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Moeava Tehei · Bruno Franzetti · Marie-Christine Maurel
Jacques Vergne · Codjo Hountondji · Giuseppe Zaccai

The search for traces of life: the protective effect of salt on biological macromolecules

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Abstract Trapping malate dehydrogenase from the extremely halophilic archaeon *Haloarcula marismortui* in "dry" salt crystals protects the enzyme against thermal denaturation. Similar protection was not observed for the homologous mesophilic enzyme. In the case of transfer RNA molecules, high salt concentration plays a protective role against thermal degradation allowing activity to be recovered. The results are discussed in the context of exploring the fate of cell-free biological macromolecules in the environment and that of orienting the search for traces of life in planetary exploration.

Key words Halophile proteins · Malate dehydrogenase · tRNA · Exobiology · Stability · Salt

Introduction

The study of cells and macromolecules that function at extremes of temperature, pressure, pH, or salinity is important because such conditions relate to primitive conditions and the study of such cells and molecules allows the limits of survival to be defined. A potential role of extreme thermophiles as early life forms has been discussed since early in the twentieth century. It has received further impetus from molecular phylogenetic analysis and the proposal to divide organisms into three main domains (Archaea, Bacteria, and Eukarya) (Woese et al. 1985), and the clustering of thermophiles close to the putative root of the phylogenetic tree (Gogarten et al. 1989; Iwabe et al. 1989). Primordial life has also been discussed in terms of hypersaline conditions (Dundas 1998). Salt and water are tightly linked, and there is evidence that primitive oceans on Earth were highly saline (Knauth 1998). Very ancient salt-tolerant bacteria have been obtained from brine inclusions in salt crystals (Vreeland et al. 2000). Salt is also abundant on planets such as Mars and Europa, one of the satellites of Jupiter (Kargel 1998). Liquid water and potential sources of energy, the key ingredients for the development of living organisms (as we know them), were present on ancient Mars. The surface of Mars today is cold and dry. If we are to search for traces of life on Mars, not enough is known of how macromolecules behave in such an environment. How are their structures, stability, dynamics, and interactions affected? This question is also of interest with respect to the conservation of free biological macromolecules in Earth environments.

Extremely halophilic Archaea are organisms that require very high salt concentrations for survival. They occur in salt flats and salt lakes such as the Dead Sea (Israel, Palestine, Jordan) or the Great Salt Lake (Utah) and compensate for the high osmotic pressure of their environment by accumulating KCl in their cytoplasm at levels close to saturation. They may not be the earliest organisms on the phylogenetic tree, but their proteins have evolved particular features of halo-adaptation, which provide them with great tolerance to decreased water activity and temperature variations (Madern et al. 2000). Their properties may make halophilic proteins good candidates for survival as biological traces in dry saline environments. The surfaces of halophilic proteins are highly acidic with negative charges that bind water and salt ions. In this respect, they are similar to nucleic acids, and it has been shown that DNA in very high salt concentrations can resist denaturation to 107°C (Marguet and Forterre 1994). RNA, however, is much more thermolabile than DNA. If life arose in thermophilic environments, the RNA world hypothesis (Gesteland et al. 1999), for example,

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M. Tehei · B. Franzetti · G. Zaccai (✉)
Institut de Biologie Structurale, 41 rue Jules Horowitz, 38027 Grenoble Cedex 1, France
Tel. +33-4-38789573; Fax +33-4-38785494
e-mail: zaccai@ibs.fr

M.-C. Maurel · J. Vergne
Institut Jacques Monod, Paris, France

C. Hountondji
Laboratoire de Biochimie, Ecole Polytechnique, Palaiseau, France

G. Zaccai
Institut Laue Langevin, Grenoble, France

would have to include mechanisms to stabilize RNA under these conditions. In this paper, results on malate dehydrogenase and transfer RNA are presented that suggest that a hypersaline environment is favorable for the preservation of certain biological macromolecular structures.

