Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 6793–6796, October 1985
Biochemistry

Electron transfer-driven ATP synthesis in *Methanococcus voltae* is not dependent on a proton electrochemical gradient

(methanogens/bioenergetics/chemiosmotic theory/substrate-level phosphorylation/electron transport)

B. P. CRIDER*, S. W. CARPER, AND J. R. LANCASTER, JR.†

Department of Chemistry and Biochemistry, Utah State University, Logan, UT 84322

Communicated by Irwin Fridovich, June 24, 1985

ABSTRACT **Intracellular ATP levels in whole cells of *Methanococcus voltae* respond to electron transfer coupled to methanogenesis. ATP synthesis can also be induced by an artificially imposed transmembrane electrical potential [formed by electrogenic movement outwards of potassium (induced by valinomycin) or of protons (induced by an uncoupler], or by a pH gradient (acid outside). These results implicate the existence of a reversible ATPase coupled to electrogenic movement of an ion(s) other than potassium or proton, and that ionophores are competent to catalyze ion movement across the cytoplasmic membrane of this organism (which is the sole membrane structure in this species). ATP synthesis driven by electron transfer is, however, insensitive to the addition of such ionophores. These results indicate that although cells possess an ion-translocating ATPase (possibly involved in the maintenance of internal ionic composition homeostasis), methanogenesis-driven ATP synthesis does not involve the intermediacy of a transmembrane ion gradient. Primarily because methane formation has been previously demonstrated to involve true electron transfer, substrate-level phosphorylation (at least in analogy to other systems) has been generally ruled out. The results presented here suggest that at least one methanogenic bacterium may use a direct linkage of ATP synthesis to electron transfer.**

Nonphotosynthetic energy conservation is currently believed to occur by two mechanisms, electron transport phosphorylation (ETP) and substrate level phosphorylation (SLP) (1). In ETP, ATP synthesis is coupled to protein-mediated electron transfer by the intermediacy of a transmembrane electrochemical ion gradient. In SLP, ATP synthesis is accomplished through phosphorylation of the carbon substrate undergoing the energy-yielding process, followed by transfer of the phosphoryl group, usually to ADP. The methanogenic bacteria are anaerobic archaebacteria that are the sole biological source of methane, most commonly produced by the eight-electron reduction of CO_2 by H_2. The demonstration of autotrophic growth by some methanogens on these gaseous species in the absence of any added carbon substrate has apparently ruled out SLP (1).

Methanococcus voltae is a halotolerant osmotically fragile organism, capable of growth and methanogenesis from either reduction of CO_2 by H_2 or by metabolism of formate to CO_2 and CH_4. We chose this organism for the studies reported here because of the fragility of the cells [probably a result of a proteinaceous cell wall (2)], which might render them ideally sensitive to ionophores. A significant complication in many studies on energy coupling in methanogens has been the sensitivity of the electron transfer pathway to halogenated hydrocarbons (3, 4), including the most commonly utilized uncouplers. Thus, we chose SF6847 for this study, which is not a halogenated compound and fully uncouples mitochondria at a concentration of 30 nM (5). Finally, *M. voltae* does not appear to possess intracellular membranous structures (2), and so results with ionophores can be interpreted in terms of a single site of action, the cytoplasmic membrane.

We report here that although cells of *M. voltae* possess an electrogenic ion-translocating ATPase (not involving either protons or potassium), electron transfer-driven ATP synthesis appears not to involve the obligatory intermediacy of a transmembrane ion gradient.

MATERIALS AND METHODS

M. voltae PS was grown in the medium described by Whitman *et al.* (6) with the addition of 1 g of sodium formate per liter. Cells were harvested in the midlogarithmic phase of growth, washed, and resuspended in 1/100th of the original culture volume in salt buffer (0.4 M NaCl/10 mM $MgSO_4$/10 mM Tricine/KOH, pH 8.0).

Three milliliters of the cell suspension was placed in 120-ml serum stoppered bottles, which were then capped. The gas phase was changed by blowing the desired gas over the cell suspension. For the measurement of methane, the cells were placed in a shaking 38°C water bath, and 50-μl head-space gas samples were removed and injected into a gas chromatograph equipped with a Porapak Q column and a flame ionization detector. For the measurement of ATP levels, the cells were placed in a shaking 38°C water bath, and 50-μl samples were removed and assayed for ATP by the luciferin–luciferase method as described by Kimmich *et al.* (7).

