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Compatible solutes of microorganisms from hot, marine environments: structure, biosynthesis, and biotechnological applications

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Abstract
Compatible solute accumulation is a frequent strategy adopted by halophiles to face fluctuations in the water activity of the environment. Among the thermophiles and hyperthermophiles that are also halotolerant or moderately halophilic, compatible solute accumulation takes place not only as an osmoregulatory mechanism, but also as a response to heat stress. Compatible solutes from hyperthermophiles have particular features, like their overall charge, that distinguishes them from solutes of mesophiles, and their role as structural protectants against heat has been often suggested. Here, we describe an overview of our knowledge on compatible solutes accumulated by (hyper)thermophiles, along with their biosynthetic routes, protein stabilizing effects and potential biotechnological applications.

1. Introduction

When confronted with adverse conditions microorganisms have to develop adaptation strategies. Halophiles, that have adapted to thrive in environments of low water activity, use mainly one of two strategies. The first one, used many extreme halophiles, is the so called “salt-in-cytoplasm” and consists in the import of inorganic salts into the intracellular environment [1]. A second, more versatile strategy, is the accumulation of small organic molecules (usually uncharged or zwiterionic) to balance the external osmolarity, thus preserving cell integrity. This strategy is widespread in the biological world, being employed by organisms of varied lineages such as archaea, bacteria, yeast, filamentous fungi, and algae that rely exclusively on solute accumulation for osmoprotection [2-3]. These compounds accumulate to high intracellular levels without disturbing cellular functions, hence the term “compatible solutes” coined by Brown in the 70’s [2]. Compatible solutes must be highly soluble and they usually belong to one of the following groups of compounds: amino acids and derivatives, sugars, polyols, betaines, and ectoines [4].

Thermophiles and hyperthermophiles (from this point on called (hyper)thermophiles), on the other hand, have no obvious way of decreasing the impact of temperature, since the temperature in the cytoplasm is always identical to the external temperature. Accordingly, temperature adaptation is expected to involve more “built-in” strategies and, indeed, most (hyper)thermophilic derived structures display high intrinsic thermal stability [5]. It would, in fact, be unreasonable to think that (hyper)thermophiles would be able to withstand high temperatures by relying heavily on extrinsic stabilization factors. However, some proteins have been isolated from thermophiles that display a much lower thermal stability than what would be expected from the temperature growth range of the organism [6-10].
Many (hyper)thermophiles have been isolated both from fresh and seawater sources. In halophilic thermophiles, compatible solute accumulation occurs not only in response to an increase in the external salinity, but also in response to supraoptimal growth temperatures [11, 12]. The latter observation seems rather intriguing; especially if we consider that the external water activity remains relatively unaltered when the temperature is raised.

On the other hand, the superior protective effect of compatible solutes from (hyper)thermophiles upon cellular structures (namely proteins) is well documented [6, 13-16]. From these two observations the inference of a link between compatible solute accumulation by (hyper)thermophiles and structural protection against heat damage appears inevitable. And if such a correlation is legitimate, this means that compatible solutes accumulated by (hyper)thermophiles may be different from those of mesophiles, and if this is the case, then we must consider what solutes are accumulated by these organisms and how are they able to confer a higher degree of added stability.

2. Compatible solutes of (hyper)thermophiles

(Hyper)thermophiles accumulate a relatively large variety of solutes. Some of these, like trehalose, α-glutamate, or proline are frequently found in non-thermophilic organisms; others like di-myoinositol phosphates are commonly found in many (hyper)thermophiles and have never been found in mesophiles (Figure 1); others still, like mannosylglycerate, are strongly associated with (hyper)thermophiles and appear only rarely in mesophiles [17].

Fig. 1. Distribution of compatible solutes in hyperthermophiles as a function of the maximum temperature for growth. BPG- cyclic 2,3-bisphosphoglycerate; DIP- di-myoinositol 1,1’-phosphate; MG- mannosylglycerate; Tre- trehalose; DGP- diglycerol phosphate; GPI- glycerophospho-inositol.

Compatible solutes exclusively or mainly found in (hyper)thermophiles will herein be named “hypersolutes” for the convenience of a short designation. In contrast to the solutes more commonly found in mesophiles, hypersolutes are generally negatively charged, and most fall into two categories: hexose derivatives with the hydroxyl group at carbon 1 blocked in an α configuration, and polyol-phosphodiesters. The most representative compound in the first category is α-mannosylglycerate (MG). Although MG was initially identified in red algae
of the order Ceramiales [18], this is one of the most widespread solutes in (hyper)thermophiles, occurring in members of bacteria and archaea belonging to distant lineages, such as the genera Rhodothermus, Thermus, Rubrobacter, Aeropyrum, Stetteria, Archaeoglobus, Methanothermus, Thermococcus, Pyrococcus, and Palaeococcus [11, 12, 19-21]. The level of MG increases primarily in response to osmotic stress, the only known exceptions to this behavior being found in bacteria of the genera Rhodothermus where it also increases with supraoptimal growth temperatures when the two stresses are combined [22].

