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MINI-REVIEW

The Na^+ Cycle of Extreme Alkalophiles: A Secondary Na^+/H^+ Antiporter and Na^+/Solute Symporters

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Abstract

Extremely alkalophilic bacteria that grow optimally at pH 10.5 and above are generally aerobic bacilli that grow at mesophilic temperatures and moderate salt levels. The adaptations to alkalophily in these organisms may be distinguished from responses to combined challenges of high pH together with other stresses such as salinity or anaerobiosis. These alkalophiles all possess a simple and physiologically crucial Na^+ cycle that accomplishes the key task of pH homeostasis. An electrogenic, secondary Na^+/H^+ antiporter is energized by the electrochemical proton gradient formed by the proton-pumping respiratory chain. The antiporter facilitates maintenance of a pH_{in} that is two or more pH units lower than pH_{out} at optimal pH values for growth. It also largely converts the initial electrochemical proton gradient formed by respiration into an electrochemical sodium gradient that energizes motility as well as a plethora of Na^+/solute symporters. These symporters catalyze solute accumulation and, importantly, reentry of Na^+. The extreme nonmarine alkalophiles exhibit no primary sodium pumping dependent upon either respiration or ATP. ATP synthesis is not part of their Na^+ cycle. Rather, the specific details of oxidative phosphorylation in these organisms are an interesting analogue of the same process in mitochondria, and may utilize some common features to optimize energy transduction.

Key Words: Alkalophily; Na^+/H^+ antiporter; Na^+/solute symporters; F_1F_0-ATPase; oxidative phosphorylation.

Introduction

What are the specific bioenergetic and biochemical adaptations associated with growth at extremely high pH values, and what role(s) does Na^+ have in

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these adaptations? The elucidation of the basis for alkalophily is best studied in bacteria that thrive in very alkaline media, but are not simultaneously challenged by additional environmental stresses such as high salt, high or low temperature, or anaerobiosis. Accordingly, obligately aerobic species of *Bacillus* that grow optimally at pH values above 10 in the presence of only 1–100 mM NaCl have been the organisms of choice for studies of alkalophily. During the past ten years, properties have come into focus that are common to all such bacteria examined to date (Krulwich *et al.*, 1988; Krulwich and Guffanti, 1989). These properties include a proton-pumping respiratory chain that exhibits high H^+/O ratios and is always present at a very high concentration of respiratory chain components in the cytoplasmic membrane. Another common property is a Na^+ cycle consisting entirely of secondary porters, which converts the conventional $\Delta\bar{\mu}_{H^+}$,[2] acid and positive out, produced by respiration into the typical pattern of the alkalophile, i.e., a large ΔpH, acid in, and a sizable $\Delta\psi$, still positive out, yielding a small, total, bulk $\Delta\bar{\mu}_{H^+}$. The conversion is essential, since energized cells of alkalophilic bacilli maintain a cytoplasmic pH at 8.5 or below during growth at pH values up to pH 10.5, and show only slightly higher cytoplasmic pH values (near pH 9.0) during growth at pH values up to pH 11.5. The conversion is accomplished by an electrogenic Na^+/H^+ antiporter that catalyzes coupled extrusion of Na^+ in exchange for a larger number of inwardly translocated protons, thus maintaining a cytoplasmic pH that is much lower than pH_{out}. In addition, the combined activities of respiration and secondary Na^+/H^+ antiport result in an inwardly directed ΔpNa^+. The Na^+ cycle is completed with the return of Na^+ to the cytoplasm via Na^+/solute symporters that take advantage of the ΔpNa^+ and $\Delta\psi$ to accumulate solutes. In those extreme alkalophiles that are aerobic, and are neither marine nor halophilic, the Na^+ cycle is thus a crucial but simple cycle. It is the facilitator of pH homeostasis, of the uptake of many solutes, and, as noted later, of motility. It contains no primary Na^+ pumps such as those found in certain anaerobic or facultative, marine bacteria. We will discuss the constituents, roles, and regulatory problems of the Na^+ cycle, and will present evidence that the Na^+ cycle consists entirely of secondary porters. Finally, we will consider the bioenergetic problem associated with the two catalysts in the alkalophile, the Na^+/H^+ antiporter and the proton-translocating F_1F_0-ATPase, that translocate protons inward from a highly alkaline *milieu* at the low $\Delta\bar{\mu}_{H^+}$. These are the only two known membrane proteins that must resolve this problem in the alkalophile, but each one is involved in a critical physiological process.

[2]Abbreviations: AIB, α-aminoisobutyric acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; PMS, phenazine methosulfate; $\Delta\bar{\mu}_{H^+}$, transmembrane electrochemical proton gradient; $\Delta\psi$, transmembrane electrical potential; ΔpH, transmembrane pH gradient; $\Delta\bar{\mu}_{Na^+}$, transmembrane electrochemical gradient of sodium; ΔpNa^+, transmembrane chemical gradient of sodium.

