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Compatible solutes of halophilic eubacteria: molecular principles, water-solute interaction, stress protection

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Abstract. Compatible solutes are best described as organic osmolytes responsible for osmotic balance and at the same time compatible with the cells' metabolism. A comprehensive survey (using HPLC and NMR methods) on halophilic/halotolerant eubacteria has revealed the full diversity of compatible solutes employed in nature. Molecular principles derived from the spectrum of compounds found in the bacterial world may be summarized as follows. Compatible solutes are polar, highly soluble molecules and uncharged at physiological pH. With the exception of proline (a proteinogenic amino acid) they are characterized as amino acid derivatives of the following types: betaines, ectoines, N-acetylated diamino acids and N-derivatized carboxamides of glutamine. Using near-infrared spectroscopy we have also been able to demonstrate that compatible solutes are strong water-structure formers and as such probably excluded from the hydration shell of proteins. This "preferential exclusion" probably explains their function as effective stabilizers of the hydration shell of native proteins (protection against heating freezing and drying). Hence these typical products of halophilic eubacteria have a considerable potential as stabilizing/protecting agents on both molecular and whole-cell level. Thorough understanding of common structural principles and fundamental water-solute interactions will ultimately enable us to design novel highly efficient stress protectants and stabilizers of biomolecules.
Key words. Halophilic/halotolerant bacteria; compatible solute; osmolyte; osmoadaptation; enzyme stabilization; stress protection; water structure former; kosmotropic solute.

Introduction

Osmotic adaptation of halophilic and halotolerant microorganisms requires osmotic equilibrium across the membrane and – as water is freely permeable – a cytoplasm of similar osmotic strength as the surrounding medium[38, 70, 24, 69, 28]. Two strategies of osmoadaptation have evolved among halophilic and halotolerant bacteria: the KCl-type and the organic-osmolyte type. Whereas for example halobacteria tolerate high cytoplasmic concentrations of KCl, due to specially adapted enzymes and cell structures[20], representatives of the second strategy use a more flexible mode of adaptation, which enables them to maintain a "normal" enzymatic machinery. Regarding modes of osmoadaptation there seems to be no clear distinction between the domains Archaea and Bacteria. All archaebacterial halobacteria examined seem to tolerate high ionic concentrations (KCl-type), but halophilic methanogenic Archaea also produce compatible solutes (glycine betaine and β-amino acids). On the other hand production and accumulation of organic osmolytes are wide-spread among halophilic eubacteria. However, one must bear in mind that mostly phototrophic and aerobic chemoheterotrophic eubacteria have been examined. The few anaerobic organisms investigated (*Haloanaerobium, Halobacteroides, Sporohalobacter, Acetohalobium* species) all seem to employ the KCl-type of osmoadaptation. This led Rengpipat et al.[54] to propose that a constrained energy conservation mechanism may have favoured the evolution of the halobacterial type of osmoadaptation (i.e. salt-dependent enzymes).

Organic osmolytes, the typical product of aerobic eubacterial halophiles, are often accumulated to cytoplasmic concentrations well above 1 mol/kg water. As these solutes – even at high concentrations – do not interfere with the cells' metabolism they have been named compatible solutes[7]. This original definition, however, does not reflect the whole potential of these osmolytes, since under conditions of osmotic equilibrium, reduced water activity may become the crucial factor for stability of enzymes and other cellular components. Hence a stabilizing effect of these solutes at the molecular and whole-cell level has long been proposed and encouraged investigations into their use as potential protectants against the destructive effects of salt[52, 51, 42, 73, 44], freezing and heating[16, 34, 1, 8] and possibly drying[1, 9, 10, 18]. Compatible solutes, therefore, have biotechnological potential as protecting agents for both industrial enzymes as well as for the conservation of microorganisms[27].

