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Bioenergetics and cytoplasmic membrane stability of the extremely acidophilic, thermophilic archaeon *Picrophilus oshimae*

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Abstract *Picrophilus oshimae* is an extremely acidophilic, thermophilic archaeon that grows optimally at 60°C and at pH 0.7. It is an obligatory acidophile that does not grow at pH values above 4.0. The proton motive force in respiring cells is composed of a large transmembrane pH gradient, inside less acid, and a reversed transmembrane electrical potential, inside positive. Cells maintain an intracellular pH at around 4.6 at extracellular pH values ranging from 0.8 to 4.0. Above pH 4.0 cells lyse rapidly and lose their viability. Liposomes prepared from lipids derived from *P. oshimae* have an extremely low proton permeability at acidic pH. However, at neutral pH, the lipids are unable to assemble into regular liposomal structures. These observations suggest that the loss of viability and cell integrity above pH 4.0 is due to an impairment of the barrier function of the cytoplasmic membrane.

Key words *Picrophilus oshimae* · Cytoplasmic membrane · Permeability · Solute transport

Introduction

The recently described, extremely acidophilic thermophile *Picrophilus oshimae* lives at an optimum pH of 0.7 at 60°C (Schleper et al. 1995). It is the most acidophilic thermophile so far described. This archaeon belongs to the order of *Thermoplasmales*, which also includes *Thermoplasma acidophilum*. *P. oshimae* is a heterotrophic organism that, in culture, can only grow on yeast extract. It requires an extreme ionic environment, specifically protons, not only for growth, but also for viability (Langworthy 1982). Acidophilic bacteria and archaea that grow at low pH values (pH 2–4) usually maintain a cytoplasmic pH close to neutrality (Michels and Bakker 1985; Moll and Schäfer 1988; Peeples and Kelly 1995).

Little is known about metabolism and energy transduction of extreme acidophiles. The cytoplasmic membrane is the only physical protection against an acid environment if a cytoplasmic pH close to neutrality has to be maintained. Then, the membrane has to withstand an extremely steep pH gradient of up to 4 or 5 pH units. Usually, this can only be realized if the membrane has a very low proton permeability at temperatures up to 60°C. In a previous study we found that thermophiles have a cytoplasmic membrane that is less permeable for protons than the membranes of organisms that grow at lower temperature. At the respective growth temperatures, the proton permeability for most of the tested organisms is in the same range. Thermophiles alter the membrane composition such that the membrane becomes less permeable to ions (Van de Vossenberg et al. 1995). For acidophiles such an impermeable membrane is even essential.

To reveal the bioenergetic features of *P. oshimae*, the magnitude and composition of the proton motive force (Δp) were studied as a function of the environmental pH. It was found that the Δp of this extremely acidophilic thermophile is equal to or higher than that of neutrophilic organisms. The proton permeability of the cytoplasmic membrane was indirectly determined in liposomes prepared from *P. oshimae* lipids. These liposomes are very impermeable to protons at the pH values that fall within the growth range. However, at neutral pH values, these lipids do not assemble into regular vesicular structures. The loss of cell viability and membrane integrity at pH values above 4.0 thus appears to be an intrinsic property of the membrane lipids and is likely to be the consequence of the adaptation of the cell envelope to extremely acidic environments.

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Materials and methods

Strains, growth conditions, and chemicals

Picrophilus oshimae DSM9789 (Schleper et al. 1995) was grown aerobically at pH 0.8 at 60°C in Brock's medium supplemented with 0.2% yeast extract. The medium contained per litre: 1.32 g $(NH_4)_2SO_4$, 0.372 g KH_2PO_4, 247 mg $MgSO_4 \cdot 7H_2O$, 74 mg $CaCl_2 \cdot 2H_2O$, 1.93 mg $FeCl_3 \cdot 6H_2O$, 1.8 mg $MnCl_2 \cdot 4H_2O$, 4.5 mg $Na_2B_4O_7 \cdot 10H_2O$, 0.22 mg $ZnSO_4 \cdot 7H_2O$, 50 µg $CuCl_2 \cdot 2H_2O$, 30 µg $Na_2MoO_4 \cdot 2H_2O$, 35 µg $VOSO_4 \cdot 5H_2O$, 10 µg $CoSO_4 \cdot 7H_2O$, and 0.2% yeast extract. The pH was adjusted with concentrated H_2SO_4. *Sulfolobus acidocaldarius* DSM639 was grown as described (Van de Vossenberg et al. 1995).

The internal volume of the cells was determined as described using ^3H_2O (1.6 GBq mmol^{-1}) and the membrane impermeable sugar inulin-[^{14}C]acetic acid (163 MBq mmol^{-1}) (Rottenberg 1979). The internal volume of the cells was 1.55 ± 0.11 µl per mg of protein (n = 15). All radiochemicals were obtained from Amersham (Buckinghamshire, UK) unless indicated otherwise.

