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MINI-REVIEW

Sodium, Protons, and Energy Coupling in the Methanogenic Bacteria

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Abstract

In this review, I focus on the bioenergetics of the methanogenic bacteria, with particular attention directed to the roles of transmembrane electrochemical gradients of sodium and proton. In addition, the mechanism of coupling ATP synthesis to methanogenic electron transfer is addressed. Evidence is reviewed which suggests that the methanogens possess great diversity in their bioenergetic machinery. In particular, in some methanogens the primary ion which is translocated coupled to metabolic energy is the proton, while others appear to utilize sodium. In addition, ATP synthesis driven by methanogenic electron transfer is accomplished in some organisms by a chemiosmotic mechanism and is coupled by a more direct mechanism in others. A possible explanation for this diversity (which is consistent with the relatedness of these organisms to each other and to other members of the Archaebacteria as determined by molecular biological techniques) is discussed.

Key Words: Methanogens; sodium; ion gradients; chemiosmotic theory; ATPase.

Introduction

Microbial systems present a remarkable diversity of metabolic coupling modes for the bioenergeticist. In addition to anaerobic respiratory and photosynthetic systems, recent work has shown that sodium is used instead of protons by some cells for energy transduction via membrane-bound primary ion translocation (Skulachev, 1985; Dimroth, 1987). A particularly

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striking example is the recent finding of an F_1F_0-type ATPase (i.e., similar in structure and inhibitor sensitivity to the proton-translocating enzymes of mitochondria, chloroplasts, and bacteria) in the obligate anaerobe *Propigenium modestum* which couples sodium (instead of proton) translocation to ATP synthesis, with the ion specificity apparently determined by the hydrophobic F_0 portion of the protein (Laubinger and Dimroth, 1987). In addition, it has been demonstrated by reconstitution into phospholipid vesicles that in some microbial systems sodium translocation can be directly driven by integrally membrane-bound exergonic metabolic reactions, e.g., electron transfer (Tokuda, 1984) and decarboxylation (Hilpert and Dimroth, 1984). The general bioenergetic significance of these findings is that they point to a more direct participation of the proteins in determining the allocation of free energy during the catalytic steps of the coupling reaction (as opposed to "ligand conduction," at least in its strictest interpretation), since, unlike protons, sodium cannot (without the intervention of the protein, directly affect oxidation–reduction of electron transfer centers or acid–base catalyzed phosphoanhydride bond formation/hydrolysis (Skulachev, 1985; Lancaster, 1987).

Phylogenetic and Bioenergetic Relationships among Methanogens

The methanogenic bacteria are a diverse group of organisms, united primarily by their ability to derive energy from the production of methane from simple compounds (CO_2, CO, formate, methylamines, simple alcohols, acetate). Methanogens are the major representatives of the Archaebacteria, an apparently ancient line of descent as unrelated to eukaryotes and prokaryotes as these two are to each other (Woese, 1987). The early divergence of the Archaebacteria in general and methanogens in particular has resulted in what has been referred to as "biochemical isolation" in this group (Wolfe, 1988; Rouviere and Wolfe, 1988), meaning numerous unique biochemical mechanisms. In addition to early divergence evolutionarily between the Archaebacteria and the Eubacteria, there is remarkable divergence within the Archaebacteria as a group (Jones *et al.*, 1987). For example, 16S rRNA comparisons reveal that the the Halobacteria are more closely related to the Methanomicrobiales than to the other two orders of methanogen (Methanobacteriales and Methanococcales) (Woese and Olsen, 1986).

There is increasing evidence that this phylogenetic relationship between the three orders of methanogens (and relatedness to Halobacteria) as determined by molecular biological techniques may be reflected also in their bioenergetics. Particularly striking, for example, are the differenes in the properties of the ion-translocating ATPases in *Ms. barkeri* (a member of the

Methanomicrobiales) and in *Mc. voltae* (a member of the Methanococcales). While the former species (commonly isolated from anaerobic digestors) possesses an F_1F_0-type ATPase (Inatomi, 1986), all detectable activity in *Mc. voltae* membranes (a marine, halotolerant organism) is insensitive to DCCD and sensitive to vanadate (characteristics of E_1E_2-type ATPases), and no hybridization is detectable when the entire *Mc. voltae* genome is probed using the complete gene for the *E. coli* F_1F_0-ATPase (Dharmavaram and Konisky, 1987). In addition, whereas *Ms. barkeri* possesses some properties typical of chemiosmotic systems, similar data with *Mc. voltae* do not support an obligatory role for ion gradients in ATP synthesis coupled to methanogenesis.

Whether chemiosmotic in energy production apparatus or not, sodium plays a critical role in the bioenergetics of members of each of the individual methanogenic taxonomic orders.[2] In this review I focus on the bioenergetic role(s) of sodium and protons in methanogens in transmembrane ion gradient formation/utilization and internal ion homeostasis. First, however, I present a brief overview of the major cellular components involved in methanogenic energy conversions.

Methanogenic Intermediary Metabolisms

Methanogens are strict anaerobes which catalyze the formation of CH_4 and the concomitant synthesis of ATP from the reduction of methyl groups, derived from C_1 metabolites (CO_2, formate, carbon monoxide, simple alcohols, methylamines) or from the methyl group of acetate. The electron donating half-cell for this oxidation–reduction may be the oxidation to CO_2 of formate, carbon monoxide, methanol, methylamines, or the carboxyl group of acetate, or (more commonly) oxidation of molecular hydrogen. Different species are capable of different combinations of these reactions (with the *Methanosarcina* being the most metabolically versatile), but these electron transfer processes are in all cases thermodynamically favorable overall due primarily to the relatively electropositive potential for the reduction of methanol (perhaps more properly, its energetic equivalent, methylcoenzyme M) to methane, the last two-electron transfer step and the only oxidation–reduction which is common to all modes. As described below, the enzyme system responsible for this step [the methylcoenzyme M methylreductase (MR)] is conserved in all species and is probably the major site for ATP synthesis common to all species.

[2] An early suggestion (Kell *et al.*, 1981) that chemiosmotic ATP synthesis occurs coupled to electron transfer at internal membrane structures has been shown to be unlikely (Sprott *et al.*, 1984; Krämer and Schönheit, 1987; Aldrich *et al.*, 1987b).

