MOLECULAR ADAPTATION OF HALOPHILIC PROTEINS

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Contents

1. Introduction
1.1. Halophilic Organisms, Halophilic Proteins
1.2. Evolution
1.3. Molecular Adaptation
2. Three-Dimensional Structures of Soluble Halophilic Proteins
2.1. Overall Structures as seen by Crystallography
2.2. Protein-Solvent as seen by Crystallography
2.3. Modeling
3. Site-Directed Mutagenesis
3.1. Mutation of Acidic Amino Acids
3.2. Salt-Bridge Clusters
4. Halophilic Enzyme Activity
4.1. Effects of Salt Concentration
4.2. Effects of Salt Type
4.3. In Vivo Activity
4.4. Detailed Description of Enzymatic Activity
5. Halophilic Protein Solubility
5.1. As a Function of the Solvent
5.2. For Purification
5.3. For Crystallization
6. Halophilic Protein Stability
6.1. Stability Measurements
6.2. Systematic Studies of Salt Type
6.3. Temperature Dependence
6.4. Isotopic Effects of Heavy Water
7. Solvent Interactions for Halophilic Proteins
8. Conclusion
8.1. Cellular Adaptation
8.2. In Vitro Studies for the Study of Solvent Interactions
8.3. Specific Binding Sites
8.4. Halophilic Proteins are Dynamic Protein-Salt-Water Complexes
Acknowledgements
Glossary
Bibliography
Biographical Sketches

Summary

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Halophilic proteins are defined as those extracted from organisms that require 2.5 mol L^{-1} NaCl or higher salt concentrations in the medium for optimum growth. Many of these proteins are themselves halophilic in that they require high salt for stability and solubility. An approach to the understanding of halophilic adaptation at a molecular level required a strategy of complementary experiments, combining molecular biology, biochemistry, and cellular approaches, on the one hand, with structural studies, biophysical chemistry, and thermodynamics, on the other. Halophilic protein sequences revealed significantly higher proportions of acidic residues than their homologues from nonhalophilic organisms. The three halophilic protein crystal structures available in early 2001 showed protein folds similar to their respective nonhalophilic homologues. Significant differences, however, appeared in the protein-solvent interactions. The excess in negative charge is found predominantly on the protein surfaces, where extensive hydration interactions are observed in the highest resolution structure. Complex salt bridges associated with solvent ion binding sites also appeared as likely halo-adaptation features. Halophilic proteins can be stabilized in a wide range of solvent conditions, making them also good models for the general study of protein-solvent interactions involved in stability, solubility, and crystallization. The effects of salt type and concentration on the activity, solubility, and stability of halophilic proteins have been characterized. Parameters pertaining to solvent-protein interactions and enzyme unfolding kinetics were found to depend strongly on solvent composition. A combination of biophysical experiments in solution revealed the important role played by water and ion-binding in these processes. An aspect of haloadaption at the cellular level has also been characterized. Cellular response to salt stress in halophilic archaea was found to involve the induction of a chaperone-like complex that protects halophilic proteins from low salt denaturation.

1. Introduction

1.1. Halophilic Organisms, Halophilic Proteins

From the Greek roots *hals*, meaning salt, and *phil*, meaning loving or friendly with, halophily represents the requirement for salt. Microorganisms that grow in salt such as the ones that cause "red-eye" or salt cod spoilage have been known since the end of the 19th century. And, in 1940, the myth of the sterility of the Dead Sea was shattered with the discovery that it supported microbiological life, which not only was adapted to the very highly concentrated salt environment but also required it for development. Three groups of halophilic microorganisms have been identified: aerobic halophilic archaea, anaerobic halophilic methanogenic archaea, and the halophilic bacteria. All the well-characterized halophilic enzymes, so far, have been purified from the first group. The retinal-binding seven α–helix membrane proteins, bacteriorhodopsin, halorhodopsin, and sensory rhodopsin have also been purified from halophilic Archaea and studied extensively, especially bacteriorhodopsin. Bacteriorhodopsin is the only protein in the purple membrane patches in *Halobacterium salinarum;* it functions as a light-activated proton pump. The retinal binding proteins in halophilic Archaea are considered more as paradigms of membrane proteins than as truly halophilic proteins and will not be discussed in this article (a review of bacteriorhodopsin is given in the bibliography).

Halophilic Archaea accumulate high concentrations of KCl in their cytoplasm, approaching saturation. All the components of the cellular machinery are therefore

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adapted to be efficient at high salt concentrations. From the first extractions of proteins from these organisms, it appeared that salt was also a requirement for the stabilization of their native structure and their solubility. Halophilic enzymes are defined either with respect to the halophily of the organism from which they are fractionated, or with respect to their own salt requirements for activity, stability, or solubility.

