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OXIDATIVE PHOSPHORYLATION BY MEMBRANE VESICLES FROM *BACILLUS ALCALOPHILUS*

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Summary

ADP and P_i-loaded membrane vesicles from L-malate-grown *Bacillus alcalophilus* synthesized ATP upon energization with ascorbate/*N,N,N',N'*-tetramethyl-*p*-phenylenediamine. ATP synthesis occurred over a range of external pH from 6.0 to 11.0, under conditions in which the total protonmotive force $\Delta\bar{\mu}_{H^+}$ was as low as −30 mV. The phosphate potentials (ΔG_p) were calculated to be 11 and 12 kcal/mol at pH 10.5 and 9.0, respectively, whereas the $\Delta\bar{\mu}_{H^+}$ values in vesicles at these two pH values were quite different (−40 ± 20 mV at pH 10.5 and −125 ± 20 mV at pH 9.0). ATP synthesis was inhibited by KCN, gramicidin, and by *N,N'*-dicyclohexylcarbodiimide. Inward translocation of protons, concomitant with ATP synthesis, was demonstrated using direct pH monitoring and fluorescence methods. No dependence upon the presence of Na^+ or K^+ was found. Thus, ATP synthesis in *B. alcalophilus* appears to involve a proton-translocating ATPase which functions at low $\Delta\bar{\mu}_{H^+}$.

Introduction

According to Mitchell's chemiosmotic hypothesis [1,2], the $\Delta\bar{\mu}_{H^+}$ (consisting of a transmembrane electrical potential, $\Delta\psi$, inside negative, and/or a transmembrane pH gradient, ΔpH, inside alkaline) energizes ATP synthesis by a

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Abbreviations: Ammediol, 2-amino-2-methyl-1,3-propanediol; DCCD, *N,N'*-dicyclohexylcarbodiimide; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; $TPMP^+$, triphenylmethylphosphonium bromide; TPP^+, tetraphenylphosphonium bromide.

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membrane-bound, proton-translocating ATPase. Mitchell has calculated that a protonmotive force of −210 mV would be required to maintain an [ATP]/[ADP] ratio of unity when the concentration of P_i is 10 mM [2,3]; this calculation is based on the assumption that two protons are translocated for each ATP formed. Evidence from studies of mitochondria as well as bacteria has generated major areas of accord on chemiosmotic principles [4], although considerable controversy remains with respect to the precise nature of the relevant transmembrane gradients, the mechanism and stoichiometry of primary proton extrusion, and the mechanism of ATP synthesis. We have been interested in using extremely alkalophilic bacteria as a system in which at least some of the cogent questions in these areas might be fruitfully explored.

Previous studies in this laboratory have shown that the obligate alkalophile, *Bacillus alcalophilus*, exhibits very low $\Delta\bar{\mu}_{H^+}$ values at optimal external pH values for growth of this aerobe on L-malate [5]. At pH values above 9.5, the organism maintains an acidified cytoplasm [5], apparently through the operation of a Na^+/H^+ antiporter [6]. Consequently, at pH 10.5, for example, the $\Delta\bar{\mu}_{H^+}$ across whole cell membranes is −80 mV, consisting of a $\Delta\psi$ of −147 mV, and a 'reversed' ΔpH of 1.15 units. At pH 11.0, at which excellent growth of the organism on L-malate occurs, the $\Delta\bar{\mu}_{H^+}$ is only −15 mV. How, then, are processes which are thought to depend upon a $\Delta\bar{\mu}_{H^+}$ energized in *B. alcalophilus*? For transport of α-aminoisobutyric acid and β-galactosides, the problem of a low $\Delta\bar{\mu}_{H^+}$ is bypassed by use of a sodium-symport mechanism [5] and a mechanism requiring the phosphate bond energy of ATP [7], respectively. It was possible that use of sodium ions rather than protons might also be involved in ATP synthesis in this bacterium. Thus we undertook to examine oxidative phosphorylation, using a vesicle system, with a view toward determining whether $\Delta\psi$-dependent and/or Na^+-dependent ATP synthesis could be demonstrated, and whether the ATP synthetic mechanism involved proton translocation. The findings indicate no special role for Na^+ in ATP synthesis, but rather, that a proton-translocating ATPase catalyzes $\Delta\psi$-dependent ATP synthesis even under conditions in which the $\Delta\bar{\mu}_{H^+}$ is low and protons are translocated against a substantial chemical gradient.

