Membrane-Associated, Energy-Linked Reactions in *Bdellovibrio bacteriovorus*

DEVORAH FRIEDBERG* AND ILAN FRIEDBERG

Department of Microbiological Chemistry, Hebrew University-Hadassah Medical School, Jerusalem,* and Department of Microbiology, Tel Aviv University, Ramat-Aviv, Israel

Received for publication 24 May 1976

Disrupted cells of *Bdellovibrio bacteriovorus* exhibited adenosine triphosphatase activity, 60 to 80% of which was in the soluble fraction. Dicyclohexylcarbodiimide did not inhibit the adenosine triphosphatase activity in membrane particles. The particles did not show energy-linked transhydrogenase activity. The activity of non-energy-linked transhydrogenase as well as the rate of oxygen consumption were higher in membrane particles of the host-independent strain than in the host-dependent strains. The uptake of amino acids and phosphate by whole cells of *Bdellovibrio* was found to be an energy-dependent process. Amino acid uptake was inhibited by cyanide and by carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone. Valinomycin, in the presence of K*, did not inhibit the uptake, and only partial inhibition was exerted by arsenate and dicyclohexylcarbodiimide. Sulfhydryl reagents inhibited amino acid uptake.

The metabolism of *Bdellovibrio* has been investigated to obtain a better understanding of its unique potential to grow in the periplasmic space of other bacteria. Most studies in this field have been concentrated on the respiration pattern of *Bdellovibrio* (13, 22, 25), its biosynthetic capacities (12, 17, 19, 21), and its energy expenditure (10, 20).

*Bdellovibrio* is an obligate aerobe, requiring oxygen for its intraperiplasmic growth (13) and for the axenic growth of the host-independent (H-I) strains (23). The endogenous respiration of *Bdellovibrio* is extremely high. No stimulation of respiration occurs in the presence of substrates such as glucose or succinate, and only a small increase in O2 consumption is observed in the presence of oxidizable substrates such as casein hydrolysate (13, 22, 25). Recently, Rittenberg and Hespell (20) have shown that *Bdellovibrio* requires very little energy for the intraperiplasmic growth. Likewise, the H-I strain of *Bdellovibrio bacteriovorus* can regulate economically its energy expenditure (10).

So far little is known of the mechanisms by which *Bdellovibrio* produces and utilizes its energy. This investigation deals with the elucidation of specific mechanisms involved in energy coupling in *Bdellovibrio*. Membrane-bound activities, mainly the properties of adenosine triphosphatase (ATPase)—one of the key components of the energy conservation system—and the role of energy in uptake processes of *Bdellovibrio* were investigated. A unique type of ATPase was found in membrane preparations of *B. bacteriovorus*, and some of its properties are described. It was shown that *B. bacteriovorus* cells take up amino acids and phosphate by energy-dependent mechanisms. Properties of these active transport systems are described and discussed.

**MATERIALS AND METHODS**

**Bacterial strains and growth procedures.** *B. bacteriovorus* host-dependent (H-D) strains 109D and 109J were grown in *Escherichia coli* B as follows. A 100-ml portion of *E. coli* B grown overnight in an enriched nutrient broth (23) was washed once and suspended in the same volume of DNB medium (22). This suspension was inoculated with 10⁶ freshly grown *Bdellovibrio* and the resultant *Bdellovibrio* culture was harvested within 24 h, when no *E. coli* spheroplasts could be observed. A strain of H-I *B. bacteriovorus* 109D, obtained from M. P. Starr, was grown in enriched nutrient broth until the late-log phase.

