Anaerobic Energy-Yielding Reaction Associated with Transhydrogenation from Glycerol 3-Phosphate to Fumarate by an *Escherichia coli* System

K. MIKI AND E. C. C. LIN*

Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115

Received for publication 16 June 1975

A particulate subcellular fraction from *Escherichia coli* K-12 induced in anaerobic sn-glycerol 3-phosphate (G3P) dehydrogenase and fumarate reductase can catalyze under anaerobic conditions the transfer of hydrogens from G3P to fumarate, with attendant generation of high-energy phosphate. The phosphorylation process is more sensitive than the transhydrogenation process to inhibition by the detergent Triton X-100. The same is true with respect to sensitivity to sodium azide, carbonyl cyanide m-chlorophenylhydrazone and *N*,*N*-dicyclohexylcarbodiimide. Such a preparation derived from cells with β-galactoside permease can accumulate thiomethyl β-D-galactoside anaerobically, and the accumulation can be stimulated twofold by adding G3P and fumarate. Mutants lacking the membrane-associated Mg**⁺**-dependent adenosine triphosphatase cannot grow anaerobically on glycerol with fumarate as the hydrogen acceptor, although they can grow aerobically on glycerol alone.

In *Escherichia coli*, anaerobic sn-glycerol 3-phosphate (G3P) dehydrogenase (gene product of *glpA*) and fumarate reductase (gene product of *frd*) each can be induced without the other, but when cells are grown anaerobically with glycerol as carbon source and fumarate as hydrogen acceptor both enzymes can be simultaneously induced. A crude extract of such cells can catalyze anaerobically the reduction of fumarate at the expense of G3P without added cofactors. Three properties of this transhydrogenation process implicate a complex of proteins associated in tight organization with the cell membrane. First, the catalytic activity is found exclusively in the pellet fraction after the crude extract is centrifuged at 200,000 × *g*. Second, the coupling activity cannot be readily constituted by mixing two crude cell extracts, one containing only anaerobic G3P dehydrogenase and the other containing only fumarate reductase. Third, the coupled activity is much more sensitive to inhibition by mild detergents than the single enzyme activities (4, 6, 9–11, 18, 25).


**MATERIALS AND METHODS**


**Chemicals.** Casein acid hydrolysate, salt-free and vitamin-free, was purchased from Nutritional Biochemicals Co., Cleveland, Ohio; 2-deoxyglucose was from General Biochemicals, Chagrin Falls, Ohio; dihydroxyacetone phosphate (DHAP), dimethylketal monooctyloxyethylene monohydrate, bovine serum albumin, *N*,*N*-dicyclohexylcarbodiimide, and *N*-tris(hydroxymethyl)-methyl-2-aminoethane sulfonic acid (TES) were from Sigma Chemical Co., St. Louis, Mo.; carbonyl cyanide *m*-chlorophenylhydrazone was...
from Calbiochem, San Diego, Calif.; Triton X-100 was from Rohm and Haas Co., Philadelphia, Pa.; yeast hexokinase was from Boehringer-Mannheim Co., Mannheim, Germany; and [3P]orthophosphate and [14C]thiomethyl β-D-galactoside (TMG) were from New England Nuclear Corp., Boston, Mass.

Preparation of the particulate subcellular fraction. Cells of strain 135 grown anaerobically at 37°C in a basal mineral medium (26) containing 0.02 M glycerol as principal carbon source, 0.02 M fumarate as hydrogen acceptor, 0.03% casein hydrolysate as growth stimulant (10), and 100 μM of threonine, 60 μM of leucine, and 4 μM of thiamine per ml as required supplements were harvested upon reaching stationary phase by centrifugation and washed once with 10 mM MgSO4 and 10 mM TES (adjusted to pH 7.5 with potassium hydroxide). They were resuspended at 0.2 g of fresh cells per ml of 10 mM TES (pH 7.5) and 250 mM sucrose for passage twice through an Aminco French pressure cell (Silver Spring, Md.) set at 20,000 lb/in². After removal of the cellular debris by centrifugation at 12,000 × g for 20 min at 5°C, the extract was recentrifuged at 200,000 × g in a model L5-50 Beckman ultracentrifuge for 2 h at 5°C. The collected pellet fraction was gently suspended at 20 mg of protein per ml of 50 mM TES, 250 mM sucrose, 10 mM MgSO4, and 0.05 mM ethylene-diaminetetraacetate (pH of mixture adjusted to 7.5).

Assay of hydrogen transfer from G3P to fumarate. The G3P-fumarate transhydrogenation reaction was measured anaerobically at 30°C by the fumarate-dependent formation of DHAP from G3P (18).

