Features of Apparent Nonchemiosmotic Energization of Oxidative Phosphorylation by Alkaliphilic Bacillus firmus OF4*

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Oxidative phosphorylation by extremely alkaliphilic Bacillus species violates two major predictions of the chemiosmotic hypothesis: the magnitude of the chemiosmotic driving force, the $\Delta p$ (electrochemical proton gradient), is too low to account for the phosphorylation potentials observed during growth at pH 10.5 without using a much higher $H^+/ATP$ stoichiometry than used during growth at pH 7.5, and artificially imposed diffusion potentials fail to energize ATP synthesis above pH 9.4. The energy-dependent symport and antiport rates are comparable with those in pH 10.5-grown cells. A model is presented for oxidative phosphorylation by the alkaliphilic Bacillus that involves a nonchemiosmotic direct intramembrane transfer of protons from specific respiratory chain complexes to the $F_0$ sector of the ATPase, whereas remaining respiratory chain complexes extrude protons into the bulk to generate the bulk potential required both for ATP synthesis and other bioenergetic work. A pH-regulated gate or a delocalized proton pathway that fails to work above pH 9.5 are suggested as possible features that account for the loss of efficacy of a bulk-imposed diffusion potential in energizing ATP synthesis above pH 9.4.

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Mitchell's chemiosmotic model (1, 2) delineates a mechanism of energization of ATP synthesis and other membrane-associated bioenergetic work during respiration and photosynthesis. This model is now widely confirmed and almost universally accepted. As applied to oxidative phosphorylation by mitochondria or aerobic bacteria, the chemiosmotic model posits that extrusion of protons across the coupling membrane during respiration establishes a $\Delta p$ (electrochemical proton gradient) that is the sole and obligatory energy intermediate between respiration and ATP synthesis. Coupling is achieved by the proton-translocating nature of the reversible $F_0$-ATP synthase. In one variation, $Na^+$ is a coupling ion for such a synthase, functioning at least well enough for modest synthesis, in an anaerobic marine bacterium, when an electrochemical $Na^+$ gradient is the intermediary energy form (3, 4).

Possible deviations from the proposed universality of the chemiosmotic mode of oxidative phosphorylation have been advanced. The complexity of the systems and the measurements involved have made it difficult to establish that there are bona fide exceptions (see Refs. 5 and 6), but oxidative phosphorylation by alkaliphilic Bacillus species is a strong candidate. Alkaliphiles violate two major predictions of the chemiosmotic model, i.e. that the phosphorylation potential and rate of ATP synthesis by oxidative phosphorylation should vary directly with the magnitude of the bulk $\Delta p$ and that an artificially imposed $\Delta p$ and respiration-generated $\Delta p$ should function identically if the forces are of the same magnitude.

Alkaliphiles such as Bacillus firmus OF4 grow aerobically on nonfermentable carbon sources, with high molar growth yield, at a constant pH of 10.5 and above (7–9). During growth at pH 10.5, a cytoplasmic pH of 8.3 is maintained through the combined actions of a proton-extruding respiratory chain and secondary $Na^+/H^+$ antiporters (9, 10). The result is a small bulk $\Delta p$ that consists of a large $\Delta V$ (transmembrane electrical potential), positive out, and the substantial detracting $\Delta p$H (transmembrane pH gradient), acid in (7–9). Yet, the ATP concentration maintained at this low $\Delta p$, as reflected in the $\Delta Gp$ of growing cells, is comparable with other aerobes and to cells of the same strain growing at pH 7.5, at which the magnitude of the $\Delta p$ is high (7–9). Earlier studies have ruled out the possibilities that the synthase is Na'-coupled (10–13) or is localized in intracytoplasmic membranes or distinct cytoplasmic membrane-associated vesicles (7, 14, 15). The alkaliphile might, then, use a variable $H^+/ATP$ stoichiometry that is unusually high at the alkaline edge of its range and manage a concomitant increase in the $H^+/O$ to account for the invariant molar growth yield as the growth pH is raised. However, a large imposed valinomycin-mediated diffusion potential energized $Na^+/AIB$ transport equally well at pH 7 and pH ≥ 9, but was completely ineffectual for ATP synthesis at pH ≥ 9 in both whole cell and membrane vesicle
were variable H+/ATP stoichiometry the solution to the quantitative discrepancy between the $\Delta p$ and the $\Delta Gp$, an imposed potential should have been effective in energizing this translocation and synthetic event at any given pH value at which respiration works. The synthesis could presumably use the same H+/ATP stoichiometry that can be used during respiration. Rather, there is an apparent qualitative difference between respiration and imposed bulk potentials. In experiments with Na+/H+ antiport activity (18), energized by imposed diffusion potentials at various external pH values, a decline in the antiport rate at pH values of about pH 10 had been observed. We had speculated that the failure of imposed potentials to function identically to respiration-generated potentials in the alkaliphile might be a reflection of the low external proton concentration at very alkaline pH, producing an effect that would apply equally to the two processes that translocated protons inward (10). During many subsequent experiments, however, a strong impression emerged that the decline in efficacy of the imposed potentials with respect to ATP synthesis was sharper and occurred at pH values that still supported full rates of antiport. Thus a detailed study was undertaken.

