Stoichiometry of the H^+-ATPase of *Escherichia coli* cells during anaerobic growth

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The H^+/ATP stoichiometry of the H^+-ATPase was investigated in *Escherichia coli* cells growing under anaerobic conditions at pH 6 and 7. The protonmotive force was determined from the intracellular accumulation of benzoate and tetraphenylphosphonium ions, as well as the accumulation of lactose in this *lac* operon inducible, but β-galactosidase negative strain. The phosphorylation potential was calculated from the cellular concentrations of ATP, ADP and inorganic phosphate. By comparing the phosphorylation potential and the proton motive force under these steady state conditions, the H^+/ATP stoichiometry was determined to be 3, similar to the value previously found in the same cells growing under aerobic conditions.

H^+-ATPase *Proton motive force* Escherichia coli *Phosphorylation potential* *Anaerobiosis* *Stoichiometry*

1. INTRODUCTION

The flux of H^+ through the proton channel within the H^+-translocating ATPase of bacterial cells occurs in the inward direction under respiratory conditions, and is coupled to the phosphorylation of ADP to ATP (reviewed in [1–3]). During anaerobic metabolism, on the other hand, the H^+-ATPase catalyzes the hydrolysis of glycolytically-generated ATP and the extrusion of H^+. Thus, this enzyme mediates a thermodynamic equilibrium between the protonmotive force and the phosphorylation potential, according to the chemiosmotic theory [4–6]. The $\Delta G_p'$ and the Δp are related by the expression $\Delta G_p' = n\Delta pF$, where $n = H^+/ATP$ and F = Faraday's constant. Thus, the H^+/ATP stoichiometry can be determined by measuring the two parameters under steady state conditions [7]. In a previous publication [8] the H^+/ATP stoichiometry of mid-exponential phase *Escherichia coli* cells during aerobic growth was determined to be 3. In the study reported here the Δp and the $\Delta G_p'$ were measured in the same strain of *E. coli* cells growing strictly anaerobically in order to calculate the H^+/ATP stoichiometry under conditions of ATP hydrolysis and H^+ extrusion.

Abbreviations: EDTA, ethylenediaminetetraacetic acid; TPP^+, tetraphenylphosphonium ion; Tris, tris(hydroxymethyl)aminomethane; XDP, xanthosine diphosphate; PMF or Δp, protonmotive force = $\Delta\Psi - Z\,\Delta pH = \Delta\tilde{\mu}_{H^+}/F$; $\Delta\Psi$, transmembrane electrical gradient; Z = 2.303 RT/F = 59 mV at 25°C; ΔpH, transmembrane chemical gradient of H^+ = pH_{out} (pH of the bulk medium)–pH_{in} (pH of the cytosol); Δp_{lac}, $\Delta\mu_{lac}/F$; $\Delta G_p'$, phosphorylation potential = Gibbs free energy of ATP synthesis = $\Delta G_0' + RT \ln[ATP]/[ADP][P_i]$; $\Delta G_0'$, standard free energy of ATP hydrolysis

2. MATERIALS AND METHODS

The measurements of the PMF and $\Delta G_p'$ were carried out as in [8], except that cell growth, addition of various radioactive probes and separation of cells from the media were carried out under strictly anaerobic conditions. *E. coli* ML 308-225 (*lac* i^- z^- y^+ a^+) cells, a gift of T.H. Wilson, were grown at 28°C within an anaerobic glove box (For-

