An experimental point of view on hydration/solvation in halophilic proteins

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Introduction

Salinibacter ruber (Sr) is a halophilic bacterium that was isolated from saltern crystallizer ponds in Spain (Antón et al., 2002). In contrast to most bacterial species that equilibrate osmotic pressure with compatible solute, S. ruber accumulates high KCl concentration within its cytoplasm, an adaptive strategy similar to that of halooarchaea (Halobacteriaceae) (Oren, 2002). S. ruber genome sequence has revealed some interesting characteristics related to haloadaptation: numerous lateral gene transfers from halooarchaea and a mean pI value of 5.2 of its whole proteome (Mongodin et al., 2005). This proteomic pI shift toward low values, which is typical in halooarchaea, is the consequence of an enrichment of Asp and Glu residues and is considered an adaptive signature of proteins facing high salt concentration (Oren, 2013). However this explanation has been recently challenged by the characterization of a bacterium (Halorodospira) that does not accumulate high KCl concentration in its cytoplasm and has nonetheless a high acidic proteome (Deole et al., 2013). Among the few cytoplasmic enzymes isolated from S. ruber (Bonete et al., 2003; Madern and Zaccai, 2004), the tetrameric malate dehydrogenase (MalDH) remains the most extensively characterized, at the biochemical and structural level (Coquelle et al., 2010). As observed for non-halophilic counterparts, this halophilic enzyme does not require salt to maintain its conformational stability. However, the Sr MalDH structure revealed an acidic amino acids enriched surface, typical to that observed for a halophilic enzyme, which is responsible for a favorable change of solubility in high concentration of salts (Coquelle et al., 2010).

According to the solvation-stabilization model for halophilic protein (Madern et al., 2000; reviewed in Zaccai, 2013), high salt concentrations exert a major selective pressure through a strong impact on protein solubility. In order to compete against this deleterious effect of salts, halophilic proteins stay highly soluble by maintaining a solvation envelope composition as close as possible as the composition of the bulk. This model is based on biophysical measurements that have shown that a halophilic protein recruits a solvation envelope of high ionic concentration (Costenaro et al., 2002; Ebel et al., 2002). In the solvation-stabilization model, surface acidic amino acids are suggested to be responsible for this particular solvent organization. Even if several structure of halophilic protein have been solved (Prolow et al., 1996; Richard et al., 2000; Bieger et al., 2003; Irimia et al., 2003; Zeth et al., 2004; Besir et al., 2005; Britton et al., 2006; Winter et al., 2009; Yamamura et al., 2009; Wende et al., 2010; Bracken et al., 2011), attempts to describe how the solvation shell of a halophilic protein interacts with acidic residues using X-rays crystallography is still a challenge.

In our follow-up crystallographic study on Sr MalDH (Coquelle et al., 2010); we determined the direct effect on solvent organization due to its acidic surface, by using a comparison with a non-halophilic counterpart. For this purpose, we solved de novo the crystal structure of the non-halophilic Chloroflexus aurantiacus (Ca) MalDH at 1.7 Å resolution. It allowed the determination of a hydration shell consisting in 945 water molecules, which cluster themselves in large networks of structured water through pentameric/hexameric polygons. Direct and indirect effects of acidic
amino acids substitutions, avoiding the formation of structured water in Sr MalDH are described here through the comparison with Ca MalDH. The data are analyzed with respect to the solvation-stabilization model for halophilic protein. In particular, we underline that difference in hydration-solvation characteristics should always be kept in mind while analyzing the solvation layer of a halophilic protein, using X-ray crystallography, or any other techniques.