**Materials and Methods**

*Bacteria and Growth Conditions*—*B. firmus* OF4 was originally isolated in this laboratory (19); it was grown on malate-containing media that have been described previously (9) and are sufficiently buffered to maintain the pH very close to the initial pH of either 10.5 or 7.5 until the late logarithmic stage of growth. Except where specifically noted, alkaliphilic cells were grown at pH 10.5. *Bacillus subtilis* BD99 was obtained from Dr. Anthony Garro and was grown in Spizizen's salts as described earlier (20). All the bacteria were grown at 30 °C with vigorous aeration.

Establishment of Diffusion Potentials of Known Magnitude or of a Respiratory-Generated $\Delta p$ in Starved Whole Cells—Cells of *B. firmus* OF4 were washed and starved by incubation in 100 mM potassium phosphate buffer, pH 7.5, plus 5 mM MgSO$_4$, to reduce the cellular concentration of ATP while preserving substantial inorganic phosphate concentrations, as described earlier (11, 16). The starved cells were concentrated to 30 mg of protein/ml in either potassium phosphate buffer, 100 mM, at pH values from 7.2 to 9.5, or potassium carbonate buffer, 100 mM, at pH values from 9.0 to 10.5. These cells also contained 10 mM NaCl and 5 mM MgSO$_4$. In some experiments the cells were washed and concentrated in 25 mM Tris, pH 7.5 or 9.0, 100 mM KCl, 5 mM MgSO$_4$, without any sodium. For experiments in which cells were to be energized by establishment of a diffusion potential, valinomycin was added to 10 $\mu$M, and the cells were respirometerically equilibrated and then treated with nigericin and equilibrated at the same pH as the equilibration buffer for a given sample of cells and then diluted to 30 mg of cell protein/ml in 100 mM potassium phosphate buffer at either pH 7.5, 8.5, 8.8, or 9.0 and containing 5 mM MgSO$_4$. NaCN (to 1 mM) and valinomycin (to 10 $\mu$M) were added for 5 min. The cells were then diluted 1:1000 into 100 mM sodium phosphate buffer, containing 5 mM MgSO$_4$, at the same pH as the equilibration buffer for a given sample of cells and containing 5 mM NaCN and 1 $\mu$M valinomycin. For cells that were energized by malate, the treatment with NaCN and valinomycin was omitted, and the concentrated cells were diluted into buffers containing 10 mM sodium malate.

Assays—The $\Delta p$ and $\Delta p$ were measured as described elsewhere (9) by the accumulation of the lipophilic cation TPP$^+$ (21) and the weak base (22) methylamine, respectively. De-energized controls were routinely included as described in the original reports. A filtration assay was employed, using 0.45-p OE filters (Schleicher and Schuell) for the $\Delta p$ determinations and GF/P glass microfilter filters (Whatman) for the $\Delta p$ determinations; cell concentrations and sample sizes were adjusted to insure rapid filtration. The filters for the $\Delta p$ assay were washed with 10 ml of the suspension buffer. Corrections for the binding errors involved in the use of TPP$^+$ were made for energized and unenergized preparations (9) using the approach of Rottenberg (22); we routinely used comparisons of the values for the filtered samples with 1, 5, and 10 $\mu$M TPP$^+$ as the theoretical $\Delta p$ determination, different cell concentrations in the range employed were shown to give the same values for $\Delta p$ and $\Delta p$.

We also routinely tested the accuracy of our measurements with diffusion potentials of known theoretical magnitude that were in the range of respiration-generated $\Delta p$ values, i.e., $-170$ to $-180$ mV. During the current study, we also compared the measured value obtained with determinations of TPP$^+$ accumulation to the theoretical value of a diffusion potential of higher magnitude than we had heretofore examined in order to better assess possible general underestimations with the TPP$^+$ assay. A second methodological control that was conducted periodically with respiring cells at pH 10.5 is a set of $\Delta p$ measurements made by the accumulation of "Rb" in the presence of valinomycin (24). Recently, Hoffmann and Dimroth (25) have presented unusually high $\Delta p$ values in strains of *Bacillus alcalophilus* using 50 nM TPP$^+$ and separation techniques that involve filtration. Their results were surprising. For example, in growing strain (50-min generation time) and a slow-growing strain (10-h generation time) with reduced cytochrome levels were found to have comparably high $\Delta p$ values. We had never used such low