Methods

Organism and growth conditions. B. alcalophilus (ATCC 27647) was grown with shaking in a sodium carbonate-buffered medium as described elsewhere [5]. The medium was supplemented with 0.1% (w/v) yeast extract (Difco), 1% (v/v) trace salts [8], and 50 mM sodium L-malate, added from separate sterile solutions. Growth was followed turbidometrically with a Klett-Summerson colorimeter (No. 42 filter).

Preparation of membrane vesicles. Isolated membrane vesicles were prepared from L-malate-grown cells of *B. alcalophilus* by a variation of the lysozyme-EDTA technique of Kaback [9]. Protoplasts were routinely lysed in 100 mM potassium carbonate buffer at pH 9.0, unless otherwise indicated, and EDTA was omitted from the procedure. Loading with ADP was accomplished by including 5 mM ADP (potassium salt, unless otherwise indicated) in the shock solution as indicated by Tsuchiya [10]. Potassium phosphate (10 mM) and

$MgSO_4$ (10 mM) were also routinely included in the shock solution, except where specified. Vesicles loaded with ADP and P_i were washed several times with 100 mM potassium carbonate, 10 mM potassium phosphate, and 10 mM $MgSO_4$, pH 9.0. In some experiments vesicles were lysed and washed in 10 mM Tris-HCl, 100 mM choline chloride, 10 mM $MgSO_4$, 5 mM Tris ADP, 10 mM Tris phosphate, pH 9.0. All experiments were performed within 4 h of vesicle preparation because of the lability of ATP synthetase activity.

The internal volume of *B. alcalophilus* vesicles was determined by the method of Stock et al. [11], using [^{14}C]inulin as a marker for extravesicular space. The intravesicular volume was found to be 1.1 μl/mg protein. Protein was determined by the method of Lowry et al. [12], using lysozyme as the standard.

Assay of ATP synthesis. Synthesis of ATP by vesicles loaded with ADP and P_i was assayed at 30°C with constant oxygenation of the assay mixture. The energy source used in most of the experiments in this paper was 20 mM ascorbate (appropriate salt) and 2 mM TMPD. Background values of ATP, in vesicles without an energy source, were always subtracted. The reaction mixture contained approx. 500 μg of vesicle protein/ml in 100 mM potassium carbonate buffer plus 10 mM phosphate and 10 mM $MgSO_4$, adjusted to the pH desired. Samples (200 μl) were withdrawn into ice-cold 30% $HClO_4$ according to the method of Cole et al. [13]. After at least 15 min on ice, the extract was neutralized with 1 N KOH. ATP was measured by the luciferin-luciferase assay in a Beckman LS-230 spectrometer, with the coincidence switch off, as described by Stanley and Williams [14]. ADP was determined by conversion to ATP with pyruvate kinase as described by Chapman et al. [15]. Phosphate was measured by the method of Fiske and SubbaRow [16]. The phosphate potential, ΔG_p, was calculated, as described by Slater [17], from the formula:

$$\Delta G_p = \Delta G^0 + RT \ln [ATP]/[ADP][P_i]$$

The value for ΔG^0 (8.6 kcal/mol) was taken from Rosing and Slater [18].

Monitoring of external pH. Membrane vesicles (1 mg protein/ml) were incubated aerobically in a reaction mixture of 3 ml, containing 25 μg/ml carbonic anhydrase. The pH was measured with a Beckman Expandomatic pH meter (Beckman combination pH electrode model 39012) connected to a strip chart recorder with full scale deflection set at 0.2 pH unit.

Measurement of quinacrine fluorescence. Fluorescence changes in right-side-out membrane vesicles were measured in a Perkin-Elmer model 650-10S Fluorescence Spectrophotometer as described previously [6].

Measurement of ΔpH and $\Delta\psi$. The internal pH of membrane vesicles was measured by the distribution of [^{14}C]methylamine in a flow dialysis assay [19] as validated previously for work in the alkaline pH range [6]. The transmembrane electrical potential ($\Delta\psi$) was determined by the distribution of ^{86}RbCl (in the presence of valinomycin) using a flow dialysis assay [19] or from the distribution of [^{3}H]$TPMP^+$ or [^{3}H]TPP^+ [20,6], using a filtration assay. When vesicles were swelled by decreasing the osmolarity of the buffer, $TPMP^+$ accumulated to a greater extent and the $\Delta\psi$ calculated from the Nernst equation was not significantly different from that in the smaller vesicles. For example, in one experiment, when the vesicle volume was increased to 2.5 μl/mg protein a

$\Delta\psi$ of −99 mV was calculated, whereas a $\Delta\psi$ of −95 mV was calculated from a vesicle preparation in which the volume was 1.1 μl/mg protein.

