Volume 232, number 2, 359–363 FEB 05881 May 1988

Chemiosmotic H^+ cycling across the plasma membrane of the thermoacidophilic archaebacterium *Sulfolobus acidocaldarius*

Ralf Moll and Günter Schäfer

Institute of Biochemistry, Medical University of Lübeck, Lübeck, FRG

Received 17 March 1988

Transitory H^+ ejection from *Sulfolobus acidocaldarius* cells induced by oxygen pulses, and anaerobic H^+ backflow were investigated. Aerobic proton extrusion is inhibited by protonophores, by nigericin and by inhibitors of respiratory electron transport; it is stimulated by DCCD. In contrast, DCCD inhibits the rate of anaerobic H^+ backflow. Aerobic proton extrusion is significantly enhanced by K^+/valinomycin. Apparent H^+/O ratios of 2.5–3 are measured. Proton extrusion generates large pH gradients (3–4 units) representing the major contribution to the total proton motive force across the plasma membrane of this thermoacidophilic archaebacterium.

Acidophile; Proton-motive force; H^+/O ratio; (Archaebacteria)

1. INTRODUCTION

The thermoacidophilic archaebacterium *Sulfolobus acidocaldarius* grows optimally at temperatures from 70 to 80°C and pH values of 2–3 under aerobic conditions. Previous studies by Anemüller et al. [1] have shown that the ATP content of *Sulfolobus* cells strictly depends on respiration. The presence of *a*- and *b*-type cytochromes and of NADH-dehydrogenase activity in the membrane was demonstrated [1,2]. In addition, an F_1-analogous ATPase could be purified and characterized by Lübben et al. [3,4]. However, despite our previous finding [1], that a significant acidification accompanies onset of respiration, it remained to be proven whether or not *Sulfolobus* generates ATP via a 'classical' chemiosmotic mechanism using protons as coupling ions. The present study presents evidence for proton cycling across the membrane as a prerequisite for generation of a proton motive force sufficient to drive ATP synthesis. The sensitivity of the proton fluxes towards inhibitors, ionophores and DCCD is demonstrated and minimum estimates for H^+/O ratios are given.

Correspondence address: G. Schäfer, Institut für Biochemie, Medizinische Universität zu Lübeck, Ratzeburger Allee 160, 2400 Lübeck, FRG

Abbreviations: DCCD, dicyclohexylcarbodiimide; SF 6847, 3,5-di(*tert*-butyl)-4-hydroxybenzylidenemalononitrile; TTFB, 4,5,6,7-tetrachloro-2′-trifluoromethylbenzimidazole

2. MATERIALS AND METHODS

S. acidocaldarius (DSM 639) was grown as described earlier [1] with the exception that cells were harvested after 18–24 h. Washings and storage of cells was performed in a buffer containing 9.8 mM ammonium sulfate, 2 mM potassium phosphate, pH 6.5 (buffer I). The final suspension was kept at 4°C containing 50–60 mg protein/ml. Incubations were carried out in buffer I supplemented with potassium sulfate as indicated with the individual experiments. Protein concentrations were determined as described by Watters [5].

For pH and oxygen recordings a specially designed multiport glass cuvette (3.3 ml) with a water jacket was used, accommodating a micro-pH electrode (Ingold LoT-275-M3-NS-6158), a Clark-type oxygen electrode and an injection/sampling channel. The reaction chamber was connected via a buffer bridge to a thermostated Ag/AgCl reference electrode. pH changes were recorded using a Knick-645 pH meter equipped with a compensating voltage device and a differential amplifier. After each experiment the buffer capacity of the respective incubation mixture was determined by calibration with aliquots of 25 mM HCl. All experiments were carried out at 60°C starting with a nitrogen gassed anaerobic cell suspension of 1–2 mg/ml final

