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Minireview

Proton-Coupled Bioenergetic Processes in Extremely Alkaliphilic Bacteria

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Oxidative phosphorylation, which involves an exclusively proton-coupled ATP synthase, and pH homeostasis, which depends upon electrogenic antiport of cytoplasmic Na^+ in exchange for H^+, are the two known bioenergetic processes that require inward proton translocation in extremely alkaliphilic bacteria. Energy coupling to oxidative phosphorylation is particularly difficult to fit to a strictly chemiosmotic model because of the low bulk electrochemical proton gradient that follows from the maintenance of a cytoplasmic pH just above 8 during growth at pH 10.5 and higher. A large quantitative and variable discrepancy between the putative chemiosmotic driving force and the phosphorylation potential results. This is compounded by a nonequivalence between respiration-dependent bulk gradients and artificially imposed ones in energizing ATP synthesis, and by an apparent requirement for specific respiratory chain complexes that do not relate solely to their role in generation of bulk gradients. Special features of the synthase may contribute to the mode of energization, just as novel features of the Na^+ cycle may relate to the extraordinary capacity of the extreme alkaliphiles to achieve pH homeostasis during growth at, or sudden shifts to, an external pH of 10.5 and above.

KEY WORDS: Alkaliphile; cytochromes; F_1F_0-ATPase; oxidative phosphorylation; pH regulation; sodium/proton antiporter; sodium binding proteins.

INTRODUCTION

Only two energy-dependent, membrane-associated processes are known to involve inward proton translocation in extreme alkaliphiles. These processes are electrogenic Na^+/H^+ antiport, an activity that is crucially involved in pH homeostasis in these organisms, and oxidative phosphorylation, which utilizes an exclusively proton-coupled F_1F_0-type of ATP synthase (Krulwich and Guffanti, 1989a, b; Guffanti and Krulwich, 1988; Hicks and Krulwich, 1990; Hoffmann and Dimroth, 1991a). Ion/solute symport systems and flagellar rotation thus far all appear to be Na^+-coupled in the extreme alkaliphiles (Krulwich and Guffanti, 1989a; Imae, 1991). This use of Na^+ bypasses a problem that arises directly from the constraints of cytoplasmic pH regulation. That is, aerobic bacteria growing actively at pH 10.5 and above maintain a cytoplasmic pH that is over two pH units below the external pH. Since the chemiosmotic driving force for proton-coupled, membrane-associated bioenergetic work (Mitchell, 1961) is the sum of the transmembrane pH gradient, ΔpH, acid out, and the transmembrane electrical gradient, $\Delta\psi$, positive out, a large ΔpH in the opposite orientation is adverse with respect to the magnitude of the total driving force, the Δp. No bioenergetic problem would exist *vis a vis* Na^+-coupled processes, because the energetically unfavorable ΔpH is not a component of the electrochemical Na^+ gradient that is the chemiosmotic driving force in these instances; this force would consist of the ΔpNa^+, the inwardly directed chemical gradient of Na^+, and the $\Delta\psi$. For proton-coupled processes, on the other hand, there would have to be some

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accommodation to the low, bulk Δp unless the $\Delta\psi$ of the alkaphiles were much higher than is found in conventional organisms and rose with increasing values of the growth pH to offset the increasingly adverse ΔpH. We will review the status of evidence with respect to a quantitative discrepancy between the Δp of extreme alkaliphiles and the bioenergetic work that is putatively coupled to that force. We will also describe a set of qualitative observations with respect to energy coupling by alkaliphiles that adds a further dimension to their energetic pattern. Then, we will discuss the state of information and existing models with respect to each of the two membrane-associated ion translocation processes that are coupled to protons.

The extreme, obligately aerobic alkaliphiles are primarily *Bacillus* species, and those that have thus far been studied from a bioenergetic point of view are nonmarine species that grow at conventional concentrations of Na^+ (Krulwich and Ivey, 1990). We have focused most of our own recent studies on alkaliphilic *Bacillus firmus* OF4, a "facultative alkaliphile" that is capable of growth at near neutral pH values (we generally use pH 7.5) and at pH values of pH 10.7 and probably higher (Guffanti and Hicks, 1991). Comparable facultative alkaliphiles have been studied by other laboratories (e.g., Koyama and Nosoh, 1985; Sugiyama *et al.*, 1986). *B. firmus* OF4 grows as well at a constant pH of 10.5 as at a constant pH of 7.5 (Guffanti and Hicks, 1991), and grows as rapidly (e.g. generation time of 40–50 min) and to the same of molar growth yields as other aerobic nonalkaliphilic *Bacillus* species on media that are comparable except for the pH. We and others have also studied "obligate alkaliphiles," taken in this field to mean strains that are capable of rapid growth to high growth yield only at pH values of about 9 and above; such strains include *Bacillus firmus* RAB and *Bacillus alcalophilus.* The basis for the inability of the obligately alkaliphilic strains to grow below about pH 9 is apparently related to the membrane lipid composition of these strains, a property that may also correlate with a somewhat better ability to grow at the very upper edge of their pH range than related, facultative strains (Clejan and Krulwich, 1988; Dunkley *et al.*, 1991). Bacteria that have been called "alkaline-tolerant" are apparently in a distinctly different bioenergetic category from the facultative or obligate, extreme alkaliphiles; alkaline-tolerant strains grow well at pH values of around 9, but do not grow as well or at all at pH values above 10. Several members of this latter group, which are often marine bacteria such as *Vibrio* species (Tokuda and Unemoto, 1982; Tokuda *et al.*, 1988) and *Bacillus* FTU (Semeykina *et al.*, 1989), have been reported to have primary Na^+ pumps that increase the $\Delta\psi$ and the total electrochemical sodium gradient when the organisms are grown at pH 9–10; they also appear to have an Na^+-coupled ATPase that can synthesize ATP under at least some experimental conditions (Sakai *et al.*, 1989; Sakai-Tomita *et al.*, 1991; Avetisyan *et al.*, 1991). Thus it is striking that the possession of mechanisms that would be expected to bypass a low Δp is not sufficient to confer the capacity for extreme alkaliphilic growth. Possible interpretations of this observation will be discussed.