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1. The abbreviations used are: PMS, phenazine methosulfate; ACMA, 9-amino-6-chloro-2-methoxyacridine; AIB, a-aminoisobutyric acid; CAPS, 3-(cyclohexylamino)propanesulfonic acid; TPP$^+$ tetraphenylphosphonium.
concentrations of TPP+ because of the study of Zaritsky et al. (26), indicating that TPP+ concentrations in that range would be largely bound to cell surface layers in at least one Bacillus species and hence would not reliably equilibrate with the transmembrane potential. Although our own methods had reliably measured diffusion potentials of known magnitude in earlier work (e.g. Refs. 16 and 17), and in view of the determinations of Hoffmann and Dimroth (25) which were presented without data on accuracy or precision, we examined the effect of using a lower probe concentration together with our usual separation technique. As shown in Table I, both concentrations of TPP+ measured a diffusion potential of -176 mV quite accurately at pH 7.5, but a considerable underestimation and enhanced variability in the measurement was observed with the lower concentration of TPP+ versus our usual concentration when the measurements were conducted at pH 10.5. When a larger diffusion potential was imposed, the measurement using 50 nM TPP+ represented a serious underestimation at both pH values, whereas measurements with 4 μM TPP+ resulted in a small underestimation with a smaller variability. In addition, the measurements of the ∆ψ of growing cells were grossly lower with 50 nM TPP+ than measurements with our usual concentration and with 4 106 Rb+ + valinomycin, which were in close agreement. Although not shown here, we also conducted a small number of side-by-side measurements of this sort with B. alcalophilus and found that with that species the low TPP+ concentrations did not underestimate the standard imposed diffusion potentials relative to our concentration. However, the ∆ψ values of respiring cells of the fast-growing strain were comparable with values obtained with our usual TPP+ concentration and with 4 106 Rb+ + valinomycin and were higher than the values obtained with the slow growing strain. That is, we did not replicate either the unusually high values reported or the comparability of the ∆ψ in the slow and fast growing strains (25) when our separation techniques were used. Perhaps an overestimation and loss of discrimination occurred in the Hoffmann and Dimroth work (25) that may have resulted from contamination of the cell pellet with residual supernatant. We plan to compare the separation methods directly. Although a comparable study of the methods used for the ∆ψ measurement was not undertaken in connection with the current study, previous studies had indicated that the method routinely used for the measurement of a ∆ψ, acid in, accurately measured imposed gradients in the relevant range (27). The change in concentration of ATP was monitored by assays of ATP using the luciferin-luciferase system (28); previous determinations had indicated that all ATP was produced accounted for by a decrease in ADP, and this was confirmed for each experimental condition in the current study. When ATP synthesis was energized by a diffusion potential, samples were taken at 15 s, 30 s and 1, 2, 5, and 10 min. When energization was by respiration, samples were taken at 5 and 10 s for determinations of rates, and the PMS concentration was adjusted so that the rates were linear during this interval. For samples to be assayed for 22Na+ efflux, the buffer in which the concentrated suspension of starved cells was equilibrated contained 22Na+ in addition to potassium and valinomycin, as described previously (18). Upon dilution to establish the diffusion potential, samples were taken for determinations of the remaining 22Na+ using a filtration assay, from which a calculation of the rate of efflux could be made (18). The samples were taken at 5, 10, 15, 20, 30, and 60 s, with linear rates generally observed between 5 and 15 s. For determinations of AIB accumulation, 500 μM [3]AIB was added to the dilution or energization buffer, and accumulation was determined using a filtration assay and de-energized controls, as described previously (29). The time points used for determinations of rate were 30 s and 1, 2, 5, and 10 min. Protein was determined by the method of Lowry et al. (30) using egg white lysozyme as a standard; this is the same protein assay and standard that had been used for cell volume measurements. Use of our values for cell volume together with other measurements based on a different protein assay and/or standard (19, 25) may introduce a small systematic error in the calculations that is best to avoid.

