# Protein AQ_1862 from the Hyperthermophilic Bacterium *Aquifex aeolicus* Is a Porin and Contains Two Conductance Pathways of Different Selectivity

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ABSTRACT The "hypothetical protein" AQ_1862 was isolated from the membrane fraction of *Aquifex aeolicus* and identified as the major porin. In experiments with one conducting unit (molecule) a conductance of 1.4 nS was observed in 0.1 M KCl at pH 7.5. This stable (basic) conductance was superimposed by conductance fluctuations of ~0.25 nS. Because both events were always observed simultaneously, it is suggested that they are caused by the same molecular entity. Nonetheless they show very different properties. The basic conductance is anion selective at neutral pH with a conductance sequence $Cl^- \approx Br^- \approx NO_3^- > F^-$ > gluconate ≈ acetate ≈ propionate and does not saturate up to 0.5 M KCl. At alkaline pH and in the presence of large anions, it becomes unselective and the conductance saturates at low concentrations ($K_m \approx 20$ mM). In contrast the fluctuating component is mainly cation selective with a conductance sequence $K^+ \approx Rb^+ > NH_4^+ > Na^+ \approx Li^+ \approx Cs^+$. It saturates at low salt concentrations ($K_m \approx 15$ mM) and is not affected by pH. In view of the diverging properties of both conductance components, it seems appropriate to assume that AQ_1862 has two different conducting pathways rather than one with two different open states.

## INTRODUCTION

Gram-negative bacteria are surrounded by an inner and an outer membrane. Permeation through the outer membrane takes place through pore-forming membrane proteins called porins. Based on the specificity and the mode of permeation, three different classes can be distinguished (1): general porins, substrate-specific porins, and active transporters. The longest known and best studied porins are the ''classical porins'' OmpF, OmpC, and PhoE from *Escherichia coli* belonging to the nonspecific class. They form water-filled pores which facilitate the diffusion of small polar substrates (<600 Da) across the outer membrane but do not have a binding site for their substrates (2,3). The substrate-specific porins such as the nucleoside transporter Tsx (4,5) or the maltose channel LamB (6) have a saturable binding side for their substrate and much lower single-channel conductances. The active transporters like FhuA bind a substrate and are able to transport it against a concentration gradient (7,8). The energy required for the transport is supplied by the inner membrane protein TonB.

The properties of the general outer membrane porins have been investigated for decades. The results show that porins of the general diffusion pathway are large channels with low selectivity; therefore, they can be described as water-filled holes in the outer membrane of bacteria (9–11) see Nikaido (1) for review. They form trimers of 16-stranded $\beta$-barrels (12–14). Only recently have more detailed studies become available which describe, for example, substates of conductance of *E. coli* OmpF (15,16), indicating that transport across the outer membrane might be more complicated than thought originally.

However, most studies have been carried out on *E. coli*, *Salmonella typhimurium*, and the different subclasses of proteobacteria whereas comparably little is known about other Gram-negative bacteria, like thermophiles, which manage to live in a very hostile environment, or autotrophes, which should have a different requirement for transport across the outer membrane. Only porins from *Thermotoga maritima* (17) and *Thermus thermophilus* (18) have been investigated, but no detailed study is available.

No study has been carried out on the outer membrane of *Aquifex aeolicus* so far. *A. aeolicus* is a Gram-negative autotrophic bacterium, which is one of the most thermophilic and evolutionary oldest bacteria known (19,20).

We have identified the ''hypothetical protein'' AQ_1862 as the most abundant protein in the solubilized membrane of *A. aeolicus*. The amino acid sequence shows no significant homology to any electrophysiologically investigated porin, but there is weak homology to several putative outer membrane porins from the short chain amide and urea porin (SAP) family (21) and the *Pseudomonas* OprP porin (POP) family. To investigate whether AQ_1862 is indeed a porin-like molecule, we investigated the pore-forming properties in planar lipid membranes. The results demonstrate that the protein has pore-forming activity which is comparable to

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known porins in some respects but also shows some peculiar features.

## MATERIALS AND METHODS

### Purification

*A. aeolicus* cells were obtained from Archeenzentrum Regensburg, Germany. Membrane isolation and solubilization were performed as described previously (22). The solubilized membrane proteins were loaded onto a Mono Q HR 10/10 column (Amersham Biosciences, Freiburg, Germany) preequilibrated with 20 mM Tris-Cl, pH 7.4, 0.05% (w/v) sodium azide, and 0.05% (w/v) dodecyl-$\beta$-D-maltoside and eluted with a linear gradient of 0–1 M NaCl. AQ_1862 containing fractions (judged by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)) were subjected to size exclusion chromatography using a Superose 6 column (Amersham Biosciences) in 20 mM Tris-Cl, pH 7.4, 150 mM NaCl, and 0.01% (w/v) dodecyl-$\beta$-D-maltoside. As the final step the protein was loaded onto a Reactive Red 120-Agarose (Sigma-Aldrich, Seelze, Germany) equilibrated with 20 mM Tris-Cl, pH 7.4, and 0.01% (w/v) dodecyl-$\beta$-D-maltoside and eluted with 1.5 M NaCl.

### Sequence comparison

A BlastP search was performed using the NCBI blast server (23) with standard settings. For signal sequence prediction the program SignalP (24) was used. The transmembrane $\beta$-barrel prediction was carried out by the program PRED-TMBB (25) and the fold compared by the program HH-PRED (26).

### Lipid bilayer experiments

Painted lipid bilayers (27) were formed across an aperture separating two half-cells (volume 1.4 ml) of a Teflon cuvette. The diameter of the aperture was 1 mm for multichannel experiments or 0.1 mm for single-channel experiments. The hole was pretreated with 0.5% (w/v) diphytanoyl phosphatidylcholine in hexane, and lipid bilayers were prepared from 1.5% (w/v) diphytanoyl-phosphatidylcholine (Avanti Polar lipids, Birmingham, UK) in *n*-decane. The cuvette was connected to the measuring circuit with Ag/AgCl electrodes via salt bridges (3 M KCl, 5% agar). One compartment was connected to a variable voltage source and the other one was connected to a current to voltage converter (Stanford Research Systems, Sunnyvale, CA; or custom made: amplification $10^9$ A/V, bandwidth dc to 1 kHz). For voltage measurements the current amplifier was replaced by a voltage amplifier with high input impedance (>10 G$\Omega$, homemade). Signals were digitized (MiniDigi 1A, Axon Instruments, Foster City, CA; sample-rate 1000 Hz) and recorded on a PC. For recording and analysis of the data the pCLAMP 9 program package (Axon Instruments) was used.