Table I. Bioenergetic Parameters of *Bacillus firmus* OF4 Growing at pH 7.5 or 10.5[a]

Parameter	pH 7.5	pH 10.5
Molar growth yield on malate, mg dry wt/nmol malate	23.2 ± 4.3	24.3 ± 3.3
pH_{in}	7.5	8.3 ± 0.1
ΔpH (mV)	0	+130 ± 5.0
$\Delta\psi$ (mV)	−136 ± 8.0	−180 ± 6.0
Δp (mV)	−136 ± 8.0	−50 ± 9.0
ΔGp (kJ/mol)	45.2 ± 0.8	46.0 ± 1.3
(mV)	468 ± 9.0	478 ± 1.3

[a] Values are taken from at least four separate determinations with means and standard deviations shown. Measurements were made on growing cells as described and reported by Guffanti and Hicks (1991).

DO EXTREME ALKALIPHILES PRESENT A BIOENERGETIC PROBLEM THAT IS NOT SIMPLY RESOLVED BY AN UNUSUALLY HIGH $\Delta\psi$?

Quantitative Considerations

Typical values for the ΔpH and the phosphorylation potentials, reflecting the [ATP]/[ADP][Pi], of *B. firmus* OF4 are shown in Table I; they are compiled from values published elsewhere (Guffanti and Hicks, 1991), and are comparable to values obtained by others (Hirota and Imae, 1983). Although not shown in Table I, the nucleotide and inorganic phosphate concentrations of alkaliphiles were in the usual range for prokaryotic cells (Guffanti and Hicks, 1991). These values were obtained from cells that were actively growing at either pH 7.5 or 10.5 in the highly buffered medium that we began to use when we started to measure Δp parameters directly in the growth

medium; at the point in the logarithmic phase at which samples were taken, the medium pH had not changed by more than 0.2 pH units. In our earliest studies of the Δp of alkaliphiles the cells were grown with less buffering and the Δp measurements were conducted in non-nutrient buffer on washed cells (e.g., Guffanti *et al.*, 1978); such preparations and others that we have used over the years give different absolute values for the Δp parameters that have been very reproducible for any given preparation. In general, at very high pH, if the $\Delta\psi$ is lower, so is the adverse ΔpH, since formation of the latter depends upon the magnitude of the former. Thus, the net bulk Δp for cells at pH 10.5 has consistently been close to the -50 mV shown in Table I. As shown there for cells growing actively at pH 10.5, the cytoplasmic pH is measured at 8.2–8.4. Although a substantial $\Delta\psi$ in the chemiosmotically productive orientation is also measured, and that $\Delta\psi$ is higher than in pH 7.5-grown cells, the net Δp of of pH 10.5-grown cells is -50 mV compared to a Δp of -136 mV in pH 7.5-grown cells (Table I). Yet, the ΔG_p values of pH 7.5- and pH 10.5-grown cells are entirely comparable to each other and those of other bacteria. Importantly, the molar growth yields of *B. firmus* OF4 at the same two pH values are also entirely comparable (Table I). Thus if one were to posit that the proton-translocating ATP synthase were functioning chemiosmotically over the entire pH range for growth, then the ratio of H^+ translocated per ATP made would rise dramatically with increasing pH (as given by the $\Delta G_p/\Delta p$ ratio, from about 3 to a value above 9); moreover, since the growth yields do not vary, a parallel increase in the H^+/O ratios would have to be posited.

Although obligately alkaliphilic *B. alcalophilus* cannot be grown to the same stage of growth over as wide a range of pH as can *B. firmus* OF4, evidence for a similar kind of discordance between the putative chemiosmotic driving force and the [ATP]/[ADP] may be found in the data of Hoffman and Dimroth (1991b). A plot of the [ATP]/[ADP] ratio, the Δp, and the $Z\Delta p\mathrm{Na}^+$ for different external pH values is presented in Fig. 1. The data are for a slow-growing variant strain of *B. alcalophilus* with an especially impaired rate of growth in unreplenished medium at pH 10 and above (Hoffmann and Dimroth, 1990, 1991b; Guffanti and Hicks, 1991). It was grown for 80 h, during which sample points were taken as the external pH value was allowed to decrease. As the pH decreased, the Δp and the $Z\Delta\mathrm{pNa}^+$ increased in parallel; by contrast, the [ATP]/[ADP] ratio did not change in parallel, and was highest during the period of fastest

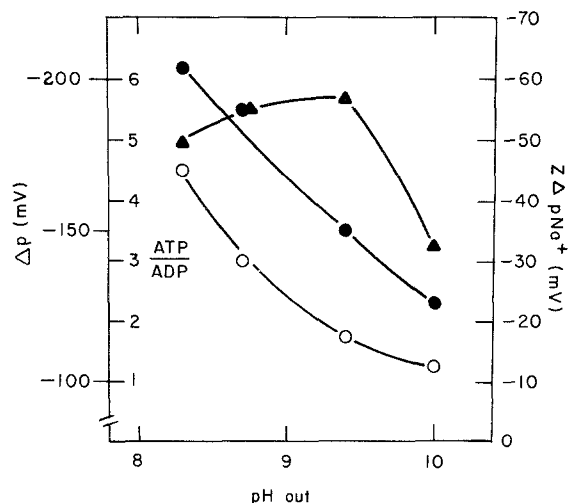

Fig. 1. The magnitude of a secondary $\Delta\mathrm{pNa}^+$ correlates better than does the ATP/ADP ratio with the magnitude of the Δp in *B. alcalophilus.* The data for the $\Delta\mathrm{pNa}^+$ (●), the ATP/ADP ratio (▲), and the Δp (○) are taken from Fig. 2 and Table 3 of the paper by Hoffmann and Dimroth (1991b) and plotted together as a function of medium pH during growth of the slow-growing *B. alcalophilus* strain DSM 485.

growth, a period in which the Δp values were not the highest (Hoffmann and Dimroth, 1991b).

But are the discordances really of great quantitative significance? Might the measurements of the Δp components be sufficiently in error (i.e., overestimation of the adverse ΔpH and/or underestimation of the $\Delta\psi$) so that the apparent discordances between the magnitude of the Δp and the ΔG_p are really very small? Hoffmann and Dimroth (1991b) have suggested that the $\Delta\psi$ generated by *B. alcalophilus* is up to 30 mV higher than the values shown in Table I and found by other investigators (e.g., Hirota and Imae, 1983). They report values of -206 to -213 mV for the $\Delta\psi$ of both fast and slow growing strains of *B. alcalophilus* at pH 10.1–10.3, using a medium with low buffering vs. the highly buffered medium used for the experiments shown in Table I. They further used 50 nM TPP^+ as the probe, vs. the usual concentration for Gram-positive bacteria, in the range of 1–10 μM (deVrij *et al.*, 1988; Kashket *et al.*, 1980; Poolman *et al.*, 1987), rapid centrifugation through silicon oils as the separation technique, and the internal binding model of Zaritsky *et al.* (1981) as the method of correcting their experimental values for probe binding. They argue that the $\Delta\psi$ values thus obtained accommodate oxidative phosphorylation under that specific

condition with an H^+/ATP of 4.4, only a little higher than the typical values around 3. Clearly, it would still be necessary to posit some variability in the H^+/ATP ratio, given the discordance shown in Fig. 1 and the fact that pH 10.3 is not the upper limit of pH for growth of *B. alcalophilus*; at pH 10.7, for example, the Δp would be even lower. Still, the applicability of these unusual measurements to more highly buffered alkaliphile cells, and their accuracy vs. those using more conventional probe concentrations, is important with respect to what the quantitative bioenergetic challenge really is.