All manipulations were performed under strictly anaerobic conditions. SF6847 was a gift from P. Hinkle of Cornell University. Ionophores were added as ethanolic solutions. Protein was determined by the method of Markwell *et al.* (8).

RESULTS

SF6847 (5 μM) had no effect on methanogenesis driven by H_2/CO_2 over a time period t of 40 min (Fig. 1). At t = 50 min, reductant (H_2) was removed and monitoring of intracellular ATP was begun. After the decline in ATP subsequent to the cessation of methanogenesis, H_2 was again added at t = 60 min. The rapid synthesis of ATP was unaffected by the presence of SF6847.

The lipids of archaebacteria are highly unusual, being composed of ether-linked polyisoprenoid (branched) chains (9); thus, it is possible that SF6847 is not competent to effectively eliminate proton movement even at this relatively high concentration. Indeed, in at least one study, protonophoric activity required concentrations of at least 1 μM

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: ETP, electron transport phosphorylation; SLP, substrate level phosphorylation.

*Present address: 151G, Veterans Administration Medical Center, Dallas, TX 75216.
†To whom reprint requests should be addressed.

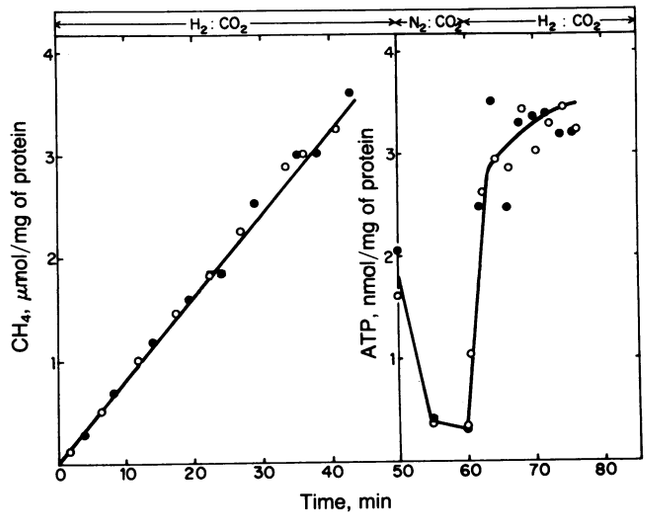

FIG. 1. Effect of SF6847 on methanogenesis and ATP synthesis. The gas phase above the cell suspension was varied as indicated at the top of the figure. ○, Ethanol control; ●, 5 μM SF6847 (0.44 μg/mg of protein) was added 9 min prior to $t = 0$. (*Left*) Methane formation. (*Right*) Methanogenesis-driven ATP synthesis.

in cells of *Halobacterium halobium* (10). In order to test this [and to detect transmembrane electrical potential-driven ATP synthesis, which has been demonstrated in another methanogen (11)], we performed the experiment shown in Fig. 2. Deenergized cells [i.e., no electron donor (H_2)] were suspended in buffer in the absence of external potassium and in the absence or presence of SF6847. Since the internal concentration of potassium in these cells has been reported to be high [>0.7 M (12)], the addition of the potassium-specific ionophore valinomycin should generate a negative membrane potential, which could be utilized to synthesize ATP by cation movement driven inwards through a membrane-bound ATPase. This was indeed the case; valinomycin addition at 5 min resulted in rapid ATP synthesis (Fig. 2). The presence of SF6847 had no effect on this synthesis, indicating that the ion moving in response to the imposed electrical field is not a proton(s).

If the function of this ion-translocating ATPase is to regulate intracellular pH [possibly by proton cotransport or countertransport with other ion(s) (13)], a pulse of acid might, under favorable conditions, result in a driving force for ATP synthesis. Such a phenomenon was demonstrated (Fig. 2) by the addition of acid at $t = 22$ min, resulting in a transient but appreciable rise in ATP levels. The prevention of this effect by SF6847 supports this interpretation and also indicates that this compound is competent to catalyze proton translocation.

