Hydrogen Sulfide Production and Fermentative Gas Production by *Salmonella typhimurium* Require F$_0$F$_1$ ATP Synthase Activity

KYLE C. SASAHARA, NINA K. HEINZINGER,† AND ERICKA L. BARRETT*

Department of Food Science and Technology, University of California, Davis, California 95616-8598

Received 5 May 1997/Accepted 30 August 1997

A previously isolated mutant of *Salmonella typhimurium* lacking hydrogen sulfide production from both thiosulfate and sulfite was shown to have a single mutation which also caused the loss of fermentative gas production and the ability to grow on nonfermentable substrates and which mapped in the vicinity of the *atp* chromosomal locus. The implication that F$_0$F$_1$ ATP synthase might be essential for H$_2$S and fermentative gas production was explored. The *phs* plasmid conferring H$_2$S production on wild-type *Escherichia coli* failed to confer this ability on seven of eight *E. coli* *atp* point mutants representing, collectively, the eight genes encoding the subunits of F$_0$F$_1$ ATP synthase. However, it did confer some thiosulfate reductase activity on all except the mutant with a lesion in the ATP synthase catalytic subunit. Localized mutagenesis of the *Salmonella atp* chromosomal region yielded 500 point mutants unable to reduce thiosulfate to H$_2$S or to produce gas from glucose, but differing in the extents of their ability to grow on succinate, to perform proton translocation as measured in a fluorescence quenching assay, and to reduce sulfite to H$_2$S. Biochemical assays showed that all mutants were completely devoid of both methyl viologen and formate-linked thiosulfate reductase and that *N,N*-dicyclohexylcarbodiimide blocked thiosulfate reductase activity by the wild type, suggesting that thiosulfate reductase activity has an absolute requirement for F$_0$F$_1$ ATP synthase. Hydrogenase-linked formate dehydrogenase was also affected, but not as severely as thiosulfate reductase. These results imply that in addition to linking oxidation with phosphorylation, F$_0$F$_1$ ATP synthase plays a key role in the proton movement accompanying certain anaerobic reductions and oxidations.

**MATERIALS AND METHODS**

**Bacterial strains, media, and chemicals.** The bacterial strains used are listed in Table 1. All bacteria were cultivated at 37°C. Aerobic cultures were grown on roller drums or a rotary shaker at 250 rpm. Anaerobic cultures were grown in standing flasks that were filled to the top. Anaerobic conditions for solid media were achieved in sealed GasPak jars (BBL Microbiology Systems) with a catalyst generating a 95% H$_2$ and 5% CO$_2$ atmosphere.

Nutrient broth, yeast extract, peptone iron agar, and triple sugar iron agar were from Difco Laboratories. The following other media were formulated as previously described: formate hydrogen lyase broth (3), thiosulfate reductase broth and nitrate broth (11), sulfite tubes (5), Luria broth (19), and M9 minimal agar base (7), to which carbon sources were supplemented at a 1% (wt/vol) concentration. Tetracycline was added to selection media at 20 μg/ml for complex media and 10 μg/ml for minimal media as noted. The 9-amino-6-chloro-2-methoxyacridine (ACMA) used for fluorescence-quenching experiments was from Molecular Probes. Carbonyl cyanide m-chlorophenylhydrazone (CCCP), *N,N*-dicyclohexylcarbodiimide (DCCD), and all other chemicals were from Sigma.

**Genetic methods.** *Escherichia coli* *atp* strains were made competent with calcium and transformed with *phs* plasmid pEB40 (9) by the methods described by Maniatis et al. (18). Hydroxylamine-localized mutagenesis was performed as described by Hong and Ames (12) with P22 HT105·201 grown on *Tu* strain (10) AK319 or AK3241. Tetracycline-resistant mutants were selected with wild-type LT2 as the recipient on Luria-Bertani–galactose with 20 μg of tetracycline per ml.