Close examination indicates, in fact, that the measurements reported by Hoffmann and Dimroth (1991b) are likely to be flawed. First, it is surprising that a *B. alcalophilus* strain growing with the usual 50 min generation time and another strain with a 10 h generation time and having a much reduced cytochrome content, exhibited the same unusually high $\Delta\psi$ (Hoffmann and Dimroth, 1991b, Table 2 and Fig. 2); with *B. firmus* OF4, for example, the differences in cytochrome content are directly reflected in the magnitude of the $\Delta\psi$ under appropriate conditions (Quirk *et al.*, 1991). Second, the use of 50 nM TPP^+, as opposed to the usual concentration in the 1–5 μM range, was argued to give higher and hence more accurate values because the $\Delta\psi$ being measured was less affected by the dissipative effect of the probe. However, TPP^+ was present during many earlier measurements of solute uptake but did not lower the steady-state accumulation ratio. Use of a very low TPP^+ concentration must, in any event, be weighed against the empirical question of which range of probe concentration better equilibrates with the $\Delta\psi$ to be measured, which is freer of idiosyncratic (e.g., pH dependent) binding artifacts that are not easily evaluated, and which correlates best with standards and with independent methods of $\Delta\psi$ evaluation. Zaritsky *et al.* (1981) had examined those issues in a Gram-positive bacterium. They concluded that for *Bacillus subtilis*, probe concentrations of less than 10 μM did not significantly affect the magnitude of the membrane potential and, importantly, that very low probe concentrations did not saturate the cell surface compartment and equilibriate fully with the $\Delta\psi$. Thus, use of 50 nM TPP^+ and Zaritsky *et al.*'s (1981) internal binding model for correction seems a particularly odd juxtaposition.

As shown recently (Guffanti and Krulwich, 1992) and included in Table II, 50 nM TPP^+ cannot be used to measure the $\Delta\psi$ of *B. firmus* OF4. By contrast, the higher concentration that we and others have used for such measurements gives precise data that correlate well both with an alternative method and with theoretical values of imposed potentials. With the two strains of *B. alcalophilus* used by Hoffmann and Dimroth (1991b), the low concentration of TPP^+ was not as inaccurate as with *B. firmus* OF4 in determinations of imposed potentials, especially when filtration was used as the method of separation. If TPP^+ can equilibrate preferentially with a surface-associated compartment when 50 nM is used, strain-specific differences in surface layers might make this kind of pronounced difference in the results. Most importantly, regardless of the method of separation or the concentration of probe used, we did not replicate the unusually high values of the $\Delta\psi$ found by Hoffmann and Dimroth (1991b); moreover, the slow-growing DSM strain generated a reproducibly lower $\Delta\psi$ in our studies than did the faster-growing ATCC strain. In general, filtration gave somewhat higher values of the $\Delta\psi$ than did centrifugation through oil, especially at pH 10.5 where we would expect the greatest sensitivity to transient anaerobiosis. Although we are troubled by our inability to account for the difference between the results of Hoffmann and Dimroth (1991b) and those shown here, the data in Table II are taken as a basis for accepting the often-replicated and validated values of the $\Delta\psi$ as the best measurements with the current technology.

What about the ΔpH measurements? Might they be overestimations? The accumulation of methylamine, assayed by a filtration assay, precisely and accurately measures imposed pH gradients in alkaliphiles, and accurately measures a ΔpH of 2 pH units when alkaliphile cells that are equilibrated at pH 8.5 are suddenly subjected to an alkaline shift of the medium to pH 10.5 in the absence of Na^+ (Krulwich *et al.*, 1985). At least one study (Ritchie and Gibson, 1987) has suggested that the presence of ammonium salts may at least transiently lower the ΔpH, and we have recently found faster growth at pH 10.5 when such salts are omitted from the medium. Independent measurement of ΔpH by NMR technology will depend upon suitable probes and conditions for higher pH than has been needed with other microorganisms (Salhany *et al.*, 1975; Kallas and Dahlquist, 1981; Slonczewski *et al.*, 1981). As with the $\Delta\psi$ measurements, though, the current methodology for the ΔpH leads to values that correlate well with standards and with appropriate inhibitor controls. In sum, there is no basis for discounting the large quantitative

Table II. Measurements of the $\Delta\psi$ in Alkaliphile Cells

A. Measurements of the $\Delta\psi$ of cells growing in highly buffered medium using different radioactive probes and difference separation techniques[a]

		$\Delta\psi$, (mV) measured by				
		$^{86}Rb^+$/valinomycin	50 nM [^{3}H]TPP$^+$		4 μM [^{3}H]TPP$^+$	
Alkaliphile	pH	Filtration	Filtration	Centrifugation	Filtration	Centrifugation
B. firmus OF4	7.5	−142 ± 8	−81 ± 41	−131 ± 6	−138 ± 8	−132 ± 10
	10.5	−181 ± 9	−108 ± 19	−115 ± 7	−180 ± 6	−159 ± 8
B. alcalophilus ATCC27647	10.5	−172 ± 15	−161 ± 10	−136 ± 2	−176 ± 13	−161 ± 3
B. alcalophilus DSM 485	10.5	−150 ± 14	−142 ± 17	−132 ± 10	−157 ± 15	−146 ± 14

B. Measurement of imposed valinomycin-mediated potassium diffusion potentials in *B. alcalophilus* DSM 485 and *B. firmus* OF4 at pH 10.5[b]

Alkaliphile	Dilution of K$^+$-loaded cells	Theoretical potential generated (mV)	Potential measured (mV)			
			50 nM TPP$^+$		4 μM TPP$^+$	
			Filtration	Centrifugation	Filtration	Centrifugation
B. alcalophilus DSM 485	1:1000	−176	−178 ± 10	−151 ± 10	−177 ± 9	−171 ± 5
	1:5000	−218	−213 ± 19	N.D.	−210 ± 7	N.D.
B. firmus OF4	1:1000	−176	−160 ± 13	−153 ± 8	−179 ± 7	−173 ± 5
	1:5000	−218	−182 ± 20	N.D.	−207 ± 6	N.D.