**RESULTS**

*Energization of Several Energy-dependent Ion-translocating Processes in B. firmus OF4 by a Potassium Diffusion Potential at Various pH Values—* A valinomycin-mediated potassium diffusion potential of -176 mV was imposed across starved respiration-inhibited cells of *B. firmus OF4* at a range of pH values, either in the absence (Fig. 1a) or presence (Fig. 1b) of a small inwardly directed Na+ gradient. All assays were conducted in duplicate and the results of at least five independent experiments were averaged for the data shown for each condition. At each pH value, under both experimental conditions, the magnitude of the potential generated was measured via TPP+ accumulation, and these measured values were identical at the various pH values and conditions, corresponding to the theoretical value within 10%. Under both conditions, the rate of ATP synthesis declined above pH 8.4 and was zero at pH 9.5. In the presence of the Na+ gradient, the decline was steeper than in its absence, with a complete failure of synthe-

### Table I

**Control experiments in connection with the measurements of the ∆ψ of alcaliphile cells**

Cells in which a diffusion potential was generated were concentrated in either 100 mM potassium phosphate, pH 7.5, or 100 mM potassium carbonate, pH 10.5 plus 10 μM valinomycin and 10 mM KCN. At pH 7.5 or 10.5 cells were diluted 1:1000 or 1:5000 into 100 mM sodium phosphate, pH 7.5, or 100 mM sodium carbonate, pH 10.5, respectively. The dilution buffers contained either 50 nM [3H]TPP+ or 4 μM [3H]TPP+. One ml samples were taken and cells were collected by filtration. Controls were dilution buffers containing 100 mM potassium phosphate or potassium carbonate. The ∆ψ of growing cells was measured by washing and suspending cells (0.05 mg of protein/ml) growing at pH 7.5 or 10.5 in 100 mM sodium phosphate 7.5 or 100 mM sodium carbonate 10.5, respectively, plus 10 mM sodium malate. Uptake of either 50 nM or 4 μM [3H]TPP+ was performed by filtration. TPP+ binding controls contained 10 μM gramicidin. Uptake of 100 μM 60Rb+ plus 1 μM valinomycin was assayed by filtration also, but binding controls were cells without valinomycin. All values are expressed as the mean ± S.D., and represent at least five independent experiments, each one done in duplicate.

| Calculated value of potential | Buffer pH | Measured value of potential |
|--------------------------------|-----------|----------------------------|
|                               | mV        | 50 nM TPP+ | 4 μM TPP+ |
| A. Valinomycin-mediated diffusion potential |           |             |             |
| 1:1000 dilution               | -176      | 7.5         | -177 ± 9   |
|                               |           | 10.5        | -160 ± 13  |
| 1:5000 dilution               | -218      | 7.5         | -180 ± 33  |
|                               |           | 10.5        | -182 ± 20  |

### ∆ψ measured using

| mV |
|-----|
| 50 nM TPP+ | 4 μM TPP+ |

| B. ∆ψ of cells growing at | mV |
|---------------------------|----|
| pH 7.5                    | -81 ± 52 |
| pH 10.5                   | -108 ± 19 |
Energy Coupling of Alkaliphile Oxidative Phosphorylation

First, cells were treated with valinomycin at pH 10.2 and then either energized by dilution at the same pH or re-equilibrated at pH 7.5 before dilution at the lower pH. This experiment was conducted to test the possibility that the valinomycin treatment, at very alkaline pH values only, inhibits the cells’ capacity to synthesize ATP and that the apparent decline in efficacy of the imposed potential actually results from this inhibition. The synthesis observed in cells treated at high pH and then subjected to imposition of the potential at low pH, showed that the treatment at high pH per se had not precluded ATP synthesis (Fig. 2a). Nor was there a changeover from phosphate to carbonate buffer responsible for the failure of the diffusion potential to energize above a given pH, since comparisons of the two buffers at pH 9.5 showed no synthesis with either (Fig. 2b). In another set of experiments, it was shown that omitting the cyanide from the pretreatment for the diffusion potential experiment did not allow ATP synthesis to occur in response to the potential at pH 9.5, eliminating some pH-dependent adverse effect of cyanide. Moreover, when the same dilution of these cells to establish a diffusion potential was made into buffer containing choline ascorbate + PMS, the electron donor energized rapid synthesis of ATP under the same conditions, again including the valinomycin treatment, in which the diffusion potential alone was inefficacious (shown in Fig. 2b for conditions without Na+; the same results were obtained in the presence of Na+).

In another set of control experiments, we examined whether the rate of ATP hydrolysis might be so much faster at very alkaline pH than at near neutral pH that this decay rate prevented the observation of ATP synthesis in the diffusion potential experiment. This control had been conducted earlier with B. firmus RAB (17). Cells that had been equilibrated at either pH 7.5 or 9.5 were diluted into electron donor-containing buffer at the same pH values and allowed to synthesize ATP for 1 min; at that time, cyanide was added, and the concentration of cellular ATP was monitored. The rates of decay of ATP at the two pH values were indistinguishable (Fig. 3). Also shown for comparison in Fig. 3, are the comparable rates of synthesis observed with the controls for this experiment and another cell sample that was equilibrated at pH 8.5 and then diluted into buffer at pH 10.4 at the point of energization with an electron donor.