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ma Scientific, Marietta OH) under an atmosphere of 5% hydrogen, 5% carbon dioxide and 90% nitrogen. Cells grown overnight in medium AA with 1% glucose [8] were inoculated into 30 ml of the same medium in 250 ml Ehrlenmeyer flasks and mixed with a magnetic stirrer. The cultures were allowed to reach mid-exponential phase, having undergone at least 2 doublings. The pH of the medium was adjusted as needed by adding deoxygenated solutions of HCl or Tris base. When the cultures had reached an absorbance of 625 nm = 1.0, radioactive probes for ΔpH ([^{14}C]benzoate), $\Delta\Psi$ ([^{3}H]TPP^{+}) and Δp_{lac} ([^{14}C]lactose) were added to the growing cultures. Just as with *E. coli* growing aerobically [8], it was necessary to treat anaerobically-growing cells with EDTA for maximal uptake of [^{3}H]TPP^{+} for the $\Delta\Psi$ measurements. In a previous publication anaerobic *E. coli* and *Klebsiella pneumoniae* cells had been reported to have no $\Delta\Psi$ [9]. In those experiments the uptake of [^{3}H]TPP^{+} was low because too little EDTA had been added. Although 5 mM EDTA rendered gentamicin more accessible to treated cells than to untreated controls, showing that the chelator had 'loosened' the cell envelope, it had no effect on TPP^{+} uptake, and hence was not used routinely. However, in the present experiments, when more EDTA was used, cellular [^{3}H]TPP^{+} uptake increased and reached a constant level over a range of EDTA concentrations, which depended on the pH. For pH 6.0 cultures, 15–25 mM EDTA was used, and for pH 7.0 cultures, 10–25 mM. Addition of 30 mM EDTA stopped growth and resulted in reduced [^{3}H]TPP^{+} accumulation. Only cultures still growing after EDTA addition are considered here. EDTA treatment had no effect on the ΔpH and Δp_{lac}-values. After further growth for 15 min the cells were separated from the medium by centrifugation through silicone oil [10] using a microfuge placed inside the glove box. The centrifuge tubes then were taken out of the glove box, samples of supernatant fluid removed for counting of radioactivity and the pellets cut into scintillation vials. Measurements of adenine nucleotide concentrations were also carried out as in [8], except that the cultures were removed from the glove box just before addition of the ice-cold trichloroacetic acid–XDP solution. Similar extracts, but without XDP, were used for ATP measurements by the luciferin-luciferase method using a photometer and reagents bought from Turner Designs (Mountain View CA). The cellular inorganic phosphate concentrations were measured in cells pelleted within the box, but extracted with trichloroacetic acid outside it.

3. RESULTS AND DISCUSSION

The protonmotive force in mid-exponential phase *E. coli* cells growing in batch culture under strictly anaerobic conditions at pH 6–7 was found to be about 150 mV (fig.1 and table 1). The ΔpH component was equivalent to 53 mV when the cells were growing at pH 7. Thus, the pH_{in} varied from 6.9–7.5 under these conditions. The $\Delta\Psi$ ranged from 93 mV (negative inside) at pH 6 to 122 mV at pH 7. Thus, the Δp calculated from the $\Delta\Psi$ and ΔpH-values was 146–152 mV at pH 6–7. Since the ML308-225 strain of *E. coli* is constitutive for the *lac* operon, but lacks β-galactosidase, the accumulation of [^{14}C]lactose was used as the other method to estimate Δp. The Δp_{lac}-values were about 131 mV at pH 6 and 140 mV at pH 7, about 15 mV lower than the Δp-values calculated from the $\Delta\Psi$ and ΔpH measurements. In contrast, during aerobic growth [8] the Δp-values are higher. For example, in aerobic cells growing at pH 6.25, the 59 ΔpH, $\Delta\Psi$, Δp and Δp_{lac}-values were 99 mV, 94 mV, 193 mV and 175 mV, respectively; in cells growing at pH 7.25 these values were 35 mV, 125 mV, 159 mV and 169 mV, respectively. It may

Table 1

Stoichiometry of the H^{+}-ATPase of mid-exponential phase, anaerobically growing *E. coli*

		External pH	
		6.0	7.0
ATP/ADP		3.1	3.7
$[P_i]$	(mM)	36	44
$\Delta G'_p$	(mV)	427	426
Δp	(mV)	146	152
Δp_{lac}	(mV)	131	140
H^{+}/ATP	(from Δp)	2.9	2.8
H^{+}/ATP	(from Δp_{lac})	3.2	3.0

The experiments were carried out as described in section 2. The standard deviations of the means were < 4 mM for P_i. The Δp and Δp_{lac}-values are from the figure