Formation of ATP. The yield of high-energy phosphate was measured by the phosphorylation of a glucose analogue. Into the side arm of a Thunberg tube was placed 0.1 ml of the particulate fraction containing about 2 mg of protein, and into the main compartment was placed 2.9 ml of 10 mM G3P, 10 mM potassium fumarate, 50 mM 2-deoxyglucose, 10 mM [3P]orthophosphate (60 to 150 counts/min per nmol), 2 mM adenosine 5′-diphosphate, 10 mM MgSO4, 1 mM dithiothreitol, 100 mM sucrose, 30 mM sodium fluoride, and 50 mM TES containing a total of 100 μM of hexokinase and 3 mg of serum albumin (both dialyzed against 1 mM TES-1 mM MgSO4 at pH 7.0). The final pH of the mixture was 7.0. After the air was evacuated from the vessel by aspiration, temperature equilibration was carried out by immersion in a water bath at 30°C for 2 min. The reaction was initiated by mixing the contents of the side arm and the main compartment and was terminated 3 min later (unless otherwise specified) by the addition of 0.5 ml of 2 N HClO4. The supernatant fluid recovered by centrifugation was neutralized by 0.5 ml of 2 N potassium hydroxide and chilled in an ice bath for 10 min. The precipitate was removed by centrifugation, and the clarified solution was analyzed for its DHAP (18) and hexose-phosphate (14) contents. The latter determination involved mixing 0.5 ml of the sample with 0.5 ml of 0.1 M unlabeled glucose 6-phosphate, 5.0 ml of isobutanol-benzene (1:1, vol/vol; saturated with water), and 1 ml of water. After vigorous shaking, 1 ml of 5% ammonium molybdate (wt/vol) in 4 N sulfuric acid was added. The mixture was again shaken vigorously, and the formed upper phase was withdrawn. After two more extractions with 5 ml of the organic solvent, 1 ml of the lower phase was mixed with 10 ml of Bray's solution (2) for measurement of radioactivity. A reaction mixture with fumarate omitted served as a blank.

Anaerobic TMG accumulation by subcellular particles. Cells of strain 150 grown anaerobically on glycerol and fumarate were disrupted, and the 200,000 × g pellet fraction was suspended at a density of 20 mg of protein per ml of 10 mM MgSO4, 250 mM sucrose, and 100 mM potassium phosphate (pH 7.0), according to the procedure described above. A 0.3-ml sample of this suspension was evacuated in a vessel for 5 min by aspiration. After equilibration at 30°C for 2 min, 0.05 ml of 5 mM [14C]TMG (2,500 counts/min per nmol) and 0.1 ml of 100 mM potassium phosphate (pH 7.0), or 0.1 ml of the same buffer containing 45 mM G3P alone, 45 mM fumarate alone, or 45 mM G3P plus 45 mM fumarate, were added to start the transport reaction. The vessel was then flushed with nitrogen gas, and at various intervals 0.05-ml samples were withdrawn, diluted with 5 ml of chilled mineral medium (26), and passed through a membrane filter (type HA, 0.45-μm pore size; Millipore Corp., Bedford, Mass.) mounted on a sintered-glass disk over a vacuum. The material retained was washed once with 5 ml of the same chilled medium.

Protein determination. Contents of protein were determined by the method of Lowry et al. (17) with bovine serum albumin as a standard.

RESULTS

Correlation of transhydrogenation and phosphorylation. Strain 135, lacking aerobic G3P dehydrogenase (gdPD−) and succinate dehydrogenase (sdh−), was used to avoid the possible participation of these two enzymes in the reactions studied. The particulate fraction prepared from this mutant catalyzed a fumarate-dependent dehydrogenation of G3P, which was accompanied by the generation of a high-energy intermediate (or state) detectable by the phosphorylation of 2-deoxyglucose to [3P]2-deoxyglucose 6-phosphate (H6P) in the presence of hexokinase, adenosine 5′-diphosphate, and [3P]orthophosphate. Both the transhydrogenation and the phosphorylation proceeded at a linear rate over a period of 10 min (Fig. 1), and the rate of both reactions was proportional to the amount of pellet protein added (Fig. 2).

Neither G3P nor fumarate alone stimulated the formation of DHAP or [3P]H6P. Moreover, the increased formation of [3P]H6P in the presence of both G3P and fumarate was not attributable to the generated DHAP and succinate, which, in turn, might have been further metabolized since the provision of these two compounds in lieu of G3P and fumarate did not
produce the same result. On the other hand, the presence of the added DHAP and succinate did not interfere with the ability of G3P and fumarate to stimulate \(^{32}P\)H6P production (Table 1). It can therefore be concluded that the net generation of high-energy phosphate is a direct consequence of the transfer of hydrogens from G3P to fumarate.