Of remaining concern were two possible problems related to cytoplasmic pH. First, it was possible that the backflow of protons that is initiated upon development of the diffusion potential more rapidly dissipates the total electrochemical proton gradient at pH values above 9 than at near neutral values. ATP synthesis might be more sensitive to such a difference in the loss of driving force than antiport. Earlier measurements of the backflow using methylamine distribution had supported the opposite conclusion, i.e. the Δp-diissipating proton backflow was somewhat slower at pH 9 than at pH 7 (17). We re-examined this issue using the quenching of ACMA fluorescence to monitor the backflow. Starved cells were washed and suspended in buffers at pH 7.5 or 9.0 and treated with valinomycin as in the other diffusion potential experiments in which sodium was omitted (e.g. in Fig. 2b). ACMA (1 μM) was added to a cuvette containing 2 ml of 100 mM choline chloride, 25 mM Tris, 5 mM MgSO4, 1 μM valinomycin, at pH 7.5 or 9.0. Fluorescence was monitored as described previously before and after the dilution (1:1000) of the starved cells into the ACMA-containing potassium- and sodium-free buffer. The fluorescence changes were quantitated at pH 7.5 and 9.0 by creation of nigericin-induced pH gradients of different magnitudes and the data were analyzed by the formula of Briskin and Reynolds-Niesman (31). The
raw data showed a tiny rate of backflow at the more alkaline pH value relative to a more rapid rate at pH 7.5. When they were corrected for pH-dependent differences in probe response, as indicated, the rate of backflow at the two pH values appeared comparable and certainly no faster at the more alkaline pH value. At pH 7.5, the pH gradient, acid in, was generated in response to the diffusion potential at a rate of about 35 mV/min, and at pH 9.0 it was generated at about 33 mV/min.

The second concern with respect to cytoplasmic pH arises from the possibility that the initial pH values above pH 9 are incompatible with rapid ATP synthesis by the starved alkaliphile cells. In the diffusion potential protocol, the cells are equilibrated so that the initial cytoplasmic pH is equal to the external pH for a given sample. The experiment depicted in Fig. 2b indicated that the conditions under which the diffusion potential was imposed at pH 9.5 allowed ATP synthesis if an electron donor was present, and earlier studies indicated that the ATPase was functional at very alkaline pH (32). A set of more complete determinations on synthesis was conducted in cells that were treated with nigericin and not with cyanide. The cells were then energized by the addition of ascorbate and a sufficiently low concentration of PMS so that an actual rate of ATP synthesis could be measured at various initial pH values.

Fig. 3. ATP synthesis by starved B. firmus OF4 cells upon addition of an electron donor and decay rates of the ATP synthesized as a function of pH. Starved cells were concentrated as described under “Materials and Methods.” ATP synthesis was initiated by a 1:1000 dilution into 25 mM Tris, 100 mM KCl, 5 mM MgSO₄ plus 10 mM potassium ascorbate/0.1 mM PMS. The values obtained were corrected by subtracting values for parallel samples that were diluted into buffers lacking the electron donor (and there was no increase in ATP in these samples over time). The pH values at which the cells were concentrated and then diluted were as follows: pH 8.5 diluted at pH 10.4 (○); pH 7.5 diluted at pH 7.5 (○, ■) and pH 9.5 diluted at pH 9.5 (Δ, ■) followed (at the arrows) by the addition of 10 mM KCN (●, ▲).

The apparent pH-dependent failure of an imposed potential to energize, specif-
when malate was used. The pH 7.5-grown cells even exhibited cells that were starved and re-energized with either ascorbate/ PMS or malate exhibited much less ATP synthesis than pH 10.5-grown cells when re-energized at pH 10.6, especially when malate was used. The pH 7.5-grown cells even exhibited a lower rate of synthesis than the pH 10.5-grown cells when both were re-energized at pH 7.5. By contrast, the Δψ values were only slightly different for re-energized cells that had been grown at pH 7.5 and 10.5. These values were substantial but not identical to the values observed in cells that have never been starved (9). The energization of other bioenergetic work supported by pH 7.5- versus pH 10.5-grown cells upon addition of malate at pH 10.6 was also comparable, as shown for formation of the ΔpH, acid in, that results from electrogenic antiport activity and for the rate of Na+/AIB symport. In fact, since the Δψ and ΔpH values were both slightly lower in the pH 7.5- than in the pH 10.5-grown cells (presumably the lower Δψ resulting in the lower ΔpH because of the energy dependence of the intermediary antiport), the bulk Δp values for the two cell preparations were almost identical at pH 10.8.