Respiration measurements

Cells were de-energized by washing twice in Brock's medium without yeast extract, supplemented with 35 mM KCl (assay medium), incubated for 1 h at 60°C, and again washed and resuspended in assay medium at OD_{660} 0.7. Various substrates [0.2% (w/v)] were added to the cell suspension, and the respiratory rate measured with a Clark-type oxygen electrode. Measurements were done at 60°C.

Cell lysis experiments

To test cell lysis, 2 ml fresh cell suspension (OD_{660} 0.4) was centrifuged for 30 s at 14000 rpm in an Eppendorf centrifuge. The cells were resuspended in 100 µl growth medium of the desired pH and immediately diluted into 1.9 ml growth medium of the desired pH at 55°C. Lysis was followed by the time-dependent increase in fluorescence intensity, monitored with the membrane-impermeable DNA probe propidium iodide (Molecular Probes, Eugene, OR, USA) (1 µg ml^{-1}). The fluorescence response of the probe varied drastically at different pH values. Therefore, the signal was expressed as percentage of maximum fluorescence change. Maximum fluorescence change was obtained by the addition of dodecyl maltoside (0.1% final concentration) to the cell suspension. The initial rate of lysis was calculated and plotted.

Intracellular ATP concentration measurements

For intracellular ATP measurements, the cells were harvested basically according to Schönheit and Perski (1983). Cells (1 ml; OD_{660} 5) were incubated for 2 h at 60°C in the presence of 0.2% (w/v) substrate. From each cell suspension, 250 µl was pipetted into 500 µl ethanol-KOH at −20°C and immediately frozen in liquid nitrogen. The ethanol-KOH contained ethanol and an empirically determined volume of 1.0 M KOH to adjust the low pH of the cells to about 7. Subsequently, the mixture was thawed on ice and frozen again in liquid nitrogen. After lyophilizing, the samples were dissolved in 750 µl 20 mM Tris-acetate, 0.2 mM Na_2 ethylenediaminetetraacetate (EDTA), 0.05 mM dithiothreitol (DTT), 0.5 mM magnesium acetate, 0.05% bovine serum albumin (BSA), pH 7.5. ATP concentrations were determined with partly purified luciferase (FLE-50, Sigma, St. Louis, MO, USA) (Lundin and Thore 1975). Portions of 0.1 ml of the resulting solution were analyzed with luciferin-luciferase reagent. Luminescence was measured with a Lumi-Tec A5-2021A luminescence meter (St. John associates, BeltgVille, MD, USA), using ATP solutions of known concentration as calibration.

Measurement of proton motive force

The transmembrane electrical potential, $\Delta\psi$, inside positive, and pH gradient, ΔpH, inside alkaline, were measured essentially as described by Michels and Bakker (1985). Cells (1.5–3.0 mg of protein) were suspended in 2 ml assay medium at pH 1.0. The pH was adjusted with concentrated H_2SO_4. At higher pH, buffers were used which contained either 100 mM potassium phosphate or 100 mM potassium phosphate supplemented with either 100 mM sulfuric acid or citric acid. To the suspensions, yeast extract (0.2% w/v) was added for energization, followed by the addition of 1.5 µM KS^{14}CN (2.1 GBq mmol^{-1}), 1.5 µM ^{14}C-acetylsalicylic acid (2.0 GBq mmol^{-1}, American Radiolabeled Chemicals, St. Louis, MO, USA), or 3.5 µM ^{14}C-DL-lactate (0.87 GBq mmol^{-1}). Cells were incubated at 60°C and aerated with humidified air preheated at 60°C. After 10 min, three samples of 600 µl were taken and centrifuged through a 500-µl layer of silicon oil (AR200/AR20 ratio of 2.5/1, v/v; Wacker Chemie, Munich, Germany) into a 150-µl layer of 14% (w/v) perchloric acid (PCA) and 9 mM EDTA. Samples (100 µl) were taken from both the layer above and the layer below the silicon. To avoid buffer effects on counting we balanced the samples obtained from the lower and upper layers with the medium used for the upper layer and PCA/EDTA, respectively. The radioactivity was determined by liquid scintillation counting. To determine the extent of nonspecific probe binding to cellular components, the cells (OD_{660} = 5) were disrupted by sonication (Soniprep 150 probe sonicator, MSE, Uxbridge, Middlesex, UK), and separated into membrane and cytosol fractions by centrifugation for 15 min at 200000 × g. From each fraction, 200 µl was transferred into the small space that is in the cap of an Eppendorf cup, and subsequently covered with dialysis tubing. From the cup top only a small ring was left, used to tighten the dialysis tubing. The samples were dialyzed in Brock's medium salts, supplemented with 35 mM KCl, and various radiolabelled tracers, at pH 1.5. After 2 h of incubation at 60°C, the radioactivity associated with the various fractions was determined.