Materials

Chemicals. Luciferin-firefly tails, ammediol, ADP, ATP (disodium salt), gramicidin, TMPD, methylamine, and DCCD were purchased from Sigma Chemical Co. $TPMP^+$ was obtained from ICN-K&K Laboratories, Inc. [^{14}C]Methylamine (52.2 mCi/mmol), ^{86}RbCl (2 mCi/mmol), and [*Me*-^{3}H]methyltriphenylphosphonium (3.59 Ci/mmol) were purchased from New England Nuclear Corp. Pyruvate kinase, and ADP (potassium salt) were from Boehringer-Mannheim. [^{3}H]TPP^+ (2.5 Ci/mmol) was the generous gift of H.R. Kaback (Roche Institute of Molecular Biology, Nutley, NJ). All other chemicals were obtained commercially at the highest purity available.

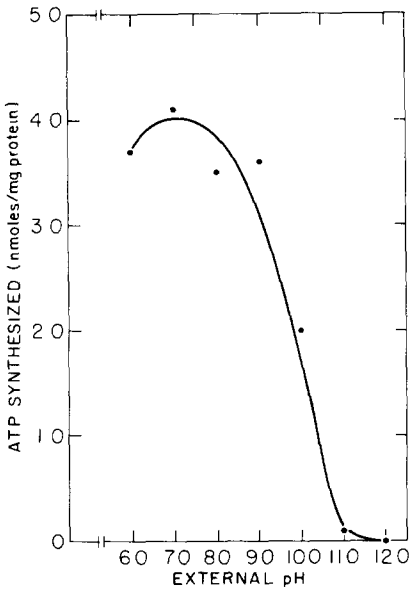
Results

ATP synthesis in right-side-out vesicles

Vesicles that were loaded with ADP and P_i, buffered at pH 9.0 during preparation, synthesized ATP when energized with ascorbate/TMPD over a range of external pH values from 6.0 to 11.0 (Fig. 1). Steady-state synthesis was maximal between pH 6.0 to 9.0. Vesicles prepared with intravesicular pHs from 7.0 to 9.0, when suddenly diluted into pH 9.0 buffer and energized with ascorbate/TMPD, also synthesized ATP. No synthesis occurred using vesicles with an intravesicular pH of 6.0. The time courses for ATP synthesis at external pHs of 9.0 and 10.5 (pH 9.0 inside) are shown in Fig. 2. For this and subsequent experiments in which an external pH of 10.5 was used, the pH was rapidly raised from 9.0 to 10.5 at the beginning of the experiment by the addition of an appropriate base. Parallel measurements of the ΔpH showed that a transmembrane ΔpH of 1.4—1.5 units was thus established, and that this ΔpH persisted for longer than the duration of the ATP synthesis experiments. Similar validations were used for adjustments to other pH values. The amount of ATP synthesis was not affected by the addition of methylamine or $TPMP^+$, or both, at the concentrations used to assay ΔpH or $\Delta\psi$.

As one assessment of the magnitude of possible errors in the measurements of $\Delta\psi$ values, three different probes were used to measure the $\Delta\psi$s generated by energized vesicles. As shown in Table I, determinations on potassium carbonate-loaded vesicles, at pH 9.0 or 10.5 outside, yielded very similar $\Delta\psi$ values, of approx. −120 mV, using either TPP^+ or $TPMP^+$ distribution. In vesicles lacking potassium (choline-Tris) the $\Delta\psi$ values, as measured by the distribution of TPP^+, $TPMP^+$, or Rb^+ in the presence of valinomycin, were within 10 mV of each other.