**Qualitative tests and enzyme assays.** Isolates were screened for H$_2$S production by staining colonies in peptone iron agar (thiosulfate reduction) and sulfite agar (sulfate reduction) and for their ability to grow on succinate with aerobically
RESULTS

Characterization of Mut1 (Ap’ lac’) fusion strain EB299. Further physiological studies of H_2S^- strain EB299 revealed that it required both a fermentable sugar and an electron acceptor for growth; although it grew on glucose minimal medium aerobically, it was unable to grow on succinate, malate, or lactate aerobically and was unable to grow on glucose anaerobically in the absence of an electron acceptor such as nitrate, trimethylamine oxide, or tetrathionate. When the electron acceptor was added to liquid cultures, the final densities obtained with strain EB299 were 40 to 60% lower than those obtained in wild-type cultures. Transductional correction of a number of growth deficiencies, e.g., by selection for the ability to grow aerobically on succinate, restored the ability of all transductants to grow under all of the conditions described as well as the ability to make H_2S from both thiosulfate and sulfite and the ability to make gas from glucose. These results strongly suggested that all of the defects could be traced to single mutation in a locus encoding something essential for the coupling of phosphorylation with respiration as well as for H_2S and H_2 production.

Conjugational and transductional mapping placed the mutation site in the vicinity of the Salmonella atp locus. The atp locus in Salmonella has not been extensively studied, but it is located at a site homologous to the E. coli atp locus, and the E. coli atp locus has been shown to encode all of the subunits of F_0F_1 ATP synthase (24). The mutation in strain EB299 was 41% cotransduced with Tn10 in strain AK3119 and 30% with Tn10 in strain AK3241, which are located, respectively, at approximately min 81 and 82 on the edition VII map (22). The uncA gene, which is now designated atp, is located at min 82 on this map. (It is located at min 84.6 on the edition VIII map [21].)

Expression of β-galactosidase by strain EB299 was assayed in cultures grown aerobically and anaerobically in unsupplemented nutrient broth or in nutrient broth supplemented with nitrate, thiosulfate, trimethylamine oxide, or glucose. Relatively strong expression (450 to 900 Miller U) was found under all conditions examined. Kasimoglou et al. (15) obtained similar results with an atp::lacZ fusion, namely, a narrow range of high expression levels under all growth conditions examined.

The results described above suggested that strain EB299 contains a mutation in the atp operon. They suggested further that ATP synthase may be required not only for the coupling of respiration with phosphorylation, but also for production of H_2S and gas from glucose. The following experiments were designed to test the latter hypothesis.

Expression of phs in an ATP synthase^- background. We showed previously that S. typhimurium on plasmid pEB40, which contains the complete phs operon, confers on E. coli the ability to produce H_2S from thiosulfate (9). If F_F_F_F ATP synthase is required for H_2S production, then phs should not confer H_2S production on an E. coli host lacking it. We transformed a series of eight E. coli atp mutants containing nitrinosguanidine-induced point mutations in each of the F_F_F_F ATP synthase subunits, respectively, with plasmid pEB40 and then assayed the transformants for H_2S production and thiosulfate reductase activity (Table 2). Only the atp^- host and the host mutated in atpC (encoding the r subunit of the F_1 portion [not at the catalytic site]) produced any H_2S, and the atpC host produced very little compared to its atp^- parent. On the other hand, all transformants except the uncD mutant exhibited significant thiosulfate reductase activity, which was measured as
TABLE 3. Effect of DCCD on formate dehydrogenase and on thiosulfate and nitrate reductases in vesicles of wild-type \textit{S. typhimurium}

<table>
<thead>
<tr>
<th>Enzyme $^{a}$</th>
<th>Sp act $^{b}$</th>
<th>−DCCD</th>
<th>+DCCD</th>
</tr>
</thead>
<tbody>
<tr>
<td>FDH$_{II}$</td>
<td>115</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>FDH$_{I}$</td>
<td>327</td>
<td>434</td>
<td></td>
</tr>
<tr>
<td>Thiosulfate reductase</td>
<td>19</td>
<td>&lt;0.4</td>
<td></td>
</tr>
<tr>
<td>Nitrate reductase</td>
<td>43</td>
<td>15</td>
<td></td>
</tr>
</tbody>
</table>

$^{a}$ FDH$_{II}$ formate as donor and benzyl viologen as acceptor; FDH$_{I}$, formate as donor and phenazine methosulfate-dichlorophenolindophenol as acceptor; thiosulfate reductase, methyl viologen as donor and thiosulfate as acceptor; nitrate reductase, methyl viologen as donor and nitrate as acceptor.

$^{b}$ Specific activity expressed as nmol oxidized or reduced artificial donor or acceptor (see footnote $a$) per minute per milligram of protein. Vesicles were either preincubated for 20 min with DCCD added at a concentration of 28 μm or were assayed without DCCD.

