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KENNETH P. MURPHY AND ERNESTO FREIRE

BIOCHEMICAL, STRUCTURAL, AND MOLECULAR GENETIC ASPECTS OF HALOPHILISM

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I. Introduction

The complicated process termed "life" is associated with a very delicate balance in the interactions among the different components of the living cell. Many of the components are macromolecules that have an extremely nonlinear response to external stimuli. Small vari-

ations in the physicochemical state of the cell could, therefore, have enormous effects on the life process. The sum total of the physicochemical conditions enabling normal functioning of the cell is termed "physiological conditions." In general, living organisms are adapted to function in a rather limited set of physiological conditions: chemical and ionic composition of the medium, pH, temperature, and pressure. Significant deviations from physiological conditions will lead to disaggregation of complex structures, denaturation of protein and DNA molecules, and consequently to cell death. In this context, it is worthwhile mentioning that although in many cases the physiological conditions as determined *in vitro* resemble the living processes, in many other cases the conditions for stability and effectiveness of biological components and interactions as determined in laboratory experiments do not necessarily apply to *in vivo* conditions for phenomena occurring in the crowded living cell (Minton, 1983; Richey *et al.*, 1987).

It is now believed that on our 4.5-billion-year-old planet, unicellular microorganisms originated about 3.5 billion years ago. Microorganisms dominated life on Earth for a considerable length of time, and higher organisms appeared only much later. In this period of time the earth cooled considerably, and oxygen appeared in the atmosphere about 2 billion years ago. Ancient microorganisms therefore had to adapt to an evolving habitat. They survive to date mostly in niches characterized by unusual environmental circumstances. A striking example of an unusual habitat, close to the topic of this article, is the Dead Sea, which was recognized only in the late 1930s [Wilkansky (Volcani), 1936]. This unique body of water, rich in ancient human history and site of biblical events, has many names, such as the Sea of Asphalt, indicating extensive organic deposits, in Hebrew, it is named the Sea of Salt. It is now known to be a dynamically evolving stratified ecological niche, hosting microbial and algal populations. An interesting aspect of the Dead Sea is that, in contrast to other well-known saltwater bodies, such as the Great Salt Lake in Utah, it is extremely rich in magnesium salts (Nissenbaum, 1975). Early studies of the Halobacteriaceae (cf. Section V,A), microorganisms that are stable and active only in extremely high concentrations of salt, have been reviewed (Larsen, 1986; Kushner, 1985).

Physiological conditions for most living organisms are very similar, but it is well documented that many organisms are adapted to grow under extreme conditions of salt concentration, pH, temperature, and pressure. It is possible to divide these organisms into two groups according to their mode of adaptation. One group of organisms de-

veloped various mechanisms to preserve a benign inner environment in extreme surroundings, for instance, bacteria that are capable of surviving in extreme pH environments by activating powerful proton pumps to maintain a close to neutral intracellular pH (Edwards, 1990). In the other group, the entire biochemical machinery is adapted to function in the particular extreme conditions. When adaptation to hypersaline conditions is considered, there are fundamental differences between the extremely halophilic archaebacteria and the other halophilic organisms regarding their mode of adaptation (see recent reviews edited by Rodriguez-Valera, 1988, and references therein). The halophilic eubacteria and eukaryotes accumulate mostly organic neutral compatible solutes and exclude most of the inorganic salts. On the other hand, the halophilic archaebacteria balance the external high salt concentration by accumulating within the cell inorganic ions at concentrations that exceed that of the medium. Therefore, all the cellular components of the halophilic archaebacteria have to be adapted to function at the extremely high intracellular salt concentration. A short explanation regarding the phylogenetic definition of the archaebacteria follows.

The original proposal by C. R. Woese and colleagues that living organisms should be classified into three different kingdoms was based on an extensive comparison of sequences of oligonucleotides derived from rRNA molecules of many organisms (Woese and Fox, 1977). This original phylogenetic distance analysis of oligonucleotides was extended to longer 16S rRNA sequences as modern, rapid DNA sequencing techniques became available, and similar results were obtained. Three unique molecular features, shared by all the members of the archaebacteria, help to distinguish these microorganisms from the eubacteria. (1) Archaebacterial lipids are made up of isopranyl glycerol ethers rather than the fatty acid ester-linked glycerol lipids that predominate in eubacteria and eukaryotes (Langworthy and Pond, 1986). (2) The DNA-dependent RNA polymerases of all archaebacteria are more complex than their eubacterial counterparts and their structure and sequence resemble more that of RNA polymerase II of eukaryotes (Zillig *et al.*, 1988). (3) Archaebacteria lack the typical eubacterial peptidoglycan cell wall and instead have an S layer composed mainly of glycoproteins (Koenig and Stetter, 1986, and references therein).