**Preparation of membrane particles.** Cells were harvested by centrifugation in a Sorvall RC2B centrifuge at 7,000 × g for 15 min at 4°C. The cells were washed once with 50 mM tris(hydroxymethyl)aminomethane (Tris), pH 7.8, containing 10 mM MgSO4, suspended in the same buffer to 1 g (wet weight) of cells per 10 ml, and disrupted in a French pressure cell at 20,000 lb/in². Nonbroken cells and debris were removed by centrifugation at 27,000 × g for 15 min at 4°C. The subsequent isolation of the membrane particles was done by the method of Fisher et al. (7); the supernatant fluid was centrifuged at 180,000 × g for 70 min at 4°C, and the pellet was homogenized in 1 ml of the Tris buffer. The homogenate of the membrane particles and the supernatant fluid were saved.

1382
ATPase, transhydrogenase, and oxidative capacity in membrane particles. The respiration and adenosine 5’-triphosphate (ATP)-driven reduction of nicotinamide adenine dinucleotide phosphate by the reduced form of nicotinamide adenine dinucleotide, as well as the non-energy-linked reduction of acetylpyridine nicotinamide adenine dinucleotide by the reduced form of nicotinamide adenine dinucleotide were measured according to the procedures of Fisher et al. (7, 8) and Kaplan (16) in a reaction volume of 3 ml, containing 1 to 4 mg of protein. A complete reaction mixture for measurements of ATPase contained 50 μmol of Tris-sulfate buffer, pH 7.8, 4 μmol of MgCl₂, membrane particles or soluble fractions (25 to 50 μg of protein), and 10 μmol of ATP in a final volume of 1 ml. The reaction mixture was preincubated for 5 min at 37°C, and the reaction was initiated by addition of the ATP. The reaction was allowed to proceed for 10 min and then was terminated by the addition of trichloroacetic acid to a final concentration of 2.5%. The inorganic phosphate released was determined by the method of Fiske and SubbaRow (9). Protein was determined according to the method of Lowry et al. (18). The oxidation velocity of membrane particles was polarographically measured in a medium containing 50 mM Tris-hydrochloride (pH 7.5) and 2.5 mM MgCl₂ with 2 mM reduced nicotinamide adenine dinucleotide as substrate at 24°C. Transport experiments. Cells were harvested by centrifugation (15,000 × g for 5 min), washed twice, and resuspended to a final concentration of 0.2 mg of protein per ml in 1 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) buffer, pH 7.6, containing 5 mM glutamate as an energy source, 50 μg of chloramphenicol per ml, and the following salts: Ca(NO₃)₂·H₂O, 0.8 mM; FeSO₄·4H₂O, 0.025 mM; and MnSO₄, 0.06 mM. Uptake of amino acids was initiated by the addition of 1.5 × 10⁻⁴ M “C-labeled L-amino acid mixture to a final concentration of amino acids of 10⁻⁴ M each (40 to 50 μCi/μmol); the uniformly “C-labeled L-amino acid mixture was a product of New England Nuclear Corp., Boston, Mass., and contained: L-alanine, L-arginine, L-aspartic acid, L-glutamic acid, L-glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tyrosine, and L-valine. Samples of 0.2 ml were removed at various intervals, filtered through membrane filters (pore size, 0.2 μm; Millipore Corp.), and washed with 5 ml of HEPES buffer. The filters were dried, and the radioactivity was determined in a Packard scintillation spectrometer, model 3330. Phosphate uptake was determined in 1 mM HEPES buffer, pH 7.8, and initiated by the addition of 32P to a final concentration of 10⁻⁴ M (50 to 100 μCi/μmol). The 32P was a product of Nuclear Research Centre, Negev, Israel.

**RESULTS**

ATPase, transhydrogenase, and oxidase activities in membrane particles of *B. bacteriovorus* strains 109J (H-D), 109D (H-D), and 109D (H-I). Table 1 illustrates the distribution of ATPase activity between membrane and supernatant fractions in three *B. bacteriovorus* strains, two H-D and one H-I. Unlike the ATPase of other bacteria, 60 to 80% of the total ATPase activity of the membrane preparations was found in the supernatant fluid; the specific activity in this fraction was found to be higher than that of the membrane-bound enzyme (whereas under similar conditions only 10 to 15% of *E. coli* ATPase activity was soluble). Furthermore, the ATPase activity in membrane preparations of the *Bdellovibrio* strains tested was not inhibited by dicyclohexylcarbodiimide (DCCD), an inhibitor of membrane-bound ATPase in other bacteria (1). Preincuba-