[a]The $\Delta\psi$ was measured in cells grown in the same highly buffered medium that yielded the data in Table I. The methods and corrections employed were exactly as described elsewhere (Guffanti and Krulwich, 1992), except for the samples in which cells were separated by centrifugation. In those measurements, the method of Hoffmann and Dimroth (1991b) was used with the following modifications: the ratio of AR200 and DC200 silicon oils used by Hoffmann and Dimroth (1991b) remained above the supernatant layer in our hands, so we used only silicon oil AR200 (d^{20} 1.049, Fluka); we cut the centrifuge tubes just below the oil layer rather than in the oil layer, cutting in the perchlorate well above the pellet, in case any of the lipophilic probe had distributed into the oil layer; and the only binding correction made for this technique was based on a gramicidin-treated control, as further correction, e.g., as we have done for the filtration measurements, would have further reduced the values. All the values from centrifugation measurements are mean values from at least three different experiments, and those in *B. firmus* OF4 were published elsewhere (Guffanti and Krulwich, 1992) and are from at least five different experiments. Since the values shown above for *B. alcalophilus* are lower than reported by Hoffmann and Dimroth (1991b), we did two sets of measurements on cells growing in the less buffered medium that they used; the results did not differ significantly from those shown above.
[b]Potentials were imposed and measured either as described elsewhere (Guffanti and Krulwich, 1992) or, when centrifugation was used, as described above. N.D. = not determined, because the cell concentration was insufficient at that dilution to be workable with the protocol employed.

bioenergetic problem presented by the Δp–ΔG_p discrepancy in the alkaliphiles.

Qualitative Considerations

In a strictly chemiosmotic model of energization, it should not matter whether the Δp was established by a natural ion extrusion system or by artificial imposition of a gradient. At a given magnitude of the Δp, the rate and extent of the process driven should be identical. Two deviations from this expectation are seen with respect to oxidative phosphorylation by extreme alkaliphiles. First, imposition of a substantial valinomycin-mediated potassium diffusion potential in starved whole cells or ADP + Pi-loaded membrane vesicles from *B. firmus* OF4 or RAB results in ATP synthesis at pH values up to about 9. However, the artificial gradient becomes completely and abruptly inefficacious for ATP synthesis at pH values above pH 9.5 (Guffanti *et al.*, 1984, 1985; Guffanti and Krulwich, 1992). Vesicles that are free of cell wall, as assessed by muramic acid content, exhibit the same phenomenon. Thus, the difference between energization mode does not arise from a difference in proton trapping that is dependent upon the outer cell surface

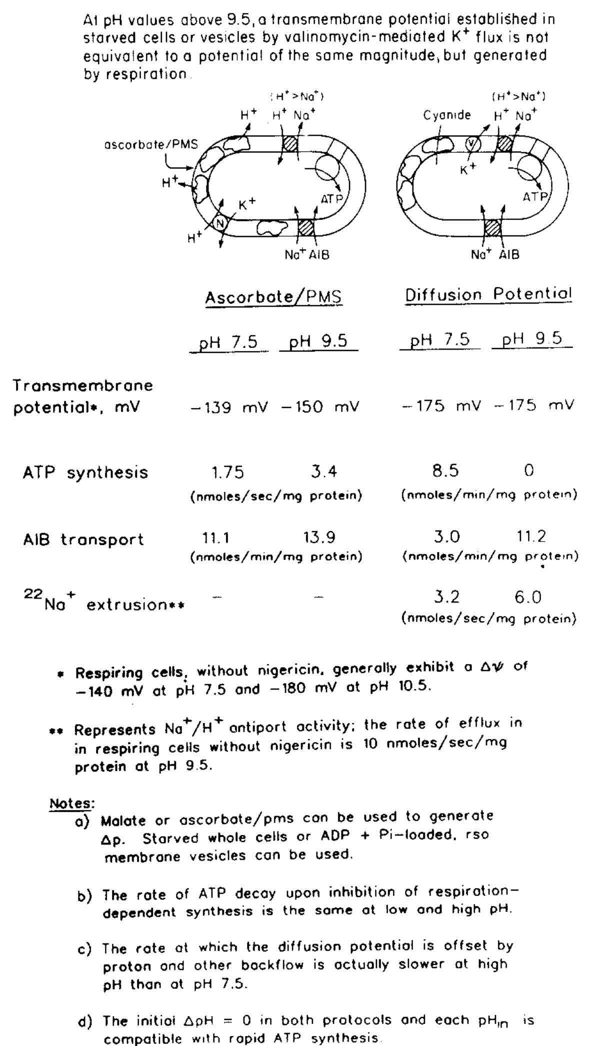

Fig. 2. A summary of a series of experiments illustrating an apparent lack of equivalence, especially for ATP synthesis, between transmembrane electrical potentials generated by respiration in the absence of a ΔpH (left) and valinomycin-mediated potassium diffusion potentials (right) of the same magnitude. N = nigericin; v = valinomycin; AIB = α-aminoisobutyric acid. The experimental details are presented in, and most of the data are taken from, Guffanti and Krulwich (1992); some additional data were gathered in our laboratory and are unpublished.

layers. The summary in Fig. 2 shows a comparison between the efficacy of an electron donor, ascorbate/phenazine methosulfate (PMS), and a diffusion potential in energizing ATP synthesis, transport of the non-metabolizable amino acid analogue α-aminoisobutyric acid (AIB), and $^{22}Na^+$ extrusion (reflecting Na^+/H^+ antiport activity) at pH 7.5 and pH 9.5. Conditions were chosen, i.e., inclusion of nigericin, for the starved cells that were energized with the electron donor, to ensure that the Δp was entirely in the form of a $\Delta\psi$; this was confirmed directly by measurements of the Δp parameters. The specificity of the failure of the artificially imposed gradient to energize ATP synthesis at very alkaline pH values is apparent; several controls are noted in the figure, and others have been presented in detail elsewhere (Guffanti and Krulwich, 1992). Although not shown in the tabular presentation in Fig. 2, the decline in efficacy of the diffusion potential as the pH is raised follows a pattern that resembles a titration curve, with a pK of about 8.5 in the presence of a small inwardly directed Na^+ gradient (Guffanti and Krulwich, 1992). The striking lack of equivalence between an artificially imposed Δp and one generated by respiration is, unless otherwise explained, a major challenge to a strictly chemiosmotic interpretation of oxidative phosphorylation by extreme alkaliphiles.