The $\Delta\tilde{\mu}_{H^+}$ Is Formed by Primary Proton Pumping During Respiration and Is Largely Converted to a $\Delta\tilde{\mu}_{Na^+}$ by a Secondary Na^+/H^+ Antiporter

The extremely alkalophilic bacilli have all been found to possess remarkably high concentrations of respiratory chain components in the membrane (Lewis *et al.*, 1980; Krulwich and Guffanti, 1989). The concentrations found in the membranes of cells grown at high pH are comparable to those found in beef heart mitochondria, and the component complexes are similarly comparable. They include, for example, a bc_1 complex and cytochrome aa_3 type terminal oxidase (Lewis *et al.*, 1981; Kitada *et al.*, 1983; Kitada and Krulwich, 1984). Interestingly, the coupling membrane of the alkalophile is also rich in cardiolipin (Koga *et al.*, 1982; Clejan *et al.*, 1986), a specific phospholipid of the mitochondrial membrane. We might speculate that the extreme alkalophiles may have convergently employed some of the same strategies as the mitochondrion to achieve effective energy transduction, out of the necessity created by the special energy costs of life at very high pH. In at least one facultatively alkalophilic strain, i.e., a strain that grows both at very alkaline and near neutral pH values, the level of cytochromes is much higher in cells grown at pH 10.5 than at pH 7.5 (Guffanti *et al.*, 1986).

The alkalophile respiratory chain pumps protons. In obligately alkalophilic *Bacillus firmus* RAB, H^+/O ratios as high as 13 were observed in oxygen pulse experiments. These experiments were conducted in the presence of valinomycin and potassium to prevent the development of a $\Delta\psi$, with respiration supported by endogenous substrates at pH 9.0 (Lewis *et al.*, 1983). The values observed with this strain were lower at pH 7.0, but that is probably because obligate alkalophiles lose membrane integrity at neutral pH (Krulwich *et al.*, 1985; Clejan and Krulwich, 1988). Again, the H^+/O ratios observed for the alkalophile at pH 9.0 are comparable to values reported for mitochondria. The observation of such values in the alkalophile supports the view that the multitude of cytochromes function effectively with respect to proton pumping. Moreover, several lines of evidence indicate that there are no Na^+-pumping respiratory chain complexes such as those observed in marine vibrios and other marine bacteria (Tokuda and Unemoto, 1982, 1984; Ken-Dror *et al.*, 1986). First, neither NADH oxidation nor respiration is stimulated appreciably by added Na^+ (Guffanti *et al.*, 1986). Second, respiration-induced efflux of $^{22}Na^+$ from preloaded cells is inhibited at pH 9.0 by protonophores, and at both pH 9.0 and much more alkaline pH by valinomycin + K^+. Were there a primary Na^+ pump associated with a respiratory chain complex, the abolition of the $\Delta\psi$ by such treatments should have stimulated, and certainly not inhibited, Na^+ efflux. On the other hand, the inhibition observed is entirely consistent with many other lines of

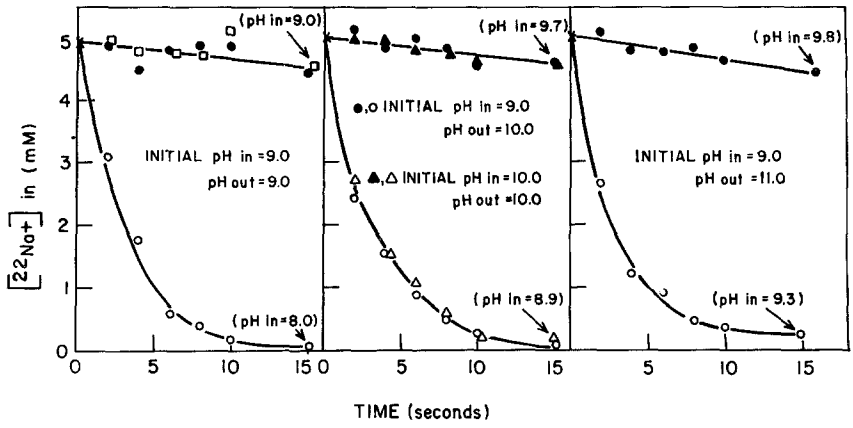


Fig. 1. The inhibition of respiration-dependent $^{22}Na^+$ efflux and concomitant H^+ uptake in alkalophilic *B. firmus* OF4 by CCCP and/or valinomycin + K^+. Logarithmically growing cells were washed and resuspended in 100 mM potassium carbonate, pH 9.0 (●, ○, □) or 10.0 (▲, △) containing 10 mM potassium malate and 5 mM $^{22}NaCl$. The cells were incubated for 2 h at room temperature under N_2. Those cells that were to be diluted into buffers containing inhibitors were treated with those inhibitors during the last 15 min of this preincubation period. Efflux was initiated by diluting the cells 200-fold into oxygenated buffer containing 50 mM sodium–50 mM potassium carbonate at the external pH indicated in the figure. The following additions were also made: (○) none, (●, ▲) 1 μM valinomycin, or (□) 10 μM CCCP. The internal pH of the diluted suspensions was determined from measurements of the ΔpH using the distribution of 4.3 μM [^{14}C]-methylamine in completely parallel sets of cells that had been preincubated with nonradioactive NaCl.

evidence that implicate a secondary, electrogenic (and hence $\Delta\psi$-dependent) Na^+/H^+ antiporter as the necessary and sufficient mediator of Na^+ extrusion in obligately aerobic, extreme alkalophiles.