Transport assays

For transport experiments, 200 µl of a concentrated cell suspension (30 mg of protein per ml) was added to 2250 µl assay medium at pH 1.0. The suspension was incubated at 60°C and aerated with humidified air preheated at 60°C. Subsequently, 0.3–5 µM of the following ^{14}C-labelled substrates was added: L-α-aminoisobutyric acid, L-glutamate, L-histidine, L-proline, L-serine, and glucose with specific activities of 2.2, 9.8, 0.37, 9.6, 2.3, and 11 GBq mmol^{-1}, respectively. At various times, samples of 300 µl were taken, diluted with 2 ml 0.1 M LiCl, and filtered over a 0.45-µm BA85 (Schleicher and Schuell, Dassel, Germany) nitrocellulose filter. Filters were rinsed with 2 ml 0.1 M LiCl, and the radioactivity retained on the filter was counted by liquid scintillation spectrometry.

Lipid extraction and purification

Lyophilized cells of *P. oshimae* were Soxhlet extracted and fractionated essentially as described by Lo and Chang (1990). The lipids were stored in chloroform/methanol/water (65/25/4, v/v/v) at −20°C. Lipids were analyzed by thin-layer chromatography using Kieselgel 60 (Merck, Darmstadt, Germany) plates and chloroform/methanol/water (65/25/4, v/v/v) as eluant. Plates were developed with iodine vapor and a molybdate reagent to reveal the presence of (phospho-)lipids (Rouser et al. 1970).

Liposome preparation and integrity

Lipids were dried by vacuum rotary evaporation, and hydrated in 50 mM (*N*-2-marpholino)propane sulfonic acid (MOPS), pH 7.0, 75 mM KCl, and 25 mM choline to a final concentration of 20 mg ml^{-1}, unless indicated otherwise. Liposomes were sized to an average diameter around 200 nm by five consecutive freezing and thawing steps, followed by extrusion through 400-nm and subsequently 200-nm polycarbonate filters using the Liposofast (Basic, Avestin, Ottawa, Canada) extrusion apparatus (Elferink et al. 1994). Liposome integrity was tested by the ability to maintain an imposed potassium diffusion gradient in the presence of valinomycin by $\Delta\psi$ (inside negative) measurements using the fluorescent probe 3,3′-diethylthiadicarbocyanine iodide [DiSC$_3$(5)] (Deamer and Nichols 1989).

Electron microscopy

The morphology of liposomes prepared at pH 7.0, 3.0, and 4.0 was studied by negative staining and cryo electron microscopy. Liposomes, 10 mg lipid ml^{-1}, were made in 50 mM potassium phosphate at the desired pH, extruded, and diluted to obtain isolated liposomes for electron microscopy. For cryo electron microscopy, a bare sample grid was dipped in the liposome suspension and withdrawn. After withdrawal from the liposome suspension, the grid was blotted against filter paper. The remaining thin film was rapidly frozen in liquid propane at −180°C. The liposome suspension was at room temperature. The preparations were analyzed at 100 kV and a sample temperature of −165°C in a Philips CM10 electron microscope.

Measurement of proton permeability

The proton permeability of the membrane was measured essentially as described by Nichols and Deamer (1980), except that the fluorescent pH dye Cl-NERF-dextran 70000 (Molecular Probes, Eugene, OR, USA) with an apparent pK_a of 4.7, was used for measurement at acidic pH. Liposomes were prepared in 50 mM citrate-KOH and 35 mM KCl pH 4.0. To reduce the buffer capacity of the external medium, liposomes were passed over a Sephadex G-25M PD10 column preequilibrated with 90 mM KCl, pH 4.0, and diluted to 1.5 mg lipid ml^{-1} in 2 ml of 90 mM KCl, pH 4.0, containing the fluorescent pH probe Cl-NERF-dextran 70000 at a concentration of 5 µg ml^{-1}. The K^+ ionophore valinomycin (1 nmol mg^{-1} lipid) was added to prevent the formation of a reversed $\Delta\psi$. The external pH was monitored from the fluorescence at 518 nm (emission 541 nm). After equilibration, the external pH was lowered by the addition of 5 µl 200 mM HCl. The proton permeability was determined from the partial recovery of the external pH due to the influx of protons into the liposomal lumen. Fluorescence measurements were done on a Perkin-Elmer LS-50B (Norwalk, CT, USA), using a thermostated, magnetically stirred sample compartment. Data were fitted to a first order kinetic rate equation as described (Van de Vossenberg et al. 1995), to yield the rate constant *k* that was used for comparison.

Results

Respirable substrates

Picrophilus oshimae grows in a complex medium with yeast extract as the main carbon and energy source. To identify the components that can act as an energy source, the rate of respiration of cells incubated in a buffer of pH 1.0 was measured in a closed vessel with a Clark-type oxygen electrode. Cells had a high endogenous respiration rate even after starvation for several hours at 60°C. Highest respiration rates were observed in the presence of yeast extract or the mixture of amino acids, casamino acids (Table 1). Only proline, glutamate, and leucine were respirable amino acids, whereas no enhancement of respiration was observed with alanine, arginine, asparagine, cysteine, glutamine, glycine, histidine, methionine, or serine. From the carbohydrates, only galactose and glucose were respired whereas no activity was found with fructose, raffinose, or glycerol. Although various substrates enhanced the respiration rate, none of them either individually or mixed could support growth, suggesting a more complex nutritional requirement. Respiration was inhibited or even completely