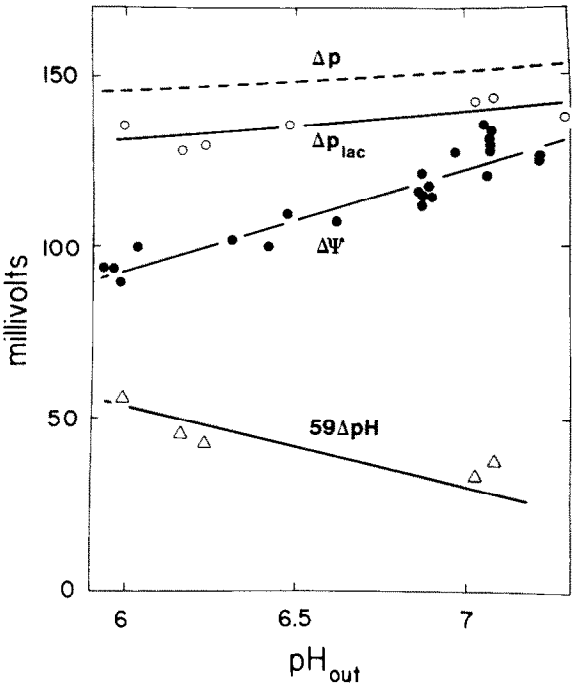

Fig.1. Effect of medium pH on ΔpH, $\Delta\Psi$ and Δp_{lac} of mid-exponential phase *E. coli* ML308-225 growing under anaerobic conditions. The experiment was carried out as described in section 2. Each value is the mean of triplicate determinations from separate cultures. The standard deviations were < 7 mV for 59 ΔpH, < 6 mV for $\Delta\Psi$, and < 1.3 mV for Δp_{lac}. The dashed Δp line was calculated from the regression lines of the $\Delta\Psi$ and 59 ΔpH points.

be noted that Δp and Δp_{lac}-values are in closer agreement in anaerobic *E. coli* than in aerobic ones [8]. The discrepancies found in the aerobic cells were attributed to Δp-dependent non-specific bindings of [^{3}H]TPP$^+$ to the cells, resulting in inaccurate $\Delta\Psi$ measurements. Such binding would be expected to be lower when the Δp is lower, giving less inaccurate $\Delta\Psi$ estimates in anaerobic cells than in respiring ones.

The difference in the poise of the proton gradient measured in anaerobically and aerobically growing cells should be reflected in a difference in the phosphorylation potential. According to the Mitchell theory, a thermodynamic equilibrium exists between these two parameters. Therefore, the $\Delta G_p'$ was estimated by measuring the cellular levels of ATP, ADP and inorganic phosphate. The ATP concentrations in the anaerobically growing cells were found to be 1.6 mM and 1.9 mM (SD = 0.5 and 0.4, n = 29 and 36) at pH 6 and 7, respectively, as determined by high pressure liquid chromatography and by the luciferin-luciferase assay. The ATP/ADP ratios were 3.1 and 3.7 at pH 6 and 7. These values are significantly different from those measured in the same cells growing under aerobic conditions [8], where ATP was present at 3.9–3.3 mM at pH 6.25–7.25, and the ATP/ADP ratios were 9.75–8.25, respectively. The ATP concentrations of anaerobic *E. coli* are lower than those found under aerobic conditions in a variety of other bacteria [11], but similar to those of *Klebsiella aerogenes* growing in continuous culture under 2.4% oxygen or less [12]. Similarly, anaerobic *E. coli* contained 36–44 mM P_i at pH 6–7, about 5–7 times more than aerobic cells [8]. Thus, the $\Delta G_p'$ of anaerobic cells was 427 mV, significantly less than the 500 mV found in aerobic cultures [8]. The difference in the $\Delta G_p'$ measured in anaerobically and aerobically growing cells suggests that there is a difference in the displacement from equilibrium of the ATPase reaction under the two steady state conditions.

The H^+/ATP stoichiometry of anaerobic cells at pH 6 or pH 7 was calculated to be 2.8–2.9 when Δp-values from $\Delta\Psi$ and ΔpH measurements were used. When Δp_{lac}-values were used, a slightly higher stoichiometry of 3.0–3.2 H^+/ATP was obtained. Considering the degree of reproducibility of the various measurements, these H^+/ATP-values are not significantly different, and may be considered to be 3. A 3 H^+/ATP stoichiometry has been reported for glycolyzing cells of the anaerobe *Streptococcus lactis* [13].

Since it is likely that the H^+/ATP stoichiometry is unchanged by the switch from aerobic to anaerobic metabolism, one can conclude from these data that under glycolytic conditions the Δp and the $\Delta G_p'$ are in thermodynamic equilibrium in cells growing anaerobically. Put another way, the H^+-ATPase functions to equilibrate the Δp and the $\Delta G_p'$ whether the H^+ flux is in the inward or outward direction.

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