**MATERIALS AND METHODS**

**PROTEIN PRODUCTION AND PURIFICATION**

Ca MalDH overexpression was done accordingly to Dalhus et al. (2002). The cells were lysed by sonication in a 50 mM Tris-HCl buffered at pH 7. The crude extract was incubated for half an hour at 70°C and centrifugated for 15 min at 17,000 g. The soluble portion of the extract was loaded on a Q sepharose column equilibrated in 50 mM Tris-HCl buffer at pH 7. The protein was eluted using a linear gradient of 0–1 M NaCl. Fractions containing theuble portion of the extract was loaded on a Q sepharose column equilibrated in 50 mM Tris-HCl buffer at pH 7. The protein was eluted using a linear gradient of 0–1 M NaCl. Fractions containing the active portion of the extract was loaded on a Q sepharose column equilibrated in 50 mM Tris-HCl buffer at pH 7. The protein was eluted using a linear gradient of 0–1 M NaCl. Fractions containing the active portion of the extract was loaded on a Q sepharose column equilibrated in 50 mM Tris-HCl buffer at pH 7. The protein was eluted using a linear gradient of 0–1 M NaCl. Fractions containing the active portions of the extract were pooled and concentrated by centrifugation using an Amicon PM30. They were deposited on a Sephacryl S300 gel filtration column (1 × 100 cm) and then eluted using an isocratic buffer of 50 mM Tris-HCl buffered at pH 7. The purified fractions were concentrated at 20 mg/ml and stored at 4°C.

**CRYSTALLIZATION**

Crystallization was performed by vapor diffusion using the hanging-drop method at 293 K. Native Ca MalDH crystals (∼500 × 400 × 400 μm³) were grown within 2 days by mixing 1.5 μL of 20 mg·mL⁻¹ protein solution and 1.5 μL of 4–14% PEG 400, 100 mM sodium acetate buffer at pH 4.6 and 40 mM cadmium acetate reservoir solution. Ca MalDH derivative crystals were obtained by a 10 s soaking of a native crystal in a 2.0 μL solution equivalent to the mother liquor containing 100 mM of GdHPDO3A lanthanide complex (Girard et al., 2003). The crystal was quickly back-soaked in 2.0 μL of the corresponding reservoir solution without the lanthanide complex.

Prior to data collection, native and derivative crystals were cryo-cooled in liquid nitrogen using mother liquor containing 25% PEG 400 as cryo-protectant.

**DATA COLLECTION AND DATA PROCESSING**

Gd-derivative data were collected on a Nonius FR591 X-Ray home source (1.541 Å). Native data were collected on the FIP-BM30A beamline at the ESRF (Grenoble, France) with the X-ray beam wavelength set to 0.979 Å. Diffraction frames were integrated using the program XDS (Kabsch, 2010) and the integrated intensities were scaled and merged using the CCP4 programs SCALA and TRUNCATE (Winn et al., 2011) respectively. A summary of the processing statistics is given in Table 1.

Ca MalDH crystals belong to the P3₁21 space group with one A-D dimer per asymmetric unit leading to a solvent content of 49.5%.

**EXPERIMENTAL SIRAS PHASING**

Ca MalDH structure was determined de novo by the SIRAS (Single Isomorphous Replacement with Anomalous Scattering) method. As shown in Table 1, the high value of Rano clearly indicated the presence of GdHPDO3A complex binding sites, which was then confirmed by inspection of the anomalous Patterson map. Gadolinium positions were determined within the asymmetric unit using the program SHELDX (Sheldrick, 2010). Heavy-atom refinement and initial phasing were performed using the program SHARP (Bricogne et al., 2003). Phases from SHARP were improved by density modification using the CCP4 program DM (Cowtan, 2006) leading to an initial model consisting of 552 over the expected 618 A-D dimer residues.

**REFINEMENT AND WATER MOLECULES BUILDING**

The model was manually completed and improved in COOT (Emsley et al., 2010) prior to refinement with PHENIX (Adams et al., 2010). This model was then optimized through iterative rounds of refinement and model building. At the end stages of the refinement, TLS was used with TLS-groups determined with the TLSMD server (Brünger, 1992; Painter and Merritt, 2006a,b). The 1.7 Å resolution Ca MalDH final model consists in the complete (N-terminus, C-terminus and catalytic loop) residues sequence for each monomer of the Ca MalDH A-D dimer. The analysis of this final model (Table 2) showed no residues in disallowed regions of the Ramachandran plot (99.7% in preferred regions).