C_1 Carriers

Depicted below are the steps involved in the eight-electron reduction of CO_2 to CH_4 by H_2 (the most common methanogenic metabolic mode), in terms of the equivalent free C_1 intermediates (described for simplicity as a linear sequence; as described below, the first and last steps are functionally coupled):

$$CO_2 \xrightarrow[\text{MFR}]{2e^-} [HCOOH(CO)] \xrightarrow[H_4\text{MPT}]{2e^-} [HCHO] \xrightarrow[H_4\text{MPT}]{2e^-} [CH_3OH] \xrightarrow[\text{CoM}]{2e^-} CH_4$$

As indicated by the use of brackets, the free intermediates are not appreciably released. Below each reaction are denoted the various carriers of the C_1 unit undergoing reduction, as delineated primarily by the elegant work of Wolfe (1985). Several of the soluble enzymes catalyzing these transformations have been isolated and purified. Methanofuran (MFR) is the initial acceptor of the CO_2 moiety, which undergoes two-electron reduction producing formylmethanofuran (Börner *et al.*, 1989). The formyl group is subsequently transferred to tetrahydromethanopterin (H_4MPT) by the enzyme formylmethanofuran : H_4-MPT formyltransferase (Donnelly and Wolfe, 1986) to produce 5-formyl-H_4MPT (as opposed to 5,10-methenyl-H_4MPT or 10-formyl-H_4MPT). After formation of 5,10-methenyl-H_4MPT by a separate enzyme (5,10-methenyl-H_4MPT cyclohydrolase (Donnelly *et al.*, 1985)), two-electron reduction by methylene-H_4MPT dehydrogenase [involving the two-electron carrier coenzyme F420 (Hartzell *et al.*, 1985)] produces methylene-H_4MPT. Two-electron reduction to methyl-H_4MPT by an as yet uncharacterized enzyme precedes methyl group transfer to yet another C_1 carrier unique to methanogens, coenzyme M (CoM; 2-mercaptoethanesulfonic acid), to produce methylcoenzyme M (MeCoM), which is catalyzed by methyl-H_4MPT methyltransferase (which has not been purified but has been demonstrated in cell extracts (Sauer, 1986; Poirot *et al.*, 1987). The conversion of each of the three C_1 derivatives of H_4MPT (prepared chemically) to methane at rates comparable to that from the physiological substrate (CO_2) (Escalante-Semerena *et al.*, 1984) and the universal presence of this cofactor (as well as MFR) in all methanogens (Jones *et al.*, 1985) demonstrates the central role of these carriers in methanogenic transformations.

Among the various oxidation–reductions in the eight-electron sequence from CO_2 to CH_4, in terms of the free intermediates it is the last step (the reduction of methanol to methane) which is the most oxidizing (i.e., the most positive reduction potential). This means that electron transfer from any of the other electron-transfer reactions catalyzed by methanogens (whether from C_1 unit or from H_2) is sufficiently negative to yield useful energy when used to provide electrons for this terminal step, which results in the formation of CH_4. It has been shown (as described below) that ATP synthesis occurs at

this step, which is catalyzed in all methanogens by the methylcoenzyme M reductase system (MR), the soluble methane-forming protein of which (component C) has been purified [MW 300,000 (Wolfe, 1985)] and shown to contain the tightly bound nickel porhinoid Factor F430. The MR system has been isolated and characterized by its activity in producing methane from the reduction of methyl coenzyme M, which remains tightly bound to the protein:

$$^{-}O_3S\text{–}CH_2\text{–}CH_2\text{–}S\text{–}CH_3 + 2e^{-} + 2H^{+} \longrightarrow {}^{-}O_3S\text{–}CH_2\text{–}CH_2\text{–}SH + CH_4$$

In addition to component C, activity requires the hydrogenase-containing protein fractions A1 and A3, as well as component A2 which has been purified to homogeneity utilizing N^6-ATP-agarose (Rouviere *et al.*, 1985). The purified protein has no detectable electron transfer or ATP hydrolytic activity. Activity also requires FAD, as well as the low-molecular-weight cofactor 7-mercaptoheptanoylthreonine phosphate (HTP), which has recently been shown to function as the immediate electron donor for the reductive demethylation of the methyl-coenzyme M, by forming a mixed disulfide with the resultant coenzyme M (Ellermann *et al.*, 1988; Bobik *et al.*, 1987). EPR studies suggest that the nickel of factor F430 undergoes oxidation–reduction during catalysis (Albracht *et al.*, 1986). Mg^{2+}/ATP addition activates the MR system; this effect is probably due to a modification phenomenon since ATP can be removed, thus retaining activity (Whitman and Wolfe, 1983). In *Mb. thermoautotrophicum* (strain Marburg), complete methyl reductase activity can be reconstituted with only purified component C, 7-HTP, vitamin B_{12}, and dithiothreitol (Ankel-Fuchs and Thauer, 1986). ATP is not required. In *Mb. thermoautotrophicum* (strain delta-H) use of titanium citrate as electron donor allows simplification of the methyl reductase also, with activity exhibited by components A2, A3, and C, and in addition catalytic amounts of ATP, vitamin B_{12}, and 7-HTP (Rouviere *et al.*, 1988).

The observation of catalytic, transient stimulation of CO_2 reduction to methane by MeCoM but *not* by CoM [the "RPG Effect" (Wolfe, 1985)] and the finding of two molecules of tightly bound coenzyme M (Hausinger *et al.*, 1984) [as well as factor F430 (Hartzell and Wolfe, 1986), HTP (Noll and Wolfe, 1986), and possibly FAD] per molecule of Component C suggests that the terminal step in this eight-electron transfer sequence is linked to the first. The mechanism of this linkage has been shown to involve the formation of the mixed disulfide of 7-HTP and coenzyme M (Bobik and Wolfe, 1988). This pathway is thus best represented as a cycle (Wolfe, 1985) (as opposed to a linear sequence), and the formation of methane from CO_2 far in excess of the added methylcoenzyme M suggests that the *in vivo* mechanism of methanogenesis does not occur by simple reduction of free methylcoenzyme M.

Electron Transfer in Methanogens

Methanogens fill an important ecological niche. In anaerobic environments, these bacteria convert the end products of biomass degradation by other organisms (primarily H_2, C_1, and C_2 compounds) into CH_4 and CO_2, making use of the energetically favorable electron transfer reactions involving the various one-carbon intermediates. Table I lists the standard reduction potentials for these transformations, in terms of the free intermediates. Although most methanogens are capable of the reduction of CO_2 with H_2 (thus carrying out the complete, eight-electron sequence through the various C_1 intermediate oxidation states), many species are capable also of catalyzing electron transfer from one C_1 unit to another. These types of studies (mixotrophic metabolism) have provided evidence for the existence of a central "pool" of relatively low reduction potential, protein-bound electrons (as described in more detail in Lancaster, 1986). Transfer of these electrons, originally provided by oxidation of various C_1 intermediates or H_2, to the terminal, most oxidizing couple (reduction of CH_3OH to CH_4), provides the free energy for the synthesis of ATP. All of the oxidation–reduction reactions shown in Table I are significantly more negative than the terminal one and so yield free energy when coupled together. As suggested by Blaut and Gottschalk (1985), however, optimization of the energetics involved would suggest that the central "pool" of electrons is sufficiently negative to drive ATP synthesis; this would mean that the oxidation of CH_3OH (to HCHO) may not be low enough potential to donate electrons to such a "pool." As described in more detail below, it appears that some methanogens utilize an electrochemical transmembrane sodium gradient to drive this reaction, by coupling oxidation of this relatively high reduction potential C_1 intermediate to the energetically downhill influx of sodium ions.