Effect of DCCD on H$_2$S production by \textit{Salmonella}. If a functional F$_0$F$_1$ ATP synthase is essential for thiosulfate reduction to H$_2$S and for the production of gas from glucose via formate hydrogen lyase in \textit{Salmonella}, then DCCD, which binds to and thereby inactivates the F$_0$ portion, should interfere with these activities. We assayed the effect of DCCD on thiosulfate reductase and formate hydrogen lyase in vesicles prepared from wild-type \textit{S. typhimurium} and compared the results with those obtained for enzymes involved in nitrate respiration, which is an activity which did not appear deficient in qualitative tests of strain EB299. The assays (Table 3) showed that DCCD had no effect on FDH$_{II}$, while it reduced the activities of nitrate reductase, methyl viologen-linked nitrate reductase, but not methyl viologen-linked nitrate reductase. In crude extracts of class I and III mutants, loss of ATP synthase activity affected the loss of ATP synthase activity (Table 4), indicating that they were, in fact, defective in this enzyme. It is interesting that so many isolates were negative for H$_2$S from thiosulfate and gas from glucose, while retaining their ability to grow on succinate, suggesting that H$_2$S and gas production may be more sensitive to small perturbations in F$_0$F$_1$ ATP synthase than is the coupling of oxidation to phosphorylation.

Proton translocation by the \textit{atp} mutants. Inverted membrane vesicles of representative mutants were assayed for proton translocation with the fluorescent probe ACMA (Fig. 1). Quenching of the fluorescence of free ACMA occurs when ATP is hydrolyzed by the F$_1$ portion of the complex on the vesicle surface and protons are pumped through the F$_0$ channel. Subsequent addition of the protonophore CCCP allows the dye accumulated inside the vesicle to move back out, restoring fluorescence which was quenched through proton translocation. The class I mutant translocated protons almost as effectively as the wild type, the class II mutant was somewhat less effective, and the class III mutant was completely devoid of translocation activity. These results are consistent with the mutant phenotypes.

Anaerobic reductase and dehydrogenase activities of the \textit{atp} point mutants. Assays of the specific reductases and dehydrogenases were performed to pinpoint the enzyme activities affected by the loss of ATP synthase activity (Table 4). In crude extracts of class I and III mutants, loss of ATP synthase activity was associated with a loss of methyl viologen-linked thiosulfate reductase, but not methyl viologen-linked nitrate reductase. In class II mutant extracts, both reductases were negatively af-

TABLE 4. Enzyme activities and other characteristics of representative \textit{atp} mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>H$_2$S from S$_2$O$_3^{2-}$</th>
<th>H$_2$S from SO$_3^{2-}$</th>
<th>Gas$^{b}$</th>
<th>Growth on succinate</th>
<th>Enzyme sp act$^{a}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ATPase</td>
</tr>
<tr>
<td>LT2 (wild type)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>5.3</td>
</tr>
<tr>
<td>EB299</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>&lt;1</td>
<td>0.12</td>
</tr>
<tr>
<td>EB600 (class I)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td></td>
<td>0.14</td>
</tr>
<tr>
<td>EB613 (class II)</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td></td>
<td>0.08</td>
</tr>
<tr>
<td>EB625 (class III)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td></td>
<td>0.10</td>
</tr>
</tbody>
</table>

$^{a}$ ATPase, micromoles of P$_i$ released; FDH$_{II}$, nanomoles of benzyl viologen reduced; FDH$_{I}$, nanomoles of dichlorophenolindophenol reduced; thiosulfate reductase (TSR), nanomoles of sulfide produced with formate or methyl viologen (MV); nitrate reductase (NaR), nanomoles of nitrite produced with formate or methyl viologen. All assays were performed with crude extracts.

$^{b}$ Production of gas during fermentation of glucose.
fected to the same extent. In all mutants, FDH₄ and FDH₅ were severely impaired. The lack of the latter enzyme probably explains the fact that class I and III mutants were unable to reduce nitrate with formate. Surprisingly, class II mutants did retain formate-linked nitrate reduction, although they had no detectable FDHN activity (with phenazine methosulfate-di-chlorophenolindophenol as the acceptor). Apparently, the defect in this class is leaky with respect to several oxidoreductase activities, including sulfite reductase, as shown in the initial mutant characterization. The assay results do not provide any clues regarding the specific sites of mutations in the three respective classes. They suggest instead that the classes represent different levels of mutation severity in the context of separate and unique interactions between native F₀F₁ ATP synthase and each of the various oxidoreductases included in the study.