There is a second qualitative observation that would seem to run counter to a strictly chemiosmotic model for oxidative phosphorylation at extremely alkaline pH. An initial set of observations with *B. firmus* OF4 indicates that one of the multiple terminal oxidases, a caa_3-type terminal oxidase, of this facultative alkaliphile is involved in some special way in oxidative phosphorylation at very alkaline pH values. This particular complex appears to be the most highly pH-regulated of the abundant respiratory chain complexes in *B. firmus* OF4, increasing about 2- to 3-fold in membrane concentration during growth at pH 10.5 as compared to growth at pH 7.5 (Quirk *et al.*, 1991; Hicks *et al.*, 1991). When starved whole cells of pH 7.5- and pH 10.5-grown *B. firmus* OF4 are re-energized by the addition of malate at pH 10.6, they generate $\Delta\psi$ values of -142 mV and -167 mV, respectively; the "reversed" ΔpH formed is correspondingly lower (the responsible antiport being energized by the $\Delta\psi$), $+102$ and $+121$ mV equivalents, respectively. Therefore the total Δp generated by pH 7.5- and pH 10.5-grown cells that are re-energized at pH 10.6 is identical. As expected, the rates of AIB uptake are similarly comparable. On the other hand, the rate of ATP synthesis is over 10 times higher in the pH 10.5-grown than in the pH 7.5-grown cells (Guffanti and Krulwich, 1992). It would seem that the specific cytochrome complement may have importance for oxidative phosphorylation at very high pH that is independent of its role in generating the bulk transmembrane gradients.

Special structural features of the alkaliphile

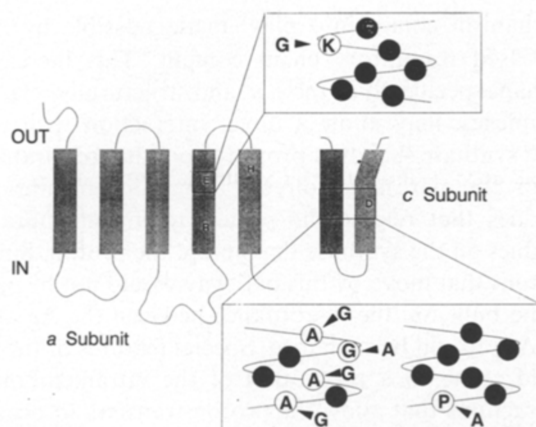

Fig. 3. Models of possible patterns of folding of the *a*- and *c*-subunits of the alkaliphile F_0 in the membrane. The enlarged boxes represent sequence detail for regions that are putatively involved in proton translocation and that have unusual substitutions in *B. firmus* OF4 and *B. alcalophilus*; the alkaliphile sequence is shown by circled residues, with the unusual ones in white circle and arrows that indicate the residue usually found in that position. The data and sources for the models have been presented elsewhere (Ivey and Krulwich, 1992).

F_1F_0-ATP synthase do not *per se* argue for a particular coupling mechanism unless they are definitively interpretable with respect to their energetic impact. Nonetheless, they are a striking part of the qualitative description of the machinery for oxidative phosphorylation and are thus noted here. Molecular biological evidence suggests that the *a* and *c* subunits of the F_0 of extreme alkaliphiles have novel deviations from the usual sequence in regions that have been shown by others, working with *Escherichia coli* and other neutrophilic organisms, to be importantly involved in the path of the proton. The specific deviations are illustrated in Fig. 3; they have thus far been found in both *B. firmus* OF4 and *B. alcalophilus*, but not in a nonalkaliphilic *B. firmus* strain or other organisms whose *atp* gene sequences have been studied in other laboratories (Ivey and Krulwich, 1991, 1992). Additional examples of unrelated extreme alkaliphiles are being sought for assessment of the generality of the comparison.

HOW MIGHT ATP SYNTHESIS BE ENERGIZED AT pH > 9.5?

Are the Synthase and Respiratory Chain Sequestered?

As part of our early work on alkaliphiles, we explored the possibility that oxidative phosphorylation is not coupled to the Δp across the cytoplasmic membrane but, rather, to gradients generated across cytoplasmic or enclosed membrane-associated vesicles or organelles. Electron microscopic examination of stained epon-embedded thin sections of alkaliphile cells revealed no cytoplasmic organelles, and nothing unusual was noted about the appearance of the cytoplasmic membrane (Krulwich, 1982). Subsequently, anti-F_1ATPase antibodies and immunogold labelling of thin sections of *B. firmus* RAB confirmed that the ATP synthase of the alkaliphile was associated with the cytoplasmic membrane and not within the cytoplasmic space, and also demonstrated that the synthase was distributed over the whole surface of the membrane (Rohde *et al.*, 1989). Nonetheless, Skulachev and his colleagues (Avetisyan *et al.*, 1991; Skulachev, 1992) have made the *a priori* proposal that the solution to the problem of oxidative phosphorylation in alkaliphiles might be the existence of "lens"-shaped, membrane-bound structures that are contiguous with the cytoplasmic membrane, making bulges therein. The structures are proposed to contain assemblies of the respiratory chain and the F_1F_0-ATP synthase and a specially buffered interior. If oxidative phosphorylation by pH 10.5-grown cells at high pH values occurred by respiration-dependent proton translocation into such structures and energization of the synthases also therein, it might explain both the discordance between values of the ΔG_p and Δp at high pH and the failure of an imposed diffusion potential to work. It would not readily explain the apparent specificity for a particular terminal oxidase *vis a vis* oxidative phosphorylation at high pH. In addition, the explanation would only hold if the structures sequestered all the synthase which should have made them hard to miss in earlier studies since the synthase is distributed all over the membrane (Rohde *et al.*, 1989). If the synthase were not all sequestered at pH 10.5, the diffusion potential should still work, and the cell would be at risk for an uncoupling cycle at high pH when "lens"-bound synthase produced and other synthases simultaneously hydrolyzed ATP. Moreover, the structures would have to be specific to cells grown at high pH, since pH 7.5-grown cells appear to function chemiosmotically, e.g., synthesize ATP in response to an imposed diffusion potential about as well as to respiration-generated gradients of the same magnitude.

