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Evidence for the Periplasmic Location of Hydrogenase in *Desulfovibrio gigas*

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Hydrogenase has been found to be located in the periplasmic space of *Desulfovibrio gigas*, and it is proposed that hydrogenase plays an important and specific role in interspecies hydrogen transfer.

The periplasmic space of gram-negative bacteria can be operationally defined as the area of the cell between the cell wall and cytoplasmic membrane. Selective release of enzymes, usually of a hydrolytic nature, and other proteins from this area of the cell has been demonstrated through the use of two techniques, "spheroplast formation" and "osmotic shock" (17). LeGall et al. (16) reported that soluble cytochrome c_3 was selectively released by washing intact cells of *Desulfovibrio gigas* with slightly alkaline buffers. After the washing procedures, the cells appeared to be intact, as indicated by microscopic examination, and little damaged by the fact that the cells would still reduce sulfate in the absence of added adenosine 5'-triphosphate (19) without an appreciable lag period (J. LeGall and H. D. Peck, Jr., unpublished data). More recently it has been found that hydrogenase (hydrogen:cytochrome c_3 oxidoreductase; EC 1.12.2.1) of *D. gigas* is also selectively released by washing with slightly alkaline buffers (G. R. Bell and J. LeGall, unpublished data). Fujita and Sato have presented evidence which indicates that cytochrome c-552 of *Escherichia coli*, a low potential cytochrome synthesized only during anaerobic growth, is located in the periplasmic area (6). These observations suggested that hydrogenase and cytochrome c_3 might be localized in the periplasmic space of *D. gigas*, and the appropriate experiments appeared to be feasible, as the genus *Desulfovibrio* is susceptible to lysis by lysozyme and ethylenediaminetetraacetic acid (5, 18). In this paper, we present results obtained by "spheroplast formation" and "osmotic shock" which demonstrate that the hydrogenase of *D. gigas* is largely localized in the periplasmic space. (This work was submitted to the Department of Biochemistry, University of Georgia, by G. R. B. in partial fulfillment of the requirement for the Ph.D. degree and was presented in part at the 73rd Annual Meeting of the American Society for Microbiology at Miami Beach, Fla, 6-11 May 1973).

D. gigas (NCIB 9332) was grown at 37 C on a lactate-sulfate medium essentially as previously described (16). Ten grams of mid-growth phase cells, harvested about 18 h after inoculation (10%), was suspended to 40 ml in degassed 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.6) which was 0.5 M in sucrose and 0.03 M in 2-mercaptoethanol. Approximately 1 μg of deoxyribonuclease I (Sigma) per ml was added to the suspension. Until use, the cells were maintained under H_2 at 4 C. The first wash could contain from 25 to 50% of the total hydrogenase activity, but subsequent washes with this medium only removed a few percent of the hydrogenase activity. Hydrogenase is determined by manometrically measuring the rate of H_2 consumption (15), and a unit of activity is defined as a micromole of H_2 oxidized per min. Best results were obtained when all reagents had been previously vacuum degassed. Bisulfite reductase (desulfoviridin) was estimated from its absorbance at 628 nm (14), and adenylylsulfate (APS) reductase was assayed using the ferricyanide assay (20). Protein was determined by the biuret method (13) with bovine serum albumin.

In order to demonstrate the localization of hydrogenase, the cell suspension was subjected to three types of lysis. For preparation of standard extracts and the separation of the soluble protein and particulate or membrane fractions, 10 ml of the cell suspension was passed twice through an Amicon French pressure cell at 5,000 lb/in^2, and the extract was centrifuged at 37,000 $\times$ *g* for 20 min at 4 C. The pellet was discarded, and the extract was diluted to 20 mg of protein/ml. This extract was layered over a discontinuous sucrose gradient of

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1.67 M (1 ml) and 0.96 M (4 ml) in 0.05 M Tris-hydrochloride buffer (pH 7.6). After centrifugation at 180,000 × *g* for 2 h, the soluble protein remained in the upper portion of the tube (corresponding to the volume of the added extract), and the reddish particulate material was located near the bottom of the 0.96 M sucrose layer. Activities were determined in both the soluble and particulate fractions.

