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Mechanisms of protein stabilization at very low pH

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ABSTRACT

Physicochemical properties of periplasmic loops exposed to low pH were determined for two membrane proteins from the bioleaching γ-proteobacterium *Acidithiobacillus ferrooxidans*. The selected proteins were an aquaporin and a potassium (K^+) channel and the properties evaluated were surface area, hydrophobicity and amino acid composition. Properties were mapped onto three dimensional protein models and subjected to Accessible Surface Area (ASA) analysis and Molecular Dynamics (MD) simulations. Results were compared to equivalent loops from homologous proteins derived from microorganisms that live in neutral pH environments. It was found that periplasmic loops of both the aquaporin and the K^+ channel protein of *At. ferrooxidans* have less surface area and exhibit greater hydrophobicity compared to their neutrophilic homologs. A reduction of the percentage of amino acids that are negatively charged (aspartic and glutamic acid) and an increase in the percentage of positively charged amino acids (histidine, arginine and lysine) was also observed in the periplasmic loops of the aquaporin and potassium (K^+) channel of *At. ferrooxidans*. Finally, an increase in the proline content of the loops of *At. ferrooxidans* was detected. Hypotheses for how these features could help stabilize the structure and function of proteins in extremely acidic condition are presented. It is noted that these proposed mechanisms are similar to those shown to be operational for the stabilization of thermophilic proteins. The data and hypotheses presented could help

provide a theoretical foundation for designing proteins with improved molecular functions for bioleaching and acid mine drainage remediation. It is also valuable information for understanding the molecular underpinnings and evolution of protein function at low pH.

1. INTRODUCTION

Whereas the molecular basis of thermostability of proteins has been extensively studied [1], [2], little is known about the mechanisms that underpin the stability and function of proteins that function at low pH. Of particular interest is how membrane embedded transporters maintain ion transport selectivity in the face of a delta pH that can be 10^7 times higher than at neutral pH, especially those that use protons as energy sources for the import or export of other ions. For example, it was recently shown that the gating of a formate channel (FocA) in *Salmonella typhimurium* operates in a pH-dependent manner [3]. Gating was shown to be mediated via the reorientation of N termini of the peptide chains and it was speculated that protonation of a histidine might be the basis for pH sensing. In addition, preliminary information is emerging for proteins that function at pH 3-4 from *Alyciclobacillus acidocaldarius* [4, 5], *Bacillus sp. Ferdowsicous* [6] and *Scytalidium acidophilum* [7], in which is was observed that positively charged and polar residues tend to be more frequent on the surface of these proteins and, conversely, a reduction of negatively charged amino acids was detected. However, it is not yet known if these mechanisms are also exploited by proteins that function at very low pHs including as low as pH0.

Regarding mechanisms for protein stabilization that operate at extremely low pH, defined here as <pH3, increased hydrophobicity is emerging as an important candidate. For example, increased hydrophobic interactions have been suggested to stabilize protein polymerization in AcoP that is required for cytochrome *c* oxidase (C*c*O) function [8]. Also, it has been proposed that proline residues can promote the stability of HIPIP in *Acidithiobacillus ferrooxidans* [9]. Higher levels than average of proline have also been reported for cytochrome c4 [10] and rusticyanin in *At. ferrooxidans* [11].

In an earlier effort to address the issue of how proteins function in extremely acidic environments (pH1), we generated models for two transmembrane proteins from the acidophile *At. ferrooxidans* that are predicted to be channels for water (model AqpF) and potassium (model KcnA), respectively [12]. AqpF was shown to have features in common with aquaporins from neutrophilic organisms including the average water occupancy of the channel and the characteristic bi-polar water orientation within the selectivity filter that reduces the intrusion of protons. However, it also exhibited particular features that might help explain its adaption to low pH, such as short periplasmic loops and the presence of an asparagine (*Asn*39) residue as part of the selectivity filter. The latter could enhance proton-blocking capability and so be critical for function at low pH. Regarding the low pH adaption of the K+ channel, it was shown it does not expose ionizable amino acids to the external surface, presumable because the protonation of these residues at pH 1 would be deleterious. The selective K+ channel also exhibited more basic residues than neutrophilic orthologs that would help protect against protonation. In this paper, we extend these models to include predictions for the surface area, hydrophobicity and presence or absence of charged amino acids and prolines in the periplasmic loops of aquaporin and the K+ channel protein of *At. ferrooxidans*. These properties are compared with those of homologous regions from neurtophilic microorganisms in order to suggest mechanisms for stabilization of proteins at very low pH.