Although earlier observations were inconsistent with an ultrastructural explanation of alkaliphily, the

proposal was sufficiently provocative to merit a reexamination, especially since some of the newer rapid freeze techniques had never been systematically applied to a comparison of the ultrastructure of pH 7.5- and pH 10.5-grown cells. Recently, therefore, we undertook a collaborative study with Dr. Shahid Khan in which freeze fracture and freeze substitution techniques were applied to pH 7.5- and pH 10.5-grown *B. firmus* OF4, pH 10.5-grown *B. alcalophilus*, and a *B. subtilis* control. The study (Khan *et al.*, 1992) confirmed earlier failures to find cytoplasmic organelles and resulted in no evidence for the kind of structures proposed by Skulachev and colleagues. On the contrary, the P-face (Khan *et al.*, 1988) of fractured membranes of the alkaliphiles vs *B. subtilis* or the alkaliphiles grown at pH 10.5 vs. pH 7.5 showed no greater differences than were observed between independent images of the same sample.

Other Chemiosmotic vs. Partially Nonchemiosmotic Options

Since alkaliphile oxidative phosphorylation at very high pH cannot be accounted for by the utilization of Na^+ as coupling ion or the sequestration of the protonmotive cycle in special structures, what models remain? As outlined diagrammatically in Fig. 4 and annotated further in Table III, a chemiosmotic model might be proposed in which the H^+/ATP and H^+/O ratios both increase as the growth pH increases; a large range for these parameters would be required for a growth range of pH 7.5–10.7. The up-regulation of the caa_3 might be importantly related to the H^+/O increase; however, that explanation for its special involvement in alkaliphily does not perfectly fit the data outlined earlier, showing that at equal values of the Δp, the pH 10.5-grown cells did better with respect to oxidative phosphorylation than pH 7.5-grown cells. Most importantly, this model does not account for the observations with respect to the failure of a diffusion potential to energize ATP synthesis at high pH; were the synthase capable of accommodating a variable and sometimes high H^+/ATP, then that high ratio should function to allow synthesis in response to an artificial gradient.

An alternative model that we have presented elsewhere (Ivey *et al.*, 1990; Guffanti and Krulwich, 1992) suggests that at pH 7.5, energization of oxidative phosphorylation could be completely chemiosmotic whereas at pH values above 9.5, a nonchemiosmotic mechanism comes into play, made possible by the increased respiratory chain content. This increase, perhaps specifically in the caa_3 and structurally related complexes, may allow a direct interaction with the ATP synthase such that protons from that respiratory chain complex move directly from intramembrane residues that release the proton to intramembrane residues on the synthase that accept the proton. Since protons that move by this pathway would not be part of the bulk Δp, the discordance between the Δp and the ΔG_p would be explained. Special features of the F_0 could relate to a promotion of the intramembrane interactions that allow the proton transfers to occur, consistent with the presence of these features in regions that are expected to be within the membrane and important in translocation of protons. The model (Fig. 4B) also shows a p*K*-regulated gate at the point of entry for bulk protons during chemiosmotic synthesis. Were that gate to be closed at pH values above 9.5, it would explain the failure of a diffusion potential to work at those pH values; an alternative explanation involving particular roles for membrane lipids in oxidative phosphorylation, as proposed by others (Haines, 1983; Teissie *et al.*, 1990), has been noted elsewhere (Guffanti and Krulwich, 1992). The value of a gate for the alkaliphile would be that protons entering the F_0 further along on the usual proton pathway, within the membrane, would be impeded by the closed gate from simply moving outward into the bulk phase. Special features of the F_0 could be involved in such a gating, but not necessarily (see Guffanti and Krulwich, 1992). It is likely that if deprotonation of a conserved residue on the F_0 stops proton conductance from the bulk, the pH at which that deprotonation occurs would make this property irrelevant for other organisms. Importantly, gating alone does not ensure productive inward movement of protons during oxidative phosphorylation at pH values above 9.5. While a subset of respiratory chain complexes at pH 9.5 and above may be handing off protons directly to the synthase within the membrane, other respiratory chain complexes continue to extrude protons into the bulk, producing a Δp and energizing all the requisite Na^+/H^+ antiport and solute symport activities of the membrane as well as oxidative phosphorylation itself. Oxidative phosphorylation has, in our hands, always required the small Δp that exists even at the highest values of the alkaliphiles' growth range. While Hoffmann and Dimroth (1991b) take such a requirement to argue for a completely chemiosmotic mechanism, we would expect this requirement with either model