If not indicated otherwise the pH of all salt solutions were adjusted to pH 7.5 and buffered with 5 mM K-HEPES or 5 mM Tris-Cl. The temperature was 20°C–22°C. Protein (0.4 mg/ml in 20 mM Tris-Cl pH 7.4, 150 mM NaCl, 0.01% dodecyl-$\beta$-D-maltoside, final concentrations 0.5–2.0 $\mu$g/ml for multichannel measurements, and 0.05–0.2 $\mu$g/ml for single-channel experiments) was added to the compartment connected to the amplifier, (*cis*-side) before forming the membrane. Usually membranes were formed in 10 mM salt solutions (both sides). Concentration gradients were established by adding small amounts of concentrated (1 M or 3 M adjusted to appropriate pH) salt solutions to the compartment connected to the amplifier (*cis*-side) while stirring. Equilibration reached its final value after 10–15 min of stirring. The values in the tables or data points in each figure represent an average of three or more membranes. The conductance of membranes (multichannel experiments) containing protein was 2 nS/mm$^2$ to 0.2 $\mu$S/mm$^2$ in 100 mM KCl. In control membranes without protein the conductance was 0.15 pS/mm$^2$.

## RESULTS

### Identification of AQ_1862 and sequence comparisons

A protein with a mass of 42 kDa could be purified from the dodecyl-$\beta$-D-maltoside solubilized membrane fraction of *A. aeolicus* by anion exchange and size exclusion chromatography followed by affinity purification on a Reactive Red dye ligand (Fig. 1). It is one of the most abundant proteins in the dodecyl-$\beta$-D-maltoside solubilized membrane fraction (Fig. 1, lane *1*). The protein was identified to be the ''hypothetical protein'' AQ_1862 (Swiss-Prot No. O67714) by matrix-assisted laser desorption ionization-time of flight mass spectrometry and peptide mass fingerprint (data not shown). Sequence analysis revealed the presence of an N-terminal signal peptide (SignalP (24)), which is cleaved behind residue number 23, and phenylalanine as the C-terminal residue. The removal of the N-terminal signal peptide could be confirmed by N-terminal sequencing (data not shown); the protein sequence starts with amino acid 24.

A sequence comparison (using BlastP (23) with standard settings) demonstrated homology to members of two families of $\beta$-barrel porins (Fig. 1 in the Supplementary Material). The first family is the SAP family (21). One member of the SAP family, the porin FmdC from *Methylophilus methylotrophus* (Swiss-Prot No. O05123), was first described as a protein encoded by a gene within the formamidase operon. It is induced by formamide, acetamide, and urea (28). The protein AQ_1862 has 25% identity (and 39% homology) for 286 aligned residues to FmdC from *M. methylotrophus* and 21% identity (and 38% homology) for 361 aligned residues to the putative FmdC (Swiss-Prot No. Q2DQD8) from *Geobacter uraniumreducens*. The second family is the

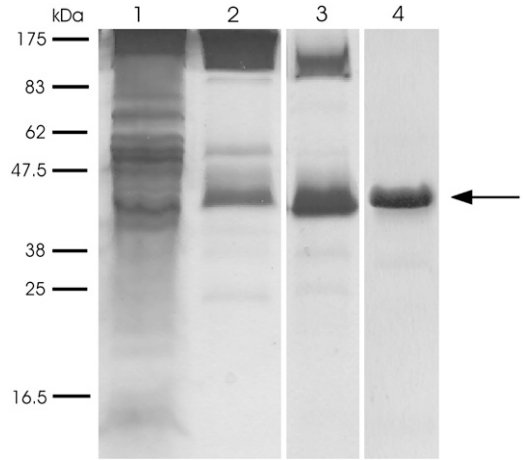


FIGURE 1 SDS-PAGE analysis of the AQ_1862 purification. (Lane *1*) Dodecyl-$\beta$-D-maltoside solubilized membrane; (lane *2*) elution from anion exchange chromatography; (lane *3*) elution from size exclusion chromatography; and (lane *4*) elution from reactive red 120 column. The arrow indicates the protein AQ_1862.

POP family (phosphate-selective porins O and P family, Pfam No. PF07396). The *Pseudomonas aeruginosa* porins OprP (Swiss-Prot No. P05695) and OprO (Swiss-Prot No. P32977) have been shown to be induced upon phosphate starvation (29). They are anion selective and show a single-channel conductance of 610 pS and 240 pS in 1 M KCl for OprO and OprP, respectively (30,31). The protein AQ_1862 has 23% identity (and 38% homology) for 267 aligned residues to the presumed phosphate-selective porin O and P from *Geobacter metallireducens* (Swiss-Prot No. Q39Y99). The sequence similarity to the *P. aeruginosa* porins is low: 16% identity (and 28% homology) for 374 aligned residues. However, tertiary structure prediction programs show high similarity to the fold of OprP. Similarity is also detected to the fold of general diffusion pathway porins OmpK36 from *Klebsiella pneumoniae* (Swiss-Prot No. Q48473) and PhoE from *E. coli* (Swiss-Prot No. P02932) and to other 16-stranded porins (HH-PRED (26)). Secondary structure prediction programs forecast a 18-stranded $\beta$-barrel (PRED-TMBB (32)). For arrangement of the $\beta$-sheets see Fig. 1 in the Supplementary Material.

## Multichannel experiments

To test whether AQ_1862 indeed is a porin we performed lipid bilayer experiments. No increase of the conductance was observed if AQ_1862 was added to preformed membranes even at high concentrations of protein (up to 50 $\mu$g/ml) which might destroy the membrane but did not induce a stable and defined conductance. On the other hand when small amounts of AQ_1862 ($\sim$1 $\mu$g) were added to the aqueous compartment of a cuvette and a membrane was formed in the presence of AQ_1862, the conductance of such membranes was much higher than in control experiments: up to $\sim$0.2 $\mu$S/mm$^2$ but only 0.15 pS/mm$^2$ in the absence of protein. The addition of detergent alone did not increase the conductance of the membrane. Once a membrane was formed, the conductance was very stable and incorporation of additional protein was a very rare event (Fig. 2, *A* and *B*). Fig. 2 *C* shows current voltage relations obtained for three membranes doped with AQ_1862—the same amount of AQ_1862 was added in each experiment—in 100 mM KCl at pH 7.5 containing different numbers of molecules and for a control membrane without addition of protein. The slope conductance is constant up to 60 mV, at higher voltages the slope increases slightly and becomes superlinear.

## Selectivity measurements

The selectivity of the conductance induced by AQ_1862 was studied by zero current membrane potential (zcp) measurements with concentration gradients of various electrolytes across the membrane. After the formation of a membrane doped with AQ_1862 the salt concentration on the *cis*-side (connected to the amplifier) of the membrane was increased up to 100 mM by adding small amounts of a concentrated stock solution (see Materials and Methods). The initial concentration was 10 mM on both sides. The zero current potential was measured either with a current amplifier applying a voltage to the *trans* side, which drives current to zero, or with a voltage amplifier.