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

protein concentration, pH 5.8–6.0. Oxygen pulses were generated by addition of small aliquots of H_2O_2 to the catalase supplemented reaction mixture. Protonophores, ionophores or inhibitors were added immediately prior to or after the oxygen pulse, except for DCCD where preincubation over 10 min was necessary to produce optimum effects. Membrane potential and pH across the cytoplasmic membrane was determined essentially as described by Michels and Bakker [6] using ^3H_2O for the measurement of total and internal water space; $KS^{14}CN$ (200 nCi/ml; 3.45 μM) and [^{14}C]benzoic acid (200 nCi/ml; 20 μM) were applied to calculate $\Delta\psi$ and ΔpH, respectively, from probe distribution. These experiments were carried out under aerobic conditions in an open reaction vessel equipped with a micro-pH electrode. The medium contained buffer I supplemented with 5 mM potassium sulfate and 10 mM glucose. For sampling, aliquots were centrifuged through 100 μl silicon oil (D = 1.026) and supernatant and pellet separated for counting; pellets were dissolved in 0.5 ml of 5% SDS.

All chemicals were purchased from Merck, Boehringer or Serva; radiochemicals from NEN or Amersham Buchler. The uncoupler TTFB was a kind gift from Professor E.P. Bakker, Osnabrück.

3. RESULTS

Anaerobic *S. acidocaldarius* cells exposed to an oxygen pulse respond by an immediate extrusion of protons, which after exhaustion of oxygen backflow into the cells with first order kinetics (fig.1). The energy of activation of the anaerobic backflow is 71.5 kJ/mol. The initial rate and amplitude of proton ejection are significantly diminished by protonophores. In line with a slight stimulation of respiration and a decrease of cellular ATP [1] this suggests the action of effective proton pumps. Fig.2 shows the effect of the uncoupler SF 6847. A residual proton production even at optimal uncoupler concentrations may be due to metabolic acidification.

Carbon monoxide, a classical inhibitor of terminal oxidases as well as azide inhibit respiration and proton ejection in straight linear correlation (fig.3). From the slope of the regression line a H^+/O ratio of 2.5 is calculated. The H^+/O values obtained obviously depend on counterfluxes of potassium ions as depicted in fig.4. In the presence

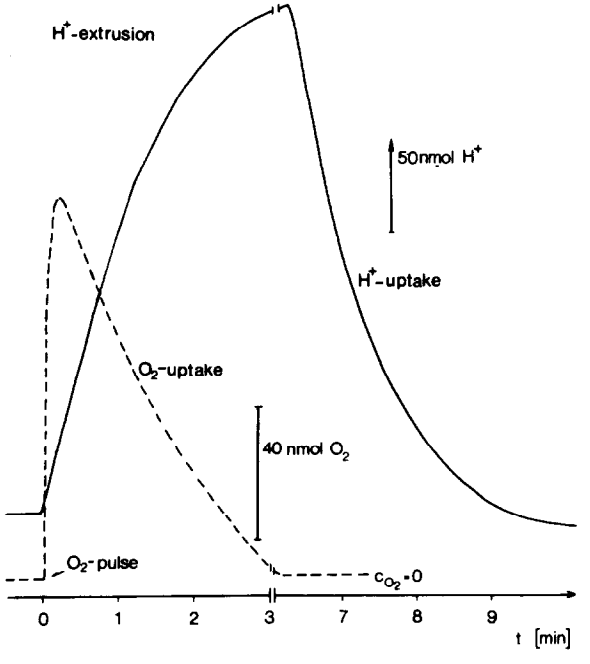

Fig.1. Oxygen induced proton extrusion and anaerobic proton backflux. *Sulfolobus* cells (1–2 mg/ml) were incubated at T = 60°C in N_2-gassed buffer I with 5 mM K_2SO_4 at an initial pH of 5.8. The oxygen pulse was generated by the addition of 100 μg bovine liver catalase and 33 μg hydrogen peroxide (485 nmol O_2).