Relatively little is known about the sequence of the various electron carriers involved in methanogenic oxidation–reduction reactions. In terms of cofactors, methanogens possess at least two unique low-molecular-weight electron transfer carriers, factor F420 (a 5-deazaflavin cofactor which is an

Table I. Standard Reduction Potentials for Electron Transfer Reactions Catalyzed by Methanogens

Oxidant	Reductant	$E^{0'}$ (V)
$2H^+ + 2e^-$	$\leftrightarrow H_2$	−0.421
$CO_2 + 2H^+ + 2e^-$	$\leftrightarrow HCOOH$	−0.414
$HCOOH + 2H^+ + 2e^-$	$\leftrightarrow HCHO + H_2O$	−0.542
$HCHO + 2H^+ + 2e^-$	$\leftrightarrow CH_3OH$	−0.189
$CO + 2H^+ + 2e^-$	$\leftrightarrow HCHO$	−0.455
$CO_2 + 2H^+ + 2e^-$	$\leftrightarrow CO + H_2O$	−0.317
$CH_3OH + 2H^+ + 2e^-$	$\leftrightarrow CH_4 + H_2O$	+0.162

obligate two-electron carrier of relatively negative potential (Jacobson and Walsh, 1984)) and 7-heptanoylthreonine phosphate (which, as described above, functions to denote electrons to methyl-coenzyme M). Other potential electron transfer components include flavin (Nagle and Wolfe, 1983), ferredoxin (Blaylock, 1968; Moura *et al.*, 1982; Hatchikian *et al.*, 1982; Terlesky and Ferry, 1988a,b), $NADP^+$ (Tzeng *et al.*, 1975; Eirich *et al.*, 1979; Yamazaki and Tsai, 1980; Eirich and Dugger, 1984), and corrin (Van der Meijden *et al.*, 1983; Ankel-Fuchs and Thauer, 1986; Poirot *et al.*, 1987; Hartzell *et al.*, 1988; Van de Wijngaard *et al.*, 1988; Schulz *et al.*, 1988). There is no evidence for a role for NADH. EPR studies have identified a minimum of three iron–sulfur centers in crude preparations from *Mb. thermoautotrophicum* which are involved in electron transfer to methyl-coenzyme M from H_2 (Rogers *et al.*, 1988).

In addition to the reactions described in the previous section involving the reduction of CO_2, several electron transfer enzymes have been purified from methanogens, including formate dehydrogenase (Barber *et al.*, 1983), hydrogenase (Albracht *et al.*, 1982; Kojima *et al.*, 1983; Fauque *et al.*, 1984), and carbon monoxide dehydrogenase (Terlesky *et al.*, 1986). Although several of these proteins are relatively hydrophobic and tend to aggregate, they appear not to be integrally membrane associated. In *Methanosarcina*, membrane-associated cytochromes (Kühn and Gottschalk, 1983), iron–sulfur centers (Kemner *et al.*, 1987), and hydrogenase (Terlesky and Ferry, 1988a) have been reported. Several immunocytochemical microscopy studies have described a subcellular localization of the methyl reductase (Ossmer *et al.*, 1986; Aldrich *et al.*, 1987a; Mayer *et al.*, 1988) and also F420-reducing hydrogenase (Müth, 1988) to the cytoplasmic membrane under some (but not all) conditions. These results must be interpreted with caution, however, since association of apparently soluble enzymes with the membrane fraction has been described for other systems, where ion translocation is not involved (Robinson *et al.*, 1987). In the case of the Methanomicrobiales, functional studies (described below) support a membrane-bound localization physiologically for the methyl reductase. Membrane-associated corrin-containing protein has been reported in *Mb. thermoautotrophicum* (Schulz *et al.*, 1988), although a functional role for this protein has not been reported. F420-reducing hydrogenase activity in *Mb. formicicum* appears to be membrane-associated (and binds to a hydrophobic column), but is also present in the soluble fraction as monomers and also aggregates (Baron *et al.*, 1987). A similar behaviour has been demonstrated for the enzyme from *Mb. thermoautotrophicum* (Wackett *et al.*, 1987) where the monomeric protein has been shown to associate into an oligomeric ring-shaped complex. Methyl reductase was also shown to form a specific oligomeric structure, as well as paracrystalline arrays at concentrations approaching those intracellularly.

This result suggests that some electron transfer enzymes in methanogens may function physiologically in a multicomponent protein complex (Lancaster, 1986; Mayer *et al.*, 1988), and that the protein–protein associations may involve hydrophobic interactions.

ATPase and ATP Synthesis

ATP hydrolyzing activity has been reported in extracts of *Methanobacterium bryantii* (Robertson and Wolfe, 1969), *Methanobacterium thermoautotrophicum* strains delta-H and Marburg (Brandis *et al.*, 1981; Doddema *et al.*, 1978), *Methanospirillum hungatei* (Sprott *et al.*, 1982), and in membranes of *Mc. voltae* and *Methanosarcina barkeri*, as described in more detail below. ATPase activity is also exhibited by component A of the methyl reductase system (Rouviere *et al.*, 1985), and may be related to the ATP activation of the MR system. However, with the exception of the ATPases in *Ms. barkeri* and *Mc. voltae*, little is known about the properties and function of these ATP hydrolyzing activities.

Sodium Gradients and Bioenergetics in Methanogens

Although earlier studies (reviewed by Daniels *et al.*, 1984) had shown that methanogens possess transmembrane ion gradients, interpretations of the effects of ionophores on energetic functions in whole cells have been hindered by the often-observed inhibition by these compounds of the ultimate driving force (methanogenic electron transfer). Unfortunately, this result *per se* provides no definitive information regarding mechanisms of coupling. A major advance in the understanding of the overall schemes of energy flow in methanogens in the past few years has been the recognition that sodium plays a special role in these processes. These results are described below for the three major lines of methanogenic descent.