Synthesis of ATP driven by valinomycin addition (at $t = 15$ min) was only slightly inhibited by SF6847 but was eliminated by the presence of potassium in the external medium (Fig. 3). This shows that the ATP synthesis is due to a potassium diffusion-generated membrane electrical potential. The lack of effect of the combination of valinomycin, potassium, and SF6847 on subsequent methanogenic electron transport-driven ATP synthesis (formate addition at $t = 45$ min) argues against the involvement of a transmembrane electrochemical potential in this process. The rapid decline in ATP 5 min after formate addition coincided with the cessation of methanogenesis because of the exhaustion of the formate (not shown).

Fig. 4 shows the results of an experiment designed to demonstrate simultaneously that the ion(s) responsible for ATP synthesis is not a proton, that SF6847 is capable of catalyzing electrogenic proton movement, and that valinomycin with external potassium will eliminate the formation of a transmembrane electrical potential. The principle is identical to that shown in Figs. 2 and 3 except that the driving force for the electrical potential formation is a

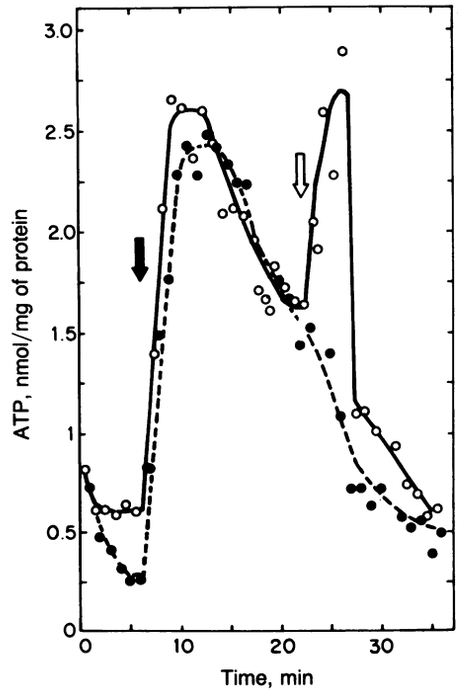

FIG. 2. Effect of SF6847 on ATP synthesis induced by imposed ion gradients. Cells were treated as described in Fig. 1 except that the salt buffer contained 5 mM Tricine. The gas phase above the cultures was 80% N_2/20% CO_2; 10 μM valinomycin (3.4 μg of valinomycin per mg of protein) was added at the time indicated by the solid arrow, and 8 mM HCl was added at the time indicated by the open arrow. Symbols are as in Fig. 1.

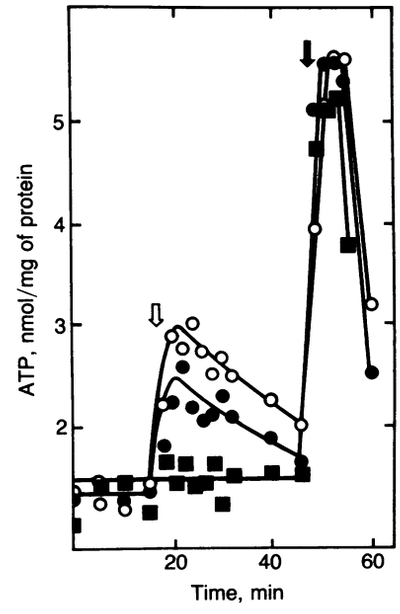

FIG. 3. Ion gradient- and methanogenesis-driven ATP synthesis. Cells were treated as in Fig. 1. Gas phase was 80% N_2/20% CO_2. At the time indicated by the open arrow, valinomycin was added to 10 μM final concentration (3.0 μg of valinomycin per mg of protein); at the solid arrow, sodium formate was added to a final concentration of 4 mg/ml. ○, Ethanol control; ●, 5 μM SF6847 (0.39 μg of SF6847 per mg of protein) added 45 min prior to $t = 0$; ■, 100 mM KCl/5 μM SF6847 added at 45 min prior to $t = 0$.