Natural diversity of compatible solutes

A comprehensive survey using high-performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) on almost all halophilic and halotolerant

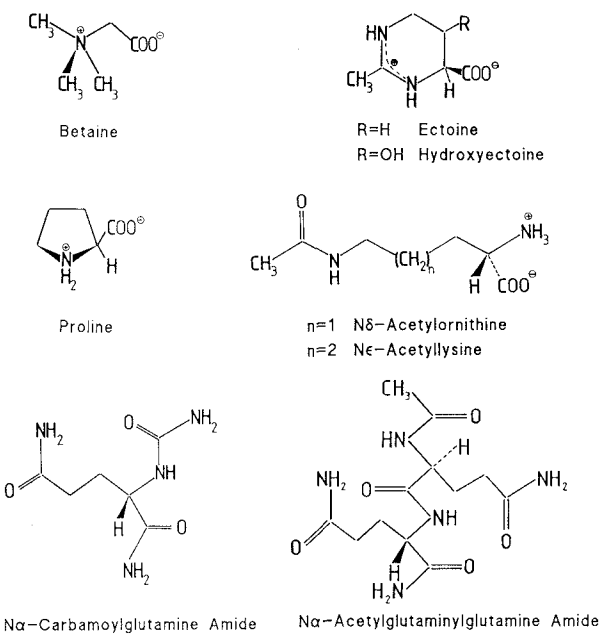

Figure 1. Compatible solutes most commonly found in halophilic/halotolerant eubacteria.

eubacteria available from culture collections (ca. 50 strains) and, in addition, on approx. 150 isolates from various biotopes like salt lakes, salinas, salt mines and similar sites of elevated salinity has revealed the full diversity of compatible solutes in nature[24, 70, 79, 65] (fig. 1). To assess the organisms' ability to synthesize compatible solutes de novo screening was performed using synthetic growth media where possible. Media supplements like yeast extract contain considerable amounts of glycine betaine (1–3% dry weight), therefore, use of even moderate concentrations (e.g. 0.05%) may lead to preferential accumulation and replacement of endogenic compatible solutes. This causality explains why heterotrophic halophiles, grown on yeast extract containing media, have erroneously been regarded as betaine producers[31]. The strains under investigation included anoxigenic phototrophic bacteria, aerobic chemoheterotrophic Proteobacteria of the α- and γ-subdivision, actinomycetes, Gram-positive cocci, bacilli and related species as well as *Staphylococcus*- and *Salinicoccus* species[24, 79, 65]. In addition, moderately halotolerant species of the genera *Brevibacterium* and *Corynebacterium* have also been examined[29, 4, 21]. On the archaebacterial side a thorough investigation of methanogenic species has similarly revealed a number of characteristic classes of compounds[58, 36, 57].

Polyols (mainly glycerol and arabitol) often found in algae, yeasts and fungi[75, 3, 30, 33] have so far not been observed in bacteria at osmotically relevant concentrations. Instead, glycerol glucosides have been described in at least one purple bacterium (*Rhodobacter sulfidophilus*)[24] and a number of moderately halophilic cyanobacteria[53, 43]. The occurrence of sugars (mainly sucrose and trehalose) as part of the solute "cocktail" seems to be very common in a wide range of microorganisms. However, they can only partly replace nitrogen-containing compatible solutes[25] and never exceed a cytoplasmic concentration of 500 mM. These solutes of lesser compatibility are, therefore, typical for organisms of relatively limited salt tolerance. They may fail to qualify as compatible solutes "sensu stricto" and a possible role as universal stress metabolites enabling survival under adverse conditions is presently under discussion[71, 77].

Although β-glutamate (like α-glutamate) is a common constituent of the solute cocktail of the *Nocardiopsis* species its maximal cytoplasmic concentration never exceeds 200 mM independent of salinity (Galinski, unpublished). Its role in osmoadaptation is therefore negligible, probably due to the fact that charged amino acids require a corresponding cation. The only uncharged and hence compatible β-amino acids involved in osmoadaptation have been reported from halophilic methanogenic archaebacteria[36]: β-glutamine and Nε-acetyl-β-lysine. Both compounds are uncharged under physiological conditions, accumulate to concentrations well above 0.5 M and show relatively low turn-over rates[57]. It is probably the higher solubility of β-glutamine which makes this osmolyte superior to the proteinogenic α-isomer glutamine (solubility: ca. 300 mM). The latter has so far only been reported as a compatible solute in moderately halotolerant members of the genus *Corynebacterium*, where it reaches cytoplasmic concentrations near saturation[21].