The permeability ratio for cations over anions $P_a/P_c$, i.e., the selectivity of the macroscopic conductance, is obtained from the Goldman-Hodgkin-Katz equation (33)

$$\mathrm{zcp} = \frac{R \times T}{F} \ln\left(\frac{P_c c^{\mathrm{trans}} + P_a c^{\mathrm{cis}}}{P_c c^{\mathrm{cis}} + P_a c^{\mathrm{trans}}}\right),$$

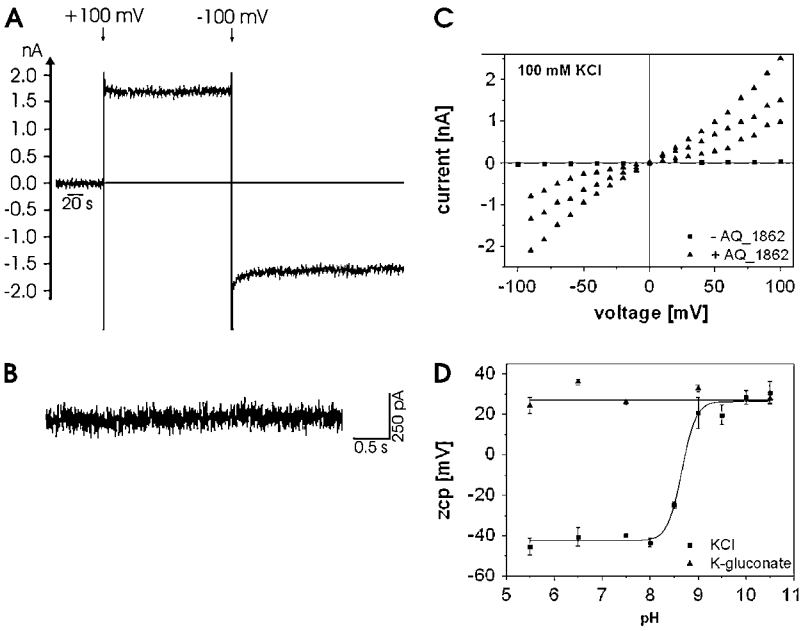


FIGURE 2 (*A*) Typical BLM experiment with AQ_1862 inserted into a diphytanoyl-phosphatidylcholine bilayer (0.1 M KCl on both sides, pH 7.5, 25°C) under conditions which favor multiple incorporation (see Materials and Methods). Switching the voltage from 0 to $\pm$100 mV induces a large current of 1.7 nA (conductance 17 nS). (*B*) Expanded current trace at +100 mV, same experiment as in *A*. (*C*) Current-voltage relations of membranes doped with AQ_1862 (▲) and a control membrane (■) without added protein (see text for details, 0.1 M KCl on both sides, pH 7.5). (*D*) The zero current potential (zcp) as a function of pH in the presence of KCl (■) or K-gluconate (▲). Each point represents the average of three membranes. For K-gluconate, the zcp is positive between pH 5.5 and 10.5 and pH independent (+35 mV). In the presence of KCl, the zcp is negative (−40 mV) below pH 8. Above pH 8 the zcp becomes more positive, i.e., the anion selectivity decreases, and above pH 9 the zcp approaches +35 mV, i.e., the membrane doped with AQ_1862 is cation selective as in the presence of gluconate.

where zcp is the zero current potential (reversal potential), $c^{cis}$ and $c^{trans}$ are the salt concentrations on the concentrated and the diluted side of the membrane, respectively, $P_a$ and $P_c$ are the permeability coefficients of the anion and the cation, respectively, and $R$, $T$, and $F$ have their usual meanings.

The observed zcp values and the calculated $P_a/P_c$ ratios are given in Table 1 for a variety of potassium salts with a 1:10 gradient (10 mM vs. 100 mM). The zcp changes from −40 mV for K-halides to +43 mV for K-propionate. This result indicates that the selectivity of the membrane switches from anion selectivity to cation selectivity when the size of the anion increases. The $P_a/P_c$ ratio decreases from 10 to 0.1 in the order fluoride, bromide, chloride, formate, nitrate, acetate, HEPES, gluconate, and propionate. Also, sulfate and phosphate have a low permeability as indicated by the zcp (Table 1).

To get further information on the permeability for cations we determined the zcp in the presence of a variety of chloride salt solutions. Table 2 shows that the membrane is anion selective for all tested chloride salts with the highest selectivity for chloride observed with the relatively large cations $Tris^+$ and $Rb^+$ and it is lowest with $Na^+$ and $Li^+$, suggesting that the permeability for cations is size dependent, too.

To investigate whether a titrable charged residue determines the selectivity of the presumed pore, the pH dependence of the zcp was studied. Fig. 2 *D* shows the pH dependence of the zcp for KCl and K-gluconate. The concentration gradient was 1:10 (10 mM vs. 100 mM) in both cases. In the presence of KCl the zcp is negative (~−40 mV) from pH 5.5 to pH 8.5, at higher pH the zcp changes its sign, and above pH 9.0 the zcp is positive (~+30 mV), indicating that the conductance changes from anion selectivity to cation selectivity. For K-gluconate the zcp is positive and pH independent between pH 5.5 and 10.5, i.e., the pore shows a pH-independent cation selectivity.

## Single-channel measurements

To obtain more detailed information on the ion selectivity and gating properties of AQ_1862, we attempted experiments with only one channel incorporated into the membrane. To increase the probability of incorporating only one channel, the area of the lipid membrane was reduced to 1% of the area used for multichannel experiments, and the final concentration of the protein added was reduced to 10%. Typically a stable conductance of ~1.4 nS in 100 mM KCl was observed under these conditions (>200 times the conductance of an undoped membrane). We will refer to this conductance as basic conductance; channel-like fluctuations of 0.25 nS were usually superimposed on this conductance. Fig. 3 *A* shows a typical experiment: switching the voltage from 0 to +100 mV induces a large current of 150 pA (indicated by 2). Superimposed on the large current step there are rapid outward fluctuations of ~25 pA (indicated by 1, see expanded scale Fig. 3 *B*). Both the basic and fluctuating conductance were observed simultaneously and eventually disappeared simultaneously (Fig. 3 *C*). Typically the magnitude of both conductances was stable for several hours.