Table 1. Respiration of *Picrophilus oshimae* cells at 60°C and pH 1 in the presence of various substrates. The following substrates did not produce a significant change in respiration up to a concentration of 0.2% or greater: glycerol, fructose, raffinose, alanine, arginine, asparagine, glutamine, glycine, histidine, methionine, serine, cysteate

Substrate	Respiration (nmol min^{-1} (mg protein)$^{-1}$)
Endogenous	22.7 (± 5.2)
Yeast extract	65.0 (± 7.2)
Casamino acids	54.6 (± 11.8)
L-Proline	50.7 (± 0.7)
L-Glutamate	40.3 (± 3.5)
L-Leucine	35.2 (± 2.5)
Glucose	34.4 (± 4.3)
Galactose	36.1 (± 4.6)
Starch	43.6 (± 1.2)
Formate	0.0
Acetate	17.6
Propionate	19.3
Lactate	0.0

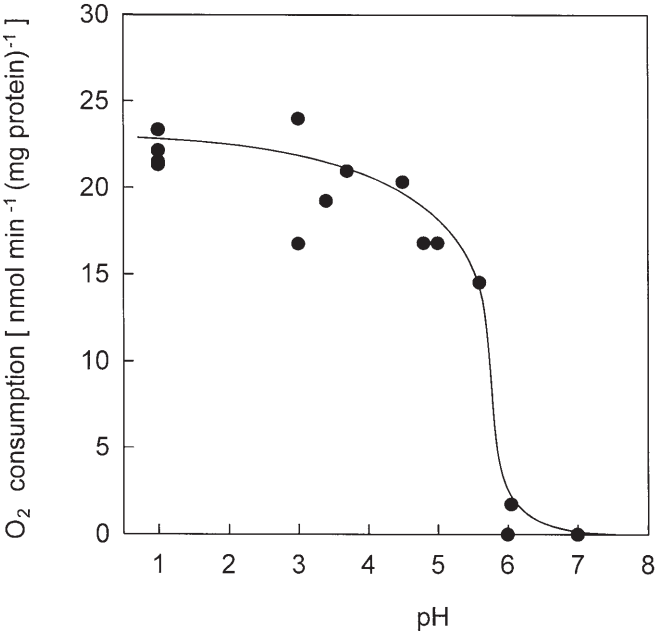

Fig. 1. pH dependency of the respiration of *Picrophilus oshimae*. Cells of *P. oshimae* were incubated in growth medium at 60°C, and energized with 0.2% yeast extract. After the experiment the exact pH values were measured. The respiration rate was calculated from the oxygen consumption as measured with a Clark electrode

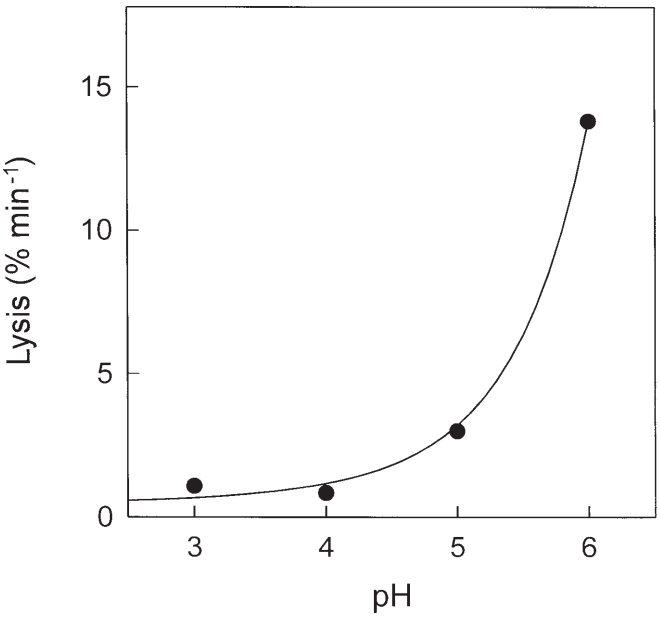

Fig. 2. pH-dependent lysis of cells. Lysis was measured as described in the Materials and Methods section

abolished by organic acids such as formate, acetate, propionate, or lactate, but not by cysteate. The internal ATP concentration of growing cells was 2.7 ± 0.3 mM (n = 3). When the medium was supplemented with starch or yeast extract, the internal ATP concentration remained equal to or became slightly higher than that in cells grown without substrate. When the cells were incubated with a mixture of amino acids (casamino acids), proline, glutamate, or 2-deoxyglucose, the ATP concentration was about half the control. Intracellular ATP was completely depleted when the cells were incubated in the presence of 30 mM lactate.

Respiration was measured as a function of the external pH. Respiration was rather insensitive to the external pH up to pH 5; respiration immediately ceased at pH values above 5 (Fig. 1). The formation of a precipitate at pH values above 5 suggested a major degree of cell lysis. The release of DNA, as observed from the fluorescence increase in the presence of the membrane-impermeable DNA probe propidium iodide (Fig. 2) is consistent with this conclusion.