2. MATERIALS AND METHODS

Comparative homology modeling of a predicted *At. ferrooxidans* ATCC 23270^{TS} potassium (K^+) channel (Kaf) and aquaporin (AqpF) proteins was performed using Modeler v4.0 [13] and ICM Molsoft [14] using the PDB 2f2b (1.68 Å) from *Methanobacterium thermoautotrophicum* and the PDB 1k4c (2 Å) from *Streptomyces coelicolor* as templates, respectively. Multiple sequence alignments of proteins were carried out using ClustalW [15]. Structures and models were analyzed using Procheck [16]. The best AqpF tetramer model was selected to be used in MD simulation. The molecular dynamics simulation was carried out using the NAMD [17] program with CHARMM27 parameter set [18]. The homotetrameric functional K^+ channel was built using the tool STAMP structural alignment in VMD software [19].

Both *At. ferrooxidans* models were submitted to ADIT validation server from PDB Database [20]. The coordinates were evaluated in terms of format coordinates, close contacts, bond distances and angles, torsion angles, chirality and missing atoms. Crystal structure coordinates for the aquaporin and K+channel were extracted for *Escherichia coli* (agpZ) and *Streptomyces lividans* (3eff) respectively from the Protein Data Bank [20]. All proteins models were submitted to suite of tools at BLUESTAR STING to evaluate the ASA (Accessible Surface Area), Hydrophobicity and ESP (Electrostatic Surface Potential) values of periplasmic predicted loops [21].

Hydrophobicity indices were deduced according to amino acid hydrophobicity values [22, 23] and weighed by accessibility to solvent.

3. RESULTS AND DISCUSSION

The predicted cell membrane locations and orientations of an aquaporin and a potassium (K+) channel from *At. ferrooxidans* and the loops that are exposed to the low pH of the periplasm and that are the subject of this study are indicated in Figure 1.

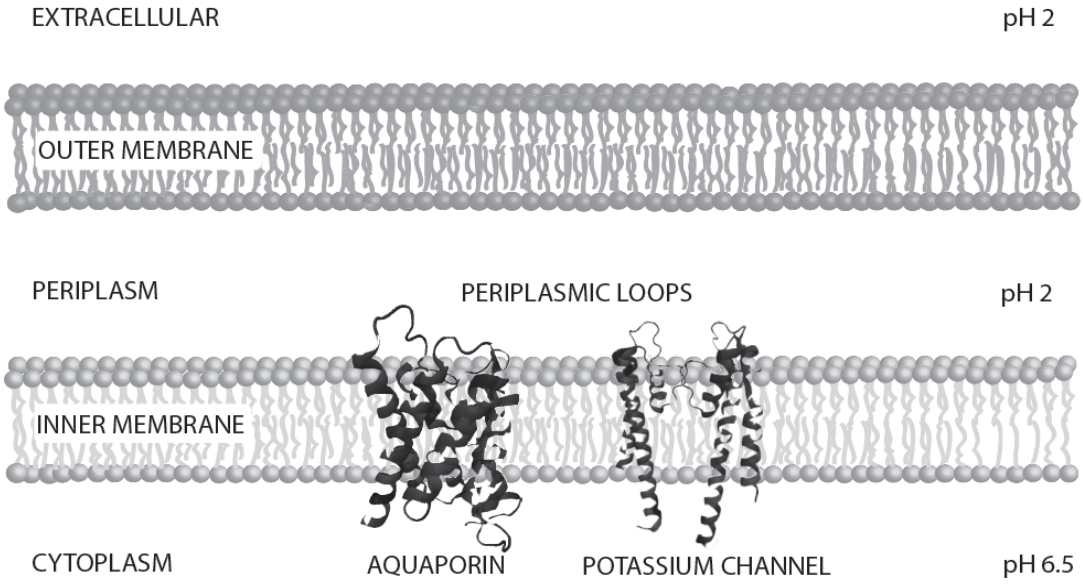

Figure 1. Scheme of the cell compartments of the Gram negative bacterium *At. ferrooxidans*, showing the predicted locations of an aquaporin and K+ channel protein embedded in the cell membrane, indicating the loops exposed to the low pH of the periplasm that are the subject of this study.

Aquaporins are homotetrameric proteins involved in the uptake of water into the cell [24]. Each monomer has six loops, four of which are exposed to the cytoplasm (pH 6.5) and three towards the periplasm (pH 2) [25]. The K^+ channel is typically composed of homotetramers with some exceptions found in eukaryotes [26]. Bacterial K^+ channels show a core composed of two transmembrane segments TM1 and TM2 and a short pore that forms the selectivity filter. Between TM1 and TM2, there is one loop exposed to the periplasm [27].