Within the observation time no reversible closure of the basic conductance was observed. We coined the terms ''basic conductance'' and ''fluctuation'' (see Fig. 3 *D* for definition) instead of using the terms ''open state'' and ''substate'' because the latter terms imply that both states are attributed to the same conductance pathway, which is not necessarily the case (see below and Discussion). The conductance of the fluctuating component shows a voltage-dependent asymmetry. In ~75% of the cases the conductance is larger at positive than at negative voltages (see Figs. 3, *B* and *E*, and 7 *A*). The remainder shows the inverse voltage dependence. It is assumed that this asymmetry indicates an intrinsic property of the protein indicating some preferential incorporation. It is not clear why the incorporation is not random despite the fact that the membrane is formed after the addition of protein. For the figures only those experiments with the larger conductance at positive voltages were used. In contrast to the fluctuating conductance the basic conductance does not show any voltage dependence. Fig. 3 *E* shows current voltage relations of the basic and the fluctuating component. The basic conductance is 1.4 nS ± 0.3 nS (mean ± SD) in 0.1 M KCl (pH 7.5), whereas the conductance of the fluctuations is 0.24 nS ± 0.014 nS (Table 3).

**TABLE 1 Zero current potential (zcp) and $P_a/P_c$ ratios calculated from the corresponding zcps for different potassium salts at pH 7.5; concentration gradients were 1:10 (10 mM vs. 100 mM)**

| Electrolyte | zcp [mV] | $P_a/P_c$ |
|---|---|---|
| KF | −43 ± 0.7 | 11 |
| KBr | −41 ± 0.3 | 10 |
| KCl | −39 ± 3.5 | 8 |
| K-formate | −34 ± 2.0 | 6 |
| $KNO_3$ | −23 ± 1.1 | 3 |
| K-acetate | +9 ± 1.9 | 0.7 |
| K-HEPES | +9 ± 1.6 | 0.6 |
| K-gluconate | +26 ± 1.0 | 0.3 |
| $K_2SO_4$ | +35 ± 1.3 | |
| K-phosphate | +42 ± 2.3 | |
| K-propionate | +43 ± 1.0 | 0.1 |

**TABLE 2 Zero-current potentials (zcp) and calculated permeability ratios obtained with different $Cl^-$ salts at pH 7.5; the concentration gradient was 1:10 (10 mM vs. 100 mM)**

| Electrolyte | zcp [mV] | $P_a/P_c$ |
|---|---|---|
| LiCl | −33 ± 2.1 | 5 |
| NaCl | −33 ± 1.5 | 5 |
| KCl | −39 ± 3.5 | 8 |
| RbCl | −42 ± 3.4 | 10 |
| CsCl | −39 ± 1.5 | 8 |
| $NH_4Cl$ | −39 ± 4.2 | 8 |
| $CaCl_2$ | −37 ± 3.5 | |
| Tris-Cl | −42 ± 1.9 | 10 |

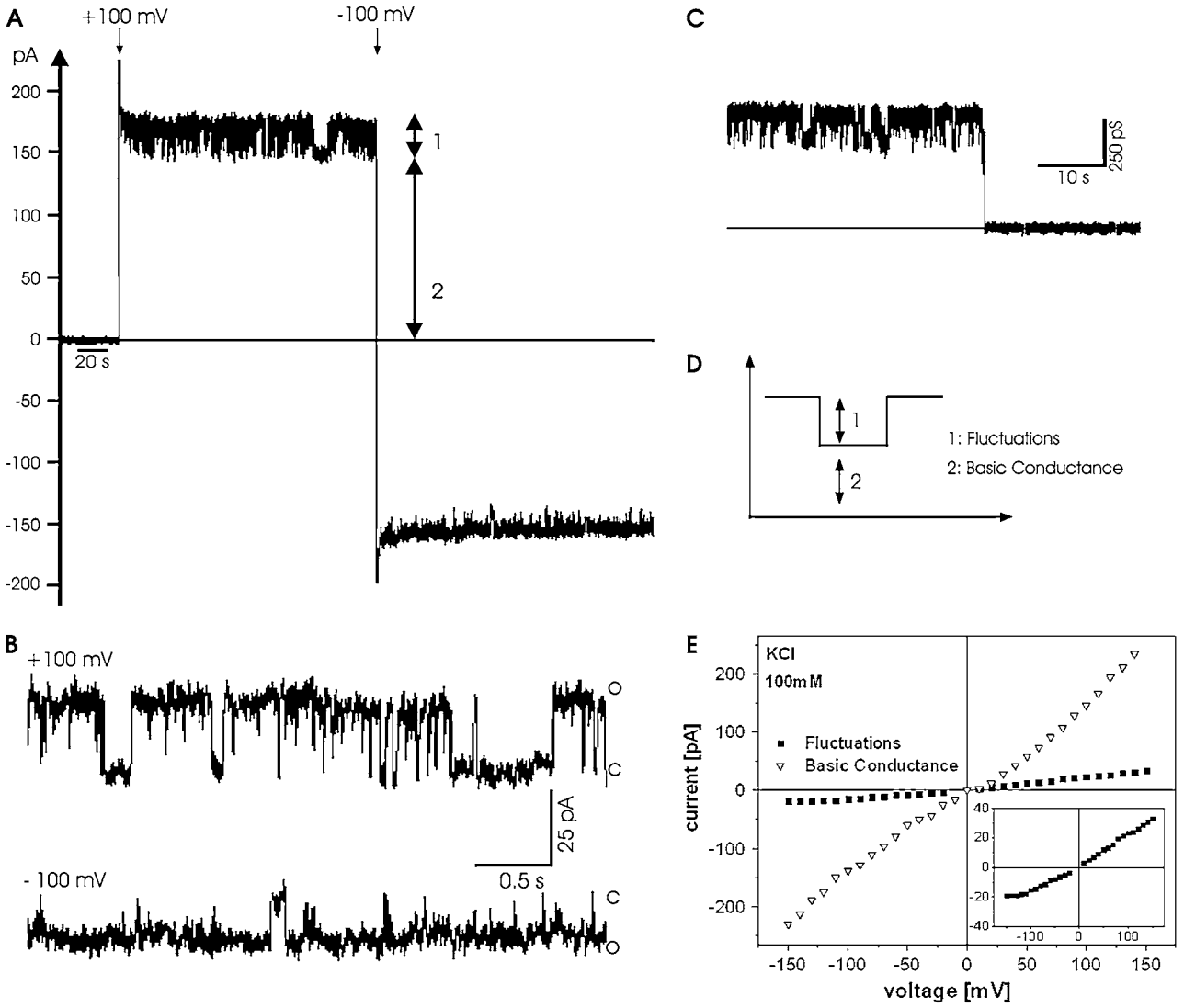


FIGURE 3 (*A*) Typical BLM experiment with a single event. AQ_1862 was inserted into a diphytanoyl-phosphatidylcholine bilayer (0.1 M KCl on both sides, pH 7.5, 25°C). Switching the voltage from 0 to ±100 mV induces a large current of 150 pA, here indicated by 2. Superimposed on the large current step there are rapid outward fluctuations of ~25 pA, here indicated by 1. (*B*) On the expanded scale, the fluctuations can be seen as channel opening and closing events. Note that the amplitude of fluctuations is smaller at negative voltage. (*C*) Simultaneous breakdown of the basic and fluctuating conductance (0.1 M KCl both sides, pH 7.5, +40 mV). (*D*) Definition of the fluctuating (*1*) and the basic (*2*) conductance. (*E*) Current-voltage relation of the basic (▽) and fluctuating (■) component in the presence of 0.1 M KCl on both sides (pH 7.5). The inset shows the current-voltage relation of the fluctuating component on an expanded scale.