Materials and methods

Stability measurements on halophilic and mesophilic malate dehydrogenases

Expression, renaturation, and purification of the recombinant malate dehydrogenase from the extreme halophile *Haloarcula marismortui* (HmMalDH) were carried out as previously described (Madern et al. 1995). Pig heart MalDH was obtained from Roche. Both enzymes were diluted 100-fold from a 5 mg/ml stock solution in 50 mM Tris, pH 8, with or without 4 M NaCl for the mesophilic MalDH and with 4 M NaCl for the halophilic MalDH. The enzymatic activity was measured as described previously (Cendrin et al. 1993) by adding 10 µl of sample to 1 ml of reaction mixture containing 2 M NaCl, 50 mM Tris (pH 8), 0.5 mM oxaloacetate, and 0.2 mM NADH for the halophilic enzyme and 50 mM Tris (pH 8), 0.5 mM pyruvate, and 0.2 mM NADH for the mesophilic enzyme. Samples were dried by evaporating the water away in a speed vacuum system. After incubating at 20°, 45°, 60°, 80°, or 100°C for 1 h, the salt crystals were transferred to another test tube and dissolved in the original volume of buffer. The residual enzymatic activity was measured under standard conditions. The same experiments were performed in parallel without the drying step.

Stability measurements on tRNA

Structural stability experiments were performed with a mixture of different specific tRNAs, isolated from *Escherichia coli* strain B (General Biochemicals GBI). The effect of salt on the resistance of tRNA to thermodegradation was studied in solution. tRNA was dissolved in water and extensively dialyzed against 1 M NaCl, 5 mM EDTA, and 10 mM cacodylate at pH 7. This treatment was to replace any remaining Mg^{2+} ions by Na^{+} counterions. The sample was then dialyzed against 10 mM NaCl. Several reaction mixtures (150 µl) containing tRNA (OD260 = 21) and NaCl (10^{-3}–3 M) were prepared. Solutions were heated to 82°C for 15 h or 31 h in well-closed microtubes. After heat treatment, samples were loaded onto 2% agarose gels in Tris acetate EDTA (TAE) buffer; the degradation of tRNA was assessed by ethidium bromide staining (See Results).

Functional stability was assessed by aminoacylation experiments (Lawrence et al. 1973). Commercial tRNA was heated under different salt conditions to 82°C for 31 h. After heat treatment, experiments were carried out at 25°C in a 20 mM Tris HCl buffer (pH 7.6) containing 10 mM 2-mercaptoethanol, 0.1 mM EDTA, 0.05 mg/ml bovine serum albumin, 7 mM $MgCl_2$, 150 mM KCl, 2 mM ATP, and 20 µM [^{14}C]L-methionine (50 mCi/mmol) or 20 µM [^{14}C]L-valine (94 mCi/mmol). The reaction was started by adding catalytic amounts of homogeneous *E. coli* methionyl-tRNA synthetase or valyl-tRNA synthetase to the incubation mixtures. After 10–15 min, when [^{14}C]amino acid incorporation into tRNA had reached a plateau value corresponding to the completion of the tRNA aminoacylation reaction, the incubation mixtures were quenched by the addition of cold 10% trichloroacetic acid containing 0.5% of nonradioactive L-methionine or L-valine. The samples were then allowed to stand in an ice bath for 30 min. Total yeast RNA (400 µg) was added as a carrier, and the precipitate of RNA was filtered on Whatman GF/C filters. The filters were washed three times with 20 ml of 5% trichloroacetic acid containing 0.5% of nonradioactive L-methionine or L-valine, and counted for [^{14}C] radioactivity in a Beckman LS 1801 liquid scintillation counter with picofluor scintillation fluid.

Results and discussion

In order to assess a possible advantage of hypersaline conditions or halophilic adaptation in the preservation of proteins, we studied the stability of pig's heart (Ph) and *Haloarcula marismortui* (Hm) malate dehydrogenase (MalDH) enzymes as a function of the presence of water and salt in the sample. PhMalDH is a mesophilic enzyme, whereas HmMalDH is extremely halophilic, that is, it requires hypersaline conditions (about 2 M) for its stability (Mevarech and Neumann 1977). Samples were incubated at different temperatures, either as solutions or after slow drying. The residual activity was then measured after dilution with the optimal reaction buffer.

We believe that slow drying is a more "natural" phenomenon than lyophilization, which may freeze active conformations and reduce the chance of kinetic destabilization during loss of water. Following slow drying, the salt-dried samples contained dry crystals about 3 mm in size (Fig. 1).