3.1 Surface area and hydrophobicity

Table 1 shows the calculated surface areas of the exposed loops for aquaporin and the K+ channel of *At. ferrooxidans* compared to the homologous regions in proteins from *E. coli* and *S. lividans*. The latter two microorganisms were chosen for comparison because (i) their periplasmic loops are exposed to neutral pH and (ii) their aquaporin and K^+ channel protein have crystal structures [28, 29] allowing detailed comparisons to made at the atomic level. The calculated total areas of the exposed loops are less in both aquaporin and the K^+ channel of *At. ferrooxidans* compared to the equivalent proteins in *E. coli* and *S. lividans*, with ratios of 1856/2389 (= 0.78) and 620/922 (= 0.67) for the aquaporin and K+ channel protein, respectively. In addition, the number of amino acids is less in both cases for *At. ferrooxidans* with ratios of 21/37 (= 0.57) and 9/14 (= 0.64), respectively. Thus, we conclude that the periplasmic loops of the *At. ferrooxidans* proteins are shorter and have smaller surface areas exposed to solvent than their neutrophilic counterparts. A trend to have shorter exposed loops is also found in proteins from thermophilic microorganisms and this property has been proposed to contribute to contribute towards entropic stabilization of the loops by increasing amino acid contact density [1].

Table 1. Surface properties of loops exposed to the periplasm of (A) an aquaporin and (B) a K^+ channel of the acidophile *At. ferrooxidans* compared to the equivalent loops of two neutrophilic microorganisms, *E. coli* and *S. lividans*, respectively.

Model ID	Organism (pH environment of exposed loops)	Surface area (Å^2)	Number amino acids	Hydrophobic index
(A) Aquaporin				
aqpF	*At. ferrooxidans* (pH 2)	1856	21	1.48
aqpZ	*E. coli* (pH 7)	2389	37	0.9
(B) K+ channel				
Kaf	*At. ferrooxidans* (pH 2)	620	9	1.25
3eff	*S. lividans* (pH 7)	922	14	0.78

The hydrophobic index for the periplasmic loops of the four proteins was calculated as described in Materials and Methods. The periplasmic loops of the aquaporin and K^+ channel protein of *At. ferrooxidans* have substantially more hydrophobicity compared to the equivalent loops in the two neutrophilic microorganisms (Table 1). Increased hydrophobicity would tend to repel protons thus protecting the entrance to the channel and pore of the aquaporin and K^+ channel. It has been previously reported that, in

contrast to other organisms, the K^+ channel protein of *At. ferrooxidans* contains an aspartic residue in the selectivity filter which, at pH 1, would probably be protonated promoting the solvation of the potassium ion [12]. This, in turn, would facilitate the discrimination between potassium and protons in the selectivity filter. We hypothesize that the observed increase in hydrophobicity of the periplasmic loops would help stabilize the structure around the selectivity filter and thus aid in proton repulsion.

3.2 Charged amino acids and proline content in periplasmic loops

The percentage of positively charged amino acids (arginine, histidine and lysine), negatively charged amino acids (glutamic and aspartic acid) and proline were calculated for the exposed loops of the aquaporins and the K+ channel of *At. ferrooxidans*, *E. coli* and *S. lividans* (Figure 2). Both the aquaporin (Figure 2A) and the K+ channel (Figure 2B) periplasmic loops of *At. ferrooxidans* contain a higher percentage of positively charged amino acids and a lower percentage of negatively charged amino acids compared to the homologous regions of the periplasmic loops of the two neutrophilic microorganisms. A similar finding has been reported for the exposed loops of a subtilisin-like protease from the thermophile *Thermoanaerobacter yonseiensis* [30].