Proton motive force

The transmembrane electrical potential ($\Delta\psi$) and pH gradient (ΔpH) were measured as a function of the extracellular pH using a probe distribution assay. Cells incubated at 60°C were energized with 0.2% yeast extract. For the ΔpH measurements, the weak acids acetylsalicylic acid (aspirin) and DL-lactic acid were used, with pK_a values of 3.48 and 3.08, respectively. Both acids accumulated to high levels when the external pH was around or below 1, yielding a ΔpH of more than 4 pH units (more than −275 mV, Fig. 3). The ΔpH steeply declined with increasing pH, whereas the internal pH remained constant at pH 4.6 ± 0.15 (n = 25) over an external pH range from 0.5 to 3.5. For the $\Delta\psi$ measurements, the anionic thiocyanate ion was used to monitor a $\Delta\psi$, inside positive, and cationic tetraphenylphosphonium ions (TPP^+) for a $\Delta\psi$, inside negative. Only thiocyanate was taken up by the cells (Fig. 3), while TPP^+ was extruded (data not shown), indicating a $\Delta\psi$ with a reversed potential (inside positive). Upon raising the extracellular pH from pH 0.7 to pH 4 the $\Delta\psi$ decreased sharply from +100 to +45 mV. Permeabilization of the cells with n-butanol, or addition of the ionophore gramicidin D, resulted in a com-

plete collapse of the ΔpH, whereas the Δψ was lowered to only +40 mV. The remaining Δψ presumably corresponds to a Donnan potential as binding studies with cytosolic and membrane fractions of lysed *P. oshimae* cells revealed no significant levels of thiocyanate binding (data not shown). These studies show that *P. oshimae* at pH 1.0 and 60°C maintains a Δp of about −175 mV, composed of a large ΔpH and a reversed Δψ. The Δp drops with increasing pH.

Uptake of amino acids

Cells of *P. oshimae* energized with glucose were tested for the uptake of various amino acids at pH 1.0 and 60°C. L-Glutamate, L-histidine, L-proline, and L-serine were rapidly accumulated, whereas no uptake was detected of alanine or its nonmetabolizable analogue L-α-aminoisobutyrate (Fig. 4a). The highest levels of uptake were observed for L-glutamate. This process was therefore further investigated. L-Glutamate uptake was strongly inhibited by the ionophore nigericin that dissipates the ΔpH (electroneutral K^+/H^+ exchange). A collapse of the Δψ by valinomycin (K^+-ionophore) had no effect (Fig. 4c). L-Glutamate uptake was completely inhibited by the addition of valinomycin and nigericin at the same time, or by gramicidin D. L-Glutamate uptake showed an optimum of pH 1.7 (Fig. 4b), and occurred with a K_m of 200 μM and a V_{max} of 0.92 nmol min^{-1} (mg protein)$^{-1}$. L-Glutamate uptake was strongly inhibited by L-aspartate and to a lesser extent by L-glutamine, while L-asparagine had no effect (data not shown). These data suggest that the uptake of L-glutamate by *P. oshimae* is dependent on the ΔpH, the main component of the Δp in respiring cells.

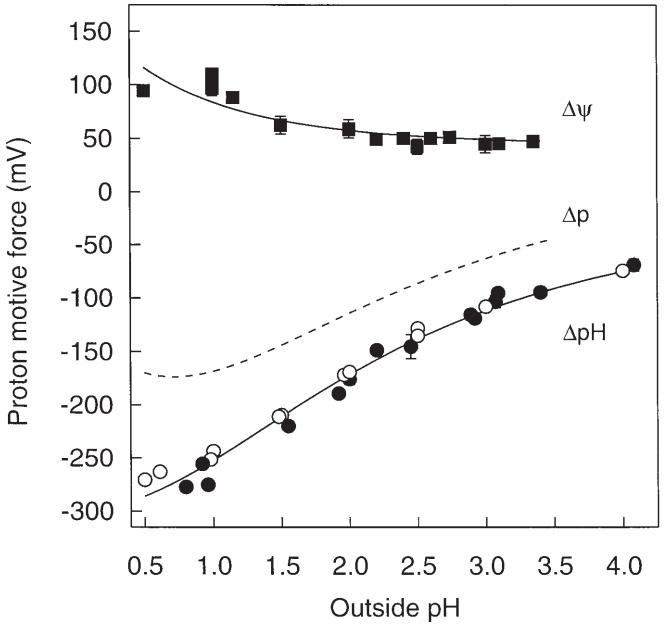

Fig. 3. pH dependency of the composition and magnitude of the proton motive force in *P. oshimae* at 60°C. Δψ (*squares*) and ΔpH (*circles*) measurements as described in the Materials and Methods section. For the ΔpH, acetylsalicylic acid (*open circles*) or lactate (*closed circles*) were used as probes. All measurements were done in triplicate

Membrane morphology

To study the characteristics of lipids of the cytoplasmic membrane, lipids were isolated from *P. oshimae* cells by Soxhlet extraction and purified by reversed phase column chromatography. Thin layer chromatography analysis revealed the presence of one main lipid component, a phospholipid. Liposomes were formed from the hydrated lipids by freeze-thaw-extrusion through a 200-nm filter and examined by cryo electron microscopy. When the procedure was carried out at pH 7.0, liposomes were formed but more than 10% of the liposomes had extensive irregular substructures (Fig. 5B). At pH 4.0, a more homogenous liposome preparation was obtained with an averaged diameter of about 200 nm (Fig. 5A), while hardly any irregular shaped liposomes were evident (less than 2%).