The rapid time course of respiration-dependent $^{22}Na^+$ extrusion from an extreme alkalophile is shown in Figs. 1 and 2. A similarly rapid time course was observed previously, when $^{22}Na^+$ efflux by the Na^+/H^+ antiporter was initiated by imposition of a diffusion potential, positive out, across the alkalophile membrane (Garcia *et al.*, 1983). Thus, it was likely that the antiporter accounts fully for Na^+ extrusion. In the experiments depicted in Fig. 1, logarithmic phase cells of *Bacillus firmus* OF4 were loaded with both $^{22}Na^+$ and malate during incubation under anaerobic conditions at the pH values indicated as "initial pH_{in}." This was usually pH 9.0, but in one set of experiments, the loading and pH equilibration was at pH 10.0. $^{22}Na^+$ efflux was initiated by diluting the anaerobic suspension into aerated buffer at the pH values indicated as "initial pH_{out}." Efflux was very rapid at pH_{in} of both 9.0 and 10.0 and external pH values from 9.0 to 11.0. At an external pH of 9.0, the protonophore CCCP completely inhibited $^{22}Na^+$ efflux, and over the whole range of external pH values, valinomycin + K^+ inhibited efflux

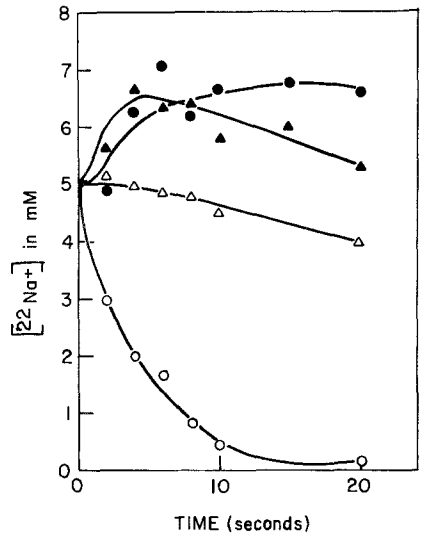


Fig. 2. The effect of CCCP or valinomycin + K^+ on electron-donor dependent efflux of $^{22}Na^+$ from cells of alkalophilic *B. firmus* OF4. Cells that were starved as described elsewhere (Guffanti and Krulwich, 1988) were equilibrated with 5 mM $^{22}Na^+$ in 100 mM potassium carbonate buffer, pH 9.0. For cells that were to be diluted into buffer containing inhibitors, the inhibitor was added to the preincubation buffer for the last 15 min before dilution. Efflux was initiated by diluting the cells 200-fold with 50 mM sodium–50 mM potassium-carbonate, pH 9.0. Where indicated, the following additions were made: (●) none; (○) 10 mM potassium ascorbate + 1 mM PMS; (△) ascorbate–PMS plus 10 μM CCCP; (▲) ascorbate–PMS plus 1 μM valinomycin.

entirely. These inhibitory effects of valinomycin and protonophore did not result from an inhibition of respiration; control experiments indicated that at the concentrations employed, the inhibitors had no effect on oxygen uptake. Nor was the effect of valinomycin + K^+ mediated by a precipitous change in pH_{in}. As shown by the numbers in parentheses in Fig. 1, $^{22}Na^+$ efflux was accompanied by an acidification of the cytoplasm, as expected for an antiporter-mediated efflux. In the presence of valinomycin + K^+, there was instead, either no change in pH_{in} or an increase from pH 9.0 up to pH 9.7 or 9.8. Since a pH_{in} of 10.0 is compatible with maximal $^{22}Na^+$ efflux (as shown in the middle panel of Fig. 1), the inhibition by valinomycin + K^+ must be mediated by its effect upon the $\Delta\psi$; as already noted, these results are compatible with a secondary Na^+/H^+ antiporter functioning subsequent to primary proton pumping by the respiratory chain, but not with the presence of a primary Na^+ pump linked to respiration.

The data shown in Fig. 2 illustrate the same findings in a protocol focusing upon the cytochrome oxidase. Cells of *B. firmus* OF4 were loaded with $^{22}Na^+$ at pH 9.0 after the cells had been depleted of endogenous electron

donors. $^{22}Na^+$ efflux was initiated by the addition of ascorbate + phenazine methosulfate at pH 9.0. Efflux of $^{22}Na^+$ was rapid and was inhibited by either CCCP or valinomycin + K^+. The same experiment was conducted at an external pH of 11.0; the rate of ascorbate-PMS-dependent $^{22}Na^+$ efflux was similar to that observed at pH 9.0, and valinomycin + K^+ completely inhibited the efflux. These results corroborate earlier findings *in vitro*, in which everted membrane vesicles prepared from *Bacillus alcalophilus* exhibited either ATP- or electron donor-dependent $^{22}Na^+$ accumulation only when $\Delta\psi$ was generated by primary proton pumping (Mandel *et al.*, 1980; Guffanti, 1983).