Although both events are strongly correlated and therefore most likely related to the same molecular entity they have different properties with respect to ion selectivity, concentration, and pH dependence, as will be shown below.

## Size and selectivity of the basic and fluctuating conductance for cations and anions

To investigate the selectivity of the basic and the fluctuating conductance, single-channel experiments with AQ_1862 were carried out in a variety of electrolytes. Fig. 4 shows recordings of the fluctuations in the presence of different potassium salt solutions (100 mM, pH 7.5, +100 mV). The amplitude of the fluctuations is identical in all solutions independent of the anion. This result suggests that the fluctuations are cation selective. On the other hand the recordings obtained in halides or nitrate appear to be more noisy than those obtained in the presence of phosphate or organic anions. We suggest that the large noise is mostly based on the large basic conductance and the related thermal noise in the presence of small anions. As Table 3 shows, the basic conductance is much higher in the presence of small anions (e.g., halides) than in the presence of organic anions or phosphate and sulfate. In addition, it might be that the frequency of the fluctuations is influenced by the kind of anion, but this question has not been addressed in detail.

**TABLE 3 Size of the basic and fluctuating conductances for different electrolytes (mean ± SD); 0.1 M salt solutions on both sides, pH 7.5, 100 mV, 25°C**

| Electrolyte | Fluctuation [pS] | ± | Basic Conductance [pS] | ± |
|---|---|---|---|---|
| KF | 241 | 17.6 | 660 | 300 |
| KCl | 241 | 13.8 | 1380 | 300 |
| KBr | 233 | 25.9 | 1370 | 320 |
| $KNO_3$ | 247 | 14.1 | 1250 | 250 |
| K-phosphate | 224 | 4.7 | 75 | 60 |
| K-formate | 255 | 25.0 | 720 | 550 |
| K-acetate | 245 | 22.6 | 300 | 170 |
| K-propionate | 230 | 10.8 | 80 | 20 |
| K-gluconate | 241 | 13.2 | 190 | 160 |
| $NH_4Cl$ | 111 | 8.7 | 990 | 260 |
| $NH_4$-acetate | 116 | 8.7 | 210 | 220 |
| NaCl | 60 | 4.1 | 1440 | 320 |
| Na-acetate | 58 | 5.2 | 200 | 80 |
| Na-gluconate | 62 | 4.7 | 190 | 100 |
| LiCl | 41 | 3.3 | 960 | 300 |
| Li-acetate | 40 | 5.5 | 200 | 90 |
| RbCl | 235 | 28.6 | 1090 | 210 |
| Rb-acetate | 203 | 1.5 | 190 | 90 |
| CsCl | 29 | 5.7 | 750 | 210 |
| Cs-acetate | 32 | 7.1 | 220 | 100 |
| NMG-Cl | – | – | 200 | 100 |
| NMG-acetate | – | – | 20 | 10 |

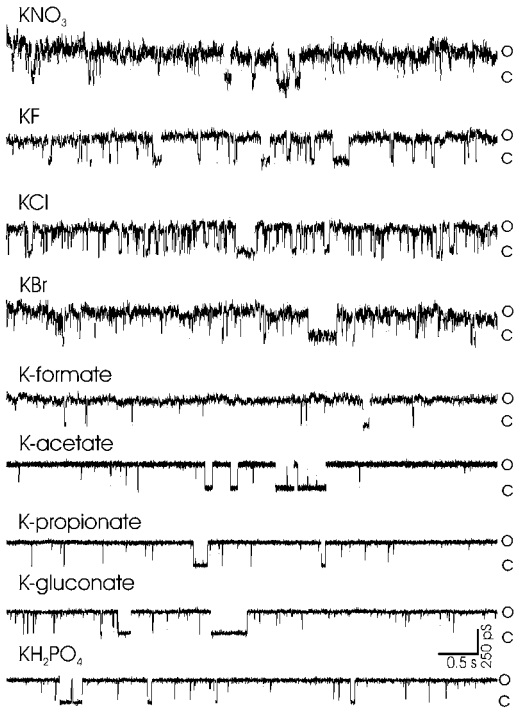


FIGURE 4 Current fluctuations recorded in different potassium salt solutions (0.1 M on both sides, 100 mV). The amplitude of the channel-like fluctuations is independent of the anion, but the traces are much noisier in the presence of small anions (see text for details). Open and closed states are marked with ''o'' and ''c'', respectively.

Table 3 shows the size of the fluctuating conductance and of the basic conductance at 100 mV and pH 7.5 for different electrolytes (100 mM on both sides). For each cation species tested ($K^+$, $Na^+$, or $Cs^+$) the magnitude of the fluctuating conductance does not depend on the anion species present (240 pS, 60 pS, and 30 pS, respectively). The rank order of the size of the fluctuating conductance for cations is $K^+ \approx Rb^+ > NH_4^+ > Na^+ \approx Li^+ \approx Cs^+$, 240 pS in $K^+$ to 30 pS in $Cs^+$. No fluctuations were observed in the presence of *N*-methylglucosamine (NMG) or $Ca^{2+}$. With respect to the basic conductance the picture is more complicated. Although in principle the anion determines the conductance, the data show that in some cases the cation has a strong effect on the conductance. E.g., all combinations of alkalines and chloride have similar elementary conductances (1–1.4 nS, 0.1 M) and the same is true for alkaline acetates (0.2–0.3 nS). On the other hand the conductances observed in the presence of NMG are extremely low: 0.2 nS for NMG-Cl and 0.02 nS for NMG-acetate, indicating that cations affect the permeability for anions of this pathway.

For the basic conductance the sequence for anions is $Cl^- \approx Br^- \approx NO_3^- > F^- >$ gluconate $\approx$ acetate $\approx$ phosphate $\approx$ propionate (100 mM, 100 mV, pH 7.5), decreasing from 1400 pS in $Cl^-$ to 80 pS in propionate. It should be noted that the variability of the basic conductance is larger than that of the fluctuating component, indicated by the large standard deviation.