Basic interest in the study of halobacteria thus relates also to a better understanding of evolutionary relationships extending to the dawn of life in a world quite unlike our present-day environment. If we consider the current processes of increasing atmospheric CO_2

levels, increasing salinity, and cooling or heating of the seas, and also the possibility of extended human journeys into space, then we must accept that adaptation of life to changing environments constitutes an important riddle deserving close examination. To understand adaptation it is of course essential to understand both the physiological and physical bases of life on a broad level, as well as the physicochemical characteristics of interactions of cell components—proteins, nucleic acids, sugars, lipids, and so forth—at a molecular and cellular level. Site-specific mutagenesis coupled to sequence analysis and macromolecular structure is becoming a powerful tool to modify the structure and properties of macromolecular components along a path of adaptive change. An important bonus in the study of modified and adapted systems is a better understanding of factors important in the function and regulation of components and systems comprising the bulk of organisms in our present-day environment.

A practical consequence of the study of proteins from organisms adapted to extreme environments consists in their utilization in biotechnological applications. Experimental dimensions are added that may make processes feasible, cheaper, or more reliable in high salt concentration or at a higher temperature, for instance. These developments are still at an early stage and will receive much stimulation with the development of suitable vectors and cloning systems in extreme environments. On a more practical level, commercial production of β-carotene and glycerol has been achieved from *Dunaliella* algae grown in open-air saline ponds (Ben-Amotz and Avron, 1990). A recent unusually successful achievement is the use of the heat-resistant *Taq* polymerase from *Thermus aquaticus* in the polymerase chain reaction (PCR), which has revolutionized processes based on the quick multiplication of minute amounts of DNA (Mullis and Faloona, 1987).

Work on extremely halophilic archaebacteria in our and other laboratories, to be described in the following review, went through a number of stages. Early work on these microorganisms was mainly aimed at developing enrichment procedures and physiological studies. Most of the biochemical studies were performed on impure enzymatic preparations. Even when some enzymes could be purified to homogeneity, the yields were too low to enable physical characterization of the proteins. The development of efficient purification protocols by which large amounts of halobacterial proteins were fractionated at high salt concentration, thus avoiding losses due to inactivation, enabled detailed biochemical and biophysical characterization of several enzymes. Studies in the ultracentrifuge and appli-

cation of thermodynamic considerations developed for multicomponent systems led to interesting results relating to salt and water binding (Pundak and Eisenberg, 1981; Pundak *et al.*, 1981). These observations were considerably extended by the use of X-ray and neutron scattering, and a model could be obtained for halophilic protein stabilization (Zaccai *et al.*, 1989; Zaccai and Eisenberg, 1990). Recent developments in molecular genetics enabled the isolation of a number of halobacterial genes, allowing quick determination of the amino acid sequences of the corresponding coded proteins. The development of transformation protocols for halobacteria (Cline and Doolittle, 1987) and the construction of halobacterial shuttle vectors (Lam and Doolittle, 1989; Holmes and Dyall-Smith, 1990) opened the way to exploitation of the methodology of site-specific mutagenesis as an extremely powerful tool in the elucidation of the relationship between the structure of the halobacterial proteins and their adaptation to function at extremely high salt concentration.

The purpose of this review article is to familiarize the reader with recent developments in the molecular characterization of halobacterial proteins, starting with the methodology of their purification. Then we describe the biochemical and biophysical structural analyses of some enzymatic systems for which extensive knowledge has been accumulated. Finally, very recent developments in the field of the molecular genetics of halobacteria are discussed. For previous descriptions of the subject, the reader is referred to recent review articles already mentioned. Other reviews dealing with more specific issues will be mentioned throughout this article.

II. Purification of Halophilic Enzymes

Halophilic enzymes are very unstable in low salt concentrations. Because some of the important fractionation methods in protein chemistry, such as electrophoresis or ion-exchange chromatography, cannot be applied at high salt concentrations, the available fractionation methods are rather limited. This basic difficulty is the main reason why the number of halophilic enzymes studied in pure form is very small.

The existing purification procedures fall into two groups: the nonhalophilic approach and the halophilic approach. In the first, at certain stages in the purification procedure, the salt concentration is reduced and techniques that are suitable to low salt concentrations are applied. Inactivation in these conditions can be overcome partially either by protecting the native enzyme with its substrate or cofactors

or by reactivating the enzyme at a later stage by exposure to high salt concentration.