The most important compatible solutes accumulating to concentrations well above 0.5 M are further characterized below:

a) Glycine betaine: Glycine betaine, originally described in the sugar beet (*Beta vulgaris*)[61], is the characteristic quaternary amide of a number of salt- and drought-resistant plants of the family *Chenopodiaceae*[67, 68]. It is also the typical product of phototrophic eubacteria, especially of those displaying a high salt tolerance[22, 32], and has further been found as a primary product in halophilic archaebacterial methanogens[58, 36]. Due to the mass development of phototrophic bacteria (e.g. cyanobacterial mats) in hypersaline environments betaine is probably abundant in natural biotopes and may serve as a convenient solute source for heterotrophic eubacteria. However, the ability to synthesize betaine de novo is rare among aerobic heterotrophic eubacteria. Of all strains examined only *Actinopolyspora halophila* is a betaine producer[65].

b) Ectoines: Ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidine carboxylic acid) was first discovered as a minor component in the phototrophic sulfur bacterium

Ectothiorhodospira halochloris[23]. This solute is without doubt the most abundant osmolyte of aerobic chemoheterotrophic eubacteria and has since been found in all halophilic/halotolerant Proteobacteria of the γ-subdivision, in all representatives of the genus *Nocardiopsis*, in all Gram-positive cocci examined so far, in brevibacteria and even in *Bacillus* species and *Sporosarcina halophila*. As much as betaine can be regarded the typical product of halophilic phototrophic bacteria, it is justified to conclude that the ability to synthesize ectoine is very common among aerobic heterotrophic eubacteria. This abundant osmolyte has remained undetected for a long time because the lone electron pair at the ring nitrogens participates in a delocalized π-bonding and does not readily react with common amino acid reagents. The hydroxy derivative of ectoine is often found as a minor component in Proteobacteria of the γ-subdivision and in *Nocardiopsis* species, where one often observes a correlation with growth temperature. Among *Marinococcus* species a growth phase dependent variation from ectoine to hydroxyectoine seems to be characteristic (Galinski, unpublished). In general the occurrence of hydroxyectoine as the major solute or as an important co-solute seems to be more typical for Gram-positive eubacteria[65].

c) Proline: Proline has been reported as a compatible solute in a number of halophilic algae (mostly *Bacillariophyceae*)[76], some halophytes[37] and marine invertebrates[64]. Among procaryotes proline was originally considered the typical solute of halophilic *Bacillus* species. This view was primarily based on investigations into *Bacillus subtilis* and closely related species[47]. A thorough screening of a whole range of halophilic/halotolerant bacilli and related species, however, has revealed that the majority of species produce ectoine, either alone or in combination with proline and/or acetylated diamino acids[48]. *Bacillus subtilis* and *Planococcus citreus* seem to be representatives of a minority of proline producers unable to synthesize ectoine. Finally, *Staphylococcus epidermidis* and two *Salinicoccus* species also belong to the group of proline producers. It is however important to note that these organisms can only tolerate elevated salinities when grown on complex media.

d) N-acetylated diamino acids: The role of Nδ-acetylornithine as an osmolyte was first shown in one of our own isolates, strain M96/12b[80]. This strain belongs to the so called bacillus-related species and, consequently, Nδ-acetylornithine was also detected, at least as a minor component, in almost all *Bacillus* species under investigation including related organisms like *Sporosarcina halophila* and *Planococcus citreus*. As some organisms display a characteristic change from proline to Nδ-acetylornithine one is tempted to assume a common biosynthetic pathway (possibly involving ornithine δ-aminotransferase). The homologous Nϵ-acetyllysine, originally isolated and identified from *Sporosarcina halophila* (Severin & Galinski, unpublished), is also found in *Planococcus citreus* and some bacilli. Therefore, at our present state of knowledge, the ability to synthesize and use N-acetylated diamino acids as compatible solutes seems to be typical for members of the phylogenetically diverse group of aerobic spore formers (bacilli) and related organisms.