The differential selectivity was investigated further by determination of the zcp in single-channel experiments (10 mM vs. 100 mM gradients using different electrolytes). Fig. 5 demonstrates exemplarily the different selectivity of basic conductance and fluctuations. Fig. 5 *A* illustrates the disappearance of the fluctuations at +40 mV indicating cation selectivity, whereas the basic conductance reverses its sign at negative potentials indicating anion selectivity. In Fig. 5 *B* current-voltage relations of the basic conductance and of the fluctuating component are shown. For all tested cations ($K^+$, $Na^+$, $Cs^+$, $NH_4^+$, $Rb^+$, $Li^+$) the zcp of the fluctuations is in the range of +40 mV independent of the type of anion. The zcp of the basic conductance is −45 mV for the relatively small anions chloride, nitrate, and formate, whereas it is close to zero (independent of the cation type) for acetate, gluconate, and propionate. It should be noted that anions which induce anion selectivity in macroscopic experiments generate high basic conductance and anion selectivity of this component in single-channel experiments. Anions which generate cation selectivity in multichannel experiments induce low basic conductance and nonselectivity of the basic conductance in single-channel experiments.

Because in multichannel experiments the zcp in the presence of KCl was strongly affected by pH, single-channel experiments were carried out at different pH values in solutions with a gradient of 10 mM vs. 100 mM KCl. The conductance of the fluctuating part and its zcp are mostly independent of the pH between 6.5 and 10.5 as depicted in Fig. 6 *A*, which shows a series of current-voltage relations.

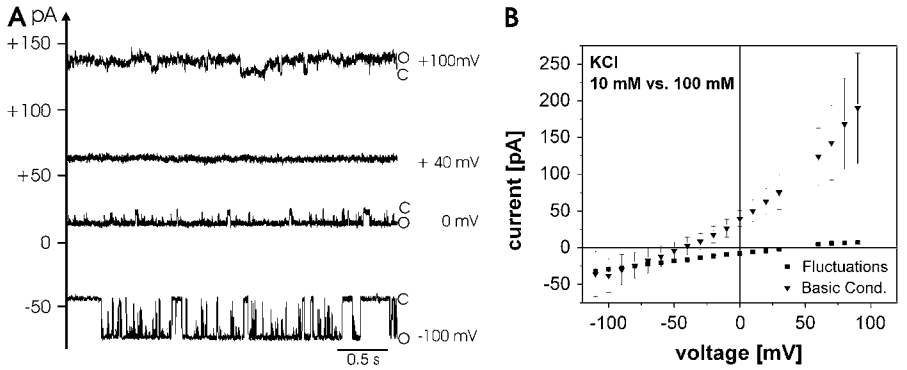


FIGURE 5 Current-voltage relation in the presence of an ion gradient (10 mM vs. 100 mM KCl). (*A*) Current traces at different applied voltages (right-hand side). ''o'' and ''c'' mark the open and closed states, respectively, of the fluctuating conductance. Note that the amplitude of the fluctuations disappears at +40 mV (zcp). Current attributed to the basic conductance may be estimated from the current level marked as closed state (*c*). This current component reverses sign at ~−45 mV: current-voltage relation of the fluctuating (■) and the basic (▼) conductance. The zero current potentials are +40 mV and −45 mV, respectively.

On the other hand the current-voltage relation of the basic component (Fig. 6 *B*) shows a remarkable shift of the reversal potential from anion selectivity at pH 6.5 (zcp = −40 mV) to nonselectivity at pH 10.5 (zcp = 0 mV).

## Concentration dependence and its pH dependence

The concentration dependence of the conductance was determined by forming a membrane in 10 mM salt solution and then increasing the salt concentration by adding small amounts from a concentrated stock solution to both sides. Fig. 7 *A* shows the concentration dependence of the fluctuating conductance for KCl at pH 7.5 and pH 10.5 and for K-acetate at pH 7.5 at ±100 mV. As mentioned above the conductance of the fluctuations is voltage dependent even in symmetric solutions. The conductances for both positive and negative voltages saturate at ~100 mM. A fit with the Michaelis-Menten equation yields a $K_m$ of ~9 mM for KCl. For all cations tested ($Na^+$, $K^+$, $Rb^+$, and $NH_4^+$ in combination with $Cl^-$ or acetate) the $K_m$ values of the fluctuating component are between 5 and 20 mM, indicating a concentration dependence that is rather insensitive to the cation species.

In contrast, the concentration dependence of the basic conductance depends on the type of anion. Since the basic conductance does not show any voltage-dependent asymmetry, only the results for +100 mV are shown. Fig. 7 *B* shows that the basic conductance at pH 7.5 increases linearly up to at least 500 mM KCl, the largest concentration measured. On the other hand in K-acetate or KCl at pH 10.5 this conductance component saturates at rather low concentrations ($K_m \approx 20$ mM, see *inset* in Fig. 7 *B*).

The difference in the concentration dependence of both conductance components in KCl explains why we never observed fluctuations at high KCl concentrations. The relatively small fluctuations which saturate at ~20 mM are not visible at high concentrations anymore because of the large noise related to the basic conductance at high concentration of small anions. All anions which induce a high basic conductance (Table 3) show a linear increase of this conductance with rising concentrations up to at least 500 mM, whereas the anions with low basic conductance (acetate, propionate, gluconate, and phosphate) saturate at low concentrations.

## Kinetic properties of AQ_1862

Both the basic conductance and the superimposed fluctuations are very stable and can be observed for several hours. Occasionally a simultaneous disappearance of both conducting components was observed (Fig. 3 *C*). A proper evaluation of the closed times of the fluctuating component is not possible because of the limited time resolution of the recording system (<300 Hz) and the number of brief openings. However, a rough estimate of the closed time indicates that the closed probability is pH dependent. The closed probability is ~20% at pH 6.5 and decreases to 1% at pH 10.5.

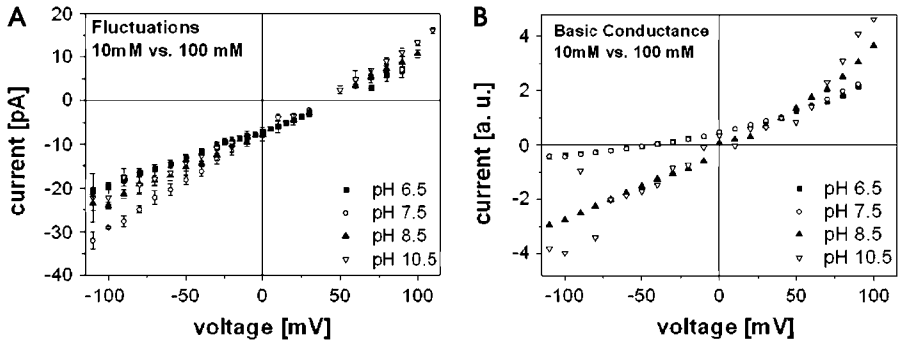


FIGURE 6 pH dependence of current-voltage relations of the fluctuating (*A*) and the basic (*B*) part of the conductance in the presence of a KCl concentration gradient (10 mM vs. 100 M). The zcp of the fluctuating component is pH independent between pH 6.5 to 10.5, whereas the zcp of the basic conductance is shifted from −45 mV at pH 6.5 to 0 mV at pH 10.5. In *B* the current is normalized to +40 mV.