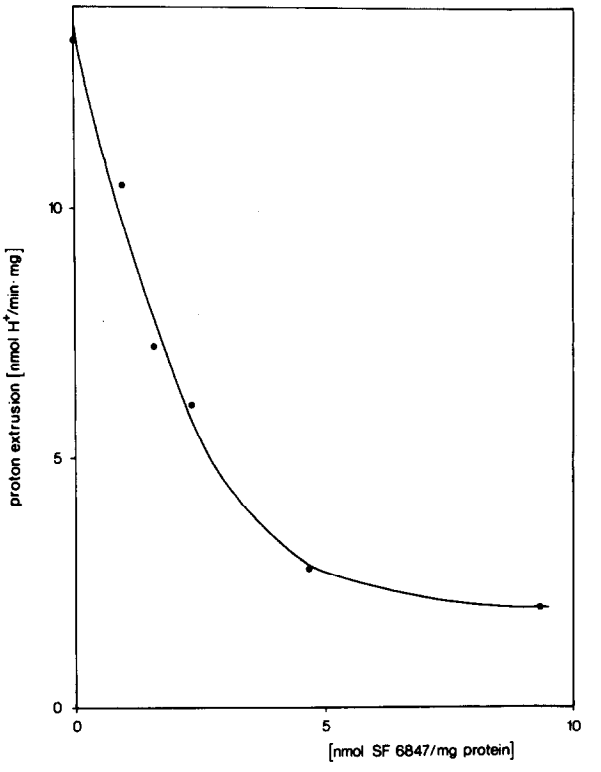

Fig.2. Sensitivity of proton extrusion to the uncoupler SF 6847. Conditions as indicated in fig.1. The protonophore was added in an ethanolic solution immediately after the oxygen pulse, and initial rates of proton extrusion were determined.

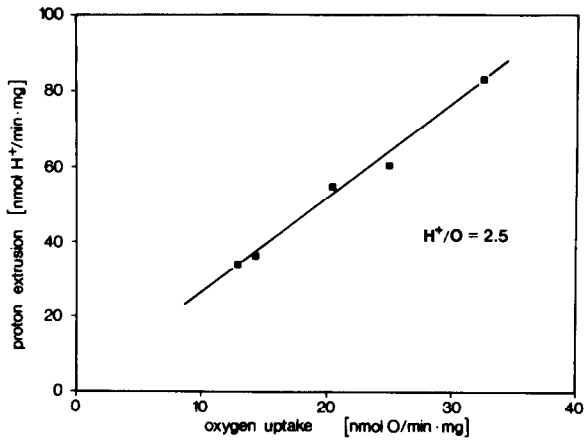

Fig.3. Correlation between initial rates of H^+ extrusion and O_2 uptake. Cells (1.1 mg/ml) were incubated at $T = 60°C$ under N_2 in a medium composed of buffer I, 75 mM K_2SO_4, 15 nmol valinomycin/mg protein, initial pH = 5.8. Immediately on addition of oxygen (121 nmol O_2), generated by H_2O_2/catalase, sodium azide was added as an inhibitor of respiration in amounts from 0 to 280 nmol/mg protein. The slope of the regression line yields directly the H^+/O ratio.

of valinomycin, values around 3 can be measured at optimum K^+ concentrations (>70 mM). In fact, at least a 2-fold increase of rate and amplitude of proton ejection is produced by valinomycin, while nigericin reduces proton extrusion to about one half (not shown). As determined in independent experiments, nigericin depletes cells significantly from potassium and like protonophores decreases ATP content. Its effect may be limited by the steady-state internal K^+ concentration of 24–40 mM [7].