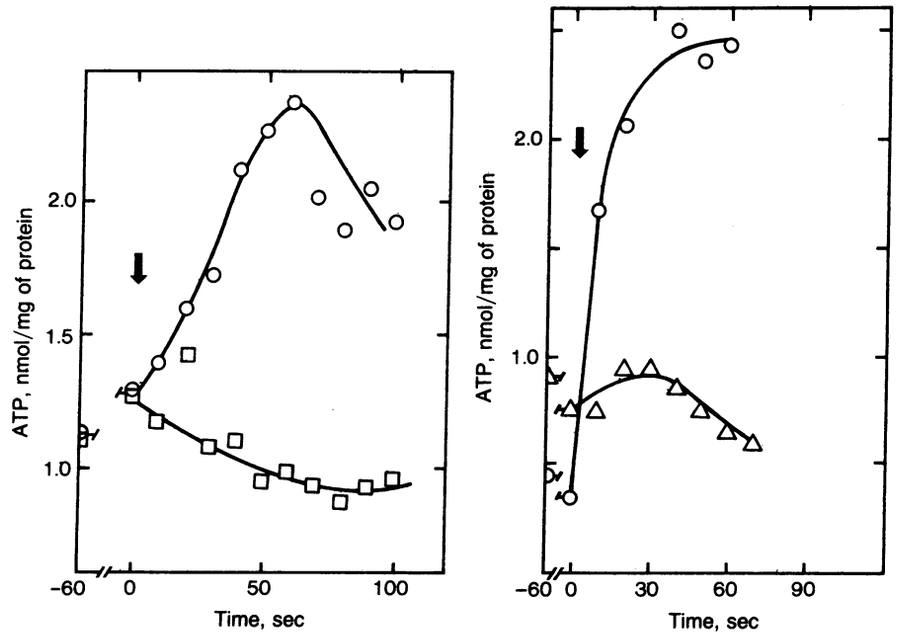

FIG. 4. Uncoupler-induced ATP synthesis. (*Left*) Cells were treated as in Fig. 1 except that the buffer was 0.4 M NaCl/0.1 M KCl/5 mM Tricine/5 mM $MgCl_2$, pH 8.0. SF6847 was added to a final concentration of 5 μM (0.23 μg of SF6847 per mg of protein). The pH was rapidly lowered to 5 by the addition of HCl, and 3 min later the pH was rapidly raised to 7.9 by the addition of KOH (indicated by the solid arrow). ○, Ethanol control; □, 10 μM valinomycin (1.8 μg of valinomycin per mg of protein) was added along with the SF6847. (*Right*) Procedure was similar to that in *Left* except that no valinomycin was added to either cell suspension. ○, SF6847 (0.40 μg of SF6847 per mg of protein) added as in *Left*; △, ethanol control.

chemical gradient of protons (instead of potassium) and the ionophore is SF6847 (instead of valinomycin). Cells were preincubated in acid (pH 5) in the presence of SF6847 and external potassium for a short time period (3 min), and the pH was then rapidly raised to 7.9 by the addition of KOH (added at $t = 0$). ATP synthesis was observed (Fig. 4 *Left*), and the presence of valinomycin eliminated the effect. This ATP synthesis required the presence of the uncoupler (Fig. 4 *Right*). The difference in the initial rate of ATP synthesis in these two experiments could be due to the difference in amount of SF6847 per mg of protein (see the legend to Fig. 4).

We interpret this experiment as follows. The preincubation allowed significant acidification of the intracellular compartment of the cells, and alkalinization of the external medium produced a chemical concentration gradient for protons, directed outwards. Catalysis of electrogenic proton movement by the protonophore resulted in the formation of a negative membrane electrical potential, to a theoretically maximum value equal to the ΔpH but opposite in polarity. Thus, there was no *net* driving force for protons. However, another cation (possibly sodium) would be driven inwards electrogenically in response to the electrical field generated, resulting in ATP synthesis. Valinomycin plus external potassium eliminated the electrical field component by rapid influx of K^+.

DISCUSSION

It has been demonstrated that separation of electrons from protons by H_2 oxidation precedes reduction of CO_2 to methane (14). This, coupled with the fact that all known systems for coupling ATP synthesis to an oxidation-reduction by SLP involve the oxidation of carbon to a carboxylic acid group (as opposed to the eight-electron reduction of CO_2 by methanogens), argues strongly for phosphorylation coupled to electron transport (ETP). An apparent lack of involvement of ion gradients in this process is supported by the absence of an indication of a location tightly bound to the membrane for any of the electron transfer enzymes isolated to date (15–17). In *Methanobacterium thermoautotrophicum*, methanogenic activity has been reported to be sedimented by high-speed ultracentrifugation (18). Separation of this activity from membranes can be obtained, however, by further subfractionation (unpublished data), suggesting that the system responsible for methanogenesis exists in the cell as a large protein complex but is not associated with the membrane.