Proton permeability

The proton permeability of *P. oshimae* liposomes was determined by the proton-pulse method. Liposomes with an

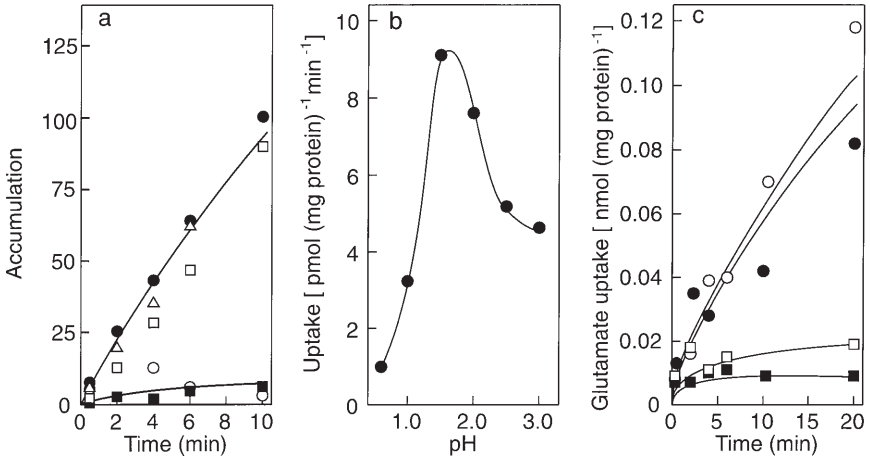

Fig. 4. a Accumulation of amino acids by *P. oshimae* cells at 60°C and pH 1.0. Uptake of L-serine (*triangles*), L-glutamate (*closed circles*), L-proline (*open squares*), L-histidine (*closed squares*), and L-α-aminoisobutyrate (*open circles*). **b** pH dependency of L-glutamate uptake at 60°C. **c** L-Glutamate uptake (*closed circles*) and the effect of the ionophores valinomycin (*open circles*), nigericin (*open squares*), and nigericin plus valinomycin (*closed squares*). Concentrations of ionophores used were 1 μM