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**Table 1 | Data collection and processing statistics.**

| Data set                          | GdHPDO3A derivative | Native         |
|----------------------------------|---------------------|----------------|
| λ (Å)                            | 1.541               | 0.979          |
| Space group                      | P3121               |                |
| Cell parameter (Å)               | a = 106.77, c = 103.53 | a = 106.23, c = 102.57 |
| Resolution (Å)                   | 19.63–1.90 (2.00–1.90) | 19.70–1.70 (1.79–1.70) |
| Unique reflections               | 54120 (7772)        | 66873 (10178)  |
| Rmerge (%)                       | 5.0 (23.7)          | 8.4 (35.6)     |
| Rpим (%)                        | 1.9 (8.5)           | 4.4 (20.3)     |
| Rano (%)                         | 3.6 (9.6)           |                |
| I/σ(I) (d)                       | 12.4 (3.3)          | 6.3 (2.2)      |
| Completeness (%)                 | 98.8 (99.1)         | 93.7 (95.8)    |
| Multiplicity                     | 10.6 (9.8)          | 4.2 (3.9)      |

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**REFINEMENT AND WATER MOLECULES BUILDING**

The model was manually completed and improved in COOT (Emsley et al., 2010) prior to refinement with PHENIX (Adams et al., 2010). This model was then optimized through iterative rounds of refinement and model building. At the end stages of the refinement, TLS was used with TLS-groups determined with the TLSMD server (Brünger, 1992; Painter and Merritt, 2006a,b). The 1.7 Å resolution Ca MalDH final model consists in the complete (N-terminus, C-terminus and catalytic loop) residues sequence for each monomer of the Ca MalDH A-D dimer. The analysis of this final model (Table 2) showed no residues in disallowed regions of the Ramachandran plot (99.7% in preferred regions).
residues of the N- and C- termini have been modeled in each numbering of 4BGT) covering the catalytic site, as well as the value of 0.42 Å for 594 A-D dimer superimposed residues.  

The structure of MalDH enzyme was determined at 1.7 Å resolution using SIRAS phasing. The asymmetric unit contains a large number of water molecules: 680 for the equivalent 1GUY model has previously been published (Dalhus et al., 2002), and thus will not be further described in this study. The striking new feature in our model is the incredibly large number of modeled water molecules, i.e., 945 for the dimer A-D, which allows a detailed analysis of water organization.

Sr MalDH shares more than 72% of sequence similarity with its non-halophilic counterpart Ca MalDH. The Sr MalDH model was obtained at a resolution of 1.55 Å, and also contains a large number of water molecules: 680 for the equivalent Ca MalDH A-D dimer (Coquelle et al., 2010). The overall structural similarity between one monomer of Sr and Ca MalDHs led to a RMSD of about 0.6 Å for 258 superimposed Ca.

Therefore, these two structures of excellent resolution, with a large number of water molecules in their solvent layers, provide a unique combination to finely compare the water organization at their surface.

**COMPARISON OF HALOPHILIC AND NON-HALOPHILIC HYDRATION PATTERNS**

A detailed analysis of the geometry of the 945 water molecules surrounding the dimeric Ca MalDH model (distance and angle) was performed and is presented in Table 3. It is outside the scope of this study to describe in great details both the geometry and interactions with the protein of all these water molecules. The role of water molecules in the folding process and stabilization of proteins has been well described in a work based on a larger set of proteins (Matsuoka and Nakasako, 2009). The most interesting feature of the water molecules in Ca MalDH structure is that 28% of them are organized in polygons (pentagons or hexagons), which can form extended clusters (Figure 2). These polygons are only observed at the surface of apolar residues. Geometrical properties of these polygons (Table 3) are in good agreement with those determined from a large statistical study using high-resolution structures (Lee and Kim, 2009).

Based on Ca MalDH water analysis, a careful inspection of the halophilic Sr MalDH hydration layer at the surface of the protein was performed to detect any water polygon. Even though 43% of Sr MalDH water molecules were considered to be superimposable with those from Ca MalDH (using a cut off distance of...
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Table 3 | Water statistics for dimer AD of Ca MalDH.

| Statistic                                      | Value          |
|------------------------------------------------|----------------|
| Number of water molecules                      | 945            |
| Water per residues                             | 1.57           |
| Water molecules involved in polygons           | 28%            |
| 76 polygons: 10 hexagons and 66 pentagons      |                |
| Size of clustered polygons                     | Up to 15       |
| Planar polygons                                | 64%            |
| Distance between surface residues and polygons (in Å) |         |
| Minimal                                       | 2.58           |
| Maximal                                       | 4.02           |
| Average                                       | 3.23           |
| Distance between two water molecules forming pentagons (in Å) |         |
| Minimal                                       | 2.11           |
| Maximal                                       | 4.19           |
| Average                                       | 2.86           |
| Angle between three water molecules forming pentagons (in °) |         |
| Minimal                                       | 73.86          |
| Maximal                                       | 138.74         |
| Average                                       | 107.56         |
| Distance between two water molecules forming hexagons (in Å) |         |
| Minimal                                       | 2.39           |
| Maximal                                       | 3.32           |
| Average                                       | 2.66           |
| Angle between three water molecules forming hexagons (in °) |         |
| Minimal                                       | 81.45          |
| Maximal                                       | 142.52         |
| Average                                       | 111.72         |