Methanobacteriales

The first indication that sodium plays a special role in methanogen bioenergetics was obtained by Perski *et al.* (1981) who showed that sodium is required for methanogenic electron transfer ($H_2 + CO_2$) in *Mb. thermoautotrophicum*, exhibiting an apparent K_s of 1 mM. This requirement for sodium was subsequently verified for a total of seven species of methanogen (Perski *et al.*, 1982).

A role for sodium in membrane bioenergetic mechanisms was subsequently suggested by Schönheit and Perski (1983), who showed that the presence of sodium results in a fivefold stimulation of ATP synthesis in whole

cells driven by a potassium diffusion potential [i.e., upon addition of valinomycin to whole cells in the absence of added potassium in the medium, as originally shown by Doddema *et al.* (1978); methanogens contain high levels of internal potassium (Daniels *et al.*, 1984), although, as described below, it is unclear what fraction is free in solution]. Butsch and Bachofen (1984) showed that sodium (> 1 mM) is required for membrane electrical potential formation, although this was most probably a result of the requirement of this ion for energy-coupled electron transfer (methanogenesis).

In 1985, Schönheit and Beimborn reported that neither methanogenic electron transfer (from H_2 plus CO_2) nor ATP synthesis in *Mb. thermoautotrophicum* are inhibited by apparent elimination of the transmembrane electrical potential, effected by either protonophores (TCS and SF6847) or valinomycin plus external potassium (Schönheit and Beimborn, 1985a). Since the magnitude of the pH gradient under coupled conditions is small energetically compared to the electrical potential [this is true of methanogens in general (Daniels *et al.*, 1984)], this argued against a transmembrane electrochemical proton gradient as the sole link between methanogenic electron transfer and ATP synthesis, and suggested that sodium might be involved instead of protons. In a more recent study (Kaesler and Schönheit, 1988), however, re-examination of this result using an apparently more accurate method for determining low values of the membrane potential demonstrated that decrease to a value of approximately -100 mV (from an uninhibited value of approximately -200 mV) indeed has no effect on either methanogenesis or intracellular ATP levels, but further hypopolarization results in a parallel inhibition in both. From this result it can only be concluded that, if ATP synthesis occurs by a chemiosmotic mechanism, the stoichiometry of protons translocated per ATP must be relatively high; a "lower limit" is 5. Using added formaldehyde as electron acceptor from H_2,[3] a similar synthesis of ATP at low values for the membrane potential occurred (also ca. -100 mV), but in this case further hypopolarization decreased the cellular ATP with no effect on electron transfer. As pointed out in more detail elsewhere (Lancaster, 1987), such an effect (decrease in ATP by elimination of the electrical potential with no effect on electron transfer) is a necessary but not sufficient condition for chemiosmotic coupling.

Kramer *et al.* (1988) have recently reported the identification of a family of diglycosyl diether lipids in *Mb. thermoautotrophicum* which contain one tightly bound alkali metal ion per molecule and comprise 8% of the total cell lipid. This raises the possibility that the total concentration of potassium (and also sodium) free in solution intracellularly is actually less than that

[3] It has been shown that formaldehyde reacts nonenzymatically with tetrahydromethanopterin to produce methylene tetrahydromethanopterin (Escalante-Semerena and Wolfe, 1984).

determined by analytical methods [for *Mb. thermoautotrophicum*, the value assumed is 0.7 M (Schönheit and Perski, 1983)]. It may be, therefore, that decreasing the transmembrane electrical potential by increasing external potassium in the presence of valinomycin will, at relatively high concentrations (low apparent values of $\Delta\Psi$), actually result in an inversion in the sign of the potential (i.e., positive inside) and thus result is inaccurate values of the calculated potential as well as inhibition of electron transfer (and thus ATP synthesis).

In *Mb. thermoautotrophicum* addition of the sodium ionophore monensin inhibits methanogenesis, but only at acid pH (Perski *et al.*, 1982; Schönheit and Beimborn, 1985b). This result suggests a role for sodium in transport (as opposed to, for example, requirement for sodium by an intracellular enzyme), and specifically internal pH regulation at acid pH (i.e., proton extrusion). The requirement for sodium could thus be due to sodium/proton antiport which, at acid pH, is responsible for maintaining relatively neutral internal pH by proton extrusion coupled to sodium uptake. Indeed, Schönheit and Beimborn (1985b) have demonstrated the presence of a Na^+/H^+ antiporter in *Mb. thermoautotrophicum* (Marburg). Sodium addition to resting cells results in H^+ extrusion which is sensitive to harmaline, an inhibitor of eukaryotic sodium translocating systems including Na^+/H^+ antiporters and which acts competitively with sodium [apparently by binding to the sodium site (Sepulveda and Robinson, 1974)]. That this antiport activity is involved in internal pH regulation is suggested by the fact that ΔpH formation at acidic external pH requires sodium and is inhibited by harmaline, which was shown to act competitively with sodium. In addition, it was shown that sodium is only required for methanogenesis at acid pH; at alkaline pH, sodium is not required. In a subsequent paper, Schönheit and Beimborn (1986) provided further evidence that sodium transport is required for methanogenesis from H_2 plus CO_2. Taken together, these results suggest that the sodium/proton antiporter in *Mb. thermoautotrophicum* functions in maintenance of neutral internal pH at acidic external pH and that methanogenic electron transfer activity requires careful maintenance of the internal pH. This sensitivity may explain the inhibition of methanogenesis by disruption of internal ion homeostasis upon the addition of ionophores (especially protonophores) (Lancaster, 1987). In addition, these results suggest the existence of a driving force for sodium which can power the efflux of protons via the sodium/proton antiport under acidic external conditions.

As originally demonstrated in *Mc. voltae* (Carper and Lancaster, 1986) (described in more detail below), Al-Mahrouq *et al.* (1986) showed that addition of sodium to resting cell suspensions of *Mb. thermoautotrophicum* results in ATP synthesis. This synthesis requires the presence of a permeant counterion (tetraphenylboron), and is eliminated by monensin but not by the

protonophore SF6847. It was also shown that harmaline addition inhibits membrane potential-driven ATP synthesis. This suggests the presence of an electrogenic, harmaline-sensitive sodium-translocating ATPase which is responsible for the membrane potential-driven sodium-stimulated ATP synthesis. In addition, the complete lack of effect under the same conditions of harmaline, monensin, or SF6847 on electron transfer-driven ATP synthesis suggests that [as had been previously proposed for *Mc. voltae* (Carper and Lancaster, 1986)], ATP synthesis is coupled to methane formation by a direct mechanism (not involving ion gradients). The deduced function of the sodium ATPase is in cellular internal ion homeostasis, including proton extrusion at acid external pH via sodium/proton antiport. In a recent report, Smigan *et al.* (1988) showed that this sodium-driven ATP synthetic activity is increased when cells are grown in media containing 50 mM (as opposed to 5 mM) sodium, suggesting that the activity is at least partially inducible.