The $\Delta\bar{\mu}_{H^+}$ of potassium-loaded vesicles energized with ascorbate/TMPD at pH 9.0 was −125 ± 15 mV, and the $\Delta\bar{\mu}_{H^+}$ of vesicles so energized at pH 10.5 was −40 ± 20 mV. The data in Fig. 3 were obtained from a series of experiments in which the steady-state $\Delta\bar{\mu}_{H^+}$ and ATP synthesis were both measured over a range of pH values. Little or no ATP synthesis occurred at a protonmotive force of −20 mV, but at higher $\Delta\bar{\mu}_{H^+}$ values, ATP synthesis increased



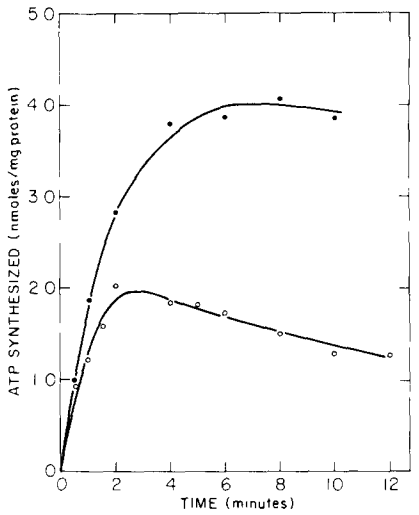


Fig. 1. The effect of external pH on the steady-state level of ATP synthesis. Vesicles prepared in potassium carbonate, pH 9.0, were concentrated and rapidly diluted 20-fold into potassium carbonate buffer, adjusted to indicated pH values, in the presence of 20 mM potassium ascorbate and 2 mM TMPD. The steady-state level of ATP synthesis at each pH was determined from a series of time points.

Fig. 2. The synthesis of ATP as a function of time at an external pH of 9.0 (●) or 10.5 (○). Vesicles were prepared and suspended in potassium carbonate buffer, pH 9.0. The synthesis of ATP was initiated by addition of 20 mM potassium ascorbate and 2 mM TMPD.

with increasing $\Delta\bar{\mu}_{H^+}$. ATP synthesis at pH 9.0 could be demonstrated using vesicles loaded with various concentrations of P_i (Table II). Energization by several natural electron donors was relatively ineffective, while ascorbate/phenazine methosulfate was almost as effective as ascorbate/TMPD (Table II).

TABLE I

MEASUREMENT OF $\Delta\psi$ BY THREE DIFFERENT PROBES

Vesicles were prepared in potassium carbonate buffer, pH 9.0, and loaded with 5 mM ADP, 10 mM phosphate, as indicated in Methods. The $\Delta\psi$ generated upon addition of 20 mM ascorbate (potassium or Tris salt) plus 2 mM TMPD, at an external pH of 9.0 or 10.5, was calculated from the steady-state distribution of 10 μM TPP^+, 12.5 μM $TPMP^+$ or 50 μM $^{86}RbCl$ plus 10 μM valinomycin. Each figure is the average of at least five separate experiments performed on different preparations of vesicles. The values represent the mean ± standard deviation.

External pH	$\Delta\psi$ (mV)				
	Potassium carbonate buffer		Choline-Tris buffer		
	TPP^+	$TPMP^+$	TPP^+	$TPMP^+$	RbCl
9.0	−125 ± 15	−119 ± 20	−78 ± 8	−74 ± 10	−81 ± 12
10.5	−109 ± 15	−107 ± 8	—	—	—

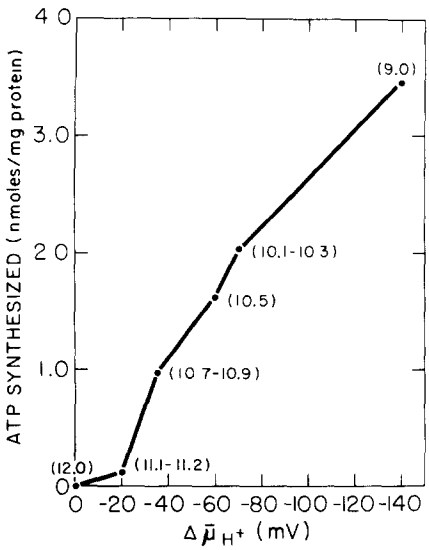


Fig. 3. The steady-state level of ATP synthesis as a function of the protonmotive force. Vesicles were prepared in potassium carbonate, pH 9.0, and loaded with potassium ADP and P_i. Synthesis of ATP was initiated by rapid dilution of vesicles into a reaction mixture containing 20 mM potassium ascorbate and 2 mM TMPD, adjusted to various pH values. The final pH was measured and is indicated in the parentheses. The steady-state level of ATP synthesis was determined at each pH from a series of time points. The $\Delta\bar{\mu}_{H^+}$ was determined by the distribution of $TPMP^+$ ($\Delta\psi$) and methylamine (ΔpH) as described in Methods.