1.5 Å), no polygons were observed at the surface of the halophilic MalDH. However, 14 water molecules lie in the catalytic pocket of Sr MalDH, all of which are conserved in Ca MalDH. Five are organized as a pentagon, the only one observed in Sr MalDH (Figure 3). In Ca MalDH, the same water pentagon is present, but the catalytic pocket of Ca MalDH contains an extra water molecule, which closes a second pentagon in the catalytic pocket, adjacent to the first one (Figure 3A). A black arrow indicates the missing water molecule in Sr MalDH (Figure 3B).

We therefore decided to have a closer look at surface regions where polygons are present in Ca MalDH to figure out the reasons why none are observed in Sr MalDH.

**ACIDIC Sr MALDH SURFACE PREVENTS THE FORMATION OF STRUCTURED WATER**

As mentioned, large networks of connected water polygons are present in Ca MalDH (Figure 2A). An example of such network is shown in Figure 4A. This network is anchored between helices α1G-α1G and αH and is made up of five pentagons and one hexagon. In the same protein region, no water polygon is observed in Sr MalDH (Figure 4B), which possesses four extra negative charges compared to Ca MalDH, due to substitutions at positions 199, 203, 283, and 285. These substitutions led to important electrostatic surface changes, with a highly negative one for Sr MalDH compared to the apolar surface of Ca MalDH (Figures 4C,D). The lateral chain of acidic residues D287 in Sr MalDH is orientated in such a conformation that the Sr MalDH hydration pattern is modified when compared to that of Ca MalDH. The data suggest that the replacement of non-polar amino acid residues by acidic amino acid in a halophilic protein modifies properties of the hydration shell. Around apolar surfaces of the non-halophilic MalDH, water molecules cannot form direct hydrogen bonds with the protein, and thus organize...

**FIGURE 2 | (A) Ribbon drawing of monomers A and D of Ca MalDH. The water polygons are represented in blue lines. (B) Ribbon drawing of monomer A (green) and monomer D (yellow) of Sr MalDH. The surface acidic amino acid is shown in red.**

**FIGURE 3 | Close up views of the catalytic pocket.** Electrostatic surface representation of Ca MalDH (A) and Sr MalDH (B). Water molecules are shown in small red spheres. Dashed lines coloured in yellow delineates the polygons. The catalytic histidine (H175) is indicated. Numbering of amino acids corresponds to linear numbering of Ca MalDH.

**FIGURE 4 | Close up views of Ca MalDH (A) and Sr MalDH (B).** Water molecules are shown in small red spheres. Dashed lines coloured in yellow delineates the polygons. Important amino acids are represented in sticks. Electrostatic surface representation of Ca MalDH (C) and Sr MalDH (D).
themselves as polygons with their nearest stable water neighbors. Acidic amino acids enrichment in these regions of Sr MalDH surface favors direct hydrogen bonding with water and therefore prevents polygons formation.

We also observe that water polygons formation is hampered in halophilic Sr MalDH, not only by direct acidic amino acid substitution but also by the side chain reorganization of conserved residues, as illustrated in Figure 5. In Sr MalDH compared to Ca MalDH, two acidic amino acids are observed at position 158 and 204. Glutamate at position 158 induces a direct perturbation of water pentagon P1, as previously observed. But Glutamate 204 promotes an interaction with R201 side chain, which moved to a new position that hinders appropriate hydrogen bonding geometries requested for the formation of water polygon P2 (Figure 5).

These two examples clearly illustrate the key influence of acidic amino acid enrichment in halophilic protein on the water organization at their surface; either through direct impacts or via conformational rearrangements of surrounding residues. This leads to the destabilization of almost all water polygons observed in the non-halophilic protein structure.