The key question in the delination of the roles of sodium in bioenergetics in the *Methanobacteriales* in particular (and indeed in methanogens in general) is whether the ultimate driving force for sodium is via a primary transport system (e.g., sodium ATPase and/or membrane-bound electron transfer chain), or by secondary transport (e.g., Na^+/H^+ antiport). It thus becomes critical to determine which ion (H^+ or Na^+) is driven across the membrane coupled to metabolic energy. The observed uncoupling of ATP synthesis and H_2/formaldehyde-driven electron transfer by elimination of the electrical potential is consistent with chemiosmotic coupling, in which case the sodium-translocating ATPase activity could be a result of either relative cation nonspecificity in a putative H^+-translocating ATPase or a specialized pump which acts under certain growth conditions. This uncoupling effect, however, is also compatible with a more direct mechanism of coupling, with ATP-driven sodium translocation functioning to maintain internal ion homeostasis, including pH (Lancaster, 1987). The demonstration of electrogenic sodium movement coupled to ATP synthesis/hydrolysis and that inhibition of transmembrane electrical potential-driven ATP synthesis by harmaline (which inhibits this ATPase) has no effect on electron transfer-driven ATP synthesis support this latter possibility. It has also been reported recently that cell-free extracts of *Mb. thermoautotrophicum* catalyze the formation of inorganic pyrophosphate stoichiometrically with methane from H_2 plus methyl coenzyme M (Keltjens *et al.*, 1988), which supports a more direct mechanism of coupling ATP synthesis to electron transfer. In this case, an important function of the Na^+/H^+ antiporter would be in internal pH maintenance under acidic conditions, resulting in the net extrusion of protons driven by ATP hydrolysis. It appears that sodium does not function as coupling ion linking electron transfer and ATP synthesis, at least in this group of methanogens.

Methanococcales

Studies of transmembrane ion gradient formation/utilization in this group of methanogens has been confined to the organism *Methanococcus voltae*, which is a halotolerant organism isolated originally from a marine sediment, and requires sodium for growth (Whitman *et al.*, 1982) and methanogenesis (Jarrell and Sprott, 1985).

Jarrell *et al.* (1984) showed that isoleucine [which is required for growth (Whitman *et al.*, 1982)] is transported by this organism via a sodium-dependent system. This sodium dependence is independent of the requirement for methanogenesis and kinetically lowers the apparent K_m for isoleucine (from 0.33 mM at 2 mM sodium to 22 μM at 25 mM) with little influence on the V_{max} (Jarrell and Sprott, 1985). This result suggests the existence of a branched-chain amino acid transport system [for which there is further evidence (Ekiel *et al.*, 1985)] which is powered by sodium movement. Additional information on the mechanisms of coupling using ionophores (monensin, nigericin, TCS, and CCCP) are unfortunately difficult to interpret due to inhibition of methanogenesis.

Crider *et al.* (1985) showed that ATP is synthesized in resting cells of *Mc. voltae* upon addition of valinomycin in the absence of added potassium; when potassium is added externally, no ATP is synthesized. As with *Mb. thermoautotrophicum*, this shows the presence of an electrogenic, non-potassium translocating ATPase and that valinomycin mediates electrogenic potassium movement. In addition, ATP is also synthesized by an acid pulse, and this effect is sensitive to the potent protonophore SF6847 (which does not inhibit methanogenesis). This result shows that proton translocation can be used to drive ATP synthesis (the two most likely possibilities are by a proton-translocating ATPase or by sodium/proton antiport in concert with a sodium-translocating ATPase), and also that SF6847 is competent to catalyze electrogenic proton translocation. Nevertheless, electron transfer-driven ATP synthesis is completely unaffected by the simultaneous addition of both valinomycin plus external potassium and high concentrations (1 μM) of SF6847. These results suggest that (1) methane formation is coupled to ATP synthesis without the intermediacy of a transmembrane electrical potential, (2) ATP hydrolysis can be used to drive the efflux of a cation other than proton or potassium, and (3) this organism possesses a Na^+/H^+ antiporter.

Additional evidence for the existence of a non-proton translocating electrogenic ATPase and for the protonophoric action of SF6847 was provided by the following experiment: Resting cells (in the presence of sodium) were subjected to a brief (3 min) incubation at acidic pH (pH 5) in the presence of SF6847, during which time the intracellular ATP levels were low. At time zero, the pH was raised to 7.9, which induced the rapid but

transient formation of internal ATP to levels approximately 80% of that driven by H_2/CO_2. This effect requires the presence of the protonophore and is eliminated by valinomycin plus external potassium. The direction of the pH shift (acid to base) and the requirement for SF6847 shows that electrogenic efflux of protons is occurring, generating a transmembrane electrical potential positive outside. The prevention of the effect by valinomycin plus potassium shows that this electrical potential is the driving force for ATP synthesis. The ion which is driven inwards in response to this electrical field resulting in ATP synthesis cannot be protons; this is because the electrical potential (which would tend to drive protons inward) is a result of an *oppositely* directed pH gradient (which would tend to drive protons outward). Since the electrical field is actually generated by the pH gradient, it can never be greater in magnitude. The *net* driving force for protons ($\Delta pH + \Delta\Psi$) will thus be zero. Any other ion, however, could be driven by the electrical field. Since this phenomenon will also occur with high internal potassium plus a potassium ionophore, the same considerations rule out this cation. Sodium is thus the most likely candidate.

Carper and Lancaster (1986) showed that *Mc. voltae* indeed possesses an electrogenic sodium-translocating ATPase: electrical potential-driven ATP synthesis requires the presence of sodium and is eliminated by addition of the sodium ionophore monensin; also, sodium addition in the presence of a permanent counterion (TPB^-) results in SF6847-insensitive ATP synthesis. Al-Mahrouq *et al.* (1986) showed that the inhibitor diethylstilbestrol (DES, which inhibits several cation-translocating ATPases) prevents ATP synthesis driven by either a transmembrane electrical potential or a chemical sodium gradient, yet has no effect on electron transfer driven ATP synthesis. This result shows that the sodium-translocating ATPase is not involved in ATP synthesis coupled to methanogenesis.

Dharmavaram and Konisky (1987) examined ATP hydrolyzing activity in membranes of *Mc. voltae*. All activity is sensitive to DES and also vanadate, but insensitive to DCCD, oligomycin, or ouabain. In addition, there is no detectable homology between the entire *E. coli* F_1F_0-ATPase and *Mc. voltae* total DNA, and no cross-reactivity is observed toward antibody directed against either *E. coli* ATPase or *Neurospora crassa* plasma membane ATPase. The enzyme studied in this report is almost certainly the sodium-translocating ATPase, which at this point seems to be of the E_1E_2 type and is the sole ATPase in this organism.