The first reported procedure for the purification of a halophilic enzyme was that of malate dehydrogenase, described by Holmes and Halvorson (1965). In this procedure, the salt concentration was reduced at the very beginning of the procedure and methods such as ion-exchange chromatography on DEAE-cellulose and electrophoresis were applied. The enzyme was recovered after reactivation by dialysis against 25% (w/v) NaCl. The yield was very poor (~0.5%). In the purification of the halophilic enzyme isocitrate dehydrogenase (Hubbard and Miller, 1969), an inactivation step was performed prior to ammonium sulfate fractionation in order to improve selectivity. In this case as well, the recovery was very low (2.7%), mainly due to the inactivation step. On the other hand, in the purification of dihydrolipoamide dehydrogenase (Danson *et al.*, 1986), although several purification steps were used at the low salt concentration at which the enzyme was inactivated, this inactivation could be completely reversed by increasing NaCl concentration to 2 *M*. In several cases large losses in activity were prevented by using a protective agent. In the purification of NADH dehydrogenase (Hochstein and Dalton, 1973), NADH was used at a concentration of 0.1 m*M* to protect the activity of the enzyme when exposed to 0.35 *M* NaCl. This protection enabled the use of ion-exchange chromatography without major loss of activity.

The enzyme DNA-dependent RNA polymerase isolated from halobacteria presents an interesting example by being active *in vitro* only at salt concentrations below 0.4 *M* and by lacking the ability to initiate transcription at the specific transcription initiation sites. It was purified from *Halobacterium halobium* (Madon and Zillig, 1983) and from *Halococcus morrhuae* (Madon *et al.*, 1983) using purification protocols that include polymer partitioning methods and heparin-cellulose and DEAE-cellulose chromatographies. No special attempts were made to keep the enzyme at high salt concentration throughout the purification procedure. The enzyme was stabilized, though, by adding glycerol to the various buffers to a final concentration of 40%. The fact that the purified enzyme is active *in vitro* only at salt concentrations much lower than that existing in the cell and the fact that it lacks specificity might indicate that some essential factors were lost during the purification.

According to the halophilic approach, all the purification steps are performed in high salt concentrations. The advantage of this approach is the high level of recovery achieved in each step. This ap-

proach was used to purify the halobacterial enzyme ornithine carbamoyltransferase (Dundas, 1970), but its applicability was for many years limited due to the lack of suitable fractionation methods for multimolar salt solutions. Subsequently, several new methods were introduced that enabled the purification of many halophilic proteins at high salt concentration. The rest of this section will be devoted to a review of these methods.

A. Ammonium Sulfate-Mediated Chromatography

Differential salting-out by ammonium sulfate salt was used for many years as a means for differential precipitation and crude fractionation of soluble proteins. This salting-out electrolyte facilitates hydrophobic interactions by reducing the solubility of amino acid side chains in salt solution. The solubility of different proteins depends, therefore, on the distribution and exposure of the various amino acids on the surface. However, because the solubility of proteins in ammonium sulfate does not vary much from one protein to another, the applicability of this method has been limited in most cases to the first steps of the purification process. Halobacterial proteins are in general more soluble in ammonium sulfate than are nonhalophilic proteins, and therefore when halobacterial proteins are to be fractionated this limitation is pronounced even more. In the early 1970s several modifications to the ammonium sulfate fractionation method were introduced. These modifications were based on the fact that high concentrations of ammonium sulfate cause the adsorption of proteins to solid surfaces or gels. Among the useful supports were Celite (King, 1972), DEAE-cellulose (Mayhew and Howell, 1971), and alkylaminoagaroses (Rimerman and Hatfield, 1973). The technique involved adsorption of the unfractionated proteins to the matrix at high ammonium sulfate concentration followed by separation of the proteins by applying a decreasing concentration gradient of the same salt. The binding of the proteins to alkylaminoagarose was interpreted to be due to the facilitation by the ammonium sulfate of "hydrophobic interactions" between the aliphatic side chains covalently bound to the matrix and the protein surface. It was surprising, therefore, to discover that unsubstituted agarose could also adsorb large quantities of proteins in the presence of concentrated ammonium sulfate solutions (von der Haar, 1976; Mevarech *et al.*, 1976). The explanation of this phenomenon given by von der Haar was that the solvation sphere on the gel surface differs from the solvation in solution and therefore the precipitation of proteins on

this surface occurs at lower salt concentration than is needed for precipitation out of solution. This explanation assumes that the proteins are precipitated at the solvation layer of the gel. Another explanation is that the proteins accumulate in the solvation layer of the hydrophilic polysaccharide matrix, in a way analogous to partition of proteins between two phases of hydrophilic polymers dissolved in salt solution (Albertsson, 1970).