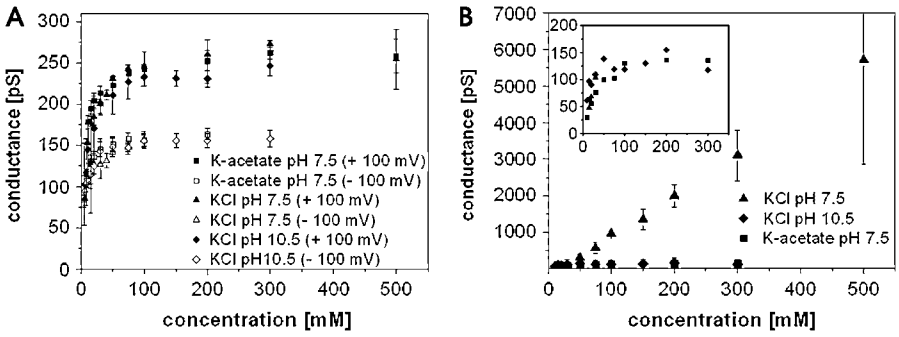


FIGURE 7 Concentration dependence of the fluctuating (*A*) and basic (*B*) conductance in the presence of K-acetate and KCl. The fluctuating component saturates at low salt concentration ($K_m \approx 15$ mM) irrespective of the anion. At negative voltages the conductance is smaller by ~1/3. Since the basic conductance does not show any voltage-dependent asymmetry, only the results for +100 mV are shown. The concentration dependence of the basic conductance differs for $Cl^-$ and acetate. In the presence of $Cl^-$, the conductance increases approximately linearly with the concentration, whereas in the presence of acetate or KCl at pH 10.5 it saturates at low salt concentration ($K_m \approx 20$ mM). In both cases, the conductance is voltage independent.

## DISCUSSION

In this study we describe the properties of the protein AQ_1862 from the marine hyperthermophile *A. aeolicus*. It shows features typical of a porin originating from a bacterial outer membrane: it has an N-terminal signal peptide, which has been cleaved off in the mature protein and a phenylalanine residue at its C-terminus (34). Sequence comparisons and fold predictions show that the protein has similarities to two families of phosphate and amide-specific porins and to OmpK36 from *K. pneumoniae* and PhoE from *E. coli*. Its presumed general function as a porin could be verified. Taking these results as well as its high abundance into account, we assume that the protein AQ_1862 is a porin originating from the outer membrane of *A. aeolicus*.

Before the properties of AQ_1862 are discussed in detail, it seems appropriate to discuss briefly the technique to incorporate the protein into the bilayer. If solved in 0.01% dodecyl-$\beta$-D-maltoside, the protein did not incorporate spontaneously in the preformed bilayer. The lack of spontaneous incorporation was confirmed with the detergent octyltetraoxyethylene. For concentrations between 0.2 and 1.8 $\mu$g/ml and observation times between 2 and 3 h, no spontaneous incorporation was detected, indicating that the behavior in dodecyl-$\beta$-D-maltoside is not an exception. We think that the method to form the bilayer in the presence of protein has some advantages: the lack of spontaneous incorporation over the time course of the experiment provides very convenient stability of the experiment. The disadvantage certainly is that incorporation cannot be observed as a step-like process which immediately gives information on the single unit conductance. However, this information was deduced from the minimal conductance observed in many experiments and from the spontaneous step-like disappearance.

The functional properties of AQ_1862 differ from the properties of the homologous proteins OprP, OprO, or PhoE in not showing any selectivity toward phosphate. Furthermore it differs from other outer membrane porins of the general diffusion pathway by showing two different conductances with different selectivities. There are several published examples for substates or ''residual conductances'' in porin conductances (15,16,35,36), but no detailed study on the selectivity has been carried out so far to our knowledge.

In multichannel experiments AQ_1862 is able to increase the conductance of a lipid membrane drastically. The conductance is anion selective at acidic and neutral pH and cation selective at basic pH. In the presence of large organic anions, cation selectivity is observed from pH 4 to pH 10. The macroscopic selectivity is dependent on the salt, varying from mainly anion selective in KCl to mainly cation selective in K-acetate. The macroscopic selectivity is similar to that of voltage-dependent anion channel (VDAC), which is also anion selective in KCl and cation selective in K-acetate (see Benz (37) for review).

For simplicity only results for KCl and K-acetate are discussed although similar data were obtained for other combinations of alkaline ions with halides and organic anions. More detailed insights into the properties of AQ_1862 were obtained with experiments designed to reduce the number of channels incorporated into the level of one channel. Although a step-like increment in conductance typical for the incorporation of porins could be observed only rarely, it is suggested that this conductance is caused by a single molecule. This suggestion is supported by the following observations: first, 1.4 nS is the lowest stable conductance in 100 mM KCl observed in more than 20 single-channel experiments; second, a shutdown of the basic and the fluctuating conductance in one step could be observed occasionally (Fig. 3); and third, in a few experiments multiples of this conductance occurred (not shown).

In single-channel experiments two different open states, a basic conductance and a fluctuating part, are observed. To emphasize some of the properties of AQ_1862 we compare its conductance pathways mainly with three porins which have been studied in detail: OmpF and PhoE from *E. coli* and OprP from *P. aeruginosa*. All three proteins form trimers. OmpF and PhoE show modest cation or anion selectivity, whereas OprP is highly selective for anions (1). The

conductance of the fluctuating part of AQ_1862 is cation selective ($P_{Cl}/P_K \approx 1{:}8$) with a conductivity sequence of $K^+ \approx Rb^+ > NH_4^+ > Na^+ \approx Li^+ \approx Cs^+$ and a maximal conductance of ~250 pS in 0.1 M KCl. Under the same conditions the selectivity of OmpF is less pronounced: $P_{Cl}/P_K \approx 1{:}3.6$ (2). The single-channel conductance for the monomer is 300 pS (38). The conductivity sequence for OmpF (38) and in aqueous solution (39) is $Cs^+ \approx Rb^+ \approx K^+ > Na^+ > Li^+$. It is remarkable that $Cs^+$ has the highest conductivity in OmpF and aqueous solution but the lowest in AQ_1862, although the other alkaline ions follow the same order. Another difference is that the fluctuating conductance of AQ_1862 saturates at low concentrations ($K_m \sim 10$ mM), whereas the conductance of OmpF increases linearly up to at least 1 M KCl (38).