The data for *Mc. voltae* demonstrates the existence of an electrogenic sodium-translocating ATPase, which is not involved in electron transfer-driven ATP synthesis and probably functions (in concert with Na^+/H^+ antiport) to maintain internal ion homeostasis. In addition, transmembrane electrochemical ion gradients are not involved in energy coupling to methane formation.

Methanomicrobiales

As alluded to above, molecular biological studies have shown that the extreme halophiles (e.g., Halobacteria) are specifically related to the *Methanomicrobiales*, to the exclusion of the other two groups of methanogen (Woese and Olsen, 1986). This alignment is also consistent with their bioenergetic mechanisms. Extensive studies have documented that electron transfer in the Halobacteria is coupled to ATP synthesis via an electrochemical proton gradient (Lanyi, 1978), and the evidence for such a chemiosmotic mechanism among methanogens is strongest for the *Methanomicrobiales*, in particular *Methanosarcina*. These are the only methanogens which contain cytochromes (Kühn *et al.*, 1979; Kühn and Gottschalk, 1983; Kühn *et al.*, 1983; Jussofie and Gottschalk, 1986) and (as described below) have been shown to possess an F_1F_0-type ATPase. However, even in this case there appears to be a special role for sodium in membrane-linked energy conservations, as is also true of the Halobacteria and the other methanogens.

Research on bioenergetic coupling mechanisms in the Methanomicrobiales in general (and *Methanosarcina* in particular) has been greatly facilitated by the ability of this organism to produce methane (coupled to ATP synthesis) from a variety of C_1 and C_2 compounds. This versatility in methane production allows "dissection" of the entire eight-electron sequence from CO_2 into various segments. For example, methane formation from CO_2 plus H_2 in *Methanosarcina* is inhibited by collapse of the transmembrane electrical potential by protonophore (Blaut and Gottschalk, 1984a); however, in the simplest methanogenic pathway, methanol is reduced by H_2 to methane [after transfer to coenzyme M by a cobalamin-containing enzyme not present in other methanogens (Van der Meijden *et al.*, 1983; Poirot *et al.*, 1987)] via the methyl reductase, with no effect on electron transfer of the addition of protonophore. This means that the protonophore-sensitive step occurs prior to the terminal electron transfer, so that ionophore effects can be investigated in this case without this complication. In a series of studies, the laboratory of Gottschalk has presented evidence that ATP synthesis is coupled to the reduction of methanol by H_2 through the intermediacy of a transmembrane ion gradient (Blaut and Gottschalk, 1985; Blaut *et al.*, 1986). The basic observations are presented below.

Addition of H_2 plus methanol to cells results in an increase in intracellular ATP levels, simultaneous with a hyperpolarization of the membrane (Blaut and Gottschalk, 1984a) and with net proton extrusion [detected by permeant weak acid distribution (Blaut and Gottschalk, 1984a) or by a pH electrode (Blaut *et al.*, 1987)]. Addition of the protonophore TCS results in a rapid depolarization of the electrical potential and depletion of intracellular ATP. In addition, electron transfer (methanogenesis) is slightly stimulated,

presumably due to the release of an inhibition of electron transfer due to the membrane potential (respiratory control).

Mountfort (1978) had originally reported that an acid pulse results in ATP synthesis in whole cells of *Methanosarcina barkeri*, and this synthesis is inhibited by DCCD. This suggested the presence of an F_1F_0-type ATPase (a conclusion which has been verified, see below). Blaut and Gottschalk (1984a, b) showed that addition of DCCD results in an inhibition of methanogenesis and decrease in intracellular ATP levels, a result which, alone, provides little information about coupling, because of the relative nonspecificity of this reactive reagent for a variety of membrane-associated systems (Azzi and Nalecz, 1984), including electron transfer (Vuokila and Hassinen, 1988) and also sodium-translocating ATPase (Laubinger and Dimroth, 1987). However, if TCS is added *after* DCCD, the membrane electrical potential is eliminated and methanogenesis is dramatically stimulated (Blaut and Gottschalk, 1984a, b; Jussofie *et al.*, 1986). The simplest interpretation of these results is that DCCD inhibits an ion-translocating ATPase which results in an increase in the membrane potential and consequent inhibition of electron transfer (analogous to inhibition of mitochondrial electron transfer by oligomycin). This would mean that electron transfer from H_2 plus methanol is directly linked to the transmembrane electrical potential (by whatever ion). Similar data have been reported for the metabolism of methylamines (Müller *et al.*, 1987b, c), which occurs analogously to methanol after deamination. An alternative possibility (in line with the data above for other methanogens) is that methane formation drives ATP synthesis by a more direct mechanism, which in turn drives ion translocation by a cation-translocating ATPase. In this case, however, inhibition of the ATPase by DCCD would be expected to result in elimination of the transmembrane potential, and subsequent stimulation of electron transfer by TCS [and also the lack of inhibition of H_2/methanol-driven proton extrusion by DCCD (Blaut *et al.*, 1987)] would not be predicted in a straightforward manner.

Inatomi (1986) and Inatomi and Maeda (1988) reported the presence in *Ms. barkeri* of an ATPase activity which sediments upon ultracentrifugation and resembles eubacterial, mitochondrial, and chloroplast H^+-translocating ATPases in that the activity is DCCD-sensitive and can be solubilized by low ionic strength buffer plus EDTA. The purified protein has a relatively simple composition, consisting of α (62k) and β (49k) subunits in a stoichiometry of 4:4. Based on subunit composition, sequence homologies, and inhibitor sensitivity patterns, it has recently been proposed that those archaebacterial membrane-bound ATPases which probably function in H^+ translocation are specifically related to the eukaryotic, vacuolar H^+-ATPases (Nelson and Nelson, 1989). Mayer *et al.* (1987) showed the presence of stalked particles (presumably ATPase) associated with the membrane in the methanogenic

organism Gö1, which is most probably a member of the *Methanomicrobiales*, based on the ability of this organism to grow autotrophically on and produce methane from methanol. Using immunoelectron microscopic techniques, membrane-associated cross-reactivity was also detected with polyclonal antiserum directed against the β subunit of *E. coli* F_1F_0 ATPase.