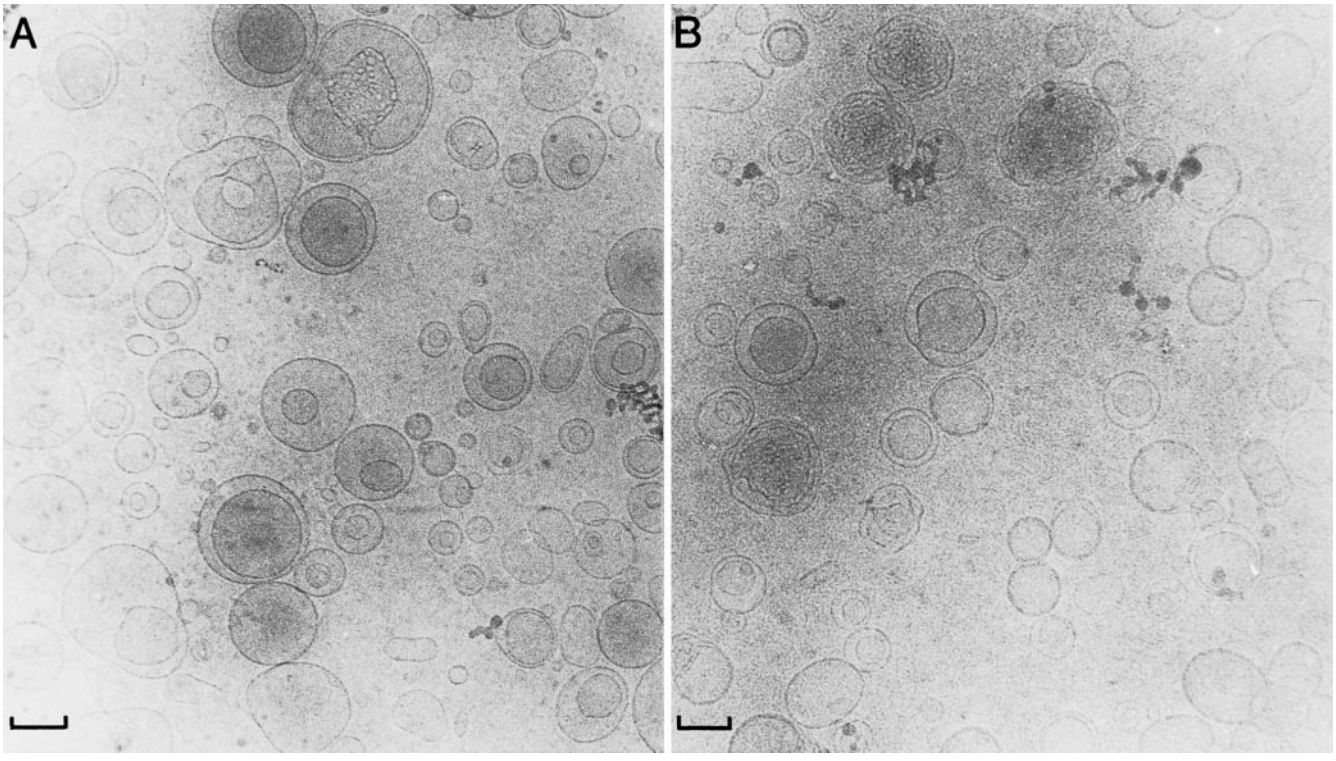

Fig. 5. Electron micrographs of liposomes of *P. oshimae* lipids at pH 4.0 (**A**) or pH 7.0 (**B**). *Bar* represents 100 nm

average size around 200 nm were prepared with a high buffer capacity on the inside and dispersed in a solution with low buffer capacity. Valinomycin, a potassium ionophore, was added to prevent the generation of a counteracting $\Delta\psi$ due to electrogenic influx of protons. The external pH was lowered by an H^+ pulse, and monitored through the fluorescence of the externally added pH dye Cl-NERF-dextran 70000. The pK_a of this dye is 4.7. Lowering of the external pH was followed by partial recovery due to the slow influx of protons into the liposomal lumen loaded with a higher buffer concentration. This phase was fitted to a first order kinetic rate equation to yield the rate constant k for H^+ influx. Rapid proton equilibration was obtained after the addition of the ionophore nigericin. The proton permeability of the liposomes was measured as a function of the temperature. At pH 4.0, the proton permeability of *P. oshimae* liposomes was nearly identical to that observed with *S. acidocaldarius* liposomes (Fig. 6). Due to the pK_a of the fluorescent dye, proton permeability measurement at lower pH values was not possible. The proton-permeability of each of the liposome preparations was plotted in an Arrhenius plot, and from the linear part of the graph, the activation energy ($\Delta G'_{H^+}$) was estimated at 36 kJ/mol. Liposomes prepared from *P. oshimae* lipids were completely permeable to protons at pH 7.0. The data demonstrate that liposomes prepared from *P. oshimae* lipids are highly stable and hardly permeable to protons at pH 4.0.

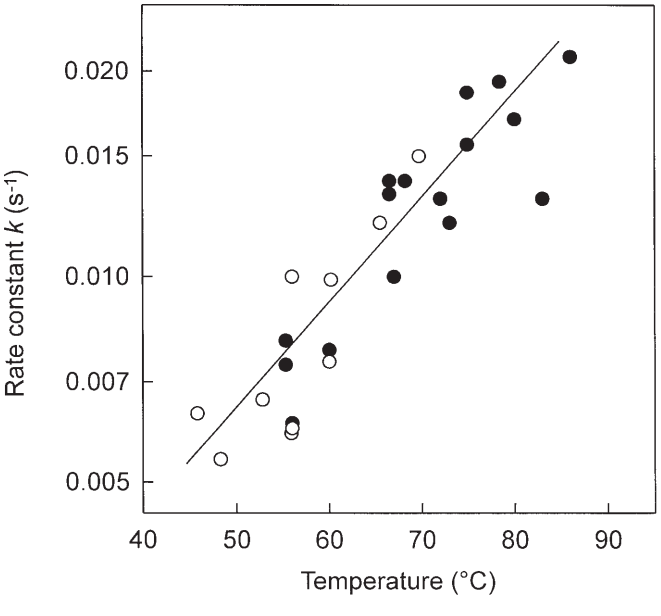

Fig. 6. Temperature dependency of the proton permeability of liposomes prepared from lipid derived from *P. oshimae* (*open circles*) and *Sulfolobus acidocaldarius* (*closed circles*). The influx of protons, as measured with the fluorescent probe Cl-NERF dextran, was fitted to a first order kinetic equation as described in the Materials and Methods section

Discussion

Picrophilus oshimae is a thermophilic archaeon that grows at pH values slightly lower than zero. Its optimal growth is at pH 0.7 and 60°C. Our data show that *P. oshimae* can use various sugars and amino acids as energy sources for respiration. The internal ATP concentration, around 3 mM in the presence and absence of substrate (yeast extract and starch) and even after starvation for 5 h, is comparable with the levels found in other prokaryotes (Kunji et al. 1993). Cells were unable to maintain a high intracellular ATP concentration in the presence of amino acids, presumably as these cannot be used as sole carbon sources for growth (Schleper et al. 1995).