---

**Table 1. ATPase activity in membrane particles and the soluble fraction of *B. bacteriovorus* strains 109J (H-D), 109D (H-D), and 109D (H-I)*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Addition</th>
<th>Membrane</th>
<th>Soluble</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total units⁸</td>
<td>Sp act (units/mg of protein)</td>
</tr>
<tr>
<td>109J (H-D)</td>
<td>None</td>
<td>18</td>
<td>140</td>
</tr>
<tr>
<td></td>
<td>Mg²⁺, 4 mM</td>
<td>18</td>
<td>187</td>
</tr>
<tr>
<td></td>
<td>Mg²⁺, 4 mM, + DCCD, 60 μM</td>
<td>180</td>
<td>180</td>
</tr>
<tr>
<td>109D (H-D)</td>
<td>None</td>
<td>19</td>
<td>123</td>
</tr>
<tr>
<td></td>
<td>Mg²⁺, 4 mM</td>
<td>123</td>
<td>141</td>
</tr>
<tr>
<td></td>
<td>Mg²⁺, 4 mM, + DCCD, 60 μM</td>
<td>125</td>
<td>153</td>
</tr>
<tr>
<td>109D (H-I)</td>
<td>None</td>
<td>20</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>Mg²⁺, 4 mM</td>
<td>87</td>
<td>180</td>
</tr>
</tbody>
</table>

* Reaction conditions were as described in Materials and Methods.

* One unit of activity = 1 nmol of P; released per min.
tion of membrane particles with concentrations of up to 960 μM DCCD did not result in any inhibition (by contrast, under similar conditions, 60 μM DCCD inhibited the ATPase activity in E. coli membrane particles by 85%). The specific activity was somewhat increased by Mg2+, the extent varying with the strain tested; Ca2+ in a concentration range of 0.1 to 10 mM had no effect, whereas higher concentrations were inhibitory.

Further characterization of the ATPase was carried out with preparations of strain 109D (H-I). The optimum pH range for both membrane-bound and soluble ATPase activities was found to be 8 to 9. The activation energy, as calculated from an Arrhenius plot in the temperature range of 25 to 45°C, was 51.5 × 103 and 33 × 103 J/mol for the membrane-bound and soluble activities, respectively. Freezing and thawing resulted in a 30% decrease in the soluble activity, whereas the membrane-bound activity was unaffected by this treatment. Fractionation of the cells at either 4 or 18°C resulted in similar specific activity of ATPase.

The specificities of the membrane-bound and soluble ATPase were similar (Table 2). The highest activity was found for ATP, whereas adenosine 5′-diphosphate, guanosine 5′-triphosphate, uridine 5′-triphosphate, and cytidine 5′-triphosphate were poorer substrates. Hydrolysis of phosphate esters such as p-nitrophenyl phosphate and glucose-6-phosphate were at assay resolution limits.

Membrane particles of the three strains tested showed reduced nicotinamide adenine dinucleotide oxidase activity (Table 3). This activity was higher in strain 109D (H-I). It was totally inhibited by 3 mM KCN in all the strains tested. Although non-energy-linked transhydrogenase could be observed in membrane particles of the three strains, the energy-linked transhydrogenase activity could not be detected except for a trace amount in strain 109D (H-I). This strain also exhibited higher values for the non-energy-linked transhydrogenase activity.