**DISCUSSION**

The challenges posed by extremely alkaliphilic Bacillus species to a strictly chemiosmotic model of the energization of oxidative phosphorylation are both quantitative and qualitative (7–10). The quantitative discrepancy between the Δp and Δψ could be explained either by a major underestimation of the latter values in respiring cells growing at high pH or by the utilization, only at very alkaline pH values, of H+/ATP stoichiometries, i.e. above 8. Some of the additional controls conducted in connection with the Δψ measurements in this study support the view that the former possibility is unlikely. For example, measured Δψ values of −180 mV in respiring cells of B. firmus OF4 are unlikely to really be underestimations of values of over −200 mV, since a −218 mV potential was measured accurately. We thus expect to measure potentials in the range of −218 mV and below accurately and to observe at least −218 mV were the true value even higher. Moreover, two different, albeit both indirect, methods of measuring the Δψ of cells growing at pH 10.5 produced the same value of −180 mV, as had been found previously in various other preparations of cells under the

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*P. G. Quirk, unpublished data.*

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**Fig. 4.** The initial rate of ATP synthesis driven by ascorbate/PMS in starved nigericin-treated cells at different pH values. Starved cells were concentrated at pH 7.5–9.5 in 100 mM potassium phosphate or pH 9.0–10.5 in 100 mM potassium carbonate. The concentrated cells were pretreated with 0.1 µM nigericin for 5 min. ATP synthesis was initiated by diluting 1:1000 into identical nigericin-containing buffers that also contained 10 mM potassium ascorbate + 2 µM PMS. Samples for ATP synthesis were taken at 5 and 10 s. The initial rates of ATP synthesis were calculated from three independent experiments with duplicate determinations.

**Fig. 5.** Synthesis of ATP by starved cells of B. subtilis energized by either imposition of a diffusion potential or addition of malate at various pH values. Cells were starved and then concentrated to 30 mg of cell protein/ml in potassium phosphate buffers at various pH values as described under "Materials and Methods." Half of each thick cell suspension was treated for 5 min with valinomycin and NaCN as described under "Materials and Methods," whereas the other half had no additions. At zero time, the cell suspensions were diluted 1:1000 into sodium phosphate buffers at the same pH values as the equilibration buffers, as indicated. For those experiments in which a diffusion potential was generated (○), the cells had been treated with and were diluted into buffers containing NaCN and valinomycin. For cells energized by an electron donor (●), the untreated cell suspensions were used and were diluted into buffers containing sodium malate. Samples were taken at various times and assayed for ATP synthesis.
**Energy Coupling of Alkaliphile Oxidative Phosphorylation**

**Table II**

ATP synthesis, AIB uptake, ΔpH and Δψ formation by cells grown at pH 7.5 or 10.5, starved and then re-energized.

| Amount of ATP synthesis | pH 7.5-grown cells re-energized with | pH 10.5-grown cells re-energized with |
|-------------------------|--------------------------------------|--------------------------------------|
|                         | Asc/PMS | Malate | Asc/PMS | Malate |
| **At pH 7.5**           |         |        |         |        |
| At pH 7.5               | 16.1 ± 2.6 | 4.2 ± 1.8 | 24.9 ± 5.6 | 6.6 ± 3.9 |
| At pH 10.6              | 6.7 ± 2.3 | 0.3 ± 0.2 | 21.8 ± 6.9 | 5.0 ± 3.2 |
| **Δψ, mV**              |         |        |         |        |
| At pH 7.5               | -146 ± 16 | -143 ± 12 | -154 ± 11 | -167 ± 16 |
| At pH 10.6              | -150 ± 14 | -142 ± 8 | -164 ± 16 | -167 ± 18 |
| **ZΔpH, mV**            |         |        |         |        |
| At pH 10.6              | +108 ± 7 | +102 ± 10 | +124 ± 11 | +121 ± 3  |
| **Rate of AIB uptake**  |         |        |         |        |
| At pH 7.5               | 12.2 ± 6 | 4.6 ± 0.8 | 10.1 ± 4 | 8.7 ± 0.4 |
| At pH 10.5              | 7.4 ± 4 | 4.3 ± 0.5 | 9.0 ± 1.7 | 5.2 ± 1.3 |