What then are the characteristics of the secondary Na^+/H^+ antiporter and how has its role in pH homeostasis been demonstrated? The antiporter has been assayed, as indicated above, via $^{22}Na^+$ efflux or accumulation in both cells and vesicles, and in proteoliposomes (Krulwich *et al.*, 1988; Seto-Young *et al.*, 1985). Na^+-dependent proton movements have also been assayed. As already described, the antiport is $\Delta\psi$-dependent, and accordingly, both the Na^+ fluxes and Na^+-dependent proton fluxes are always found to be sensitive to agents that abolish the $\Delta\psi$. Among different alkalophilic bacilli there is considerable variability in the affinity of the antiporter for Na^+, and this affinity correlates well with the concentration of Na^+ required for growth (Krulwich *et al.*, 1988). All the Na^+/H^+ antiporters of the alkalophilic bacilli can use Li^+ as a substrate instead of Na^+ (Sugiyama *et al.*, 1985). The antiporter has not been shown to be sensitive to amiloride or its analogues in whole cells or right-side-out vesicles (Garcia and Krulwich, unpublished data), but this may result from the inaccessibility of the Na^+ site on the inside of the cell or vesicle. It would be of interest to examine amiloride sensitivity in an everted system.

Consistent with its role in acidifying the cytoplasm, the Na^+/H^+ antiporter of the extreme alkalophiles is generally found to be inactive or much less active at values of $pH_{in} < 8.5$ than at higher cytoplasmic pH values (Garcia *et al.*, 1983; Kitada *et al.*, 1982). An analogous inhibition of the Na^+/H^+ antiporter of *Escherichia coli* by low cytoplasmic pH has been demonstrated (Bassalina *et al.*, 1984). The relationship of antiporter activity in the alkalophilic bacilli to external pH is more complex and depends upon how the antiport is energized. As can be seen in Fig. 1, when the Na^+/H^+ antiporter is energized by respiration, the rate of Na^+ efflux is independent of external pH over a broad range of alkaline pH values. This is completely consistent with the antiporter's apparently crucial role throughout this range of pH. On the other hand, an interesting anomaly is observed when antiport, assayed for example as $^{22}Na^+$ efflux from starved cells (Garcia *et al.*, 1983), is energized by a valinomycin-mediated K^+ diffusion potential. Using that mode of energization, antiport is rapid at near neutral pH to pH 9.0, as long

as the cytoplasm is above pH 8.0. At higher values of pH_{out}, however, the rate of antiport falls off. For example, with an initial pH_{in} of 9.0, the rate of antiport at $pH_{out} = 10$ is lower than that at $pH_{out} = 9.0$ even though respiration-driven antiport activity is the same at those two external pH values and growth of the alkalophile is better at pH 10.0 than at 9.0. This discrepancy between energization of the Na^+/H^+ antiporter via respiration and an artificial diffusion potential that does not entail proton pumping is also seen in connection with the one other alkalophile function known to require inward proton translocation, i.e., ATP synthesis during oxidative phosphorylation. A model accounting for the discrepancy will be discussed below.

If the Na^+/H^+ antiporter is necessary for pH homeostasis, then removal of Na^+ should result in immediate alkalinization of the cytoplasm during growth or energized resting conditions. Accordingly, mutants that lack Na^+/H^+ antiporter activity should be unable to grow at extremely alkaline pH values. If the antiporter is sufficient for pH homeostasis, then the magnitude of the steady state, reversed ΔpH should be achievable given the $\Delta\psi$ and some reasonable H^+/Na^+ stoichiometry; also, the typical alkalophile $\Delta\bar{\mu}_{H^+}$ patterns should be generated in vesicles upon energization in the presence of Na^+ as the sole cation. These criteria are all met. A challenge of alkalophile cells, by incubation at their usual growth pH in the absence of Na^+, using any one of several protocols, results in immediate alkalinization of the cytoplasm so that $pH_{in} = pH_{out}$ (McLaggan *et al.*, 1984). For example, cells of *B. firmus* RAB growing on malate at pH 10.5, with $pH_{in} = 8.5$, show an immediate rise in pH_{in} to 10.5 upon rapid resuspension in malate-containing medium in the absence of Na^+ (Kitada *et al.*, 1982; Krulwich *et al.*, 1984). Mutants have been isolated from alkalophilic bacilli that have lost the ability to grow above pH 9.0—approximately the cut-off between commonly observed alkaline tolerance and true alkalophily. Several of these mutants in a number of different strains have been shown to have lost Na^+-dependent pH regulation and all the activities associated with the Na^+/H^+ antiporter (Koyama *et al.*, 1986; Krulwich *et al.*, 1988). At least one alkalophilic *Bacillus*, *B. firmus* RAB, generates variant strains that actually grow better than the parent at the most alkaline edge of the pH range (e.g., pH 11.5–12) and grow on somewhat lower Na^+ concentrations than the parent. Such variants appear to have enhanced activity of the Na^+/H^+ antiporter (Krulwich *et al.*, 1986).