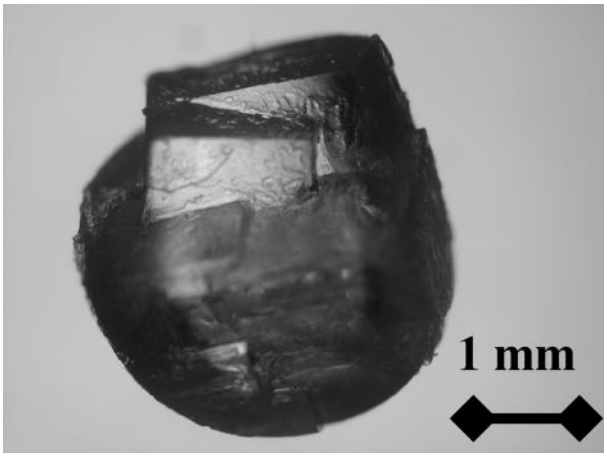

Fig. 1. Photograph of a salt crystal obtained by water evaporation under conditions described in the text. The dry material shown contained the *Haloarcula marismortui* malate dehydrogenase (HmMalDH) enzyme trapped inside

Salt crystals were examined by optical microscopy under normal and polarized light. They appeared to have no liquid inclusions and to be globally isotropic. A stepwise dissolution of the crystal followed by activity measurements showed that the enzyme was totally localized inside the crystal (data not shown).

The histogram in Fig. 2A displays the data obtained for solutions of PhMalDH in high- and low-salt buffers, and for solutions of HmMalDH in high-salt buffers. The mesophilic enzyme under low-salt conditions and the halophilic enzyme under high-salt conditions showed similar thermostability (about 15%) upon heating to 60°C. At 80° and 100°C, residual activity could not be detected in the mesophilic enzyme, whereas a residual activity of about 10% was measured for the halophilic protein. On the other hand, 4 M salt in the incubation buffer significantly destabilized the mesophilic enzyme with respect to heat denaturation, even though it did not inhibit its activity at room temperature.

The data shown in Fig. 2B are from samples dried by slow evaporation at room temperature. Loss of water, with or without salt, led to drastic destabilization of the non halophilic enzyme, even at room temperature (20°C). In contrast, the halophilic enzyme resisted dehydration perfectly well and even appeared to be stabilized by the salt crystals, since about 90% of its activity was recovered at 60°C. At the same temperature, activity of only 15% was detected when the enzyme was kept in salt solution, suggesting that the protection of the halophilic protein is related to the crystalline state and not to microscopic liquid inclusions, for example, or to stabilization by nonspecific "salting-out" effects (Bonnete et al. 1994).

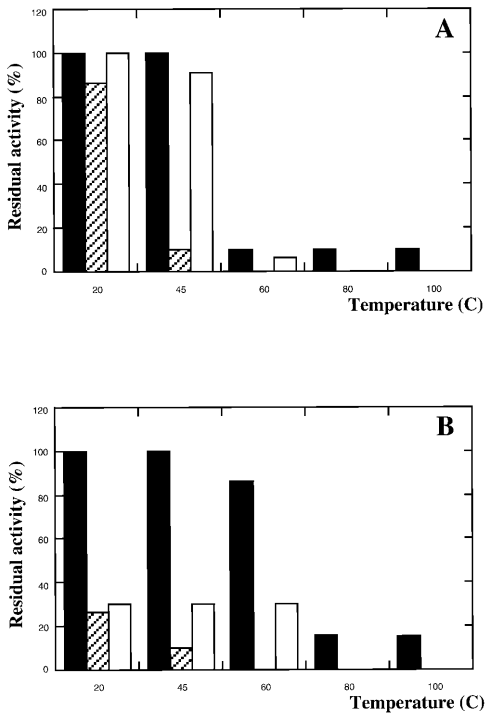

Fig. 2A,B. Residual activities of HmMalDH and pig heart MalDH after incubation under various conditions. HmMalDH was prepared in a 4 M NaCl solution (*solid bars*). Pig heart MalDH was prepared either in a 4 M NaCl solution (*hatched bars*) or in the absence of salt (*open bars*). Both enzymes were buffered with 50 mM Tris, pH 8. The residual activities presented in **A** were measured at room temperature after incubating for 1 h at various temperatures. **B** Data from samples dried by evaporating the water in a speed vacuum system for 2 h at room temperature. After incubation (1 h) at various temperatures, the dry material was resuspended in the original volume (100 µl) of buffer, and the residual enzymatic activity was determined by using the standard enzyme essay as described in Materials and methods

Special "dry" environments have been shown to protect protein structures against denaturation (the quotation marks are included because it is unknown how much bound water is still associated with the protein under such conditions). Trehalose, for example, has been shown by neutron scattering experiments to protect myoglobin under dry conditions by maintaining its structure in a hard dynamic state (Cordone et al. 1999). Even though halophilic proteins have been known to require highly concentrated salt solutions for stability, we believe that this is the first report showing that they can survive "dry" conditions in salt crystals. We suggest that the protection is related to special halo-adaptation features, which have been extensively studied for HmMalDH (Eisenberg et al. 1992; Madern et al. 2000; Richard et al. 2000). Halophilic proteins display peculiar solvent interactions; a highly concentrated layer of hydrated salt ions from the solvent is anchored to the protein by charged residues on the protein surface. This adaptation may allow a halophilic protein to maintain a folded active structure, even in very low water activity. Unlike in the case of mesophilic proteins, salt crystals do not constitute a hostile environment for halophilic proteins; in fact, they even increase their thermostability.