Synthesis of ATP was inhibited by arsenate and by DCCD at both pH 9.0 and 10.5 (Table III). Inhibition by DCCD was particularly marked when the inhibitor was included in the shock medium during vesicle preparation. Gramicidin and KCN also inhibited ATP synthesis strongly. DCCD had no effect on the $\Delta\psi$, while gramicidin completely abolished the $\Delta\psi$ (data not shown).

TABLE II

EFFECT OF ELECTRON DONOR AND P_i CONCENTRATION ON ATP SYNTHESIS

Vesicles were prepared in potassium carbonate buffer, pH 9.0, and loaded with 5 mM potassium ADP and P_i, as indicated. ATP synthesis was energized by addition of potassium ascorbate (20 mM)/TMPD(2 mM), potassium ascorbate (20 mM)/phenazine methosulfate (0.1 mM), or other electron donors at 10 mM.

Electron donor	[P_i] mM	ATP synthesized (nmol/mg protein)	
		One minute	Steady state *
Ascorbate/TMPD	10	2.02	3.50
Ascorbate/TMPD	5	—	2.28
Ascorbate/TMPD	2	—	1.20
Ascorbate/TMPD	1	—	0.88
Ascorbate/phenazine methosulfate	10	1.72	—
D-Lactate	10	0.32	—
L-Malate	10	0.08	—
Succinate	10	0.10	—
NADH	10	0.02	—

* Steady-state values, expressed as nmol ATP synthesized/mg vesicle protein, were determined from time course experiments of the kind shown in Fig. 2.

TABLE III

THE EFFECT OF INHIBITORS ON ATP SYNTHESIS

Vesicles were prepared in potassium carbonate buffer, pH 9.0, and loaded with 5 mM potassium ADP and 10 mM P_i. ATP synthesis, energized by ascorbate/TMPD, was assayed at external pH values of 9.0 and 10.5, in the presence of the indicated inhibitors. Vesicles were preincubated with the inhibitors for 5 min, except for DCCD, which was either included in the shock medium during vesicle preparation or preincubated with vesicles for 30 min.

Inhibitor	Concentration of inhibitor	ATP synthesized (nmol/min/mg protein)	
		pH_{out} 9.0	pH_{out} 10.5
None, —electron donor	—	0.17	0.16
None	—	2.16	1.87
Arsenate	10 mM	0.89	0.79
DCCD	100 μM	0.65	0.73
DCCD (shocked into vesicle)	100 μM	0.16	0.30
Gramicidin	10 μM	0.19	0.11
KCN	1 mM	0.15	0.15
NaN_3	5 mM	0.93	0.80

In view of the substantial ATP synthesis at low $\Delta\bar{\mu}_{H^+}$, a more detailed analysis of the synthesis at external pH values of 9.0 and 10.5 was undertaken. At steady-state, ADP and P_i-loaded vesicles whose internal pH was buffered to 9.0 exhibited approximately twice as much ATP synthesis at an external pH of 9.0 than at an external pH of 10.5 (Table IV). The phosphate potentials calculated from these experimental data differed by only 1 kcal/mol for the two pH values. Most notably, the theoretical mV equivalents of the ΔG_p values were considerably greater than the observed $\Delta\bar{\mu}_{H^+}$. The discrepancy at an external pH of 10.5 was particularly striking; the theoretical $\Delta\bar{\mu}_{H^+}$ (assuming an H^+/ATP ratio of 2) required for synthesis was in excess of —200 mV, while the force actually measured was —40 ± 20 mV.