The fluctuating conductance shows no pH dependence regarding selectivity, conductance, or saturation. It shows a voltage-dependent asymmetry: in one direction the conductance is one-third smaller than in the other. A similar effect was described for OmpF at lower ion concentrations (100 mM KCl) (40). Assuming that this asymmetry is an intrinsic property of the protein, this result indicates a preferential orientation of the incorporated porin. Possibly this asymmetry is due to a differential distribution of charges at both ends of the pore, leading to different ion concentrations at opposite openings of the pore which might result in different conductances. No voltage-dependent closing of the channel was observed as described for many porins including VDACs (41,42).

To our knowledge, fluctuations of this kind have only been described for OmpA (43). Mitochondrial VDACs also show channel-like fluctuations between their open (= basic conductance plus fluctuations) and their closed (= basic conductance) states (44,45). The selectivity of the mixed open state is anion selective and that of the closed state cation selective. The basic conductance shows a variety of peculiar features. The elementary conductance is rather high: 1.4 nS at 0.1 M KCl in combination with a relatively high selectivity, indicated by zero current experiments ($P_{Cl}/P_K \approx 8{:}1$) at neutral pH (7.5). Other porins of comparable molecular weight like the anion-selective porins PhoE and OprP have a much lower conductance, and porins with a comparable conductance have a much larger molecular weight (18). With respect to the selectivity for anions, the basic conductance of AQ_1862 is between PhoE and OprP (e.g., $P_{Cl}/P_K$: PhoE ≈ 3, OprP > 100), but the basic conductance is much higher than that of the classical porins: 1.4 nS vs. 0.2 nS for PhoE (46) or 0.1–0.16 for OprP (47). So far we have no reasonable explanation for this high conductance, which occurs simultaneously with high selectivity.

At pH 7.5 the basic conductance increases linearly with the concentration of KCl up to at least 0.5 M. An increase of pH from 7.5 to 10.5 leads to loss of selectivity ($P_{Cl}/P_K \approx 1{:}1$) in parallel with a decrease in the conductance and dramatic changes of the concentration dependence. At pH 10.5 the $K_m$ for KCl is ~20 mM, compared to a $K_m \gg 0.5$ M at pH 7.5. Saturation at low concentrations at pH 7.5 is also observed with K-acetate or other organic anions simultaneously with cation selectivity ($P_{anion}/P_{cation} = 1/8$). The concentration dependence of the conductance of PhoE is comparable to that of the basic conductance at neutral pH: in the presence of KCl the conductance of PhoE increases linearly up to 0.5 M. On the other hand the conductance of OprP saturates with a $K_m \approx 50$ mM, which is comparable to the concentration dependence of the AQ_1862 basic conductance at basic pH (10.5). With respect to the concentration dependencies observed in AQ_1862, it may be of interest that in OprP the mutation of a single lysine into glutamate relieves saturation of OprP (47): the concentration dependence of the conductance is linear up to 3 M KCl.

Intuitively the effect of large anions or high pH on the concentration dependence of the basic conductance is not easily understood if cations as well as anions move independently in the pore. One possibility is that there is interaction between cations and anions such that slowing down of anion translocation by size and/or modified interaction with charged residues also limits cation translocation. Another hint for the interaction between anions and cations is the reduction of the conductivity observed if $K^+$ is replaced by NMG. In 0.1 M KCl at pH 7.5, the basic conductance is ~1.4 nS. Replacing KCl with NMG-Cl reduces this conductance sevenfold to 0.2 nS (similar results were obtained with Tris-Cl). The profound effect of the size of the cation species also hints in the direction of an interaction between the flows of cations and anions. The concentration dependencies indicate that mobile anions as well as mobile cations are a prerequisite to enable high translocation rates, i.e., high conductivity.

At the moment it is not clear how these interactions are mediated. Im and Roux (48) described a model for ion translocation in the cation-selective porin OmpF based on structural data indicating interactions between cations and anions by the formation of ion pairs which facilitate the passage of anions. In this model cations move independently but anions need charge compensation by cations. The conductance pathway responsible for the basic conductance seems to be more complicated as both cations and anions seem to need ions of opposite charge to achieve high translocation rates.

Since no structures of homologous molecules are available and predictions of $\beta$-barrel structures are still challenging (note the large deviation in the $\beta$-barrel arrangement using two different prediction programs, Supplementary Material Fig. 1), an experimental determination of the three-dimensional structure of AQ_1862 is required to identify the amino acid(s) responsible for this effect. The selectivity observed in macroscopic experiments can be explained by the relative conductances and selectivities of the basic conductance and the fluctuating part. It is not determined by the properties of the different ions. The selectivity is also influenced by the

different open probabilities, i.e., the macroscopic selectivity is a time average, but this question has not been investigated in detail. The basic conductance in KCl at neutral pH is more than five times higher than the fluctuating conductance and anion selective; therefore, the macroscopic anion selectivity is determined by the basic conductance. The basic conductance in K-acetate and KCl at alkaline pH is in the same range as the fluctuating conductance and nonselective, whereas the fluctuating conductance shows cation selectivity so that a macroscopic cation selectivity is observed.

Although it cannot be definitively excluded that we observe two channels or one channel with different states, we think that there are good arguments in favor of a single molecule with two different conductance pathways. The single-molecule hypothesis is based on SDS-PAGE, which shows only one band, as well as by the statistics of the black lipid membrane (BLM) measurements, which show that both conductance components occur and disappear simultaneously (see Fig. 3). Nonetheless the two ''pores'' differ on a bundle of properties, i.e., the pH dependences of selectivity and conductance and concentration and voltage dependence of conductance. Therefore we suggest that AQ_1862 does not just posses one pore-like structure which can adopt two different states but that it rather contains two different conductance pathways.

From a physiological viewpoint it is apparent that *A. aeolicus* has a tightly regulated ion flux across the outer membrane. The bacterium is able to discriminate ions and even react to changes in the environment before ions reach the periplasm. For example, when the external milieu becomes more alkaline, the pores acquire cation selectivity and allow ions like $NH_4^+$ to pass and to neutralize the periplasm. Porins from evolutionary ancient bacteria have not been studied in detail so far, but obviously many features of AQ_1862 resemble the mitochondrial VDACs more than the porins of the proteobacteria.

## SUPPLEMENTARY MATERIAL

To view all of the supplemental files associated with this article, visit www.biophysj.org.

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