**Morphology of membrane preparations of strains 109J (H-D), 109D (H-D), and 109D (H-I).** The membrane particles obtained by disrupting cells of strain 109D (H-I) in a French pressure cell consisted of a heterogeneous population of “vesicle-like” particles, 40 to 160 nm in diameter (Fig. 1A). Likewise, cells disrupted by sonic oscillation (1 min) gave rise to similar vesicle-like particles. Subsequent exposure of the French pressure preparation to further sonic oscillation yielded a population of smaller vesicle-like particles 40 to 100 nm in diameter (Fig. 1B). Cells ruptured by these three methods exhibited similar values of ATPase activity. Membrane particles of strains 109D (H-D) and 109J (H-D) were similar in shape and diameter to the vesicle-like particles of strain 109D (H-I). However, E. coli particles obtained by the same procedures were smaller (15 to 20 nm in diameter) and more homogeneous (Fig. 1C).

**Transport of amino acids and phosphate in whole cells of strains 109J (H-D), 109D (H-D), and 109D (H-I).** To gain more insight into the energy metabolism of Bdellovibrio in whole cells, the transport of amino acids and phosphate was investigated. A number of inhibitors that are generally assumed to affect electron transport, KCN, DCCD and arsenate (ATP-
ENERGY-LINKED REACTIONS IN B. BACTERIOVORUS

Fig. 1. Membrane particles of B. bacteriovorus strain 109D (H-I) and E. coli B. (A) French pressure cell particles of Bdellovibrio prepared as described in Materials and Methods. (B) Particles of Bdellovibrio, prepared as the particles in (A) and then sonically disrupted for 2 min and treated as described in Materials and Methods. (C) French pressure cell particles of E. coli B. The preparations were negatively contrasted with uranyl acetate. Magnification, ×60,000.

linked reaction inhibitors), carbonyl cyanide p-trifluoromethoxyphenyl hydrozone and valinomycin (uncouplers), and the sulfhydryl reagents iodoacetate, N-ethylmaleimide, and p-hydroxymercuribenzoate, were tested for their effect on amino acid uptake by strain 109D (H-I). The results presented in Fig. 2 illustrate the uptake of amino acids in whole cells of strain 109D (H-I). Arsenate at a concentration of 10 mM reduced the level of amino acid uptake by 45%, suggesting that their uptake can be driven by a high-energy phosphate bond (probably ATP). Furthermore, the uptake of amino acids was reduced to various levels by different concentrations of DCCD (Fig. 3).

The results presented in Table 4 show that KCN at a minimal concentration of 0.5 mM reduced both the initial rate and the level of amino acid uptake by 86 and 91%, respectively, implicating electron transport as an energy source for uptake. At lower concentrations, the inhibition by KCN was smaller. The combina-
tion of cyanide and DCCD at low concentrations was more effective than either inhibitor alone. The inhibition observed was as expected on the basis of the inhibitors acting independently. The uncoupler carbonyl cyanide p-trifluoromethoxyphenyl hydrazone almost completely inhibited the uptake of amino acids, whereas valinomycin, either alone or in the presence of 10, 50, or 100 mM KCl, did not inhibit, but slightly stimulated, the uptake in ethylenediaminetetraacetic acid-treated cells. Since KCl itself (100 mM) inhibited transport by 30 to 40%, the effect of valinomycin in the presence of higher concentrations of KCl was not examined. Sulphydryl reagents inhibited the uptake of amino acids to different degrees depending on the inhibitor used, suggesting that SH groups may participate in the uptake process. The effect of dithiothreitol, which par-

![Figure 2](http://jb.asm.org/)

**Figure 2.** Effect of arsenate on amino acid uptake in cells of *B. bacteriovorus* strain 109D (H-I). Assays were performed as described in Materials and Methods. Cells were preincubated with arsenate for 5 min. Symbols: Uptake in the absence (○) and presence of 2 mM (□) or 10 mM (●) arsenate.

![Figure 3](http://jb.asm.org/)

**Figure 3.** Amino acid uptake in cells and ATPase activity in membrane particles of *B. bacteriovorus*. Assays were performed as described in Materials and Methods. Cells or membrane particles were preincubated with DCCD for 30 min. Symbols: Uptake (○); ATPase (●).

**Table 4.** Effect of energy inhibitors and sulphydryl reagents on amino acid uptake by *B. bacteriovorus* strain 109D (H-I).