In contrast to the frequent occurrence of MG, its uncharged derivative, mannosylglyceramide (MGA) is extremely rare and was found in only a few strains of Rhodothermus marinus. Other variations upon the MG theme include mannosylglucosylglycerate (MGG) and glucosylglucosylglycerate (GGG), compounds that have only been found in Petrotoga miotherma, and Persephonella marina, respectively. Another related compound is glucosylglycerate (GG), which is relatively common in halotolerant mesophilic bacteria and has been found in one thermophile, the bacterium Persephonella marina (unpublished results).

Within the polyol-phosphodiester group, the most notable member is di-myoinositol phosphate (DIP). This solute has never been found in organisms with optimal growth temperature below 50ºC, and usually accumulates in response to supraoptimal growth temperatures [17]. Examples of other polyol-phosphodiesters are diglycerol phosphate (DGP), only found in members of the genus Archaeoglobus, and glycerophospho-inositol, a structural chimera of DIP and DGP that was found only in two hyperthermophiles. A derivative of DIP, di-mannosyl-di-myoinositol phosphate, is present in species of the genus Thermotoga, where its level increases mainly in response to heat stress [23].

Although (hyper)thermophiles use a variety of compatible solutes during thermo- or osmoadaptation, there are some general trends in their response. Among them is the differential pattern of solute accumulation in response to different stress factors. Often, MG, DGP and amino acids accumulate preferentially in response to increased salinity while the level of DIP and DIP-derivatives responds to heat stress [11, 19].

3. Biosynthesis of compatible solutes

Although mannosylglycerate was not the first hypersolute to be discovered it is by far the most studied. Biosynthetic routes and key enzymes for MG synthesis are known in many organisms, and its stabilizing properties have been examined in a number of systems [11, 14, 16, 24-31]. There are two pathways for the synthesis of this solute (Fig. 2).

![Fig. 2. The two pathways for the synthesis of mannosylglycerate in Rhodothermus marinus. Single-step pathway uses mannosylglycerate synthase (MGS), while the two-step pathway involves the actions of mannosyl-3-phosphoglycerate synthase (MPGS) and mannosyl-3-phosphoglycerate phosphatase (MPGP).]
One of these pathways, also called the single-step pathway, consists on the direct condensation of GDP-mannose with D-glycerate by the action of mannosylglycerate synthase (MGS); while the other, also called the two-step pathway, involves a phosphorylated intermediate synthesized by mannosyl-3-phosphoglycerate synthase (MPGS) from GDP-mannose and D-3-phosphoglycerate, and produces mannosyl-3-phosphoglycerate, which is subsequently dephosphorylated by a specific phosphatase (MPGP).

The two-step pathway appears to be much more widely distributed in Nature than the single-step pathway. In fact, all thermophiles examined to date, from either the domains Bacteria or Archaea are able to synthesize MG via the two-step pathway, while the single-step pathway has been found in mesophilic algae of the order Ceramiales (unpublished results). However, in the thermophile Rhodothermus marinus the two pathways coexist [25]. This pathway duality is rather intriguing and has only recently been explained [29]. The single-step pathway is selectively expressed in response to heat stress, whereas the two-step pathway is predominantly active under osmotic stress. Therefore, the two biosynthetic pathways play specialized roles in the adaptation to heat and osmotic stress in R. marinus.

In spite of being the first hypersolute to be discovered di-myoinositol phosphate (DIP) biosynthesis is still unclear, with two different pathways proposed in two investigated organisms. The first step is common to both proposals and consists in the conversion of glucose-6-phosphate to inositol-1-phosphate, after this Chen et al. [32] propose that in Methanotorris igneus part of the inositol-1-phosphate is dephosphorylated into inositol while another part is activated to CDP-inositol, both molecules being then condensed to yield DIP by the action of a DIP synthase. In Pyrococcus woesei, however, Scholz et al. [33] found a different route. In this case, two molecules of inositol-1-phosphate are condensed to yield DIP at the expense of NTP. Both proposed pathways were partially demonstrated in cell extracts, but the key enzymes were not characterized, neither the respective genes were identified.

Diglycerol phosphate (DGP) biosynthesis was investigated in Archaeoglobus fulgidus. The respective key enzymes are not yet characterized, but 31P-NMR experiments in cell extracts have proved it to be strictly dependent on CDP-glycerol and glycerol-3-phosphate (unpublished results).