In growing, energized alkalophile cells, the total, bulk $\Delta\bar{\mu}_{H^+}$ is low but never zero, because a $\Delta\psi$, positive out, that is greater than the reversed ΔpH is always observed (Krulwich and Guffanti, 1989). A Na^+/H^+ antiporter translocating more H^+ than Na^+ per cycle, by some small multiple could achieve the steady-state values observed in energized cells. The precise

stoichiometry will be demonstrable once the antiporter can be studied in proteoliposomes in the absence of other Na^+- or H^+-porters. Finally, isolated membrane vesicles generate patterns of the $\Delta\bar{\mu}_{H+}$ that are comparable to those of whole alkalophile cells when energized with an electron donor in the presence, but not in the absence, of Na^+ (or Li^+) as sole monovalent cation (Mandel *et al.*, 1980; Krulwich *et al.*, 1982). Nor is there any specific anion requirement (Kitada *et al.*, 1982). Thus, at the first approximation, the Na^+/H^+ antiporter is both necessary and sufficient for pH homeostasis in extreme alkalophiles. In some manner that is not yet completely understood, there is also some effect of Na^+ on the permeability of alkalophile cells to K^+ (Koyama *et al.*, 1987; Matsukura and Imae, 1987), and it is possible that the interplay between the two monovalent cations has some modulating effect on pH homeostasis.

Na^+/Solute Symporters Complete the Na^+ Cycle

Since pH homeostasis in cells that are extruding protons during respiration depends upon the constant efflux of Na^+ in exchange for protons, there must be an effective mechanism for the reentry of Na^+ to complete the cycle. Moreover, the combined activities of respiration and the Na^+/H^+ antiporter produce an inwardly directed $\Delta\bar{\mu}_{Na+}$ that could energize Na^+ reentry and perform bioenergetic work. Indeed, the functions of Na^+ reentry and accumulation of many different solutes appear to be served together via Na^+-coupled solute symporters (Koyama *et al.*, 1976; Kitada and Horikoshi, 1977; Guffanti *et al.*, 1978; Krulwich *et al.*, 1984). Some carbohydrate substrates appear to be transported by an ATP-dependent mechanism in the extreme alkalophiles (Guffanti *et al.*, 1979), but amino and organic acids and at least some ions are transported by Na^+-dependent processes. The Na^+/solute symporters, unlike the Na^+/H^+ antiporter, do not utilize Li^+ in place of Na^+ (Sugiyama *et al.*, 1985).

The specific evidence that Na^+/solute symporters actually play a role, presumably via Na^+ reentry, in pH homeostasis comes from experiments in which cells of *B. firmus* RAB were first equilibrated at pH 8.5 and then subjected to a sudden shift in pH_{out} to 10.5 (Krulwich *et al.*, 1984). In the presence of Na^+, but not in its absence, the internal pH was maintained at 8.5 and then gradually drifted upward. The addition of AIB, a nonmetabolizable amino acid analogue whose transport is coupled to Na^+ entry, markedly enhanced the time during which the pH_{in} remained steady at or below pH 8.5; the presence of AIB also enhanced the pH homeostasis observed in the presence of otherwise limitingly low concentrations of Na^+. It is most likely that the Na^+/solute symporters of alkalophiles have some form of pH

regulation so that pH homeostasis is facilitated during fluctuations in the concentration of solutes in the medium. None of the alkalophile symporters has yet been examined in sufficient molecular detail to assess this likelihood empirically.

Another bioenergetic function in the extreme alkalophiles that takes advantage of the $\Delta\bar{\mu}_{Na^+}$ is motility. Na^+ has clearly been shown to be required for motility in these organisms (Hirota *et al.*, 1981; Sugiyama *et al.*, 1986). The swimming speed of an alkalophilic *Bacillus* varied linearly with the magnitude of the $\Delta\bar{\mu}_{Na^+}$, and with the magnitude of the $\Delta\psi$ above a threshold of -90 mV, when the experiment was done in the presence of a fixed concentration of Na^+ (Hirota and Imae, 1983). The characteristics of the Na^+-dependent flagellar rotor as compared to those of the proton-driven rotors of most other bacteria are not yet known, but amiloride has been found to inhibit Na^+-driven motility of an alkalophilic *Bacillus* (Sugiyama *et al.*, 1988).