same conditions. We have used several different cell growth and preparation conditions over the years, including washed cells in buffer with (36) and without (29) energy source, cells growing in our original less buffered medium (e.g. Ref. 19), cells grown in more highly buffered medium (9, 33), and cells that were starved and re-energized under various conditions as in the current study; the measured Δψ values have been remarkably consistent for any given preparation and have agreed with independent measurements of similar organisms in other laboratories (37) using probe concentrations that are in the range customarily used by investigators working with Bacillus and other Gram-positive species (38-40). As indicated by Zaritsky et al. (26) and shown in Table I, use of much lower probe concentrations is problematic in Bacillus species, which lack the outer membrane of Escherichia coli but often have strain-specific charged polymers in the cell wall layer. In addition, it is possible that use of low probe concentrations exacerbates the problem of overestimation of the Δψ when centrifugation is used as the separation technique. In sum, a Δψ of ~280 mV would be needed in B. firmus OF4 at pH 10.5 to achieve the observed Δp values using only the bulk gradients and the same apparent stoichiometry of H+/ATP as that calculated for the same cells at pH 7.5 (9). The quantitative data presented here and elsewhere (8, 9) are inconsistent with that possibility. Only variable and, in the most alkaline growth range, high H+/ATP and H+/O ratios can account for the quantitative bioenergetic profile of the alkaliphiles in a chemiosmotic fashion. In fact, the results shown for the slow-growing B. alcalophilus strain during a growth curve in the Hoffmann and Dimroth study (25) present the same bioenergetic profile observed by us with B. firmus OF4 and other alkaliphiles, i.e. whereas the magnitude of the inwardly directed Na⁺ gradient (reflective of antiport activity) followed the pattern of the total bulk Δp during growth, the highest growth rates and the highest [ATP]/[ADP] ratios did not correspond with the period in which the bulk chemiosmotic driving force was the highest. The authors noted this striking observation, but did not discuss the issues it might raise vis-à-vis their proposal of a chemiosmotic mode of ATP synthesis without unusual stoichiometric considerations (25, 41).

Whatever the mechanistic H⁺/ATP stoichiometry may be at either near neutral or very alkaline pH, an explanation of the quantitative discrepancy that posits an increase in the stoichiometry at increased pH cannot explain the failure of an imposed diffusion potential to energize ATP synthesis above about pH 9. This striking failure is demonstrable both in cells and in membrane vesicles that lack cell wall markers as well as cytoplasm (17). It is now clearly shown to be specific for ATP synthesis as opposed to a general phenomenon affecting both processes, i.e. ATP synthesis and Na⁺/H⁺ antiport, that catalyze inward proton movement. The decline in the efficacy of the imposed potential with respect to energization of ATP synthesis exhibits a titration-like pattern that is markedly sharper (fewer deprotonations involved?) in the presence of an inwardly directed Na⁺ gradient. Given the data in Fig. 4, showing rapid ATP synthesis over a broad range of cytoplasmic pH values, it is unlikely that the Na⁺ effect represents an indirect effect resulting from inhibited antiport, and a resulting slightly elevated pHₐ, in the presence versus the absence of the small Na⁺ gradient. Extreme alkaliphiles lack a Na⁺-coupling option for oxidative phosphorylation to bypass the low Δp, possibly because Na⁺-driven synthesis does not work well enough to support aerobic growth on nonfermentative carbon sources, particularly when the aerobe has special energetic needs. It is notable that the presence of the inwardly directed Na⁺ gradient, which would be the situation in growing cells, actually decreases the pH range over which a diffusion potential is effective.

A model that encompasses the known characteristics of oxidative phosphorylation in extreme alkaliphiles is shown in Fig. 6. It features a pK-regulated gate in the FₐFₒ-ATP synthase. At pH 7.5, the gate is proposed to be open so that an imposed potential can energize ATP synthesis as well as other bioenergetic work. Under those circumstances the H⁺/ATP ratio calculated from the ΔGp/Δp ratio of respiring pH 7.5-grown cells is near 3 (8, 9), consistent with commonly observed stoichiometries and a completely chemiosmotic mode of energization. At pH values above 9.4, the gate on the ATP synthase is proposed to shut, thereby precluding inward proton flow from the bulk and, hence, precluding energization by an imposed diffusion potential. As noted above, the closing of the gate occurs at lower pH values and more sharply in the presence of the physiologically normal gradient of Na⁺, out >
in, consistent with the proposed importance of this property during growth at pH values in the highly alkaline range. A gate could be a major kinetic factor in preventing protons that arrive at the Fo-ATPase via an alternate intramembranal entry route from simply flowing outward into the bulk rather than flowing inward productively through the synthase. The small bulk Δp that exists across the alkaliphile membrane even at the alkaline extreme for growth and the substantial ΔV component are also clearly necessary factors in preventing the loss of protons from the putative intramembrane pathway to the bulk and energizing their inward productive translocation. In all the experiments that we have conducted on alkaliphile cells and vesicles, ATP synthesis at high pH has never been observed at Δp = 0 (7, 8); this is confirmed in the current study. It has occasionally been suggested (e.g. Ref. 25) that a requirement for the bulk force would run counter to a model in which a bulk force was not the sole intermediary in coupling. By contrast, we have always predicted and shown that a bulk force was necessary for oxidative phosphorylation by the alkaliphile at high pH even if it proved not to be sufficient by itself.