In addition to H_2/CO_2, elimination of the membrane electrical potential with ionophores inhibits methane formation from methanol alone (Blaut and Gottschalk, 1984a). In this metabolic mode, the six-electron oxidation of one mole of methanol to CO_2 provides electrons for the reduction of three other moles of methanol to methane:

$$CH_3OH + H_2O \longrightarrow CO_2 + 6H$$

$$3CH_3OH + 6H \longrightarrow 3CH_4 + 3H_2O$$

As described above, evidence has been presented that all methanogenic oxidations (C_1 units or H_2) transfer electrons to a central, low-potential "pool" [perhaps the iron–sulfur centers recently detected by EPR spectroscopy in *Mb. thermoautotrophicum* (Rogers *et al.*, 1988)] which funnels these low-potential electrons to the methyl reductase, which in turn produces CH_4 and accomplishes ATP synthesis.[4] Among the various C_1 oxidation–reductions methanol oxidation may well be too positive in potential to donate electrons to this pool. Indeed, Gottschalk has provided data for *Methanosarcina barkeri* which demonstrates that this apparently uphill reduction is driven by coupling to the energetically downhill electrogenic influx of sodium ions (Blaut *et al.*, 1986):

Blaut *et al.* (1985) showed that, while collapse of the electrical potential (upon addition of protonophore) inhibits methanogenic electron transfer from methanol alone, the reduction of methanol by formaldehyde (the two-electron oxidation product of methanol) is not sensitive to this treatment. In this case, the electrons for the reduction of methanol to methane are provided by oxidation of the formaldehyde to CO_2, which suggests that it is the two-electron oxidation of methanol to formaldehyde which is the sensitive step. It was also shown that methanogenesis from methanol alone is strictly dependent on the presence of sodium, whereas disproportionation of formaldehyde (oxidation of HCHO to CO_2 which reduces other molecules of HCHO to CH_4) occurs without sodium. These results suggest that the

[4] It is important to point out that if electrons from an energetically unfavorable oxidation (e.g., methanol) are provided to a more negative potential "pool" which ultimately drives coupled ATP synthesis by further electron transfer to an even more positive acceptor such as methyl coenzyme M (by whatever mechanism), the free energy invested by initially driving this reverse electron transfer coupled to electrogenic cation influx can at least theoretically be conserved. The free energy gained from the electrogenic influx of cation is thus conserved by lowering the effective reduction potential of the electrons derived from methanol oxidation.

oxidation of methanol requires the presence of both sodium and a transmembrane electrical potential.

Müller *et al.* (1987a) showed that sodium is extruded by cells concomitant with methanogenesis and ATP synthesis. In addition, the presence of Na^+/H^+ antiport activity was demonstrated, by detecting medium acidification upon the addition of sodium to resting cells. This antiporter was sensitive to inhibitors of sodium-linked transport systems (harmaline and amiloride).

The above data, alone, would not discriminate between (A) a chemiosmotic (i.e., primary, electron transfer-driven proton extrusion) system which drives sodium efflux via Na^+/H^+ antiport and (B) primary sodium extrusion (e.g., ATPase) which is also sensitive to these inhibitors and which maintains internal pH homeostasis by the Na^+/H^+ antiporter, similar to the proposal for the two other orders of methanogen as described above. However, unlike *Mb. thermoautotrophicum*, Müller *et al.* (1987a) showed that the maintenance of internal relatively alkaline pH at acid external pH values in *Ms. barkeri* is not dependent on the presence of sodium, which argues against a role for sodium in internal pH maintenance under acidic conditions in this organism. In addition, while amiloride inhibits methanogenesis-driven sodium efflux, under relatively acidic external pH the addition of amiloride actually increases energetically uphill proton extrusion. This latter result shows that there must be a primary driving force for proton translocation which is independent of sodium. Taken together, then, the data of Müller *et al.* (1987a) demonstrate that the sodium gradient in *Ms. barkeri* is generated by Na^+/H^+ antiport and that the primary ion translocated coupled to metabolic energy is the proton. These results correlate nicely with the previous work cited above utilizing effects of DCCD and TCS on electrical potential formation and proton translocation driven by electron transfer from H_2 plus methanol.

Additional recent studies from Gottschalk's laboratory have supported this overall scheme for ion translocation in *Ms. barkeri*. Müller *et al.* (1988a) showed that methanogenesis from methanol is inhibited by (a) inhibition of Na^+/H^+ antiport (with amiloride or K^+), (b) monensin, or (c) inversion of the normal direction of the sodium gradient, such that $Na^+_{in} > Na^+_{out}$. Under these same conditions, methanogenesis from H_2 plus methanol is unaffected. These results indicate that a sodium gradient is required for methanol oxidation. In addition, when the Na^+/H^+ antiporter is inhibited by amiloride, addition of methanol alone results in sodium influx, while H_2 plus methanol addition shows no such effect. Finally, Müller *et al.* (1988b) examined ion translocation phenomena in cells driven by the addition of formaldehyde or formaldehyde plus H_2. Under these conditions, the oxidation of methanol occurs in reverse (i.e., reduction of formaldehyde),

and so sodium extrusion should occur. Indeed, such extrusion of sodium was detected, and this translocation is insensitive to protonophore but sensitive to monensin. In addition, a protonophore-insensitive transmembrane electrical potential of -60 to -70 mV was detected.

Several species of methanogen are capable of acetoclastic metabolism, which is the production of methane and carbon dioxide from acetate:

$$CH_3COOH \longrightarrow CH_4 + CO_2$$

This reaction is coupled to the synthesis of ATP. It has been shown that the process occurs in two steps, the cleavage of the carbon–carbon bond with the formation of a bound carbonyl of unknown structure and methyl coenzyme M:

$$CH_3COOH + CoM\text{–}SH \longrightarrow [CO] + CoM\text{–}CH_3 + H_2O$$

It has recently been shown that activation of acetate by ATP with the production of acetyl phosphate and acetyl-CoA are required before scission of the carbon–carbon bond (Krzycki and Zeikus, 1984; Krzycki *et al.*, 1985; Grahame and Stadtman, 1987; Fischer and Thauer, 1988). Methane is subsequently produced from the reduction of methyl coenzyme M with electrons generated from the oxidation of the CO group, which is catalyzed by carbon monoxide dehydrogenase. This enzyme, similar to its counterpart in *Clostridium thermoaceticum* (Ragsdale and Wood, 1985; Wood *et al.*, 1986), probably catalyzes physiologically the cleavage of acetyl-CoA with the oxidation of the CO group and production of a bound methyl moiety, which is subsequently transferred to CoM–SH (Lovley *et al.*, 1984; Krzycki *et al.*, 1985; Terlesky *et al.*, 1987). Thauer's laboratory has provided evidence that the electron transfer from the CO group to methylcoenzyme M generates ATP by a chemiosmotic mechanism, involving the production of a proton gradient. The data which support this conclusion are summarized below.