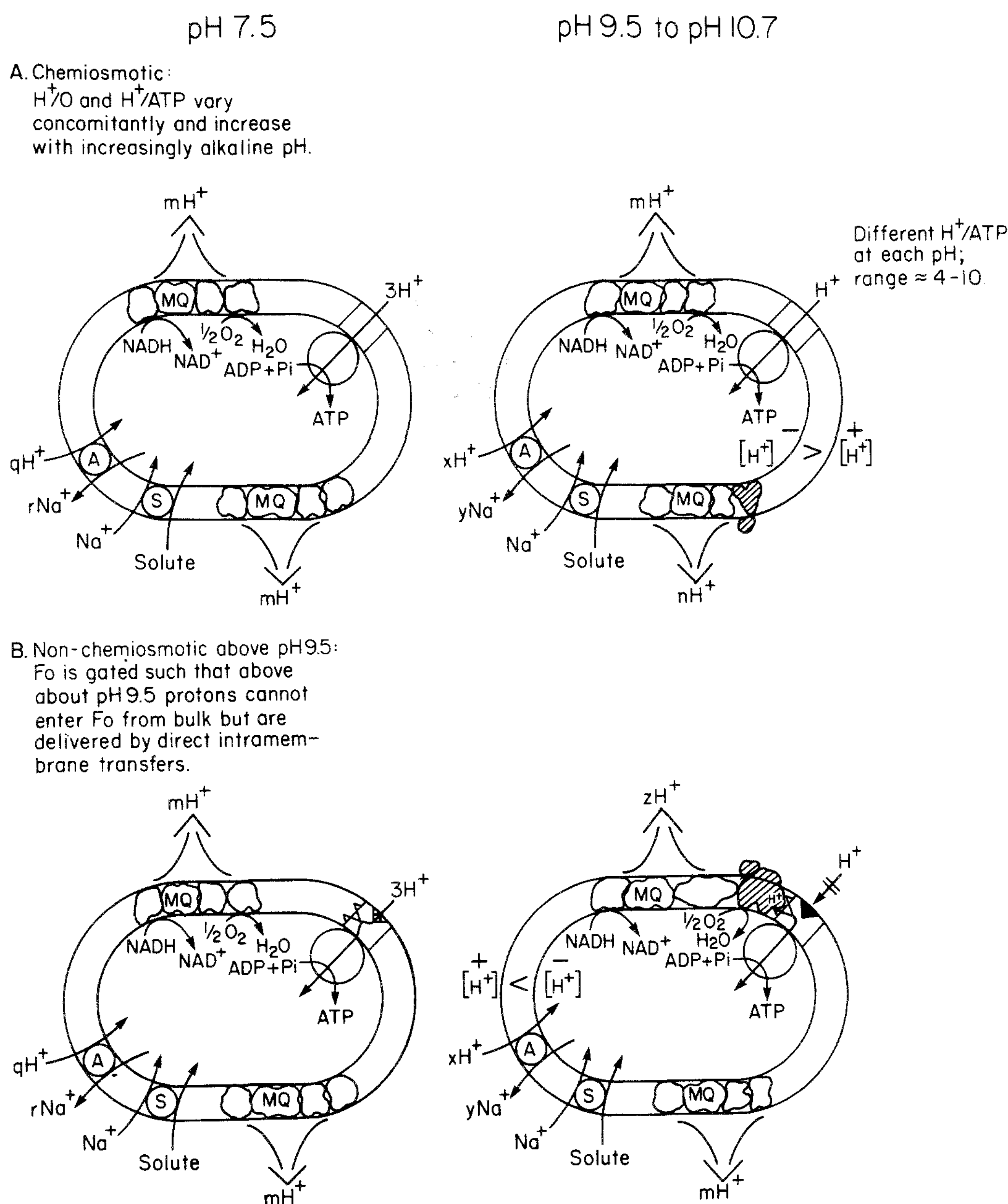

Fig. 4. Attempts to fit the experimental data on oxidative phosphorylation to a chemiosmotic model and an alternative, partially nonchemiosmotic model. The models are as described in the text, model B having been presented earlier (Guffanti and Krulwich, 1992). Further predictions are detailed in Table III, a companion to this figure. The hatched respiratory chain complex represents a pH-regulated terminal oxidase. pH-dependent changes in the respiratory chain may have specific interactive roles and may increase the H^+/O and Δ^+ at high pH. MQ = menaquinone, shown by Meganathan and Coffell (1985) to be the quinone in the alkaliphile, correcting an initial report by Lewis *et al.* (1981).

(Table III). The bulk $\Delta\psi$ is a major part of the total energetic driving force for inward proton movement.

Na^+/H^+ ANTIPORT AND pH HOMEOSTASIS

The Na^+ cycle of alkaliphilic *Bacillus* species has recently been reviewed (Ivey *et al.*, 1992a). Direct documentation currently exists for the following elements, which together may account for pH homeostasis, generation of an inwardly directed sodium gradient, and the uptake of numerous solutes: (a) electrogenic exchange of cytoplasmic Na^+ for H^+ from

Table III. Features and Predictions of the Models in Fig. 4

Model	H^+/ATP, H^+/O	Explains specific failure of imposed diffusion potential to energize ATP synthesis above pH 9.5?	Bulk $\Delta\psi$ required for ATP synthesis	Possible role of special alkaliphile F_0 motifs	Other predictions
A: Chemiosmotic	Both must vary in concert since molar growth yields on malate are the same at pH 7.5 and pH 10.5. A single F_1, F_0-ATPase species can couple at $3H^+$ to $10H^+$/ATP.	*No*. There must be some as yet undetected problem with that experiment since the imposed potential would be expected to be efficacious at high pH, the synthase simply using a high H^+/ATP.	Yes. All relevant forces are bulk.	Could relate to capacity of F_0 to couple at a wide range of H^+/ATP. Some of regions are clearly in intramembrane regions; provide a variable proton well?	Might be able to directly show different H^+/ATP at pH 7.5 vs. pH 10.5. If alterations in F_0 motifs compromise growth at very alkaline pH, should alter H^+/ATP ratio. Expect no lipid mutations to prevent oxidative phosphorylation unless they compromise Δp formation or the function of specific catalysts.
B: Nonchemiosmotic, F_0 is pK-gated	No specific predictions. There need not be a change in coupling stoichiometry with pH, but one is not precluded.	Yes. Closure of the F_0 gate prevents loss to the bulk of protons that arrive at the F_0 through intramembrane pathway. It also precludes synthesis via bulk protons in response to imposed potential.	Yes. Bulk $\Delta\psi$ is a major component of the total driving force and is required for protons to move inward in the synthetic direction.	Could relate to optimization of intramembrane proton transfer from residues on (a) respiratory chain component(s). Some motif(s) might relate to gate, although that could be a property of all F_0's.	Proton flow through reconstituted F_0 should show gating property as a function of pH. Some mutations in special F_0 motifs might affect this property. Specific lipids as well as specific F_0 motifs might be involved in optimizing intramembrane protein: protein interactions that allow proton transfer, i.e., mutable to a pH-conditional phenotype.

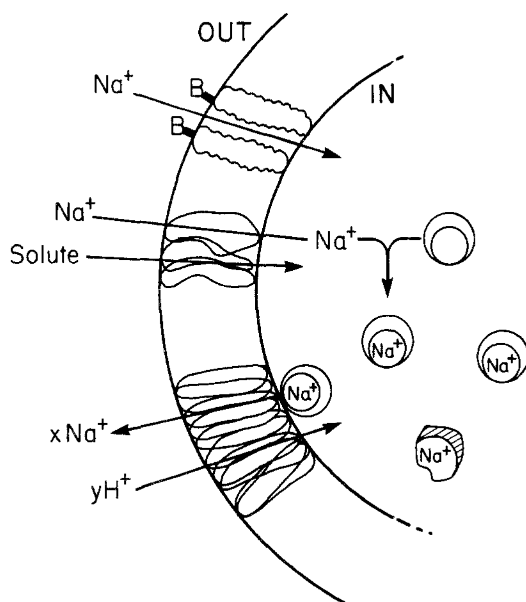

Fig. 5. Demonstrated and proposed elements of the alkaliphile Na^+ cycle. Secondary to primary proton extrusion by respiration, which is not shown in the figure, a Na^+/H^+ antiporter (representing a composite of at least two distinct antiporters) catalyzes electrogenic Na^+/H^+ exchange, with the H^+/Na^+ ratio being greater than 1. A sodium-binding protein is shown interacting with the antiporter; others, and one hatched binding protein that represents a CadC that apparently also binds Na^+ (Ivey *et al.*, 1992b), are shown in the cytoplasm. Na^+ is returned to the cytoplasm after antiport-dependent extrusion, by Na^+/solute symporters and, perhaps, by a pH-regulated channel as proposed by McLaggan *et al.* (1984, 1991).

the medium mediated by at least two Na^+/H^+ antiporters; (b) return of Na^+ to the cytoplasm via Na^+/solute symporters that catalyze uphill solute transport coupled to downhill Na^+ transport; and (c) one or more Na^+ binding proteins that sequester Na^+ (perhaps minimizing its cytotoxicity while the export machinery does its job) and also make the cytoplasmic substrate more available to the integral membrane, secondary antiporters. These entities are diagrammatically shown in Fig. 5; the figure shows, in addition, a pH-regulated channel for Na^+ that has been postulated to exist by McLaggan *et al.* (1984, 1991). Although no direct evidence has yet been obtained for such a channel, the proposal is based on observations of sustained and rapid cycling of Na^+ in support of antiport activity at high pH in the apparent absence of solutes that could provide a symporter-based Na^+ re-entry route.