Apart from the special case of the retinal-binding membrane proteins, the best characterized halophilic protein from the structural and biophysical points of view, malate dehydrogenase from *Haloarcula marismortui* (*Hm* MalDH), is an enzyme for which both definitions apply, since it is unstable below 2 mol L^{-1} NaCl. However, because solvent effects can be easily modulated, a soluble halophilic protein is now considered as one extracted from a halophilic microorganism that requires at least 2.5 mol L^{-1} NaCl in the medium for optimum growth. Proteins from microorganisms with lower salt requirements are therefore excluded from this definition, even if they appear to require high salt concentration for activity or stability.

1.2. Evolution

From the first evaluations of their global amino acid compositions, it appeared that halophilic proteins present a higher proportion of acidic residues than their nonhalophilic homologues. This was confirmed by a statistical analysis of the amino-acid compositions of 26 halophilic soluble proteins and those of their nonhalophilic homologues. The analysis also revealed a significantly lower content in basic residue content, and an increase in the content of small hydrophobic residues (glycine, alanine, valine) together with a decrease in the content of the largest ones (isoleucine, leucine). The evolutionary process that leads to the acidic nature of halophilic proteins has been studied on the enzyme, superoxide dismutase (SOD). In a general evolutionary process, when two gene sequences evolve independently from their ancestor, they accumulate synonymous mutations at the third codon position. Such mutations usually do not change the sense of the codon, and are therefore accumulated without inducing deleterious effects at the protein level. The divergence thus appears to be greater at the DNA level than at the protein level. Pairwise comparisons between nonhalophilic and halophilic SOD have been performed at both the DNA and protein levels. A high frequency of nonsynonymous nucleotides was found at the first and/or second codon position substitutions. Such mutations result in a change in the corresponding amino acid in the protein. Due to this, a halophilic gene is more divergent at the protein level (the gene product) than at the DNA level when compared with a non-halophilic homologous gene. Such intensive selection might be explained by specific constraints exerted at the structural level, in relation to the high salt environment. The first complete genome sequence of an extreme halophile, Halobacterium NRC1 was reported in 2000. It confirmed the very acidic character of halophilic proteins, with an Isoelectric Point (pI) of 5.1. The full exploitation of the genomic information now available in evolutive terms remains to be done.

1.3. Molecular Adaptation

To be active, stable, and soluble in high salt are major challenges facing proteins in halophilic microorganisms. High salt concentrations affect the conformational stability of proteins. In general, salt conditions that favor solubility are destabilizing of the

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folded form and vice-versa. Halophilic proteins have, therefore, evolved specific molecular mechanisms that allow them to be both stable and soluble in the high-KCl concentration of the cytoplasm. At the same time, high salt has become an absolute requirement, since halophilic proteins in general unfold at low salt. These adaptive mechanisms are reflected in the solvent interactions and three-dimensional structures of halophilic proteins. Stability and activity are also strongly related with protein dynamics, which is itself solvent-environment dependent. Dynamics-activity relations in bacteriorhodosin and purple membranes have been very well characterized and experimental studies of soluble halophilic protein dynamics were initiated in the year 2000. First results established the correlation among atomic fluctuation dynamics, solvent environment, and stability.

2. Three-Dimensional Structures of Soluble Halophilic Proteins

2.1. Overall Structures as seen by Crystallography

Only three soluble halophilic protein crystallographic structures have been published before May 2001. Two of them are monomeric proteins: ferredoxin from *H. marismortui* (*Hm* Fd) and dihydrofolate reductase from *H. volcanii* (*Hv* DHFR). These two proteins are moderately sensitive to low salt since still active and folded in 0.4–0.5 mol L^{-1} NaCl or KCl. Malate dehydrogenase from *Haloarcula marismortui* (*Hm* MalDH), which unfolds below 2 mol L^{-1} NaCl, was the first halophilic protein to be solved and the only oligomeric one so far. In contrast to the common dimeric forms that had been observed for other malate dehydrogenases, *Hm* MalDH forms a tetramer, which can be analyzed as a dimer of dimers. In fact, *Hm* MalDH belongs to a new malate dehydrogenase protein family that is closely related to the tetrameric lactate dehydrogenases.

In the crystallographic structures of halophilic proteins, the most notable structural feature related to high-salt molecular adaptation is the very acidic character of the protein surface. Most of the acidic residues are localized in patches at the surface. This is illustrated for *Hm* MalDH on Figure 1, Panel A and B. For *Hm* Fd, the entire surface is coated with acidic residues, except in the vicinity of the iron cluster. Compared to other ferredoxins, *Hm* Fd has an N-terminal extension containing 15 negative charges and forming an hyper acidic domain. The amino-acid composition of *Hv* DHFR is not typically halophilic, since it is not significantly different from its nonhalophilic homologues, especially with respect to charged residue content. However, clusters of acidic residues are found also in this structure. The high charge density on the surface of halophilic proteins contributes to their low salt instability. The "halophilic addition" on *Hm* Fd was proposed to play a major role in haloadaptation by providing a higher solvent-accessible acidic surface area.