ATP Synthesis Is $\Delta\tilde{\mu}_{H^+}$-Driven, and Not Part of the Na^+ Cycle of Extreme Alkalophiles

A priori, it might be expected that extreme alkalophiles would use the $\Delta\bar{\mu}_{Na^+}$ that arises from respiration and antiport activity to energize ATP synthesis just as it uses the $\Delta\bar{\mu}_{Na^+}$ to energize solute uptake systems and motility. Thus far, however, there are no proven examples of Na^+-coupled ATP synthases that use Na^+ to produce the ATP that is formed during oxidative phosphorylation. The strongest case for possible, physiologically relevant synthesis of ATP by a Na^+-coupled enzyme is in a nonalkalophile. In the anaerobic fermentative organism, *Propionigenium modestum*, the Na^+-coupled synthase may well take advantage of the $\Delta\bar{\mu}_{Na^+}$ established by an Na^+-translocating decarboxylase to make some ATP (Laubinger and Dimroth, 1987, 1988), but it is not yet clear whether the enzyme utilizes only Na^+ or does so preferentially to protons. In facultatively anaerobic, marine vibrios, primary pumping of Na^+ via the respiratory chain augments the $\Delta\psi$ at moderately alkaline pH values (below pH 9.0), in addition to extruding Na^+ (Tokuda *et al.*, 1988). It is possible that the Na^+-coupled ATPase of these organisms similarly augments proton-coupled oxidative phosphorylation at moderately alkaline pH values (Dibrov *et al.*, 1986). These are, however, nonalkalophiles that occasionally encounter a somewhat alkaline *milieu* and do not grow at all at extremely alkaline pH. In the absence of data from mutants lacking the proton translocating ATP synthase activity, it is likely that the Na^+-coupled ATPase of the vibrios functions in Na^+ extrusion during those optimal, fermentative growth conditions in which respiratory

chain activity would be depressed, as proposed in the anaerobic methanogens (Lancaster, 1988).

Certainly, the extreme alkalophiles—truly adapted for life at pH values 1.5–3 units above pH 9.0—do not use Na^+ as a coupling ion for ATP synthesis during oxidative phosphorylation. In common with other aerobic cells and organelles that are most highly adapted to carry out oxidative phosphorylation, the alkalophilic bacilli couple ATP synthesis to protons utilizing an F_1F_0-ATPase in spite of the low bulk $\Delta\bar{\mu}_{H^+}$. These findings make it tempting to speculate that the use of protons confers specific advantages in energy coupling to ATP synthesis relative to the use of Na^+.

The lines of evidence supporting the use of protons as the coupling ion for ATP synthesis in oxidative phosphorylation by extreme alkalophiles are numerous. Na^+ is neither required nor stimulatory to ATP synthesis by starved whole cells or ADP + Pi-loaded vesicles (Guffanti *et al.*, 1981, 1985). Nor is Na^+ stimulatory or required for ATP-^{32}Pi exchange by proteoliposomes containing purified ATPase (Hicks *et al.*, unpublished data). Rather, those processes are sensitive to protonophores. Moreover, DCCD-sensitive proton movements have been demonstrated concomitant with ATP synthesis in the vesicle experiments (Guffanti *et al.*, 1981). An F_1-ATPase has been purified and characterized from two alkalophilic bacilli (Koyama *et al.*, 1980; Hicks and Krulwich, 1986), and the F_1F_0-ATPase from *B. firmus* OF4 has been purified and partially characterized. As with the total membrane-associated ATPase activity, there is no Na^+-stimulated ATPase in any of these preparations. Moreover, a Na^+-coupled synthase that might have escaped these purification and assay attempts should nonetheless have been demonstrable upon imposition of large chemical and electrochemical gradients of Na^+ across the membrane of ATP-depleted cells of *B. firmus* OF4; on the contrary, imposition of artificial proton gradients but not Na^+ gradients resulted in ATP synthesis (Guffanti and Krulwich, 1988). Data from this latter kind of experiment are illustrated in Fig. 3. In these experiments, cells are starved to reduce the ATP levels under conditions in which the cells retain their full respiratory capacity, their capacity to generate a $\Delta\psi$, and their capacity to restore normal levels of cellular ATP when appropriately energized. They are then treated with cyanide so that the $\Delta\psi$ is completely dissipated. If this is not achieved, there remains the possibility that a small $\Delta\psi$, like the small $\Delta\bar{\mu}_{H^+}$ of the growing cells, is quite competent to energize ATP synthesis. This may in fact be a problem in interpreting experiments conducted in other cell systems in which ostensible ΔpNa^+-dependent ATP synthesis was demonstrated without inhibition of the respiratory chain (Dibrov *et al.*, 1986), so that a residual $\Delta\psi$ may play a role in the synthesis observed. As shown in Fig. 3 with *B. firmus* OF4 at an initial pH of 10.2, imposition of an artificial ΔpNa^+ alone can cause the uptake of AIB, presumably via Na^+/solute