In mitochondria or *E. coli* cells proton backflow competent for ATP synthesis occurs through the DCCD sensitive proton channel F_0. Thus, the inhibition of anaerobic proton flux by DCCD is usually taken as indicative for a pathway via an F_0F_1-ATP synthase. Actually, fig.5 shows that not only anaerobic backflow of protons into *Sulfolobus* cells is inhibited by DCCD (a), but also that after preincubation with DCCD the oxygen induced proton extrusion is drastically increased up to concentrations of 80 nmol DCCD/mg protein

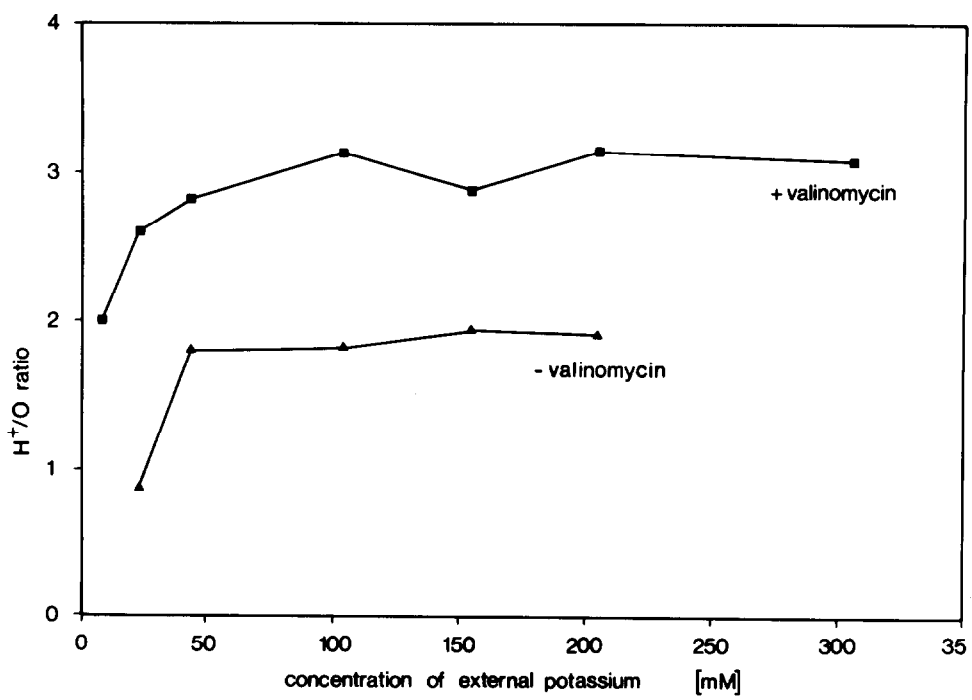

Fig.4. Influence of extracellular potassium concentration on H^+/O ratios. Conditions as shown in fig.3 with buffer I supplemented, however, with varying amounts of K_2SO_4. H^+/O ratios in these experiments were determined according to Mitchell et al. [11] from the total amplitude of H^+ extrusion.