To estimate the possible advantage of hypersaline conditions in preserving RNA stability against thermodegradation, tRNA samples were heated to high temperature in aqueous solutions with various NaCl concentrations. After incubation for 31 h at 82°C, tRNA in low-salt solutions was highly damaged. An increasing salt protective effect on the macromolecule was observed that tended toward a plateau from 0.5 to 3 M NaCl (Fig. 3). Stability studies were also performed by measuring the residual specific tRNA charge capacity after heat treatment for 15 h at 82°C (Fig. 4). Two specific tRNA molecules were tested: tRNA-Met and tRNA-Val. At NaCl concentrations of 1 or 2 M, the amino

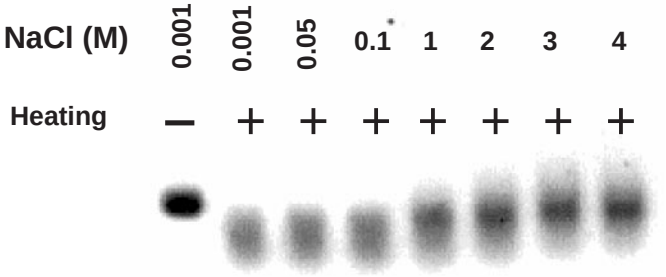

Fig. 3. Structural integrity study of tRNA at high temperature in various salt concentrations. tRNA in NaCl solutions of increasing concentration was heated to 82°C for 31 h (see Materials and methods). The products were then analyzed by electrophoresis on a 2% agarose gel

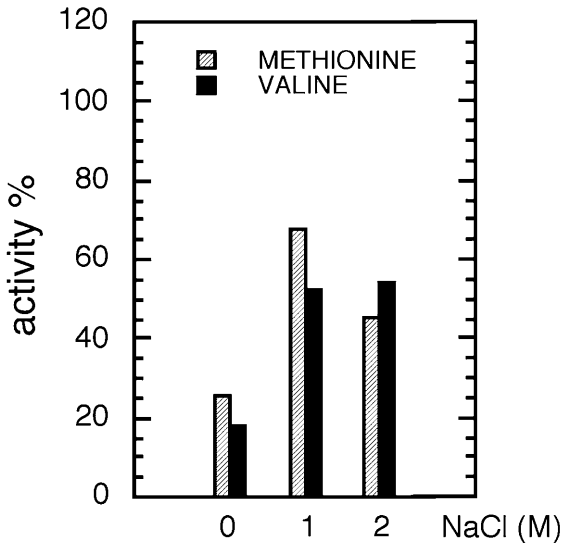

Fig. 4. NaCl protective effect against thermodegradation of tRNA in solution. tRNA dissolved in water, with or without the addition of 1–2 M NaCl, was heated to 82°C for 15 h. Samples were analyzed by measuring the specific amino acid charge capacity of methionine and valine (activity is expressed as the proportion of picomoles of amino acid charged per OD unit of RNA after treatment compared with an untreated RNA sample)

acid acceptance of the tRNAs was 50%–70% that of the untreated control tRNA. In contrast, the charge capacity of the tRNAs heated in the absence of salt was reduced to about 20% that of the control (Fig. 4).

Conclusion

We report here that a halophilic enzyme can survive dry conditions and heat denaturation in salt crystals, and that tRNA molecules are stabilized by high salt concentrations against heat degradation. These results suggest that these macromolecules could well survive in dry saline environments on Earth such as evaporated lakes or seas. Mars also is believed to have supported oceans over a sufficiently long time to allow high salt concentrations to become established. This period was followed by a period during which it is believed that water progressively disappeared from the surface of the planet. The hypothesis can be formulated that this process was prolonged enough to allow a cellular life form to adapt to conditions under which salt was concentrated by water evaporation. If this is true, our data suggest that it may be possible to detect traces of halophilic proteins and RNA on Mars. We conclude that a salt environment is an important parameter to be considered when sampling sites are chosen.

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