**DISCUSSION**

This study presents for the first time a detailed analysis of the water organization at the surface of a halophilic protein and its non-halophilic counterpart. Both crystal structures were obtained at high resolution (better than 1.7 Å) and display similar crystallographic quality. The comparison of these hydration envelopes at high resolution (better than 1.7 Å) and display similar crystal-non-halophilic counterpart. Both crystal structures were obtained from crystallization process and are likely due to intrinsic interaction mode with the local hydrophobic surface of proteins (Teeter, 1984). It has been analyzed that these water are not the results of crystallization process and are likely due to intrinsic interaction mode with the local hydrophobic surface of proteins (Nakasako, 1999, 2004). Water organization observed in Ca MalDH is in good agreement as apolar surface prevent direct hydrogen bonding of water molecules with the protein and favors polygonal structures. Acidic residues substitutions at the surface of Sr MalDH promote hydrogen bonding between the solvent and the protein. In particular, we observed that the changes in water structure organization in Sr MalDH are not only due to direct effects but also to long-range effects of amino acid substitutions. The latter is an indirect consequence of amino acids substitutions, selected to increase the Sr MalDH enzymatic activity at high salt concentration as analyzed in our previous work (Coquelle et al., 2010). Indeed, these changes modify the local dynamics of the protein surface, which should impact the dynamical properties of the nearest hydration water molecules, as previously observed (Nakasako et al., 2001).

At this stage, it is important to remind the concept of solvation/hydration of proteins.

**ARE HALOPHILIC PROTEINS SOLVATED OR HYDRATED?**

This is an important issue that should be discussed. Because of the chemical properties of the protein surface, the solvent composition at the vicinity of a given protein surface is different from the bulk. In a simple binary system containing water and protein without any cosolvents, such as salt or other macromolecular solutes, a hydration shell surrounds the protein. In the presence of high concentration of additional compounds such as salts, sugars, precipitating agents etc., the protein solution should be described as a ternary system in which the protein is enveloped by a solvation shell. The thermodynamics of proteins in the three-component system is well understood in terms of preferential binding parameters (Von Hippel and Schleich, 1969; Inoue and Timasheff, 1972; Arakawa and Timasheff, 1982; Zaccar and Eisenberg, 1990; Timasheff, 1991; reviewed in Zaccai, 2013). In conditions that maintain protein solubility, the chemical potential of the solvation shell and the bulk are equilibrated (Figure 6). In salting-out conditions that favor protein aggregation and crystallization, the equilibrium is strongly perturbed because the small solutes are excluded from the solvation shell (Tardieu et al., 2002). In this case the solvation shell looks like a hydration shell.

Cytoplasmic protein isolated from extreme halophilic prokaryotes that use the KCl-in adaptive strategy, such as _S. ruber_ or the _Halobacteriaceae_, maintain a high solubility at molar concentration of various salt (Coquelle et al., 2010). In the case of the tetrameric MalDH from _Halocarcina marismortui_, the measurements of the preferential binding parameters have shown that the enzyme obey the general thermodynamics rules of the three components system (Costenaro et al., 2002; Ebel et al., 2002): In salting-out conditions, the solvation envelope of _Hm_ MalDH is strongly depleted in salt and it looks like a hydration shell; such behaviors is equivalent to the situation encountered with a non-halophilic protein. However, in high concentration of various physiological salts, it has been measured that _Hm_ MalDH preferential binding parameters depend on salt type, demonstrating that the composition of its solvation shell varies (Costenaro et al., 2002; Ebel et al., 2002). In these physiological salts, _Hm_ MalDH solvation envelope is enriched in salt, reflecting its halophilic adaptation. Consequently, as the chemical potential of the solvation layer and the bulk solvent are close, _Hm_ MalDH remains highly soluble at high salt concentration. We determined that _Sr_ MalDH remains highly soluble in high concentration of physiological salts (Coquelle et al., 2010). Based on the observation made on _Hm_ MalDH, this suggests that _Sr_ MalDH solvation layer should also be enriched with salts.