<table>
<thead>
<tr>
<th>Additions*</th>
<th>Conc</th>
<th>Initial rate (nmol of amino acids/min per mg of protein)</th>
<th>%</th>
<th>Level at 30 min (nmol of amino acids/mg of protein)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>1.45</td>
<td>100</td>
<td>10.0</td>
<td>100</td>
</tr>
<tr>
<td>KCN</td>
<td>0.1 mM</td>
<td>0.77</td>
<td>53</td>
<td>6.7</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>0.25 mM</td>
<td>0.54</td>
<td>37</td>
<td>3.5</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>0.5 mM</td>
<td>0.21</td>
<td>14</td>
<td>0.9</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>10 mM</td>
<td>0.21</td>
<td>14</td>
<td>0.7</td>
<td>7</td>
</tr>
<tr>
<td>DCCD</td>
<td>60 µM</td>
<td>1.10</td>
<td>76</td>
<td>3.6</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>120 µM</td>
<td>0.57</td>
<td>39</td>
<td>2.7</td>
<td>27</td>
</tr>
<tr>
<td>KCN</td>
<td>0.1 mM</td>
<td>0.54</td>
<td>37 (70)*</td>
<td>3.3</td>
<td>33</td>
</tr>
<tr>
<td>+ DCCD</td>
<td>60 µM</td>
<td>0.28</td>
<td>19 (36)</td>
<td>1.2</td>
<td>12</td>
</tr>
<tr>
<td>+ DCCD</td>
<td>120 µM</td>
<td>0.09</td>
<td>6</td>
<td>1.1</td>
<td>11</td>
</tr>
<tr>
<td>FCCP</td>
<td>10 µM</td>
<td>0.31</td>
<td>21</td>
<td>4.2</td>
<td>42</td>
</tr>
<tr>
<td>Valinomycin + KCl</td>
<td>5 µM</td>
<td>101</td>
<td>11.5</td>
<td>115</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 mM</td>
<td>0.31</td>
<td>21</td>
<td>4.2</td>
<td>42</td>
</tr>
<tr>
<td>Iodoacetate</td>
<td>5 mM</td>
<td>0.20</td>
<td>14</td>
<td>1.0</td>
<td>10</td>
</tr>
<tr>
<td>N-ethylmaleimide</td>
<td>10 µM</td>
<td>0.22</td>
<td>15</td>
<td>3.1</td>
<td>31</td>
</tr>
<tr>
<td>p-Hydroxymercuribenzoate</td>
<td>100 µM</td>
<td>0.45</td>
<td>31</td>
<td>6.2</td>
<td>62</td>
</tr>
<tr>
<td>p-Hydroxymercuribenzoate + DTT</td>
<td>100 µM</td>
<td>0.51</td>
<td>35</td>
<td>5.6</td>
<td>56</td>
</tr>
</tbody>
</table>

* Abbreviations: FCCP, carbonyl cyanide p-trifluoromethoxyphenyl hydrazone; DTT, dithiothreitol.
* Number in parentheses is the percentage of the KCN-treated value. Preincubation: KCN and FCCP, 5 min; DTT, iodoacetate, N-ethylmaleimide, and p-hydroxymercuribenzoate, 15 min; p-hydroxymercuribenzoate + DTT, 15 min with p-hydroxymercuribenzoate followed by the addition of DTT and further incubation for 15 min. Ethylenediaminetetraacetic acid-valinomycin-treated cells (29) were stored on ice for 30 to 240 min.
tially reversed the inhibition exerted by p-hydroxymercuribenzoate, is in accordance with this suggestion.

The results in Fig. 4 illustrate phosphate uptake in whole cells of strains 109D (H-D) and 109J (H-D). Similarly to amino acid uptake in strain 109D (H-I), the uptake of phosphate by 109D (H-D) and 109J (H-D) cells is completely inhibited by 10 mM KCN. A similar inhibition was exerted by 1 mM KCN.