TABLE IV

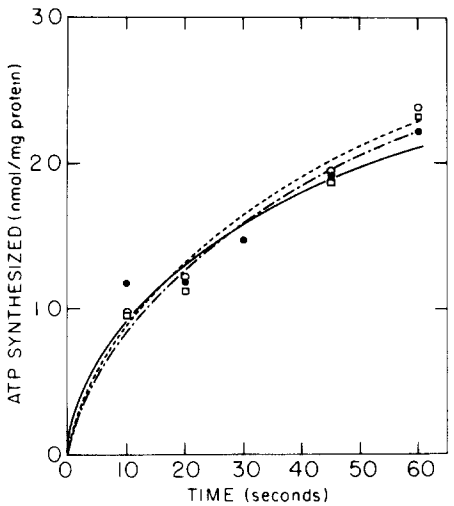
THE PHOSPHATE POTENTIAL (ΔG_p) AND PROTONMOTIVE FORCE FOR ATP SYNTHESIS AT EXTERNAL pH VALUES OF 9.0 AND 10.5

Vesicles were prepared and loaded with 5 mM potassium ADP, 10 mM $MgSO_4$, and 10 mM P_i in potassium carbonate buffer, pH 9.0, as described in Methods. Steady-state ATP synthesis was assayed at an external pH of 9.0 or 10.5. At pH 9.0, time points of 2—4 min were used, and at pH 10.5, time points of 7—10 min were used. The values for [ATP] and [ADP] are the average of seven separate determinations. Theoretical values of ΔG_p and the protonmotive force needed for ATP synthesis (for a H^+/ATP ratio of 2) were calculated from the formula: $\Delta G_p = \Delta G^0 + RT \ln [ATP]/[ADP][P_i]$ by inserting the values for [ATP] and [ADP] shown. The concentration of P_i was 10 mM, as confirmed by assay of the vesicle extract. The actual $\Delta\bar{\mu}_{H^+}$ was determined from steady-state measurement of $TPMP^+$ accumulation ($\Delta\psi$) and methylamine accumulation (ΔpH) as described in Methods.

External pH	ATP (mM)	ADP (mM)	ΔG_p		Actual $\Delta\bar{\mu}_{H^+}$ (mV)	$\Delta\psi$ (mV)	ΔpH (mV)
			kcal/mol	mV equiv.			
9.0	3.2	1.3	12	—252	—125 ± 15	—125 ± 15	0
10.5	1.6	2.9	11	—231	—40 ± 20	—122 ± 15	+82 ± 5

We have previously shown that the transport of at least one substrate is energized by the $\Delta\psi$ plus a $\Delta\bar{\mu}_{Na^+}$ in *B. alcalophilus* [5]. It was therefore of particular interest to examine the effect of Na^+ on ATP synthesis. No stimulation of ATP synthesis by added Na^+ or K^+ could be demonstrated, as shown for NaCl and KCl in Fig. 4. Other sodium and potassium salts, including NaSCN and KSCN, were tested, both with and without ascorbate/TMPD. No stimulation of ATP synthesis was observed. Vesicles prepared in choline-Tris buffer, pH 9.0, rather than ammediol buffer, also showed no difference in the initial rate or steady-state synthesis of ATP upon the addition of NaCl or KCl (data not shown).

If the $\Delta\bar{\mu}_{H^+}$, albeit low, energized ATP synthesis in *B. alcalophilus*, then proton translocation should be demonstrable concomitant with ATP synthesis. A series of experiments was conducted in which proton movements were monitored in suspensions of ADP and P_i-loaded vesicles that were energized with ascorbate/TMPD. Right-side-out vesicles containing ADP and P_i in choline-Tris buffer showed essentially no acidification of the external milieu upon energization, whereas parallel DCCD-treated vesicles exhibited a small, reproducible acidification (Fig. 5A). Energization of vesicles in potassium-containing buffer



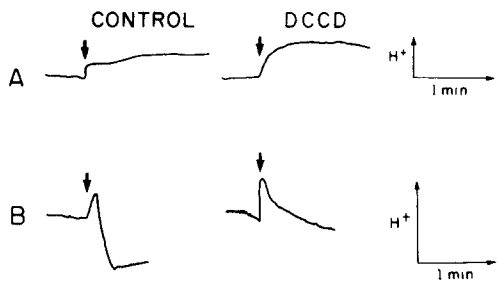


Fig. 4. The effect of added K^+ or Na^+ on ATP synthesis. Vesicles were prepared and suspended in 100 mM ammediol and 10 mM $MgSO_4$, pH 9.0. Synthesis of ATP was initiated by the addition of 20 mM ascorbic acid (dissolved in ammediol buffer so as not to lower the pH of the reaction mixture) and 2 mM TMPD. An additional 10 mM choline chloride was added to control vesicles (●), and either 10 mM NaCl (○) or 10 mM KCl (□) was added to other preparations at zero time.