Because ammonium sulfate-mediated chromatography requires high salt concentrations and halobacterial proteins are very stable in ammonium sulfate concentrations higher than 1 *M*, this method is very useful for the purification of halobacterial enzymes. Detailed analyses of the adsorption properties of the halobacterial enzymes malate dehydrogenase (*h*MDH) and glutamate dehydrogenase (*h*GDH) on various supports were performed by Mevarech *et al.* (1976). A crude protein extract of sonicated *Haloarcula marismortui* was dialyzed against 2.5 *M* ammonium sulfate and applied on columns prepared from various materials. A decreasing concentration gradient of ammonium sulfate was then applied, and fractions were collected and assayed for enzymatic activity. On Sepharose 4B this procedure enabled the separation of several enzymes, with purification factors ranging between four- and sixfold. The ammonium sulfate concentrations at which the enzymes *h*GDH and *h*MDH were eluted from various solid supports are compared in Table I together with the ammonium sulfate concentration at the midpoint of the solubility curve. From this comparison it is clear that the elution from Celite is governed mainly by the solubility properties of the two halophilic enzymes in ammonium sulfate. As for the other solid supports, the ammonium sulfate concentrations at which the two enzymes are eluted are much lower than those at which they are precipitated, although the order of elution is related to that of decreasing solubility. It is worth noting that the elution concentration depends on the charge of the matrix. This observation is very surprising—it is totally unexpected that at concentrations of salt as high as 2 *M*, ionic interactions between the matrix and the proteins are effective, and this might be related to hydration-mediated interactions such as the ones postulated to contribute to the stabilization of the proteins (Zaccai *et al.*, 1989).

To summarize this set of observations, halophilic proteins adsorb to polysaccharide matrices at high ammonium sulfate concentration. When the matrix is charged the adsorption of proteins having the same charge on the matrix is reduced whereas the adsorption of proteins having opposite charge is greatly facilitated. These observations enabled the development of several powerful purification

TABLE I
Elution of Two Halophilic Enzymes by Decreasing Concentration Gradients of Ammonium Sulfate[a]

Column	GDH (*M*)	MDH (*M*)
Sepharose 4B	1.44	1.70
CM-cellulose	1.84	2.06
HMD-agarose	1.18	
DEAE-cellulose	ne[b]	ne[b]
Celite	3.11	3.53
Solubility[c]	3.01	3.32

[a]Ammonium sulfate concentration at which the enzymes eluted from various solid supports. From Mevarech *et al.* (1976), with permission.

[b]ne, Not eluted: the enzymes did not elute until the concentration was 0.4 *M* $(NH_4)_2SO_4$ [0.3 *M* $(NH_4)_2SO_4$ in the case of GDH]. They could, however, be eluted by a NaCl gradient.

[c]Ammonium sulfate concentration at which 50% of the activity was found in the supernatant.

schemes by which halophilic proteins were purified to homogeneity. In order to demonstrate the full potential of the ammonium sulfate-dependent chromatographies, the applicability of these methods to the purification of some halophilic enzymes will be reviewed.

The capacity of Sepharose to adsorb proteins in 2.5 *M* ammonium sulfate is at least 30 mg/ml gel (Leicht and Pundak, 1981). Even higher amounts of protein can be adsorbed on DEAE-cellulose under the same conditions. It is, therefore, advantageous to use Sepharose or DEAE-cellulose, either batchwise (Zusman *et al.*, 1989) or by loading on a column (Leicht and Pundak, 1981; Mevarech *et al.*, 1977), in the early stages of the purification. In addition to the severalfold purification achieved in this step, it is possible to eliminate most of the cellular debris as well as viscous material, which interferes in later stages. The desorption of the proteins from the Sepharose can be achieved by an ammonium sulfate decreasing concentration gradient. The adsorption of proteins to DEAE-cellulose is governed by both the interaction with the polysaccharide backbone, which is facilitated by the salt, and the interaction with the positive charges of the matrix, which are enhanced as the salt concentration is reduced. Therefore, when decreasing concentration gradients are applied to a DEAE-cellulose column, the proteins will start to move as soon as the interactions with the polysaccharide gel weaken. However, when the

