO production and consumption was not detectable during growth of *T. commune*.

To assess whether H₂ production was formed as a consequence of fermentative metabolism to gain ATP needed for sulfate activation, we compared hydrogen transformation during growth of *D. vulgaris* on organic electron donors or on thiosulfate or sulfate as electron acceptors. Both hydrogen and CO were produced and consumed during growth with either thiosulfate or sulfate as the electron acceptor (Fig. 6). More hydrogen was produced, however, by both

FIG. 6. Comparison of H₂ and CO transformations during the growth of *D. vulgaris* on organic electron donors and sulfate or on thiosulfate. Cultures were grown in serum vials with 50 ml of medium at 30°C.

occurring on pyruvate plus sulfate and the lowest rates on lactate plus sulfate (Fig. 3A). CO addition significantly stimulated H₂ production in *D. vulgaris* cells grown on lactate plus sulfate more than in pyruvate plus sulfate-grown cells (Fig. 3B). The stimulation of hydrogen production by CO addition to pyruvate-grown *D. vulgaris* suggests that CO as well as organic substrates are donors for hydrogen production.

Figure 4 compares the influence of the electron donor on hydrogen evolution by Desulfovibrio cell suspensions in the

FIG. 7. Effect of exogenous hydrogen on growth and substrate utilization by *D. vulgaris*. *D. vulgaris* was grown with shaking at 30°C on either pyruvate alone or pyruvate plus sulfate or lactate plus sulfate. Hydrogen, when indicated, was added (50% of culture headspace) to cultures after 1 day.
lactate- and pyruvate-grown cultures with sulfate than by cultures grown with thiosulfate as the electron acceptor.

**Influence of exogenous hydrogen on organic substrate utilization.** Experiments were initiated to determine whether hydrogen was an obligate intermediate during growth on organic substrates by assessing the influence of exogenous hydrogen on substrate consumption. Exogenous hydrogen addition did not inhibit growth or substrate consumption when *D. vulgaris* was cultured on organic substrates plus sulfate; however, growth and substrate utilization were apparently inhibited by hydrogen when cells were grown on pyruvate alone (Fig. 7).

**DISCUSSION**

These data support the general hypothesis that the physiological function of hydrogen metabolism during the growth of sulfidogenic bacteria on lactate or pyruvate as the electron donor and on thiosulfate or sulfate as the electron acceptor is to control the redox level of internal electron carriers involved in energy metabolism (21, 23). Furthermore, the data do not support the hypothesis that an obligate hydrogen cycle is required for energy conservation during growth on lactate or pyruvate plus sulfate.

Figure 8 compares the model for obligate hydrogen cycling linked to chemiosmotic energy metabolism described by Odom and Peck (12) with a model for trace H₂ transformation linked to control of the redox state of internal electron carriers involved in chemiosmotic energy conservation. The trace H₂ transformation model (Fig. 8B) is proposed to account for the present data and supports previous suggestions on the role of hydrogenase in regulating electron flow and preventing energy loss (23). In this model, hydrogen production occurs as a side reaction of regulating the levels of reduced electron carriers which link to electron transport-mediated phosphorylation via sulfate reduction. The functions of hydrogenase in the trace H₂ transformation model differ significantly from those in the obligate H₂ cycling model because the cytoplasmic hydrogenase which indirectly couples to ferredoxin oxidation maintains the redox state of ferredoxin so as not to overreduce electron carriers involved in other reactions (e.g., lactate oxidation), and hence it plays a role in trace H₂ production. Consequently, the periplasmic hydrogenase can consume this H₂ and prevent energy loss. The periplasmic hydrogenase may play a major catabolic role, however, when sulfidogens grow on H₂ plus sulfate (1-3). The present data suggest a minor role...
for the periplasmic hydrogenase during growth on lactate or pyruvate plus sulfate.

The following lines of evidence support the trace H$_2$ transformation redox model in lieu of other proposed functions for hydrogen metabolism during growth of sulfate-reducing bacteria on lactate or pyruvate plus sulfate. (i) The finding that H$_2$ did not competitively inhibit organic substrate oxidation in the presence of sulfate implies that H$_2$ is not an obligate intermediate. The oxidation of pyruvate in the absence of sulfate was, however, inhibited by its product, hydrogen. Presumably, this occurs because of the reversibility of cytoplasmic hydrogenase and H$_2$ being an obligate end product of growth under these conditions. (ii) Higher levels of hydrogen were formed from lactate-sulfate than pyruvate-sulfate, suggesting that the more positive lactate-pyruvate half reaction ($E^{0'} = -190$ mV) requires more H$_2$ production than the pyruvate-acetyl coenzyme A half reaction ($E^{0'} = -540$ mV) to prevent overreduction of the electron carrier(s) involved in substrate oxidation-sulfate reduction. Since pyruvate oxidation is a thermodynamically better electron-donating reaction than lactate oxidation, higher levels of H$_2$ from lactate may imply redox regulations. (iii) H$_2$ was produced from organic electron donors and either sulfate or thiosulfate, indicating the need for redox regulation in lieu of sulfate activation (21, 23). The obligate H$_2$ cycling model requires the coordinated function of two spatially differentiated hydrogenases (i.e., periplasmic and cytoplasmic) with high activity for generation of a proton motive force. Notably, the trace H$_2$ transformation model suggests that during growth on organic substrates, a proton gradient (interior alkaline) is established by a membrane-bound vectorial electron-hydrogen transfer linked to cytoplasmic substrate oxidation reactions; whereas in the obligate H$_2$ cycling model, the periplasmic hydrogenase itself generates the proton motive force.

Finally, the data suggest the presence of two functionally distinct hydrogenase activities in _D. vulgaris_ (i.e., a CO-inhibited and a CO-stimulated hydrogenase). A CO-stimulated hydrogenase activity and an uptake hydrogen are important to growth of _D. vulgaris_ Madison on CO because H$_2$ appears as an initial stoichiometric product of CO transformation which is further coupled to sulfate reduction and energy conservation (Lupton and Peck, in press). Notably, growth of _D. vulgaris_ with CO as the electron donor may be in line with the obligate H$_2$ cycling chemiosmotic model proposed by Odom and Peck (12) for growth of sulfidogens on organic electron donors and sulfate, but this appears as a consequence of H$_2$ generation by an undescribed hydrogenase activity associated with CO oxidation. CO dehydrogenase activities purified from anaerobes do not produce H$_2$ (28). Evidence for a CO-stimulated hydrogenase in _T. commune_ is not apparent from the data presented here because the CO-dependent stimulation of hydrogen production from lactate could be a consequence of inhibiting the organisms of sulfate reductase, which contains a CO-inhibited siroheme (8). Thus, it is apparent that further studies at the biochemical and genetic level are required to prove the role proposed here for multiple hydrogenase activities during growth of sulfidogens on different organic electron donors plus sulfate.

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**LITERATURE CITED**