e) N-derivatized glutamine amides: This very unusual class of compounds has amidated glutamine as a common structural characteristic. To render the molecule polar but uncharged the amino residue is typically acetylated or carbamoylated. A novel representative of this class of compatible solutes, Nα-carbamoyl-L-glutamine-1-amide (CGA), has so far only been found in the phototrophic bacterium *Ectothiorhodospira marismortui*, where it amounts to as much as 30% of the solute pool[26]. As its cytoplasmic concentration reaches more than 0.5 M, it certainly serves an important function as an osmolyte[49]. A similar structural principle is realized with Nα-acetylglutaminylglutamine amide (AGGA), another representative of this class of osmolytes and so far the only neutral dipeptide of osmotic function. This osmolyte is observed as a minor component in *Rhizobium meliloti*[66], in two *Chromatium* species, in *Thiocapsa halophila*, *Rhodopseudomonas marina* and *Azospirillum brasilense*[65]. It follows that Nα-acetylglutaminylglutamine amide has to date only been detected in Proteobacteria of the α-subclass and in phototrophic bacteria of the γ-subclass.

A brief summary of the distribution of compatible solutes within the microbial world is given in the table.

Table. Typical occurrence of predominant compatible solutes in halophilic/halotolerant microorganisms

Polyols	algae, fungi
Betaines	phototrophic bacteria, methanogenic bacteria, *Actinopolyspora halophila*
Ectoines	Proteobacteria γ-subdivision, *Nocardiopsis* sp., brevibacteria, Gram-positive cocci, many bacilli
Proline	algae, some bacilli and related species (*Planococcus citreus*), *Staphylococcus epidermidis**, *Salinicoccus* sp.*
NAc-O, NAc-L	strain M96/12b and *Sporosarcina halophila* minor compounds in many Bacilli and related organisms
β-Amino acids	methanogenic bacteria (β-glutamine, Nϵ-acetyl-β-lysine)
Carboxamides	*Ectothiorhodospira marismortui* (CGA), other anoxigenic phototrophic bacteria, *Azospirillum brasilense*, *Rhizobium meliloti* (AGGA)

NAc-O = Nδ-Acetylornithine, NAc-L = Nϵ-Acetyllysine, CGA = Nα-Carbamoylglutamine amide, AGGA = Nα-Acetylglutaminylglutamine amide, * = growth on complex media only.

Molecular principles

The following structural principles are possibly deduced from the spectrum of solutes employed in nature:
1) All bacterial compatible solutes described so far are polar, highly soluble, uncharged amino acids and/or derivatives.
2) Of the natural proteinogenic amino acids only proline is an important compatible solute. Other potential candidates of sufficiently high solubility are glycine, serine and alanine (3.3, 2.4 and 1.9 mol/kg water, respectively). However, only alanine is occasionally found as a minor component of the solute "cocktail" in halophiles.
3) Amino acid derivatives belong to one of the following classes of compounds:
– N-methylated compounds (betaines)
– Nα-acetylated or Nα-carbamoylated carboxamides (CGA, AGGA)
– terminally acetylated diamino acids (Nδ-Acetylornithine, Nε-Acetyllysine)
– ectoines (which may be compared to cyclic condensation products of N-acetylated diamino acids).

In all cases derivatization seems to adopt the following strategy:
1) Methylation of the amino nitrogen reduces the charge density and confers partial hydrophobicity to the zwitterionic molecule. This type of derivatization also increases the size of the hydration shell[15,74].
2) Amidation of a carboxyl group converts a charged moiety to a polar but uncharged carboxamide. Natural semi-carboxamides are the amino acids asparagine and glutamine, the low solubility of which probably excludes use as a major compatible solute. Amidation of glutamine (yielding glutamine amide) requires additional modification of the amino group in order to maintain an uncharged molecule, e.g. carbamoylation (CGA) and acetylation of the dipeptide (AGGA).
3) Terminal acetylation of a diamino acid transforms a cation into an uncharged molecule characterized by a zwitterionic amino acid moiety and a polar uncharged N-acetyl group (comparable to semi-carboxamides).
4) The zwitterionic ectoines combine structural elements of betaine (charge density and charge separation), proline (ring structure) and acetylated diamino acids (NH—C(CH$_3$)=X group).