Blaut and Gottschalk (19) have demonstrated that whole cells of *Methanosarcina barkeri* exhibit respiratory control—i.e., addition of uncoupler stimulates electron transfer. Although this has typically been a characteristic feature of chemiosmotic ETP systems, similar behavior could be exhibited if the uncoupler increases the activity of an ion-translocating ATPase (normally functioning to maintain intracellular ionic composition) by a "futile cycle" of proton translocation. The resulting lowering of the intracellular energy charge would be expected in general to stimulate the rate of catabolic processes (20).

Finally, the data presented here cannot absolutely rule out the existence in methanogens (21) of "localized" proton gradients resistant to ionophore addition. For all such systems known, however, ATP synthesis is eliminated under conditions where ionophores are capable of dissipation of transmembrane ion gradients by catalyzing electrogenic ion movements. Such a possibility is thus argued against by findings reported here that ionophores are effective in mediating ion movement across the cytoplasmic membrane of *M. voltae* (which is apparently the sole membrane structure in this species) and that addition of these compounds has no effect on electron transfer-driven ATP synthesis. Also supporting our interpretation is the recent finding that the intracellular membrane structures observed in some species are not involved in methane formation (22), and the recent demonstration of methanogenesis-driven ATP synthesis in *Methanobacterium thermoautotrophicum* in the absence of a

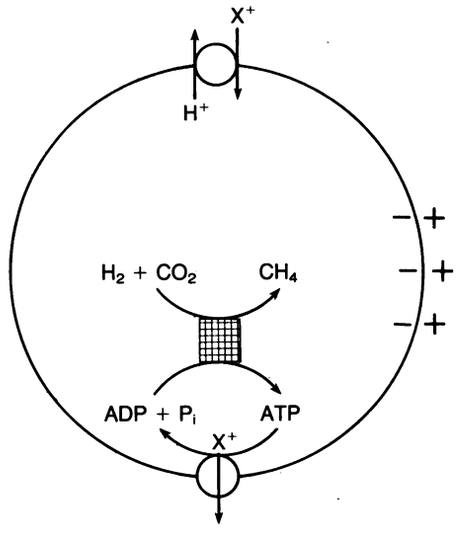

FIG. 5. A possible scheme for bioenergetics of *M. voltae*.

measurable proton gradient (23). Additionally, there are at least two reported instances of a direct coupling between electron transfer and ATP—in nitrogenase (24) and clostridial glycine reductase [(25); however, in the latter case, ATP synthesis by an analogous reaction (hydrogenation of 2-enoates) is uncoupler sensitive (26)].

Fig. 5 depicts a scheme that is most consistent with our results. Methanogenic electron transfer is coupled to ATP synthesis by a direct mechanism not involving the intermediacy of a transmembrane ion gradient. In addition, however, *M. voltae* cells possess an ion-translocating ATPase, which could serve to regulate internal ionic composition including pH. The ion translocated by this enzyme is not proton(s) or potassium; sodium is the most likely candidate, since ATP synthesis in whole cells of *Methanobacterium thermoautotrophicum* driven by a membrane potential has been shown to be stimulated by this ion (11). This ATPase could also be the source of the transmembrane electrical potentials that have been documented in several studies (27) and also are exhibited in analogous fashion by some strictly fermentative organisms (e.g., *Streptococcus faecalis*) in which ATP synthesis is also not accomplished by ETP (28) and which have been reported to possess a sodium-translocating ATPase (29, 30). The uncoupler-sensitive rise in cellular ATP induced by an acid pulse implicates a mechanism for the coupling of proton translocation to ATP synthesis. Since Fig. 4 shows that the primary ion being moved by such a pump is not a proton, the simplest explanation for this result is a proton–cation antiporter, by which the imposed proton gradient induces a gradient of the cation, which is ultimately responsible for ATP synthesis.