DISCUSSION

Results obtained in this investigation demonstrate that B. bacteriovorus has a unique type of ATPase. Most of the enzyme activity was found in the soluble fraction of disrupted cells, whereas generally this activity remains membrane bound (1).

In addition, unlike the ATPase of most bacteria, the membrane-bound activity of ATPase in membrane preparations of B. bacteriovorus is insensitive to inhibition by DCCD, an inhibitor of membrane-bound ATPase from various sources (5, 11). Studies with mutants of E. coli and Streptococcus faecalis indicate that DCCD sensitivity in these organisms appears to be conferred by a specific membrane component (2, 6). Alternatively, DCCD-resistant ATPase activities were found in certain mutants in which the binding of ATPase to the membrane was defective (15). Unlike mutants of E. coli (15) and S. faecalis (11), the uptake of amino acids by intact cells of B. bacteriovorus is DCCD sensitive. This may mean that in B. bacteriovorus both structural and functional roles of ATPase are maintained in vivo, whereas during cell fractionation, i.e., in vitro, the binding of the enzyme to the membrane is altered in such a way that the membrane-bound activity of ATPase becomes DCCD resistant. This change might result from weak binding of the ATPase to the membrane, as previously suggested for certain E. coli mutants (15).

The data show that membrane particles of B. bacteriovorus constitute a heterogeneous population of various sizes, much larger than E. coli membrane particles and of different membrane morphology.

Energy-dependent transhydrogenase could not be detected in membrane particles of the H-D strains, and only trace values of a respiration-driven activity were revealed in membrane particles of the H-I strain tested. Membrane particles of the H-I strain exhibited higher non-energy-linked transhydrogenase activity as well as higher oxygen consumption. These differences might originate because of the different growth conditions of the H-D and H-I strains, or may reflect a general difference between the membrane structure of the H-I and H-D strains.

Intact cells of B. bacteriovorus obtain amino acids and phosphate from the medium via an energy-dependent process, as shown by the use of energy metabolism inhibitors. The uptake of phosphate and amino acids seems to be energized mainly by electron transport, since it is virtually inhibited by KCN. The amino acid uptake may be energized by ATP hydrolysis, presumably via ATPase. However, this energy source is not the main one, since uptake was only partially inhibited by the ATPase inhibitors DCCD and arsenate. The total inhibition exerted by the high concentration of DCCD (1 mM) might be due to nonspecific toxic effects of DCCD on the membrane (26). The synergistic effect of DCCD and cyanide indicates that two pathways of energy supply to amino acid uptake might operate in Bdellovibrio. There is now evidence that bacterial transport is energized by the "high energy state" of the membrane, generated by either electron transport or ATP hydrolysis (24). Recently it has been shown that the transport of some amino acids in E. coli is driven solely by phosphate-bound energy, derived from substrate or oxidative phosphorylation (3, 4, 14). The data indicate that the two pathways of energy coupled to amino acid uptake might also operate in Bdellovibrio. However, since Bdellovibrio is an obligate aerobe, it is very likely that substrate-level ATP synthesis would not function in the

---

**Fig. 4.** Phosphate uptake in cells of B. bacteriovorus strains 109J (H-D) (O) and 109D (H-D) (C, ■) in the absence (open symbols) or presence (closed symbols) of 10 mM KCN. Assays were performed as described in Materials and Methods.
presence of respiration inhibitors. Thus, a marked inhibition of uptake by cyanide is possible even though arsenate and DCCD exert a partial inhibition as well.

ACKNOWLEDGMENTS

We wish to thank David L. Gutnick and Moше Shilo for their critical reviews of this manuscript and to express our gratitude to Moše Shilo for encouragement and helpful discussions during the course of this work. The excellent technical assistance of Anat Paz, Ruth Brucker and Rivkah Schwartzkopff is gratefully acknowledged.

This study was supported by a grant from the U.S.-Israel Binational Science Foundation.

LITERATURE CITED