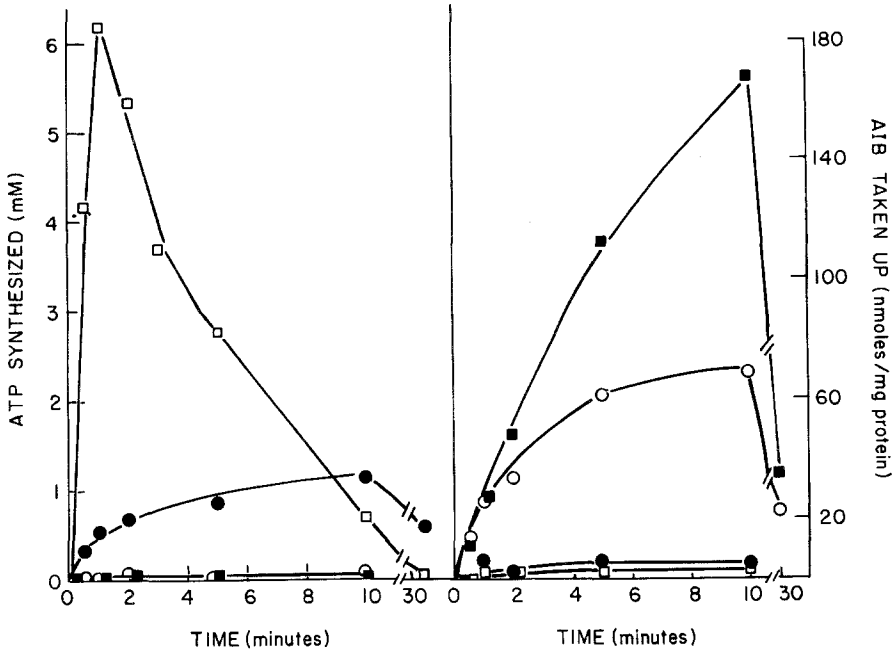


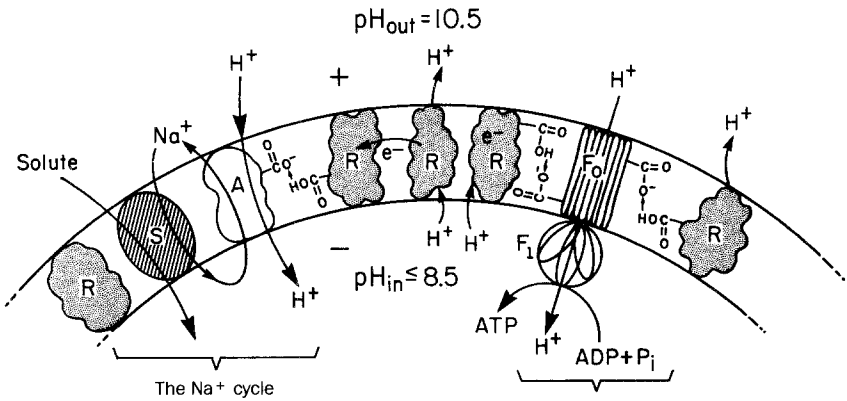
Fig. 3. ATP synthesis and AIB uptake by starved, cyanide-treated cells of *B. firmus* OF4 in response to an imposed ΔpH, $\Delta\bar{\mu}_{H+}$, ΔpNa^+, or $\Delta\bar{\mu}_{Na+}$. Cells of *B. firmus* OF4 were starved as described previously (Guffanti and Krulwich, 1988) and were suspended in 25 mM Tris, 10 mM $MgSO_4$, and 100 mM KCl at pH 10.2 (●, □) or pH 9.5 (○, ■). The thick cell suspensions were incubated for 30 min with 10 mM KCN. Reactions were initiated by diluting the cells 200-fold into the following buffers all of which contained 10 mM cyanide, Na^+ or K^+ salt as appropriate. (●) 25 mM Tris–100 mM KCl, pH 7.5 (ΔpH imposed); (○) 25 mM Tris–100 mM–100 mM NaCl, pH 9.5 (ΔpNa^+ imposed); (■) 25 mM Tris–100 mM NaCl, 1 μM valinomycin, pH 9.5 ($\Delta\bar{\mu}_{Na+}$ imposed); or (□) 25 mM Tris–100 mM choline chloride, 1 μM valinomycin, pH 7.5 ($\Delta\bar{\mu}_{H+}$ imposed). Incubation mixtures that were used for measurements of AIB uptake contained 250 μM [^{14}C]AIB. Samples were removed at various times for measurements of ATP synthesis or AIB uptake described previously (Guffanti *et al.*, 1984).

symport; simultaneous imposition of a valinomycin-mediated K^+ diffusion potential and the ΔpNa^+ is even more effective for AIB uptake. By contrast, imposition of a ΔpH either alone or together with a diffusion potential does not result in any AIB uptake. The findings, in the same cells, for ATP synthesis are precisely the opposite (Fig. 3). As shown before (Guffanti and Krulwich, 1988), an imposed ΔpH alone results in some synthesis of ATP, and imposition of a ΔpH together with a diffusion potential results in synthesis of ATP up to the levels observed in growing cells. Imposition of the same chemical or electrochemical gradients of Na^+ that were effective for AIB uptake by the same cells results in no ATP synthesis whatsoever. There is, therefore, no synthase in the membrane that is capable of coupling ATP

synthesis even to a very large, and demonstrably competent, electrochemical gradient of Na^+.