Alkaliphile cells that are subjected to an upward shift in pH, e.g., from equilibration at pH 8.5 to a sudden shift in the medium pH to 10.5, maintain their cytoplasmic pH near 8.5 if adequate Na^+ is present. Sustained maintenance of pH_{in} below pH 8.5 is observed in such an experiment if Na^+ and a solute that is co-transported with Na^+ (even a nonmetabolizable one such as AIB) are both present (Krulwich *et al.*, 1985). If, on the other hand, Na^+ is not present during the upward shift in external pH, the pH_{in} immediately rises to 10.5. The Na^+/H^+ antiport activity that has been measured in cells, vesicles, and prteoliposomes that were reconstituted with crude membrane extracts, have allowed certain general properties to be deduced: antiport is electrogenic, being inhibited by agents that dissipate the transmembrane $\Delta\psi$; it is competitively inhibited by Li^+; and it is inhibited by high cytoplasmic proton concentrations, e.g., $pH_{in} = 7$ (Krulwich *et al.*, 1988; Imae, 1991). In these respects, the overall antiport activity resembles that observed in other eubacteria (Bassilana *et al.*, 1984; Schuldiner and Padan, 1992).

Are there particular problems in connection with inward proton translocation, clearly crucial to pH homeostasis during growth on nonfermentative carbon sources, at remarkably low external proton concentrations and low Δp? In experiments in which kinetics of Na^+/H^+ antiport was studied upon imposition of a valinomycin-mediated potassium diffusion potential across cells with a starting cytoplasmic pH of 9, the rate of antiport, as measured by potential-dependent $^{22}Na^+$ efflux, was not as rapid at an external pH of 10 as it was at lower pH values (Garcia *et al.*, 1983). Kitada and Horikoshi (1987) also reported a discordance between a diffusion potential and respiration in energizing Na^+/H^+ antiport. We originally suggested that the greater apparent efficacy of respiration was comparable to the phenomenon discussed in connection with ATP synthesis (Krulwich and Guffanti, 1989b). Detailed recent experiments, however, have made it clear that antiport continues to be energized by a diffusion potential at all alkaline pH values measured (up to pH 10.6) and way above the value of pH 9.5 at which a diffusion potential abruptly becomes inefficacious in energizing ATP synthesis (Guffanti and krulwich, 1992). Whether the specific antiporter(s) that function at the highest pH values in the alkaliphiles' growth range have unusual kinetic properties and stoichiometries awaits the availability of proteoliposomes with individual, purified antiporters. This has thus far only been accomplished with one of the *E. coli* antiporters, NhaA (Taglicht *et al.*, 1991). Taking advantage of the progress in the

molecular biology of the Na^+/H^+ antiporters of *E. coli* (Schuldiner and Padan, 1992), we have cloned a gene for one apparent antiporter (NhaC) from *B. firmus* OF4, and obtained indications of at least one other antiporter in that strain (Ivey *et al.*, 1991). In addition, evidence has emerged for a novel Na^+ binding protein, shown in Fig. 5, that may enhance antiport activity in the alkaliphile by delivering substrate to the secondary antiporter(s) in the membrane (Ivey *et al., unpublished data*). Another feature of pH homeostasis that may be special to the alkaliphiles is the generation of genetic variants that have an even greater capacity for antiport and maintenance of pH_{in} than the parent strains (Krulwich *et al.*, 1986); recent studies have moved us closer to genetic elements that might play a role in this phenomenon (Ivey *et al.*, 1992b).

WHY DON'T ALKALINE-TOLERANT BACTERIA WITH PRIMARY Na^+ CYCLES GROW AT THE SAME HIGH pH VALUES AS EXTREME ALKALIPHILES?

If a bacterium has a respiration or substrate-coupled Na^+ extrusion system and an ATP synthase that can utilize Na^+, then it might be expected to have an advantage with respect to growth at very alkaline pH values since the ΔpH, acid in, would not be energetically adverse to Na^+-coupled ATP synthesis. Why then do all the extreme alkaliphiles thus far appear to have H^+-coupled oxidative phosphorylation, and why don't those bacteria that have primary Na^+ cycles grow well at extremely alkaline pH values? Since there is no detailed information about the mechanisms of pH homeostasis in alkaline-tolerant bacteria that have primary Na^+ cycles, it is possible that these mechanisms are simply inadequate to allow growth at the same extreme pH values at which the alkaliphiles thrive. However, additional possibilities should be considered, especially in view of the failure, to date, to find extreme aerobic alkaliphiles that have primary Na^+ cycles. As noted elsewhere (Ivey *et al.*, 1992a), these include the possibility that Na^+-coupled synthases, even of the F_1F_0-type as only documented in *Propionigenium modestum* so far (Laubinger and Dimroth, 1987), may not work well enough to support oxidative phosphorylation by a strict aerobe at pH values that entail the extra energetic stress of pH homeostasis. It is also possible that if the kind of non-chemiosmotic mechanism shown in Fig. 4B exists, then this option of a direct proton pathway might offer a selective advantage to an organism (or organelle?) that is especially adapted to maximise the conservation of energy from respiration-dependent proton movements. Proposals of direct intramembrane pathways of energy transduction during oxidative phosphorylation, involving protein–protein interaction within the membrane, have been advanced by others for mitochondria (Gupte *et al.*, 1991; Rottenberg, 1990; Slater, 1987; Westerhoff *et al.*, 1984). The mitochondrial coupling membrane and that of the extremely alkaliphilic *Bacillus* species have in common a concentration of cytochromes that is higher than other bacterial species (Krulwich and Guffanti, 1989a), a substantial cardiolipin concentration (Clejan *et al.*, 1986), and the likelihood that energy transduction has been the focus of intense selective pressure.

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