The molecular structure of ectoines is, therefore, a good example for terminal acetylation in combination with charge delocalization.

It can be concluded from the above that the prerequisites neutrality of (net) charge and high solubility alone do not suffice as criteria for good compatible solutes. Furthermore, as both zwitterionic and polar uncharged

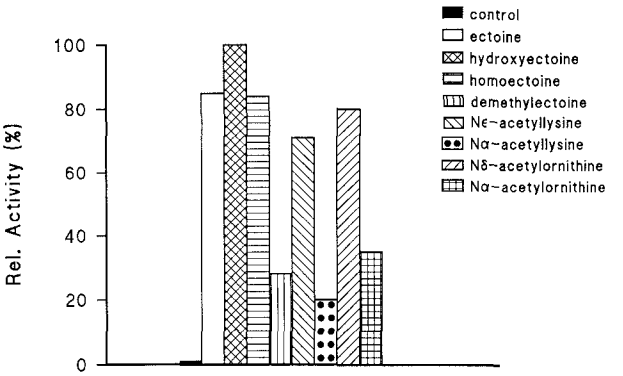

Figure 2. Freeze-thaw protection of various ectoines (including artificial derivatives) and N-acetylated diamino acids on lactate dehydrogenase. Residual activity after four freeze-thaw cycles in 1 M concentration of protectants (the unprotected enzyme shows no residual activity), average of three experiments, standard deviation: 5–10%. It is seen that demethylation of ectoine markedly reduces its stabilizing properties. A comparison of N-acetylated diamino acids further reveals that the natural solutes (terminally acetylated) are much better protectants than their α-isomers.

solutes are employed as compatible solutes, the presence of local charges at specific sites is no characteristic criterion to explain compatible solute properties at the molecular level. The only common characteristic observed so far in all solutes is a combination of polar and relatively hydrophobic moieties. Hydroxyproline for example proved less effective as a stabilizer than its more hydrophobic analog proline[63,60]. It has further been shown in the series glycine betaine – dimethylglycine – sarcosine – glycine that successive demethylation of betaine results in gradual loss of its protecting abilities[52]. A comparison of a number of artificial ectoine derivatives (fig. 2) with respect to freeze stabilization has confirmed that the methyl group is the most critical factor affecting stabilizing properties. In addition, it was shown that the terminally acetylated derivatives of ornithine and lysine (both occurring in nature) are better stabilizers than the α-acetylated isomers (fig. 2). Possibly the molecular basis of solute compatibility is explained by a delicate arrangement and balance of hydrophilic/hydrophobic structural elements and the resulting steric conformation effecting water solute interactions. Further elucidation of common characteristic features is under present investigation as a thorough understanding of the underlying principles should enable us to possibly design new and more powerful compatible solutes for biotechnological applications.

Protein stability and water-solute interaction

A comprehensive overview on dominant forces involved in protein stability has been given by Schellman and Dill[62,19]. Non-polar residues of the peptide chain are sequestered into a core where they largely avoid contact with water. These hydrophobic residues in the core of proteins appear to be more strongly conserved and

correlated with structure than other types of interactions. A non-polar transfer to water is *unusual* as it is principally opposed by an excess in entropy (water structure forming). In addition, the heat capacity change upon transfer is large and positive, possibly explained by an "iceberg-like" ordering of water molecules subject to melting with increasing temperature. The entropy change caused by hydrophobic solvation is partly counterbalanced by an increase in entropy due to backbone expansion and side chain unfreezing with increasing temperature. This implies that enthalpy and entropy are strong functions of temperature and that free energy vs. temperature is a curved function. Therefore, proteins have a specific maximum of stability and denature (unfold) both at higher and lower temperatures.