In the presence of bromoethanesulfonate (a specific inhibitor of methyl coenzyme M reduction), oxidation of carbon monoxide (CO) is coupled to the synthesis of ATP (Bott *et al.*, 1986). Under these conditions, the electron acceptor is H^+, producing H_2 (an exergonic reaction). Concomitantly, a membrane electrical potential of -100 to -150 mV is produced, which is eliminated by the addition of protonophore. ATP synthesis is also eliminated, and CO oxidation is stimulated some 70%. Addition of DCCD inhibits ATP synthesis, retards electron transfer, and has little effect on the electrical potential. Subsequent addition of protonophore relieves the inhibition by DCCD of CO oxidation. These results suggest that the transmembrane electrical potential is directly generated by electron transfer, and that ATP is synthesized by a DCCD-sensitive, H^+-translocating ATP synthase. In a subsequent study (Bott and Thauer, 1987), it was shown that reduction of

CH_3OH by H_2 to CH_4 will provide energy for the production of CO from H_2 plus CO_2. Presumably, the outward translocation of protons coupled to methanol reduction by H_2 drives the reaction above in reverse, since protons would be driven *inwards*. This was demonstrated by showing that protonophore eliminates the membrane electrical potential, ATP synthesis, and CO production, while not affecting methanogenesis. DCCD has the expected effects, i.e., inhibition of ATP synthesis, but has little effect on electron transfer (production of CH_4 and CO); subsequent addition of protonophore collapses the membrane potential and inhibits CO synthesis but has little effect on CH_4 formation. In addition, it was found that neither production of CO from H_2 plus CO_2 nor the reverse reaction is dependent on the presence of sodium ion. In a recent paper, DCCD-insensitive, electrogenic proton translocation outwards was demonstrated coupled to CO oxidation (Bott and Thauer, 1989). These results show that CO oxidation is reversibly coupled to proton translocation.

Peinemann *et al.* (1988) have shown that addition of acetate to resting cells of *Ms. barkeri* results in methanogenesis-driven ATP synthesis and increase in the membrane electrical potential. Protonophore addition results in depolarization of the membrane, decrease in ATP levels, and also inhibition of methane formation. DCCD decreased ATP levels and also inhibited methanogenesis. It is likely that the inhibition of methanogenesis is due to the depletion of ATP, which is required for the initial activation of acetate prior to metabolism. Interestingly, sodium was found to be required for methanogenesis from acetate, and a transmembrane sodium gradient is generated. The site(s) in the acetoclastic reaction sequence which requires sodium is at present not known, but is apparently not CO dehydrogenase, methyl reductase, or the conversion of acetate to acetyl phosphate or acetyl-CoA.

The cellular enzymatic components involved in these acetoclastic electron transfer reactions in *Methanosarcina* are beginning to be identifed. In addition to CO dehydrogenase, a ferredoxin and a membrane-bound hydrogenase have been shown to be involved in electron transfer, coupling CO oxidation to methylcoenzyme M reduction (Terlesky and Ferry, 1988a, b). Also, a methyl group-transferring, protein-bound corrinoid has been reported to be involved in transfer to coenzyme M (van de Wijngaard *et al.* 1988).

These data show that the Methanomicrobiales in general (and *Methanosarcina* in particular) couple electron transfer to ATP synthesis by a chemiosmotic mechanism. In particular, proton translocation outwards is coupled to the reduction of CH_3OH by H_2 and to the oxidation of CO to CO_2 plus H_2. In addition, however, a sodium gradient is produced by Na^+/H^+ antiport, which can be directly coupled to electron transfer to drive the oxidation of methanol to formaldehyde. In addition, there may be an involvement of sodium in the energetics of acetate catabolism.

Possible Evolutionary Significance of Methanogen Bioenergetic Diversity

The methanogenic bacteria display a remarkable diversity in the overall patterns of free-energy transduction driven by electron transfer. In particular, the Methanococcales couple ATP synthesis to methane formation by a direct mechanism, not involving the intermediacy of a transmembrane electrochemical gradient. Internal ion homeostasis (as well as ion–solute cotransport) is powered by an electrogenic sodium-translocating ATPase. The Methanomicrobiales, on the other hand, are capable of ATP synthesis via a DCCD-sensitive, F_1F_0-type ATPase which is coupled to methanogenic electron transfer (specifically, H_2 reduction of CH_3OH) through the intermediacy of a transmembrane electrochemical proton gradient. In addition, energetically unfavorable electron transfer (methanol oxidation and reduction of CO_2 by H_2 to CO) is driven by coupling to the energetically downhill influx of sodium and of proton, respectively. In the case of sodium, the driving force is provided by Na^+/H^+ antiport. The Methanobacteriales possess a sodium-translocating ATPase and Na^+/H^+ antiporter (which functions in internal pH maintenance), and sodium plays a special role in bioenergetics, although the precise role(s) has yet to be delineated. At this point, it appears that this order of methanogen resembles the Methanococcales bioenergetically more closely than the Methanomicrobiales.

In all methanogens, the evidence is strong that the principal site of ATP synthesis is the terminal, methane-producing, most energetically favorable electron transfer step in all metabolic modes, the methyl reductase system. A puzzling question, then, is how can this enzyme, which catalyzes in all methanogens the same basic electron transfer reaction, apparently function to accomplish ATP synthesis in two cases (Methanococcales and Methanomicrobiales) by different mechanisms? In the former, ATP synthesis is coupled by a direct mechanism; in the latter, the enzyme couples electron transfer to the electrogenic translocation of proton. One possibility is that ATP synthesis by the "direct" mechanism occurs in all methanogens, but that the Methanomicrobiales have evolved in addition a direct link between electron transfer and ion gradient formation/utilization. The former mechanism may thus be a more ancient adaptation for energy coupling to methane formation primarily from CO_2, which would be present in early ecosystems where autotrophic metabolisms would be required. Methanogenesis utilizing C_1 units as reductant instead of H_2, provided by biomass degradation (presumably a later-appearing environment), in some cases required a mechanism for "priming the pump," by reverse electron transfer from a relatively positive potential redox couple driven by cation influx. Such bioenergetic considerations are also consistent with molecular biological evidence suggesting specific relatedness of the Halobacteria to the Methanomicrobiales, to the

exclusion of the Methanococcales and the Methanobacteriales (Woese and Olsen, 1986), including comparisons of the genes for the methyl reductase (Allmansberger *et al.*, 1989; Weil *et al.*, 1989). An intriguing possibility is that the "direct" mechanism of ATP synthesis presently employed by the Methanococcales is an evolutionary forerunner of energetic coupling mechanisms utilizing transmembrane ion gradients.

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