It is particularly notable in the left-hand panel of Fig. 3, and has been consistently observed, that not only is Na^+ totally ineffective as a coupling ion for ATP synthesis in extreme alkalophiles, but a transmembrane diffusion potential that is generated by K^+ fluxes rather than by proton pumping is also ineffective at very alkaline pH values (Guffanti *et al.*, 1984, 1985; Guffanti and Krulwich, 1988). Earlier studies have shown that this incompetence of a valinomycin-mediated K^+ diffusion potential is not observed with alkalophile cells at neutral pH values, but only at the very alkaline pH values at which they grow best. At very alkaline pH values protons need to be added when the diffusion potential is imposed, in order to observe ATP synthesis that is comparable to that observed with the proton-pumping respiratory chain (Guffanti and Krulwich, 1988). The incompetence of the diffusion potential alone at high pH does not relate to trivial buffer effects, the presence or absence of Na^+, changes in pH_{in}, or increases in the rate of ATP hydrolysis at high pH (Guffanti *et al.*, 1985), but reflects a difference between a $\Delta\bar{\mu}_{H^+}$ composed entirely of transmembrane potential that did not involve primary proton pumping and one that did. These observations completely parallel observations described in an earlier section with respect to the energization of the Na^+/H^+ antiporter.

As we have discussed elsewhere (Krulwich and Guffanti, 1989), these observations incline us toward a working hypothesis for energization of the two proton-translocating functions of the extreme alkalophiles that incorporates: the high concentration of respiratory chain components; the membrane lipid characteristics; and the apparent need for actual proton pumping in order for ATP synthesis or rapid antiport activity to occur at very high pH in the presence of an appropriate $\Delta\psi$. This parallel coupling model utilizes many concepts and proposals developed by other investigators of oxidative phosphorylation (e.g., Ferguson, 1985; Westerhoff *et al.*, 1984), especially proposals by Rottenberg (1985, 1988). As shown for the extreme alkalophile in Fig. 4, respiratory chain components that pump protons may release those protons to the external medium or may "hand off" a proton directly to an appropriate proton-binding residue on the Na^+/H^+ antiporter or the F_1F_0-ATPase. At pH 7.0, the external proton concentration is sufficiently high that protons derived from the medium may be the major source of the protons translocated by the latter two enzymes, and that is clearly the situation when a diffusion potential energizes the antiport or ATP synthesis at pH values below 9.0. The "hand off" pathway, whereby a proton released by a respiratory component reaches the antiporter or synthase without first appearing in the bulk, may become crucial at the more alkaline pH values. This would account for the incompetence of the diffusion



The Na^+ cycle

Electrogenic Na^+/H^+ Antiporter (A) extrudes Na^+ in exchange for protons, some of which may be delivered through collisions with proton-translocating respiratory chain components (R). The A catalyzes net proton accumulation and transduces the electrochemical proton gradient that is produced by respiration into an electrochemical sodium gradient. Na^+/solute symporters (S) utilize the latter gradient for solute accumulation, and also provide the re-entry routes for Na^+.

At the very high values of pH_{out} that are optimal for growth the protons that energize ATP synthesis via the F_1F_o ATPase are largely (and may be entirely) delivered "locally", upon collision with respiratory chain components (R). Only some of the protons pumped by R appear in the bulk. The $[H^+]_{out}$ at the very alkaline pH is apparently too low to support ATP synthesis upon imposition of a K^+ diffusion potential. The localized flow of protons is thus crucial.

Fig. 4. A schematic diagram illustrating the Na^+ cycle of extreme alkalophilic bacilli and a working hypothesis with respect to parallel coupling pathways for the two membrane functions that must translocate protons inward from the highly alkaline bulk phase.

potential alone at these pH values unless protons are actually added. It could also account for ATP synthesis at the low bulk $\Delta\bar{\mu}_{H^+}$ values generated during optimal growth, but also encompass the failure to observe synthesis when that low $\Delta\bar{\mu}_{H^+}$ is completely dissipated. Even at the most alkaline pH values at which growth occurs, some protons are released into the bulk during respiration and the large $\Delta\psi$ is developed; this $\Delta\psi$ plays a role in all bioenergetic work, including the Na^+/H^+ antiport and ATP synthesis. Were the $\Delta\psi$ to collapse, protons would presumably leak out of the membrane through the proton-translocating pathways.

In this model, the frequency of intramembrane "hand off" of a proton vs release of the proton to the medium might depend upon the frequency of productive collision of the relevant intramembrane proteins, as has been proposed for electron transfer between mitochondrial electron carriers (Hackenbrock *et al.*, 1986). The extremely high concentrations of respiratory chain components and some of the membrane lipid properties of the alkalophile, that facilitate diffusional collisions might offer opportunities for energy

transduction that other organisms that cannot grow at very alkaline pH lack. It will be of interest both to test this working model and to contemplate the possible advantage(s) of retaining proton-coupled oxidative phosphorylation side by side with a Na^+ cycle that mediates other major cell functions. It may be that parallel coupling offers a flexibility so that a cell can order the priority of different bioenergetic work to meet variable physiological circumstances.

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