Fig. 5. Proton translocation by energized, ADP and P_i-loaded vesicles. Membrane vesicles were prepared from protoplasts lysed in 1 mM Tris, 10 mM $MgSO_4$, 5 mM Tris ADP, 10 mM Tris phosphate, with the addition of 90 mM of either choline chloride (A) or KCl (B). The pH of the preparation buffer was adjusted to 8.5 with choline hydroxide or KOH, respectively. In each experiment, half the vesicles were prepared in the presence of 100 μM DCCD. The external pH was adjusted to 8.5 within one min prior to the addition of ascorbate (10 mM) and TMPD (1 mM), which occurred at the points indicated by the arrows. Although the pH of the ascorbate/TMPD was adjusted to pH 8.5, an initial acidification of 0.003 pH units upon energization was due to a slight difference between the pH values of the solutions. The arrow indicating H^+ points toward acidification of the external milieu, as monitored by a pH electrode. The length of the arrow indicates a change in pH of 0.01 units.

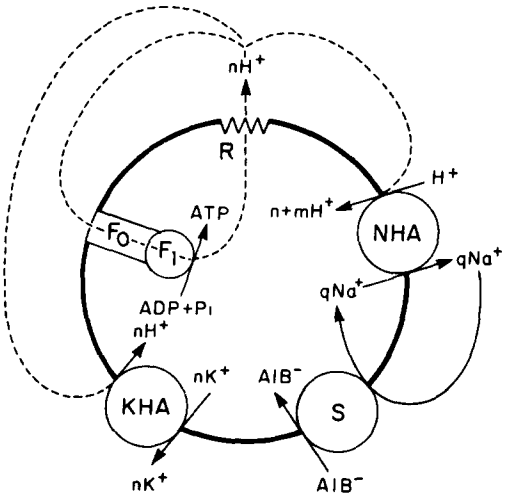


Fig. 6. A summary of ion and proton movements in *Bacillus alcalophilus*. The sodium/proton antiporter is represented by NHA and the potassium/proton antiporter by KHA. The symporter for α-aminoisobutyrate (AIB) and Na^+ is represented by S. R designates the respiratory chain, and F_0F_1 is the membrane-bound ATPase. The values of n, m and q are unknown, but since the NHA is energized by the $\Delta\psi$ [6], q is probably less than $n + m$.

caused an alkalinization of the external medium (Fig. 5B). Parallel vesicles that also contained DCCD exhibited a slight initial acidification followed by a return to the initial pH, without alkalinization. Another experimental approach was then employed to demonstrate proton movements during ATP synthesis. As shown elsewhere [6], potassium carbonate vesicles at pH 9.0 demonstrated no enhancement of quinacrine fluorescence upon energization with ascorbate/TMPD. The absence of a ΔpH presumably reflects the presence of the potassium-proton antiporter [6]. When such vesicles were loaded with ADP and P_i, energization with ascorbate/TMPD exhibited marked quenching of quinacrine fluorescence (21%); whereas, the same vesicles, treated with 100 μM DCCD, exhibited little or no quenching.

Discussion

In Fig. 6, a summary of the ion movements studied and proposed thus far in *B. alcalophilus* is presented. These include primary proton extrusion via respiration (R), followed by electrogenic inward proton translocation concomitant with ATP synthesis by an F_0-F_1 ATPase, or electroneutral exchange of the extruded protons for potassium ions (K^+/H^+ antiporter). The Na^+/H^+ antiporter is shown to acidify the intravesicular space [6]. The circulation of Na^+ is completed, at least in part, by solute transport systems such as that for aminoisobutyrate [5] which occur in symport with Na^+.

Although the participation of low concentrations of Na^+ or some other cation cannot be excluded, the involvement of a proton-translocating ATPase in ATP synthesis is indicated by the following: (a) DCCD inhibited ATP synthesis, especially when the inhibitor was shocked into the intravesicular space;

(b) added Na^+ and K^+ failed to stimulate ATP synthesis; and (c) a DCCD-sensitive inward translocation of protons could be demonstrated in ADP and P_i-loaded vesicles using fluorescence methods and direct pH monitoring. The difference in proton translocation patterns between choline-Tris vesicles (Fig. 5A) and K^+-containing vesicles (Fig. 5B) was consistent with the activity of the K^+/H^+ antiporter in the latter preparation [6]. This activity, in the absence of ATPase-mediated proton translocation (i.e., in the presence of DCCD), exchanged potassium ions for protons to abolish the gradient of protons produced upon energization. In the choline-Tris vesicles neither the Na^+/H^+ nor K^+/H^+ antiporter is operative [6]. Such vesicles, therefore, sustained a small ΔpH, outside acid, when DCCD was present. It should be noted that since cation/solute symport systems in *B. alcalophilus* appear to be coupled to Na^+ [21] rather than protons, vesicles in choline-Tris buffer would have fewer proton entry systems than most bacteria to interfere with the measurement of proton flux through the ATPase.