DISCUSSION

The results described above strongly suggest that a fully functional F₀F₁ ATP synthase is an absolute requirement for the production of H₂S from thiosulfate and gas (H₂ and CO₂) from glucose. Two lines of evidence strongly support this conclusion: (i) the observation that the phs operon fails to confer H₂S production on E. coli atp mutants and (ii) the isolation of more than 500 point mutants defective in F₀F₁ ATP synthase, all of which failed to produce H₂S from thiosulfate and gas from glucose, even though all of them produced nitrite from nitrate in qualitative tests (and thus did not have a pleiotropic mutation affecting all molybdoenzymes). The finding that DCCD interferes with thiosulfate reduction and, to a lesser extent, FDHH activity is also consistent with the requirement for F₀F₁ ATP synthase in H₂S and gas production, although direct inhibition of these enzymes by DCCD could not be ruled out by the experimental results. Assays of specific reductases and dehydrogenases in the mutant atp background revealed electron transfer from either formate or methyl viologen to thiosulfate to be completely blocked. In the same background, both FDH₄ and FDH₅ were severely impaired but not inactive. The complete loss of fermentative gas production in the mutant atp background could be the result of a more serious impairment in the activity of the associated hydrogenase.
The role of \( F_{0}F_{1} \) ATP synthase in maintaining the protonic potential of bacterial cell membranes in the context of ATP synthesis and hydrolysis is now well-established (see reference \( 8 \) for a review). However, this complex has not previously been implicated as an essential part of proton movement associated with oxidation-reduction enzymes in the absence of phosphorylation. Perhaps the proton movement accompanying thiosulfate reduction to \( H_2S \) and formate oxidation to \( H_2 \) and \( CO_2 \) resembles that of proton-dependent transport systems, such as some of the proton-dependent multidrug efflux systems (20), more than it resembles proton export via electron transport chains. One possible model for the role of \( F_{0}F_{1} \) ATP synthase in thiosulfate reduction as well as sulfite reduction (a soluble system) would include the separate transport of \( S^{2-} \) and \( H^{+} \) to the exterior to form \( H_2S \), with the proton movement relying on the \( F_{0}F_{1} \) complex. Similarly, hydrogen gas might be formed outside the cell after protons are exported via the \( F_{0}F_{1} \) complex and electrons are brought to the exterior via membrane electron transport.

Three phenotypically distinct classes of mutants defective in \( F_{0}F_{1} \) ATP synthase and thiosulfate reductase were isolated, but the respective phenotypes did not suggest specific defects in \( F_{0}F_{1} \) ATP synthase subunits. Analysis of the phenotypes at this stage is difficult because the \( atp \) operon in \( S. typhimurium \) has not been analyzed and specific interactions of \( F_{0}F_{1} \) ATP synthase with anaerobic oxidoreductases have not been previously described. The range of defects regarding the anaerobic enzymes assayed suggests that ATP synthase may interact with several anaerobic enzymes in different ways. The thiosulfate reductase activities of the mutants isolated in this study were lower than the activities determined for most of the \( E. coli \) \( atp \) mutants transformed with the \( phs \) plasmid. This difference may reflect the multicopy nature of the \( phs \) plasmid and/or different regulatory systems operating in the \( E. coli \) background compared to the \( S. typhimurium \) background.

The requirement for thiosulfate reductase for \( F_{0}F_{1} \) ATP synthase is yet another feature that distinguishes thiosulfate reduction from other anaerobic reductions which involve structurally similar membrane-bound electron transport systems, such as nitrate and trimethylamine oxide reductions. Unlike these other systems, thiosulfate reduction is extremely sensitive to catabolite repression (6) and does not support growth on nonfermentative substrates (11). We have hypothesized that the production of \( H_2S \) as a toxic molecule may instead play a specific role in gastroenteritis (2). Experiments to test this hypothesis are in progress.

ACKNOWLEDGMENTS

Early phases of this work were supported by Public Health Services grant AI-22685 from the National Institutes of Health, and later phases were supported by funds from the California Agricultural Experiment Station.

We thank Lyndall Hatch for providing the \( E. coli \) ATP synthase mutants. We thank Daniel Klionsky for guidance in the ATP synthase assay procedures and Anh Nguyen for help in performing the assays.

REFERENCES