For the formation and fractionation of spheroplasts, 20 ml of the cell suspension was centrifuged at 37,000 × *g* for 5 min at 4 C, and the cells were resuspended to 250 ml in the sucrose-Tris-hydrochloride buffer to which had been added ethylenediaminetetraacetic acid (to 1 mM) and lysozyme (to 50 μg/ml). After incubation at 30 C for 30 min, maximal formation of spheroplasts was obtained. As shown in Fig. 1, 15-fold dilution of the spheroplast preparation in the sucrose-Tris-hydrochloride buffer resulted in only a slight decrease in 660-nm absorbance over a 20-min period (curve a), indicating their stability in hypertonic solutions. However, when the same dilution was made in distilled water, a rapid decrease in 660-nm absorbance resulted, indicating lysis of the spheroplasts (Fig. 1, curve b). The spheroplast preparation was centrifuged at 10,000 × *g* for 20 min at 4 C, and the supernatant fraction was designated the "spheroplast medium." The sedimented spheroplasts were lysed by suspension in 1 vol of 0.05 M Tris-hydrochloride buffer (pH 7.6) and designated the "spheroplast lysate."

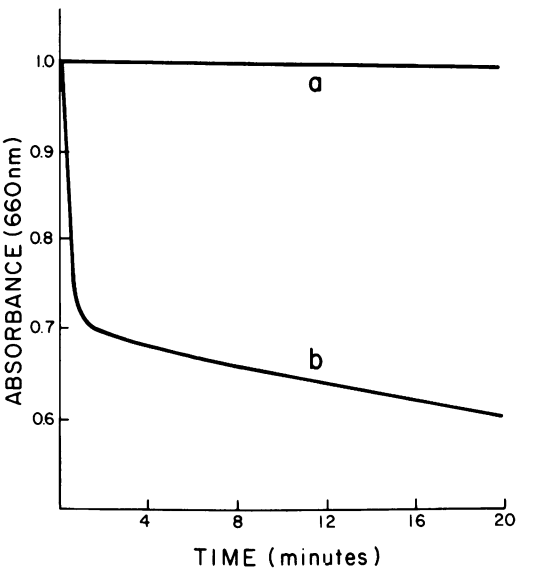

FIG. 1. *Lysis of spheroplasts of D. gigas. Cells were treated for spheroplast formation with ethylenediaminetetraacetic acid and lysozyme. Absorbance was monitored with a Gilford 200 spectrophotometer. Curve a: Dilution with sucrose-Tris medium (15-fold). Curve b: Dilution with water (15-fold).*

For osmotic shock fractionation, 10 ml of the cell suspension was diluted to 200 ml with the sucrose-Tris-hydrochloride buffer containing ethylenediaminetetraacetic acid (to 0.1 mM). The dilution represents 80 parts of buffer to 1 part of cells (vol/wt). The suspension was centrifuged at 10,000 × *g* for 20 min at 4 C, the supernatant fluid was decanted, and the cells were rapidly resuspended to 200 ml in cold 1 mM $MgCl_2$. The suspension was next centrifuged at 10,000 × *g* for 20 min at 4 C and the supernatant was termed the "shock fluid." The pellet was suspended in 200 ml of 0.05 M Tris-hydrochloride (pH 7.6) and passed through the French pressure cell to yield an extract termed the "shocked cell lysate."

The results of the assay of these fractions for protein, hydrogenase, bisulfite reductase, and APS reductase are shown in Table 1. The soluble fraction prepared by means of the French pressure cell contained most of the protein and hydrogenase and all of the bisulfite reductase and APS reductase. With spheroplast formation, 21% of the cellular protein and 100% of the hydrogenase are found in the "spheroplast medium," but only 12 and 10% of bisulfite reductase and APS reductase are found in this fraction. Most of the protein, bisulfite reductase, and APS reductase remain in the spheroplasts, i.e., the "spheroplast lysate." With shocked cells, about 15% of the cellular protein and 90% of the hydrogenase are released into the shock fluids, but only 5% of the bisulfite reductase and none of the APS reductase are found in this fraction. Although bisulfite reductase and APS reductase have molecular weights of about 200,000 and hydrogenase has a molecular weight of 60,000, only very small amounts of ferredoxin, flavodoxin, and rubredoxin were found in selective washes containing the hydrogenase. This also indicates that the selective loss of hydrogenase is not due to a general loss of low-molecular-weight proteins. Although *Desulfovibrio desulfuricans* has been reported to have multiple forms of hydrogenase (1), electrophoresis of the spheroplast medium, shock fluid, lysate of shocked cells, soluble protein fraction, and crude extracts on polyacrylamide gels (2) always revealed only one activity band (1) which migrated at the same R_f (0.37 at pH 8.3; 0.57 at pH 8.9).