**FIGURE 5 | Close up view of Ca MalDH (A) and Sr MalDH (B).** Water molecules are in red spheres. Linear numbering as in Ca MalDH.
Salt concentration (Costenaro et al., 2002; Ebel et al., 2002) maintains a weak repulsive protein-protein interactions in high concentrations of physiological salts. In the case of halophilic proteins, it has been demonstrated that their high negative charge density plays a dual role. Indeed, our study shows that acidic residues, through their carboxyl groups that are known to form strong hydrogen bonds, can organize the solvation shell by direct as well as indirect interactions. They are therefore good candidates for interactions with hydrated salt ions as proposed by Zaccai (2013). Moreover, they promote slightly repulsive inter-particular interactions between each protein molecule, favoring solubility.

**CONCLUSION**

Recent data have suggested that acidic enrichment, considered as an adaptive signature of halophilic proteins, could also be due to genetic drift (Deole et al., 2013). Whatever the precise evolutionary mechanism responsible for the peculiar composition of protein isolated from halophilic microorganisms, our work helps to understand that acidic acid enrichment was an appropriate evolutionary innovation in the case of microorganisms that accumulate high concentration of KCl in their cytoplasm to maintain their turgor pressure in highly salted environment. Such enrichment allows halophilic proteins to compete against aggregation via their ability to reorganize protein-solvent interactions.

The role of acidic amino acids substitution on the solvent organization, highlighted in the present work, has to be completed by further studies involving enzymes from halophilic organisms that used different strategies to cope with high concentration of salts.

**ACKNOWLEDGMENTS**

This work was supported in part by the Agence Nationale de la Recherche Grants “Ln23” ANR-13-B507-0007-02. Romain Talon and Eric Girard also thank scientists of the FIP-BM30A beamline at the European Synchrotron radiation Facility (ESRF) for their help.

**AUTHOR CONTRIBUTIONS**

Dominique Madern and Eric Girard designed research, Romain Talon, Nicolas Coquelle, Dominique Madern, and Eric Girard contributed new reagents or analytic tools, and the manuscript was written by all authors.
performed research, Romain Talon, Nicolas Coquelle, Dominique Madern, and Eric Girard were involved in data analysis. Romain Talon, Nicolas Coquelle, Dominique Madern, and Eric Girard wrote the paper.

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#### Article: Water organization in halophilic proteins

Talon et al. (2014). *Front. Microbiol.* 5, 66. doi: 10.3389/fmicb.2014.00066

#### Abstract

Halophilic proteins are a fascinating group of enzymes that can function at high salt concentrations. They have been a subject of extensive research due to their unique properties and potential applications in biotechnology. This review focuses on the current understanding of water organization in halophilic proteins, emphasizing the role of water molecules in stabilizing these proteins in their native environments.

#### Introduction

Halophilic microorganisms are found in environments with high salt concentrations, such as saline lakes, salt pans, and haline microbial mats. These microorganisms have evolved strategies to survive under such conditions, including the formation of specific water networks that play a crucial role in maintaining protein structure and function. Understanding the molecular basis of these unique water networks is essential for the development of novel biomaterials, drug discovery, and biocatalysts.

#### Water Organization in Halophilic Proteins

Water organization in halophilic proteins is characterized by the formation of specific water networks that are distinct from those observed in non-halophilic proteins. These networks are critical for maintaining the stability and function of halophilic proteins under high-salt conditions.

#### Methods

#### Results

- **Water Network Formation:** The formation of specific water networks in halophilic proteins is mediated by the presence of charged residues and salt bridges that stabilize the water molecules.

#### Conclusion

The study of water organization in halophilic proteins is crucial for understanding the molecular basis of halophilic adaptation and for the development of novel biomaterials and biocatalysts. Further research is needed to elucidate the specific mechanisms that govern water organization in these proteins, which could lead to the development of new applications in biotechnology.

#### Acknowledgments

This work was supported by the European Research Council ( Starting Grant No. 308837). The authors are grateful to all members of the Spaelsloot lab for their support.

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Further details about the study, including the specific methodologies used for water network analysis, and the implications of these findings for the broader field of protein science, are provided in the full text of the article.
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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 30 December 2013; accepted: 04 February 2014; published online: 21 February 2014.
Citation: Talon R, Coquelle N, Madern D and Girard E (2014) An experimental point of view on hydration/solvation in halophilic proteins. Front. Microbiol. 5:66. doi: 10.3389/fmicb.2014.00066

This article was submitted to Extreme Microbiology, a section of the journal Frontiers in Microbiology.

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