The bioenergetic parameters are typical for an acidophilic organism, i.e., a very large ΔpH and a reversed Δψ, to yield a Δp with a magnitude that is in the range found also in neutrophiles and other acidophiles (Booth 1985; Michels and Bakker 1985; De Vrij et al. 1988; Moll and Schäfer 1988; Matin 1990; Peeples and Kelly 1995). Both the Δp and ΔpH decline steeply with increasing pH. The ΔpH is the main constituent of the Δp at pH 1.0, and uptake of L-glutamate and presumably of most other substrates is mainly driven by the ΔpH. This implies that *P. oshimae* is well adapted for growth at extreme acidic conditions.

The effect of ionophores and uncouplers on the transport activities has to be carefully interpreted. Valinomycin collapses the Δψ, nigericin collapses the ΔpH. Both ionophores together uncouple the cells; this can also be accomplished with gramicidin D. Compounds such as nigericin and gramicidin D, that collapse the ΔpH, induce an acidification of the internal pH that results in inhibition of respiration and other cellular functions. The best system for a detailed energetic analysis of transport processes would be an isolated membrane vesicles system. So far, preparing functional membrane vesicles from these archaea has not been possible. The importance of pH homeostasis is evident from observations that weak acids are potent inhibitors of the growth of *P. oshimae*. At pH 1.0, 30 mM lactate and formate completely block respiration. At this pH value, these acids are almost solely present in their protonated form. Apparently, the cytoplasmic membrane of *P. oshimae* is, just like membranes of other organisms, highly permeable to the protonated weak acids. These acids not only cause an inhibition of respiration but also a complete dissipation of the ΔpH and depletion of the cellular ATP. The reason why formate and lactate are better inhibitors of respiration than acetate and propionate is not clear.

Lactate and acetyl salicylic acid were both used as internal pH indicators. The results obtained with both probes were similar. These studies demonstrated that *P. oshimae* maintains its intracellular pH around pH 4.6 when the extracellular pH is varied between pH 0.7 and 4.0. Internal pH measurements in *Thermoplasma acidophilum* have shown that this organism maintains its intracellular pH at 6.0 when the pH is varied between 0.5 and 3.0 (Michels and Bakker 1985). The values of the internal pH are lower than those reported for nonthermophilic acidophiles (Matin 1990). In both *P. oshimae* and *T. acidophilum*, the ΔpH is large whereas the Δψ is reversed. The ΔpH can be completely dissipated by *n*-butanol and gramicidin D, whereas the Δψ remains clamped at +45 mV. The latter may be due to a Donnan potential at these low pH values resulting from the immobilized charges of macromolecules in the cell (McLaggan et al. 1989; Matin, 1990).

Since *P. oshimae* cells have to maintain a steep pH gradient across the membrane, we have analyzed the proton permeability of liposomes prepared from lipids derived from their cytoplasmic membrane. When the liposomes were prepared at pH 7.0, many liposomes revealed irregular substructures. These structures are possibly interlamellar attachments due to membrane fusion (Frederik et al. 1991), or another form of nonbilayer structure. Liposomes prepared at pH 3 and 4 lacked the substructures. These liposomes exhibited a very low proton permeability at pH 4.0, but became leaky when exposed to pH 7.0. The permeability coefficient, P (in $cm\,s^{-1}$) can be derived from the first order rate constant k (Yamauchi et al. 1993). For protons, the values for P measured in this study range from 10^{-11} to $10^{-10}\,cm\,s^{-1}$, depending on temperature. P values reported for other lipids range from 10^{-10} to $10^{-4}\,cm\,s^{-1}$, depending on the lipids employed in the study (Deamer and Bramhall 1986). The lipid composition of *P. oshimae* largely resembles that of *T. acidophilum* (Langworthy 1982; Schleper et al. 1995). Both organisms contain tetraether lipids with cyclized hydrocarbon chains, but lack the typical *Sulfolobus* nonitol ethers. The proton permeability of *S. acidocaldarius* liposomes at pH 4.0 was about 2-fold lower than that determined at pH 7.0 in a previous study (Van de Vossenberg et al. 1995). Liposomes prepared from *P. oshimae* lipids were completely permeable to protons at pH 7.0. The inability of the *P. oshimae* lipids to assemble into regular membrane structures at neutral pH may be a main reason that cells are extremely susceptible to lysis when exposed to pH values above pH 4. *S. acidocaldarius* tetraether lipids and synthetic bolaform lipids primarily adopt a membrane-spanning shape upon hydration (Elferink et al. 1992; Thompson et al. 1992). When assayed at pH 4.0 and 60°C, liposomes prepared from *P. oshimae* or *S. acidocaldarius* lipids showed nearly identical proton permeability. This proton permeability is significantly lower than that observed for mesophilic or thermophilic organisms at their respective growth temperatures. This is required to withstand the high proton concentration at pH values below 1.0. In addition, the reversed Δψ will counteract the proton influx, and thus help in maintaining a ΔpH, inside alkaline versus outside, under extreme conditions. Our studies with the liposomes prepared from P. *oshimae* lipids indicate an intrinsic instability of the cytoplasmic membrane at higher pH values. *P. oshimae* is not only well adapted to endure the extreme acidic environment, but even requires an acidic pH for membrane stability and cell integrity.

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