**Inhibition of the reactions.** Although the transhydrogenation and phosphorylation reactions are connected, the functional requirements of the latter are evidently more stringent than those of the former. Triton X-100, previously shown to inhibit the hydrogen transfer reaction, presumably by deranging the protein and membrane phospholipid interaction (18), was found to be even more potent in disrupting the phosphorylation process (Fig. 3).

Azide, carbonyl cyanide \(m\)-chlorophenylhydrazone, and \(N,N'\)-dicyclohexylcarbodiimide, generally known to inhibit respiratory phospho-

**TABLE 1. Dependence of DHAP and H6P formation on the presence of G3P and fumarate**

<table>
<thead>
<tr>
<th>Additions to reactions mixture</th>
<th>nmol of product formed/min per mg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>G3P Fu-</td>
<td>DHAP</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

\(\textsuperscript{a}\) Where indicated as a "+", 1.2 \(\mu\)mol was added.

\(\textsuperscript{b}\) A blank value of 4 U was subtracted.

\(\textsuperscript{c}\) A blank value of 18 U was subtracted.

\(\textsuperscript{d}\) Two hundred and five units should be subtracted from all numbers in parentheses to correct for the DHAP added to the system.

---

**FIG. 1.** Proportionality of the amounts of DHAP (O) or \(^{32}P\)H6P (●) formed to the time of incubation. The reaction mixture contained 0.92 mg of protein.

**FIG. 2.** Rates of fumarate-dependent G3P dehydrogenation (O) and associated formation of \(^{32}P\)H6P (●) as functions of protein concentration. The complete mixture, with fumarate omitted, served as a reaction blank; the amount of DHAP formed was 5 nmol/min per mg of protein and the amount of \(^{32}P\)H6P formed was 14 nmol/min per mg of protein.

**FIG. 3.** Effect of Triton X-100 on activities of the fumarate-dependent G3P dehydrogenation (striped bar) and phosphorylation (white bar). The uninhibited dehydrogenation activity was 251 nmol/min per mg of protein, and that of \(^{32}P\)H6P formation was 39 nmol/min per mg of protein. Blank values obtained with G3P addition alone were subtracted in all cases.
rylation, including systems of *E. coli* (8, 12, 21, 24), were also more effective in interfering with phosphorylation than transhydrogenation (Table 2).

**Anaerobic stimulation of TMG accumulation.** The subcellular pellet fraction prepared from strain 150, constitutive in β-galactoside permease, showed some ability to take up [14C]TMG. This process was stimulated more than twofold by the addition of G3P and fumarate (Fig. 4). It thus appears that the energy generated by the transhydrogenation reaction could also be harnessed for active transport by this permeation system.

**Growth behavior of ATPase mutants.** Two mutants defective in their membrane-associated ATPase (uncA) and their parents were compared for their abilities to grow on glycerol, with either fumarate or molecular oxygen as the exogenous hydrogen acceptor. Whereas all four strains grew under the aerobic condition (through the mediation of their aerobic G3P dehydrogenase), only the parental ATPase-positive strains grew with fumarate (Table 3).

**TABLE 2. Inhibition of transhydrogenation versus phosphorylation reactions**

<table>
<thead>
<tr>
<th>Inhibitor added</th>
<th>Conc (mM)</th>
<th>DHAP formed (%)</th>
<th>[14C]H6P formed (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>NaN₄</td>
<td>2</td>
<td>100</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>55</td>
<td>10</td>
</tr>
<tr>
<td>CCCP</td>
<td>0.01</td>
<td>102</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>64</td>
<td>33</td>
</tr>
<tr>
<td>DCCD</td>
<td>0.05</td>
<td>106</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>99</td>
<td>20</td>
</tr>
</tbody>
</table>

* CCCP, Carbonyl cyanide m-chlorophenylhydrazone; DCCD, N,N'-dicyclohexylcarbodiimide.

**DISCUSSION**

The $E'_{\text{m}}$ (midpoint potential) of the G3P-DHAP redox reaction is $-0.19 \text{ V}$, and that of the succinate-fumarate redox reaction is 0.00 V. The coupling of these two systems should therefore yield a $-\Delta G^\circ$ of about 8.8 kcal. The actual ability of the cell to harness this potential source of energy has been revealed in several ways.