We gratefully acknowledge the help of W. B. Whitman and R. S. Wolfe for help with cultures. This work was supported by a research grant from the Division of Biological Energy Research, U.S. Department of Energy, and the Department of Chemistry and Biochemistry of Utah State University. This work was done during the tenure of an Established Investigatorship of the American Heart Association (to J.R.L.) with funds contributed in part by the Utah Heart Association.

1. Thauer, R. K., Jungermann, K. & Decker, K. (1977) *Bacteriol. Rev.* **41**, 100–180.
2. Balch, W. E., Fox, G. E., Magrum, L. J., Woese, C. R. & Wolfe, R. S. (1979) *Microbiol. Rev.* **43**, 260–296.
3. Roberton, A. M. & Wolfe, R. S. (1970) *J. Bacteriol.* **102**, 43–51.
4. Whitman, W. B. & Wolfe, R. S. (1983) *J. Bacteriol.* **154**, 640–649.
5. Terada, H. & Van Dam, K. (1975) *Biochim. Biophys. Acta* **387**, 507–518.
6. Whitman, W. B., Ankwanda, E. & Wolfe, R. S. (1982) *J. Bacteriol.* **149**, 852–863.
7. Kimmich, G. A., Randles, J. & Brand, J. S. (1975) *Anal. Biochem.* **69**, 187–206.
8. Markwell, M. K., Haas, S. M., Bieber, L. L. & Tolbert, N. E. (1978) *Anal. Biochem.* **87**, 206–210.
9. Makula, R. A. & Singer, M. E. (1978) *Biochem. Biophys. Res. Commun.* **82**, 716–722.
10. Bakker, E. P., Rottenberg, H. & Caplan, S. R. (1976) *Biochim. Biophys. Acta* **440**, 557–572.
11. Schonheit, P. & Perski, H. J. (1983) *FEMS Microbiol. Lett.* **20**, 263–267.
12. Jarrell, K. F., Sprott, G. D. & Matheson, A. T. (1984) *Can. J. Microbiol.* **30**, 663–668.
13. Padan, E., Zilberstein, D. & Schuldiner, S. (1981) *Biochim. Biophys. Acta* **650**, 151–166.
14. Spencer, R. W., Daniels, L., Fulton, G. & Orme-Johnson, W. H. (1980) *Biochemistry* **19**, 3678–3683.
15. Gunsalus, R. P. & Wolfe, R. S. (1978) *J. Bacteriol.* **135**, 851–857
16. Escalante-Semerena, J. C. (1983) Dissertation (Univ. of Illinois, Urbana).
17. Krzycki, J. A. & Zeikus, J. G. (1984) *FEMS Microbiol. Lett.* **25**, 27–32.
18. Sauer, F. D., Mahadevan, S. & Erfle, J. D. (1984) *Biochem. J.* **221**, 61–69.
19. Blaut, M. & Gottschalk, G. (1984) *FEMS Microbiol. Lett.* **24**, 103–107.
20. Atkinson, D. E. (1968) *Biochemistry* **7**, 4030–4034.
21. Westerhoff, H. V., Simonetti, A. L. & Van Dam, K. (1981) *Biochem. J.* **200**, 193–202.
22. Sprott, G. D., Sowden, L. C., Colvin, J. R. & Jarrell, K. F. (1984) *Can. J. Microbiol.* **30**, 594–604.
23. Schonheit, P. & Beimborn, D. B. (1985) *Eur. J. Biochem.* **148**, 545–550.
24. Watt, G. D., Bulen, W. A., Burns, A. & Hadfield, K. L. (1975) *Biochemistry* **14**, 4266–4272.
25. Tanaka, H. & Stadtman, T. C. (1979) *J. Biol. Chem.* **254**, 447–452.
26. Bader, J. & Simon, H. (1983) *FEMS Microbiol. Lett.* **20**, 171–175.
27. Daniels, L., Sparling, R. & Sprott, G. D. (1984) *Biochim. Biophys. Acta* **768**, 113–163.
28. Harold, F. M. & Papineau, D. (1972) *J. Membr. Biol.* **8**, 27–44.
29. Heefner, D. L. & Harold, F. M. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 2798–2802.
30. Kinoshita, N., Unemoto, T. & Kobayashi, H. J. (1984) *J. Bacteriol.* **158**, 844–848.