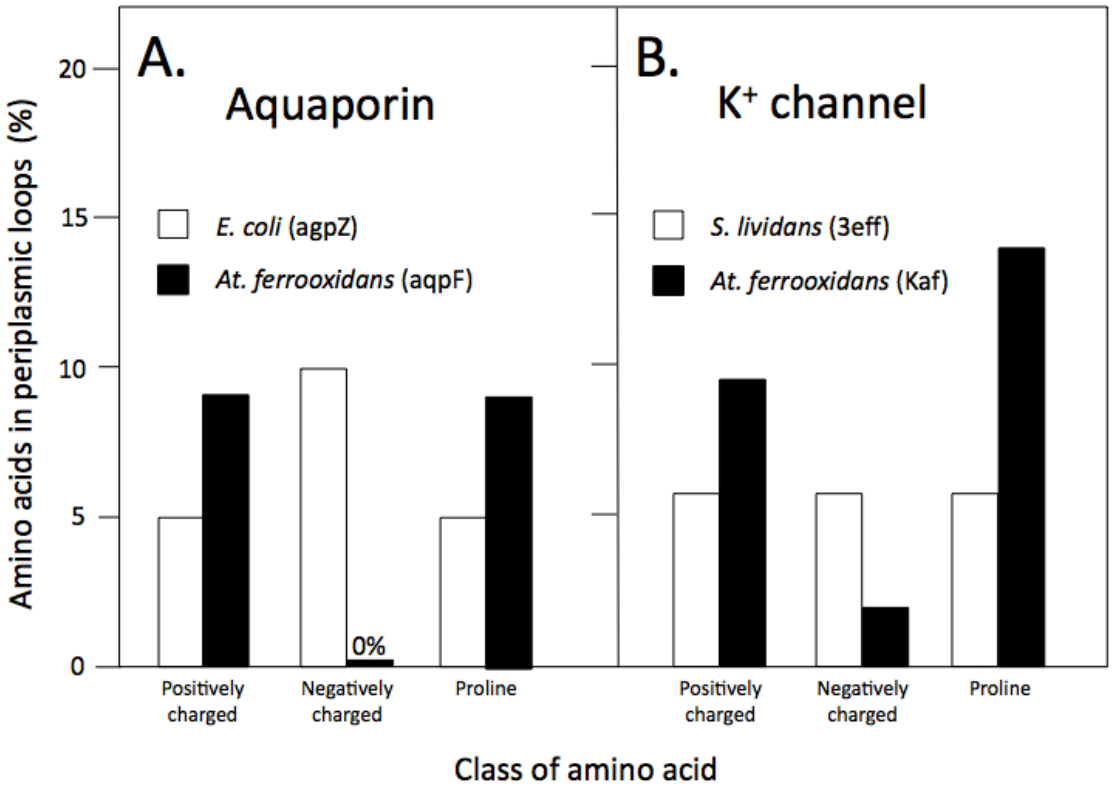

Figure 2. The frequency (%) of positively charged (arginine, histidine and lysine) and negatively charged (glutamic and aspartic acid) amino acids in the periplasmic loops of (A) aquaporin and (B) the K+ channel of *At. ferrooxidans* compared to the homologous regions of these proteins from the neutrophilic microorganisms *E. coli* and *S. lividans,* respectively.

In acidic conditions (pH 2), aspartate and glutamate could be in a protonation-deprotonation equilibrium (pK 3.6 and 4.3 respectively) leading to destabilization of folds. This suggests that the reduced content of these amino acids in the loops of *At. ferrooxidans* is an adaption to minimize this potential problem. The observed increase

in positively charged amino acids could be an additional strategy to stabilize proteins at low pH. Lysine and asparagine at pH 2 are expected to be protonated and would aid in the electrostatic repulsion of protons. An alternate hypothesis is that the positively charged amino acids could form hydrogen bonds or salt bridges with negatively charged amino acids to maintain the conformation of the loops. However, our models show that there are no readily accessible negatively charged residues in the neighborhood of the positively charged amino acids, reducing the likelihood that this mechanism is important in stabilizing the periplasmic loops of the aquaporin and K^+ channel.

Regarding the proline content, higher frequencies were found in the periplasmic loops of the two proteins from *At. ferrooxidans* compared to homologous regions from the neutrophilc microorganisms (Figure 2). A similar increase in proline content has been observed in proteins from thermophiles and it was suggested that proline can increase thermostability as a result of its ability to increase amino acid interactions [31–34]. It is hypothesized that a similar mechanism exists in acidophiles whereby proline, by virtue of its ability to potentiate more amino acid contacts, can assist in stabilization of protein structure at low pH.

3.3 Critical placement of two prolines.

In addition to the general mechanism whereby prolines can potentially increased stabilization, we propose that two of the prolines in the K+ channel of *At. ferrooxidans* carry out a local role in the stabilization of the lobby or entrance zone of this protein. The lobby has a critically located aspartic acid that is part of the characteristic GYGD motif found in many K^+ channel proteins. This aspartic acid in the K+ channel of other organisms aids is the deprotonization of the solvated K^+ ion. However, at pH2 this residue would be expected to be protonated. We propose that the additional two prolines found in the *At. ferrooxidans* K^+ channel help maintain a very hydrophobic environment repelling hydrophilic molecules like water that could protonate the aspartate residue and block the potassium desolvatation.

4. CONCLUSIONS

Molecular modeling of two membrane embedded proteins, a predicted aquaporin and K+ channel, from *At. ferrooxidans* reveals general and specific properties that we propose help stabilize the periplasmic loops of these proteins at very low pH (<pH3). The general properties include (i) reduced loop size and loop surface area, (ii) increased hydrophobicity, (iii) increased positively charged amino acids and decreased negatively charged amino acids and (iv) increased proline content. The specific properties include the occurrence of critically located amino acids. Many of these properties have also been suggested to stabilize proteins against heat denaturation in thermophilic microorganisms. Knowledge of the properties that help stabilize proteins at low pH is not only useful for understanding protein function and evolution but is also a prerequisite for redesigning proteins for biotechnological applications.

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