At the level of intact cells, it was found that the initiation of *E. coli* K-12 killing by colicin K has an energy prerequisite, which can be satisfied anaerobically by providing cells induced in anaerobic G3P dehydrogenase and fumarate reductase with the two relevant substrates (7). In the present study it was observed that mutants defective in the membrane ATPase, an

**TABLE 3. Hydrogen acceptor dependence of wild-type and ATPase mutants for growth on glycerol as carbon source**

<table>
<thead>
<tr>
<th>Strain</th>
<th>glpA</th>
<th>glpD</th>
<th>uncA</th>
<th>Fumarate</th>
<th>Molecular oxygen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>134</td>
<td>290</td>
</tr>
<tr>
<td>135</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>148</td>
<td>9</td>
</tr>
<tr>
<td>AN120</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>10</td>
<td>240</td>
</tr>
<tr>
<td>AN180</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>125</td>
<td>280</td>
</tr>
<tr>
<td>N144</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>44</td>
<td>235</td>
</tr>
<tr>
<td>A428</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>132</td>
<td>265</td>
</tr>
</tbody>
</table>

* Strains AN120 and AN180 were grown in mineral media supplemented with 0.2 mM arginine and 0.5 µM thiamine. Strains N144 and A428 were grown in mineral media supplemented with 0.2 mM proline, 0.2 mM histidine, and 0.5 µM thiamine. For anaerobic growth, 0.63% casein hydrolysate was added as a stimulant.

* One Klett unit corresponds about $2 \times 10^6$ cells/ml.

* Exogenous hydrogen acceptor.
enzyme with a crucial role in energy transduction (3, 8, 12, 24), could not grow anaerobically on glycerol with fumarate as the hydrogen acceptor, although they could grow aerobically on glycerol alone.

At the level of Kaback vesicles, membranes prepared from E. coli ML grown anaerobically on glycerol and fumarate increased their rate of anaerobic lactose transport when G3P was added with fumarate (13).

At the level of the subcellular particles used in the present study, two energy-yielding activities were manifested in the presence of G3P and fumarate: the net formation of ATP and the stimulation of TMG accumulation. The interference of the ATP-generating process by inhibitors of energy metabolism, such as azide, carbonyl cyanide m-chlorophenylhydrazone, and N,N'-dicyclohexylcarbodiimide, and the implication of ATPase in this process suggest that the mechanism of energy generation is akin to that of oxidative phosphorylation, mediated by a proton motive force according to the model of Mitchell (5, 19, 20, 28). The presence of closed membrane vesicles would then be required to explain the results. Electron microscopic examination of the preparation indeed revealed numerous vesicular structures with diameters of about 0.1 μm, some apparently sealed and others not, amidst a background of ribosomal particles (courtesy of D. W. Fawcett, Department of Anatomy, Harvard Medical School).

The presence of unsealed vesicles may explain the low yield of 1 mol of ATP formed per 10 mol of G3P dehydrogenated ([14C]H6P/DHAP of about 0.1), since a leaky membrane cannot be expected to maintain a proton gradient. Both the heterogeneity of the vesicles and the presence of ribosomes might account for the low specific TMG transport activity of the preparation used in this study relative to that observed for lactose accumulation in Kaback vesicles. On the other hand, this TMG transport activity compared more favorably with the ascorbate and phenazine methosulfate-stimulated aerobic accumulation of β-D-galactosyl-l-thio-β-D-galactoside in another subcellular preparation of E. coli K-12 disrupted by decompression (1).

To account for the ability of our preparation to catalyze both TMG accumulation and ATP generation in the framework of Mitchell's hypothesis (27, 28), it might be postulated that some vesicles were normally oriented with respect to the phospholipid bilayer, whereas others were everted. The former accumulated TMG (Fig. 5), whereas the latter generated ATP (Fig. 6). In the case of the normal vesicles, TMG, G3P, and fumarate (15, 16, 23) entered the vesicles through their respective permeases. In the case of the everted vesicles, no permeation system was necessary, since the complete enzymatic machinery was exposed to the reactants in the incubation medium.

Finally, it is tempting to imagine that the G3P-fumarate system was one of the earliest energy transformation mechanisms to appear during evolution, since the reaction required only a few proteins and no molecular oxygen. To this "one-site phosphorylation" machine additional electron (or hydrogen) carriers might have been sequentially added during the natural history of the elongation of the electron transfer chain. Sulfite and nitrate respiratory systems would thus appear before the final emergence of true aerobic respiration.
ACKNOWLEDGMENTS

This investigation was supported by Public Health Service research grant 5 ROI GM11983 from the National Institute of General Medical Sciences and by grant GB-43288X from the National Science Foundation.

We wish to thank T. H. Wilson for helpful discussions.

LITERATURE CITED