A gating property has been proposed in other experimental systems (42, 43) and need not be special to the alkaliphiles. One might imagine, for example, that a conserved basic residue on the α-subunit could, upon deprotonation, no longer allow proton entry at the usual bulk entry point. Such a deprotonation event might never occur in the physiological range of conventional organisms, but would be serendipitously useful to the alkaliphile. The experiments conducted on B. subtilis may indicate that whatever is titrated as the pH is raised in the diffusion potential experiment (the putative gating residue?) is similarly titrated in B. subtilis. The difference might be that the alternative coupling option that is proposed to be available to the alkaliphile, because of its particular concentration, cation and constellation of respiratory components and special features of the Fo (44), is not available to respiring B. subtilis cells; therefore, the two energization modes show the same pattern of decline in B. subtilis (Fig. 5).

It should be noted that a pK-regulated gate on the Fo is only one possible interpretation of the apparent titration in Fig. 1 that is consistent with all the current data. An alternative, for example, would be that there is a delocalized but nonbulk pathway for proton delivery to the Fo that is physiologically important at conventional pH values. Such a pathway has been proposed by others in connection with their work on membrane lipids (45, 46). If that sort of proton delivery were an important part of proton delivery to the synthase when a diffusion potential is imposed, and only functioned below pH 9.5, then at more alkaline pH values the diffusion potential might not work.

The major feature of the model is a direct transfer of protons from a respiratory chain complex, perhaps specifically the caa₃ oxidase, and the Fo within the membrane during electron transport. The alkaliphile Fo might well have special properties that promote the proposed direct protein-protein interactions within the membrane so that transfer of a proton could occur without its loss to the bulk (44). Moreover, when the concentration of the directly interacting complex(es) is sufficiently high, such interactions may enhance oxidative phosphorylation even at pH values at which the proposed gate is open, since pH 10.5-grown cells synthesized significantly more ATP at pH 7.5 than did pH 7.5-grown cells (Table II). Also, respiration supported much more rapid rates of ATP synthesis than a comparable diffusion potential even at near neutral pH (Figs. 1 and 4). Importantly, although some protons are proposed to be delivered directly to the ATP synthase, without first equilibrating with the bulk, other respiration-linked proton extrusion events continue to result in generation of a bulk Δp. The Δp is used for other bioenergetic work, and as noted above, for the requisite backpressure to facilitate inward proton movement through the ATP synthase.

The current model could be consistent with conventional H⁺/ATP stoichiometries, but argues neither for nor against other stoichiometries. Nor does the model encompass specific features that might illuminate the significance of the low redox potentials that are found for the respiratory chain components of the alkaliphiles (47, 48). The model also lacks detail about the nature of the intramembranal proton pathway, a concept first proposed by Williams (49). We do posit, however, that the intramembrane pathway involves a direct protein-protein interaction between one or more specific respiratory chain elements and the ATP synthase. We are further inclined toward the expectation that the residues specifically involved in the transfer are normally part of the protonic pathway, but that they are points in the pathway that are within the membrane, between sites of proton uptake or release from the cytoplasmic and bulk sides of the membrane. It is of note in this connection that two regions of interesting deviation from the prokaryotic consensus sequence for the c-subunit of the B. firmus OF4 Fo are in the middle of the two transmembrane regions of this polypeptide in regions proposed by others to be involved in proton translocation (44). The protein-protein interactions that are central to this model could result from collisional events such as those proposed by Slater (50), and recently by Gupte et al. (54), perhaps facilitated by the especially fluid membrane of the alkaliphile (33) or from the formation of dynamic or stable complexes between the interacting partners in the proton transfer (51–53). It is of interest that Rottenberg specifically proposed the possibility of a complex between helices of the cytochrome oxidase.
and the ATP synthase (53), an interaction that we propose here.

We are currently endeavoring to select pH conditional mutants of the alkaliphile that have lost the ability to grow at pH 10.5; those mutants will be screened for possible loss of the putative gating property and/or deviations from the novel alkaliphile \( F_o \) sequence motifs. If suitable mutants are found, it then might be possible to examine the properties of second-site mutations that restore alkaliphily as an approach to possible protein-protein interactions. We are also studying the effects of mutations that alter the levels of terminal oxidases on growth and oxidative phosphorylation at pH 7.5 versus pH 10.5. It will be important to seek such biochemical and molecular biological modes of testing the current hypothesis. The postulation of a nonchemiosmotic direct proton transfer is based on the failure of a diffusion potential to act equivalently to a respiration-generated potential, specifically for ATP synthesis at high pH. For all our attempts to rule out trivial explanations for this qualitative aspect of the alkaliphile's bioenergetic dilemma, it remains possible that an undetected technical explanation underlies these observations, making independent modes of examining the model particularly important.

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