Structural features that can be related to *Hm* MalDH stability are also found in thermophilic proteins, including the stabilization of helices by incorporation of alanine, and acidic residues in their N-terminal ends, and, in a more impressive way, the large number of salt bridges, particularly multiple salt bridges localized at the subunit interfaces. In the dimer-dimer interface of the tetramer, two clusters of complex salt bridges were found in which the basic residues interact with more than one acidic residue. Crystal structures of a number of hyperthermophilic proteins have now been

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published, in which extensive intersubunit salt-bridge networks are frequently found, in contrast to mesophilic proteins.

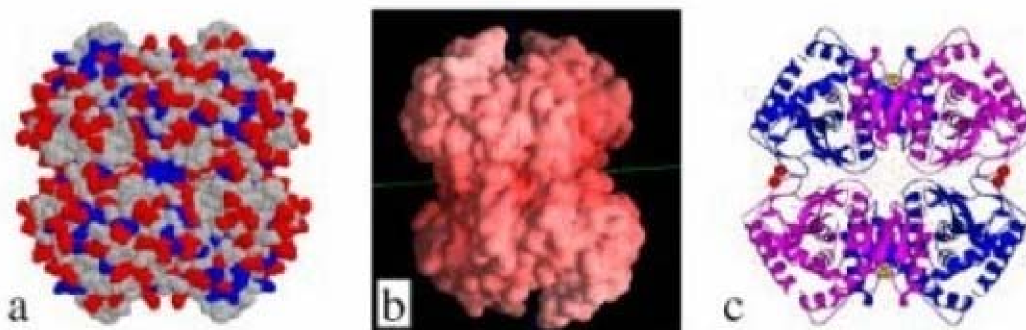

Figure 1. Three-dimensional structure of halophilic malate dehydrogenase (Hm MalDH)

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Biographical Sketches

Christine Ebel was born on 12 July 1958 in Strasbourg, France. She is a civil service scientist of the Centre National de la Recherche Scientifique, the French national research organization, and works at the Institut de Biologie Structurale, Grenoble, France, where she heads the "Molecular Interactions" group in the Laboratory of Molecular Biophysics. Christine Ebel was educated at the University of Grenoble as a physical organic chemist and obtained her doctorate degree (Doctorat d'Etat) in 1988. Dr. Christine Ebel specializes in the study of the solution structure of macromolecules by biophysical methods, in particular, neutron scattering, analytical ultracentrifugation, and high-precision density measurements. She has contributed to understanding molecular adaptation to extreme saline conditions, protein solvation, and stabilization, as well as strong and weak protein-protein interactions and protein crystallization. Christine Ebel teaches at the University of Grenoble. In 2001, she had published about 40 original research papers, and held three patents. She is married and has three children.

Dominique Madern was born on 28 December 1960, at Bourg de Péage, France. From 1983 to 1991, he was employed as a technician by the European Molecular Biology Laboratory in Grenoble, France, to work on structure-function relationships of various aminoacyl-tRNA synthetase, key enzymes in protein synthesis. In 1991, he joined the Centre National de la Recherche Scientifique, the French national research organization. He works as research engineer at the Institute of Structural Biology, Grenoble, in the Molecular Biophysics Laboratory. The research interests of this laboratory are centered on proteins from organisms adapted to extreme conditions, in particular halophilic proteins from hypersaline environments. Based on his research achievements in this field (5 years), Dominique Madern was awarded a doctorate degree in biology by the University of Grenoble in 2000. In the last three years, he has started to study various hyperthermophilic proteins using molecular biology and biophysical methods. He also developed an interest in the mechanisms of biological evolution, and has contributed to the understanding of phylogenetic relationships within a particular protein family. In 2001, he was the author of 15 original research papers. Dominique Madern is married and has one child.

Giuseppe Zaccai was born on 20 January 1947, in Alexandria, Egypt. He was educated at the University of Edinburgh in Scotland, where he obtained a PhD degree in physics in 1968. He is employed by Centre National de la Recherche Scientifique, the French national research organization, and heads the Laboratory of Molecular Biophysics of the Institute of Structural Biology, Grenoble, France. The research interests of the laboratory are centered on proteins from organisms adapted to extreme conditions, such as halophilic proteins from hypersaline environments, and thermophilic proteins adapted to very high temperatures. He pioneered neutron scattering applications to structural biology, in particular, to study membranes and protein dynamics, and has a joint appointment with the Institut Laue Langevin in Grenoble, which has a high flux neutron source for research applications. Giuseppe Zaccai teaches at the University of Grenoble and until recently directed the graduate program in Biological

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Crystallography and NMR. In 2001, G. Zaccai had about 140 original research publications. G. Zaccai is married and has three grown up children.

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