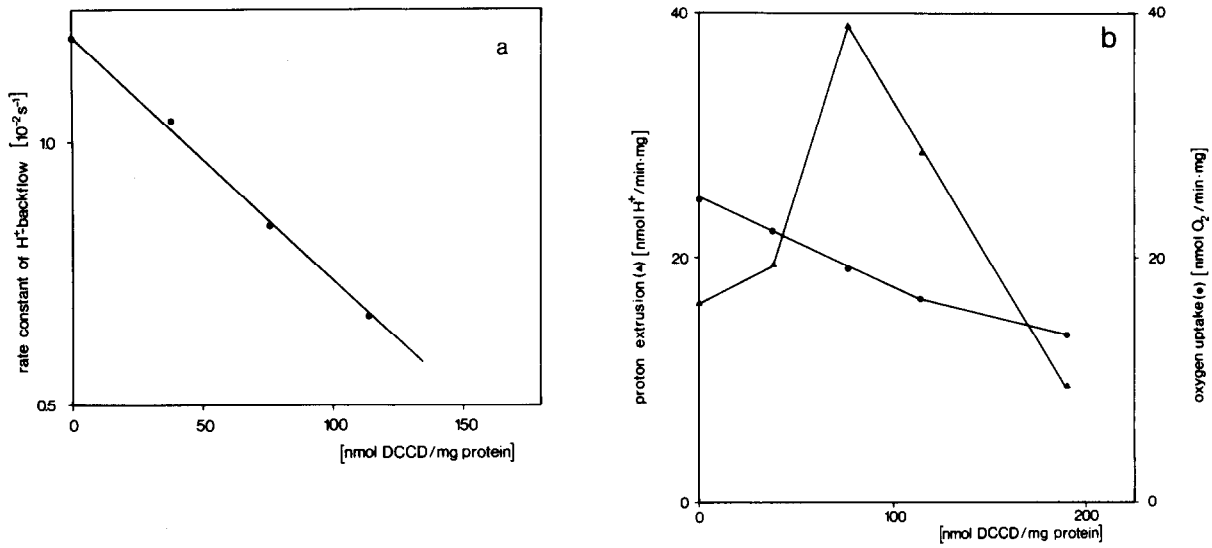

Fig.5. Effect of DCCD on H^+ backflow (a) and on initial rates of H^+ extrusion (b). Conditions as in fig.1; initial pH = 6.1. Cells were incubated with DCCD for 10 min prior to the oxygen pulse (485 nmol O_2). Kinetics of anaerobic H^+ backflow (a) expressed as first order rate constants were determined from semilogarithmic plots.

(b); higher concentrations cause a progressive inhibition presumably due to unspecific protein modifications. This effect is accompanied by a simultaneous depletion of cellular ATP content as shown by Lübben et al. [8]. Up to 120 nmol DCCD/mg protein the amplitude of H^+ backflow is unaffected.

The above results greatly suggest the participation of proton cycles in energy conservation according to the concept of Mitchell [9]. Preliminary

Table 1

Determination of pmf across the plasma membrane of *Sulfolobus acidocaldarius* cells under various conditions

Additions	pH_{out}	pH_{in}	$-Z\Delta pH$ (mV)	$\Delta\psi$ (mV)	Δp (mV)
a					
–	5.7	7.44 ± 0.06	−113.5 ± 5.7	6.8 ± 1.4	−106.7 ± 7.1
0.75 mM NaN_3	5.7	6.63 ± 0.06	−63.5 ± 2.9	9.0 ± 3.0	−54.4 ± 0.3
b					
–	5.6	7.43 ± 0.02	−116.8 ± 1.5	8.0 ± 4.5	−109.0 ± 6.0
Acid pulse	3.5	6.55 ± 0.01	−199.0 ± 0.8	30.0 ± 2.8	−170.0 ± 0.6
c					
–	6.1	7.51 ± 0.02	−96.7 ± 2.4	2.0 ± 3.0	−94.7 ± 0.5
Acid pulse	3.4	6.56 ± 0.06	−210.2 ± 2.8	39.2 ± 1.7	−171.0 ± 4.3
6.2 μM TTFB	3.4	5.96	−169.6	91.9	−77.7
12.5 μM gramicidin D	3.4	5.35	−129.6	110.9	−18.7

The three sets of experiments show (a) the effect of azide as a respiratory inhibitor, (b) the effect of an acid pulse with 0.5 M H_2SO_4 and (c) the additional effects of the protonophores TTFB and gramicidin D. All experiments were carried out at 60°C and a protein concentration of 0.6 mg/ml; $Z = 2.3RT/F$. When standard deviations are given averages were calculated from several samples withdrawn from the incubation mixture in 3 min intervals during steady-state conditions

measurements of proton motive forces by distribution of ΔpH and $\Delta\psi$ responsive probes substantially support this view. Table 1 gives values of membrane potentials and pH gradients under conditions applied also during the continuous pH registrations of figs 1–4. The influence of a respiratory inhibitor is shown, as well as the effect of an external pH jump with or without TTFB or gramicidin D present. These data are uncorrected for binding of the probes. After correction [10], mainly decreasing the readings of the membrane potential by about 20–30 mV, the maximum total proton motive force would amount to about 200 mV. Thus, a significantly positive inside membrane potential would result only under conditions of uncoupling and massive proton influx.