The results of the spheroplast and osmotic shock experiments demonstrate that the hydro-

TABLE 1. *Cellular localization of hydrogenase, bisulfite reductase, and APS reductase in D. gigas*

Fraction	Protein		Hydrogenase[a]		Bisulfite reductase[b] (desulfoviridin) (% Content)	APS reductase[c] (% Content)
	mg	%	Total units	%		
Soluble fraction	407	94	1,650	99	100	100
Membrane fraction	26	6	23	1	0	0
Spheroplast medium	153	21	750	100	12	10
Spheroplast lysate	580	79	0	0	88	90
Shock fluid	57	15	950	90	5	0
Lysate of shocked cells	310	85	105	10	95	100

[a] H_2 uptake with benzyl viologen as electron acceptor.
[b] Calculated from absorption at 628 nm.
[c] Based on ferricyanide assay.

genase of *D. gigas* is localized within the periplasmic space of the cell. Using the spheroplast technique, all of the hydrogenase of the cell can be liberated; however, some lysis inevitably occurs and, as a consequence, there is some contamination of the periplasmic fraction with intracytoplasmic proteins. On the other hand, the osmotic shock technique did not completely release the hydrogenase, but the preservation of cellular integrity minimized the contamination problem.

Although the sulfate-reducing bacteria can oxidize molecular hydrogen in the presence of organic and inorganic electron acceptors, sulfate-reducing bacteria grown with organic substrates, such as lactate, ethanol, and formate, in the presence of sulfate still contain high levels of hydrogenase. Hydrogen does not appear to be involved in this latter type of respiration, as hydrogen is not produced or utilized in significant quantities and a hydrogenase-free strain of *D. desulfuricans* has been reported to grow normally on lactate or pyruvate and sulfate (21). Furthermore, the oxidation of H_2 has been reported to contribute little to the energy metabolism of *D. vulgaris* growing on lactate (10). The demonstration that the hydrogenase of *D. gigas* is primarily of periplasmic origin only intensifies the question of the physiological role of hydrogenase and the advantage this specialized enzyme compartmentalization and concentration may offer to these bacteria.

We suggest that hydrogenase, in addition to its established role as a dehydrogenase, also serves a hydrogen-binding function in the utilization of low levels of H_2 and possibly the production of H_2 in thermodynamically unfavorable reactions. Thus, hydrogenase may function essentially as a hydrogen-binding protein required for the transfer of low levels of H_2 between microorganisms growing in a mutual relation, i.e., interspecies hydrogen transfer (3, 22), and for the utilization of low levels of hydrogen produced by cathodic depolarization in the corrosion of iron by the sulfate-reducing bacteria (11). With regard to this idea, it should be noted that little or no H_2 is found in gases produced by mature fresh-water (4, 8, 12) sediments and that the concentration of H_2 in the rumen, where there appears to be interspecies hydrogen transfer coupled to methane formation, is of the order of 10^{-6} M (9). There are a number of reports which suggest that hydrogen transfer does occur between certain microorganisms and that the molecule actually transferred is molecular hydrogen. *Methanobacillus omelianskii* has been demonstrated to be a mixed culture consisting of a hydrogen-oxidizing, methane-forming bacterium (H organism) and a second bacterium (S organism) which oxidizes ethanol to acetate and H_2 (3). The methane bacterium grows well on H_2 and CO_2, but the S organism grows poorly on ethanol even when the H_2 is continuously removed (22). When the organisms are grown together on pyruvate, there is a 20-fold increase in amount of H_2 produced (calculated from methane formation), and it is postulated that the process of methane formation acts as a highly efficient hydrogen trap which pulls pyruvate oxidation in the direction of acetate formation. The sulfate-reducing bacteria, *D. vulgaris* and *D. desulfuricans*, do not grow or metabolize lactate or ethanol in the absence of sulfate or fumarate;

however, these organisms will grow on lactate or ethanol alone in the presence of a methane-forming bacterium (H organism) (M. P. Bryant, Amer. Chem. Soc. Abstr., Microbiol. Sect., 1969, p. 18). Methane is formed from CO_2 presumably by reducing power, probably in the form of H_2 derived from the oxidation of both lactate and ethanol to acetate. Here again, the methane-forming system appears to act as a highly efficient hydrogen trap in "pulling" these oxidations. Another symbiotic-like association that may involve intercellular hydrogen transfer includes that of *Chloropseudomonas ethylica*, which is reported to be a mixed culture consisting of *Chlorobium limicola* and an unidentified *Desulfovibrio* (7). Symbiotic associations such as those mentioned above may allow much greater flexibility for growth for those anaerobic bacteria which have evolved enzyme systems which permit such interaction. The exhaustion of specific nutrients required for growth of the isolated organism need not result in a failure to survive, provided that it can associate symbiotically with other bacteria in such a manner that it is able to metabolize substrates that could not be utilized in pure culture. Thus, we wish to propose that the periplasmic location of hydrogenase represents a specific adaptation of anaerobic microorganisms for symbiotic-like growth with other anaerobic bacteria. It would be predicted that all bacteria capable of participating in interspecies hydrogen transfer would have hydrogenase located in the periplasmic space or on the surface of the cell membrane.

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