4. Compatible solutes as protein stabilizers

Because of its pattern of accumulation in response to supraoptimal growth temperatures, hypersolutes were soon envisioned as thermoprotectants of biological materials. Since then, many in vitro studies have proved their ability as superior protein stabilizers when compared to their mesophilic counterparts [13-16, 30-31, 34-36]. Just as an example, 0.5 M MG is able to increase by 8°C the melting temperature of staphylococcal nuclease, while 2.5 M glycerol, a canonical protein stabilizer, produces only a 3°C increase (unpublished results from our team).

Hypersolutes in general display a considerable specificity of the stabilizing effect, with the extent of stabilization being highly modulated by the solute/protein pair under consideration [35, 37]. For instance DGP, a highly effective stabilizer of Desulfovibrio gigas rubredoxin, is ineffective towards a similar rubredoxin from D. desulfuricans [35]. Another curious hypersolute feature is their global charge. Solutes derived from mesophiles are usually non-charged, or zwiterionic, hypersolutes however tend to be negatively charged. Moreover, the charge seems to play a role in the stabilizing action. In an illustrating example, Faria et al. [30] proved that the melting temperature of bovine ribonuclease A in the presence of DIP is increased by 4°C in the presence of 6 M DGP.

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and absence of MG depends on the ionization state of the solute; with higher stabilization achieved at pH values above 5, when the solute is fully ionized, and no added stability when the solute is uncharged.

These features are difficult to explain with the present models for solute action. The most widespread of these theories, the preferential exclusion model \[38, 39\], states that the stabilizing solutes are excluded from the solvent/protein interface, in a process which is, in itself, entropically disadvantageous. Upon denaturation, there is usually an increase in protein surface, which necessarily causes an increase in the extent of exclusion. Thus, in the presence of an excluded solute, denaturation becomes thermodynamically more difficult. However, to evaluate the extent of stabilization one has to determine the degree of exclusion, which is different for any solute/protein pair and a consequence of an ensemble of fine protein/solvent interactions, making it almost impossible to predict the stabilizing effect.

In another approach, Bolen and co-workers performed a systematic study of the energy involved in the transfer of the 20 amino acids and an analogue of the peptide backbone from pure water to solutions with various concentrations of sucrose, sarcosine or urea \[40-41\]. The transfer free energies obtained are quite small, showing that the interactions of those solutes with the protein components are modest and that water is a strong competitor for interactions with the protein. The determination of the transfer free energy of the peptide backbone from water to solutions with stabilizing solutes was found to be largely positive (thermodynamically unfavorable) \[41\]. This approach identifies the solvophobic effect of the backbone as the main driving force for stabilization, and explains the general action of stabilizing solutes, with a certain degree of solute/protein specificity being due to the opposing contributions of the amino acid side chains \[40\].

Concerning the stabilization mechanism, at the molecular level, we are even further away from explaining the phenomenon. The first hypothesis on this subject suggested small structural alterations able to promote more stable conformations. However, NMR studies have shown that, at least in the studied cases, no measurable structural changes take place \[42-43\], strengthening the view that stabilizing compatible solutes exert their action through changes in the solvent structure and/or by changing dynamic properties of the protein. In an \(^{15}\)N-NMR study on the dynamics of \textit{D. gigas} rubredoxin, Lamosa \textit{et al}. \[42\] showed that, although no changes could be detected in the protein structure upon solute addition (100 mM DGP), a small but generalized increase in the order parameters took place. This overall rigidification became increasingly more pronounced when moving from fast to slower time scales, indicating that, although the vibrational modes of individual atoms and small groups may be barely affected by the presence of solutes, it is clear that the concerted motion of larger protein segments becomes more restricted and this may be an important factor in protein stabilization \[42\].

5. Potential applications for solutes from (hyper)thermophiles

Due to the enhanced ability to stabilize biological materials, the application of hypersolutes in industrial applications was soon envisioned, and several industrial patents on their uses have been filed \[44-45\].

Since a major drawback of enzyme usage in industrial processes is its relative instability leading to degradation during long-term storage and repetitive use, there is a high interest in methodologies that increase enzyme stability. Therefore, high performance solutes able to stabilize enzymes at lower concentrations than conventional stabilizers are currently
envisioned as additives in analytical/clinical test-kits, development of heat-stable vaccines, biosensors, cosmetics, DNA amplification and cell preservation. Moreover, the ability of these solutes to interfere with protein aggregation and fibril formation may provide clues for the design of chemical chaperones useful in the treatment and/or prevention of protein-misfolding associated diseases.

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7. References