4. DISCUSSION

Our experiments clearly demonstrate essential bioenergetic properties of the thermoacidophilic archaebacterium *S. acidocaldarius*. First, its respiratory system is capable of driving active proton extrusion even against a large pH gradient, though the assignment of individual pumps to specific complexes of its membrane-bound electron transport system has to be clarified. Second, its plasma membrane withstands gradients of 3–4 pH units, maintaining an internal pH around 6.5; under these conditions even in acidic environments the actual membrane potential is close to zero and turns positive only in the presence of protonophores, collapsing the proton motive force. Third, the efficiency of proton extrusion critically depends on K^+ concentration. Therefore, it is likely, that additional K^+ transport systems participate in proton cycling, allowing to balance the membrane potential of a large pH gradient as suggested for other acidophilic organisms by Booth [12] and by Bakker et al. [13,14]. The nature of these postulated systems is unknown, however. In this context it has to be stressed, that the observed H^+/O ratios appear low as compared to mitochondria or other bacteria [15]; most likely they are underestimated due to the function of other proton dependent symports or antiports not identified so far. Further investigations using rapid kinetic methods are in progress. Finally, as suggested by the DCCD effects, we have to conclude that protons are indeed the coupling ions for primary energy conservation in *S. acidocaldarius*. Recently a DCCD-binding low molecular mass proteolipid from its plasma membrane similar to the F_0-subunit c of *E. coli* has been identified [8]. Together with the characterization of an ATPase closely related to known F_1-type ATPases [4], this lends strong support to the view that the chemiosmotic principle of energy conservation as well as its catalysts are early products of evolution, because archaebacteria are considered to be the least distant of organisms from the lowest branching points on the evolutionary scale [16].

REFERENCES

[1] Anemüller, S., Lübben, M. and Schäfer, G. (1985) FEBS Lett. 193, 83–87.
[2] Wakao, H., Wakagi, T. and Oshima, T. (1987) J. Biochem. 102, 255–262.
[3] Lübben, M. and Schäfer, G. (1987) Eur. J. Biochem. 164, 533–540.
[4] Lübben, M., Lünsdorf, H. and Schäfer, G. (1987) Eur. J. Biochem. 167, 211–219.
[5] Watters, C. (1978) Anal. Biochem. 88, 695–698.
[6] Michels, M. and Bakker, E.P. (1985) J. Bacteriol. 161, 231–237.
[7] Moll, R. (1987) Diplomarbeit, University of Hannover.
[8] Lübben, M., Anemüller, S. and Schäfer, G. (1987) Biol. Chem. Hoppe-Seyler 368, 555.
[9] Mitchell, P. (1966) Biol. Rev. 49, 359–378.
[10] Zaritsky, A., Kihara, M. and Macnab, R.M. (1981) J. Membrane Biol. 63, 215–231.
[11] Mitchell, P. and Moyle, J. (1967) Biochem. J. 105, 1147–1162.
[12] Booth, I.R. (1985) Microbiol. Rev. 49, 359–378.
[13] Michels, M. and Bakker, E.P. (1987) J. Bacteriol. 169, 4335–4341.
[14] Bakker, E.P., Borchard, A., Michels, M., Altendorf, K. and Siebers, A. (1987) J. Bacteriol. 169, 4342–4348.
[15] Jones, W.C., Brice, J.M., Downs, A.J. and Drozd, J.W. (1975) Eur. J. Biochem. 52, 265–271.
[16] Woese, C.R. and Fox, G.E. (1977) Proc. Natl. Acad. Sci. USA 74, 5088–5090.