salt concentration is reduced too much, the interaction with the positive charges becomes stronger and the proteins are retarded again on the gel. There are two ways to desorb the proteins from the positively charged gel, either by eluting the protein with ammonium sulfate solution at the concentration at which the protein is bound most weakly to the gel, or by using solutions having lower ammonium sulfate concentrations, to which sodium chloride is added to overcome the electrostatic interactions. For every protein there is a different optimum combination of the two interactions, thus the ammonium sulfate concentration at which the protein moves most quickly in the gel is different. Using this principle, it was possible to purify the ferredoxin of *H. marismortui* (Werber and Mevarech, 1978) and the dihydrofolate reductase of *Haloferax volcanii* (Zusman *et al.*, 1989) to homogeneity using only three purification steps. In the first case, after a step of adsorption to Sepharose 4B at 2.3 *M* ammonium sulfate and elution with a decreasing concentration gradient from 2.3 to 1.3 *M*, there were two steps at which the protein was adsorbed on DEAE-cellulose at 2.3 *M* ammonium sulfate and eluted by 1 *M* ammonium sulfate. In the case of the dihydrofolate reductase, the order of the steps was reversed. In the first two steps the enzyme was adsorbed on a DEAE-cellulose column at 2.5 *M* ammonium sulfate and eluted with 1.5 *M* ammonium sulfate. These steps were followed by adsorption to Sepharose 4B at 2.5 *M* ammonium sulfate and elution with a decreasing concentration gradient of 2.5 to 1 *M*.

The other principle was used in the large-scale purification of *h*MDH and *h*GDH (Leicht and Pundak, 1981). After fractionation on Sepharose 4B, the two enzymes were adsorbed on DEAE-cellulose and eluted with a concentration gradient ranging from 1.3 *M* ammonium sulfate to 1.3 *M* ammonium sulfate containing 2 *M* sodium chloride. In the purification of superoxide dismutase from *Halobacterium cutirubrum*, the enzyme was eluted from DEAE-Sepharose using a NaCl concentration gradient ranging from 0 to 0.8 *M* in the presence of 0.8 *M* ammonium sulfate (May and Dennis, 1987).

Although ammonium sulfate-mediated adsorption to Sepharose has been widely used in the purification of many other halophilic enzymes [i.e., halobacterial translation elongation factors by Kessel and Klink (1981); NAD-dependent glutamate dehydrogenase (E.C. 1.4.1.2) by Bonete *et al.* (1986); 2-oxoacid:ferredoxin oxidoreductases by Kerscher and Oesterhelt (1981a)], the systemic exploitation of ionic interactions between the proteins and charged gels at high ammonium sulfate concentrations still lags behind the other purification methods.

B. Affinity Chromatography

The principle of affinity chromatography, by which proteins are purified according to their specific ability to bind immobilized ligands, is particularly suitable to halophilic enzymes. Because halophilic enzymes are active at high salt concentrations, it is reasonable to assume that they will also bind their substrates and cofactors in these high salt concentrations. The enzyme *h*MDH of *H. marismortui* was purified to homogeneity using 8-(6-aminohexyl)amino-NAD-agarose (Mevarech *et al.*, 1977) and the *h*GDH of the same organism was purified using 8-(6-aminohexyl)amino-NADP-agarose (Leicht *et al.*, 1978). Leicht (1978) has shown that not only does the ammonium sulfate not interfere with the binding of the enzyme to the ligand, it actually enhances the interactions. This enhancement was shown to be biospecific, suggesting that the strength of the interaction between the enzyme and the immobilized coenzyme is a function of the sulfate concentration.

A very interesting application of affinity chromatography to the purification of halophilic enzymes was reported by Sundquist and Fahey (1988). These authors have purified the enzymes bis-γ-glutamylcysteine reductase and dihydrolipoamide dehydrogenase from *H. halobium* using immobilized metal ion affinity chromatography in high-salt buffers.

C. Other Methods

Two widely used methods that are not affected by high salt concentrations are gel-permeation chromatography and chromatography on hydroxylapatite gels. Columns for gel-permeation chromatography are prepared in either NaCl or KCl, usually in low phosphate concentration buffer. These salts are neutral in the sense that the migration of the proteins in the gel is dictated mostly by their Stokes radii. However, due to the rather low resolution of regular gel-permeation matrices in several cases [i.e., malate dehydrogenase (Mevarech *et al.*, 1977) and the two 2-oxoacid:ferredoxin oxidoreductases (Kerscher and Oesterhelt, 1981a)], the enzyme is recycled three or more times in the column in order to increase the effective length of the columns.

Chromatography in hydroxylapatite gel is particularly suitable for application after the gel-permeation chromatography steps. The adsorption of proteins to the matrix is affected specifically by the pres-