Recently, Koyama et al. [22] reported that the ATPase from a different alkalophilic *Bacillus* resembles those from other bacteria with respect to the types of F_1 subunits found. Various properties of the *B. alcalophilus* vesicle system, in addition to the translocation of protons, resemble those of ATP synthesis in other bacterial vesicles. The amount and time course of ATP synthesis by right-side-out vesicles of *B. alcalophilus* were quite similar to those observed by Tsuchiya [10] using analogous preparations from *Escherichia coli*.

However, the initial dilemma is unresolved, and even underscored, by the findings reported here. ATP synthesis by vesicles of *B. alcalophilus* occurred at pH values at which the vesicles, like the parent cells, exhibited very low $\Delta\bar{\mu}_{H^+}$. At least some relationship of ATP synthesis to these small $\Delta\bar{\mu}_{H^+}$ values is suggested by the data in Fig. 3, and by the lack of synthesis under conditions in which the $\Delta\bar{\mu}_{H^+}$ is completely abolished by inhibitors.

How can a proton-translocating ATPase synthesize ATP at a low $\Delta\bar{\mu}_{H^+}$? In view of the extensive validation of the methods by ourselves and others [23, 24] it is unlikely that 4-fold errors in the $\Delta\bar{\mu}_{H^+}$ measurements at pH 10.5, for example, account for the data obtained. Moreover, there are other examples in prokaryotes as well as eukaryotes in which the ΔG_p does not correlate with the $\Delta\bar{\mu}_{H^+}$. The work of Maloney [25] with *Streptococcus lactis* and Wilson et al. [26] with *E. coli* has indicated a 'gating' mechanism for ATP synthesis driven by an artificially-imposed pH drop or potassium diffusion potential. In those species the 'gate' appeared to be close to −200 mV, whereas Van der Drift et al. [27] found the 'gate' to open at approx. −145 mV in a different strain of *Streptococcus*. By contrast, Decker and Lang [28] have reported L-malate-dependent ATP synthesis by whole cells of an uncoupler-resistant mutant of *Bacillus megaterium* at extraordinarly low $\Delta\bar{\mu}_{H^+}$ values. Another bacterium, *Paracoccus denitrificans* exhibits ATP synthesis at $\Delta\bar{\mu}_{H^+}$ values in the same low range that we have found in *B. alcalophilus* [29,30]. Moreover, several investigators have found a discrepancy between the magnitude of the $\Delta\bar{\mu}_{H^+}$ and the ΔGp in mitochondrial preparations [31] and chloroplasts [32].

In view of these findings, as well as certain kinetic experiments by others, there have been suggestions that bulk transmembrane gradients are not the relevant ones. The involvement of intramembranal gradients [33] or localized

gradients [31,34—36] would make the observed discrepancies between the ΔGp and the $\Delta\bar{\mu}_{H^+}$ values for bulk transmembrane gradients irrelevant. Alternatively, or in addition, some ATPases may utilize especially high H^+/ATP ratios such that a small protonmotive force could account for the synthesis observed. A high stoichiometry of this kind has been suggested for *P. denitrificans* [29,30]. In *B. alcalophilus* vesicles a stoichiometry of $4H^+$/ATP at pH 9.0 and of $8H^+$/ATP at pH 10.5 would be required. Such stoichiometries would raise interesting questions with respect to proton pumping and/or the efficiency of proton utilization in *B. alcalophilus*. This organism has recently been shown to have an extraordinarily high cytochrome content (especially *b*- and *c*-type cytochromes) without a correspondingly high rate of oxygen consumption [37]. Perhaps the respiratory chain of *B. alcalophilus* is especially adapted for efficient energy transduction. We are continuing our studies of ATP synthesis in *B. alcalophilus* with the goal of further elucidating the basis for and properties of ATP synthesis at apparently low driving forces.

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