A number of models have been proposed to explain the stabilizing action of compatible solutes: water replacement[14], hydrophobic interaction[63] and preferential exclusion model[1]. Although supporters of the preferential exclusion model have gathered considerable experimental evidence to support the view of an uneven distribution of compatible solutes in a protein solution, it still lacks a molecular explanation. According to this model compatible solutes are excluded from the hydration sphere of proteins. This exclusion from part of the solvent available is consistent with a decrease in entropy of the system (higher ordering), and this entropically unfavourable situation in turn causes minimization of the excluded volume and subsequently stabilizes the conformation of a protein and/or promotes the association into oligomers. Provided this model applies to all compatible solutes (most of which have not yet been included in these studies) it would help to explain why a wide variety of different substances display similar stabilizing effects. However, the question still remains as to *why* compatible solutes are so effectively excluded from the hydration shell of proteins.

An explanation of this unusual behaviour of compatible solutes is possibly found in their characteristic water-solute interaction. Whereas according to the "continuum model" water assumes an uninterrupted three-dimensional lattice of tetrahedrally coordinated hydrogen-bonded molecules (with stretching and bending deformations, but no bond breakage), mixture models ("vacant lattice point" and "flickering cluster") propose water molecules fluctuating through states where none, one or both of its hydrogens are engaged in hydrogen bonding by means of a cooperative process. The finding that near-infrared overtones of O–H vibrations display a characteristic shift to the left with increasing temperature, whereas the ice spectrum is markedly displaced to longer wavelengths (fig. 3A), has been used in favour for a model of discernible water populations of different structural properties (e.g. hydration water and bulk water). The spectroscopic shift is explained by varying

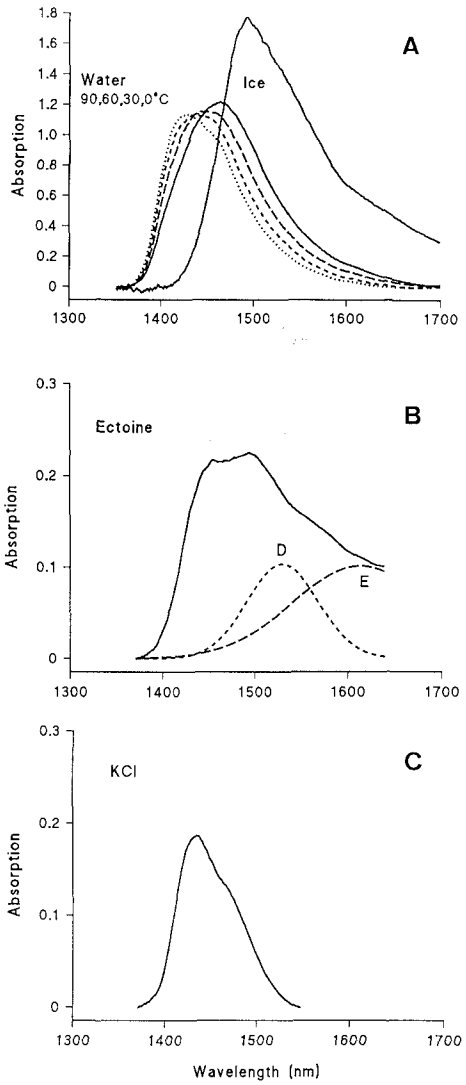

Figure 3. Near-infrared (NIR) absorption of ice and water at different temperatures (A) and NIR difference spectra of ectoine (B) and KCl (C) obtained from solutions of 2 and 1 mol/kg water, respectively. The shift towards shorter wavelengths with increasing temperature (A) is caused by weaker hydrogen bonding, whereas water at lower temperatures contains higher proportions of strongly bonded ice-like clusters. Difference spectra, reflecting strength of hydrogen bonding in the hydration shell of solutes therefore prove that natural compatible solutes (in contrast to denaturants like salt and urea) are strong water structure formers as shown by their ice-typical absorption bands (designated bands D and E).

proportions of strongly bonded hydrogens and seems to support the view that ice-like structures (clusters) are maintained even in liquid water[45,46,5,6]. As a solution of solutes is spectroscopically comprised of both bulk and hydration water, which differ with respect to their absorption maximum, near-infrared spectroscopy can be used to determine hydration numbers as well as characteristic hydration spectra of solutes by means of difference spectroscopy[55,56,72]. The hydration numbers obtained for natural compatible solutes in 2 M solution range between 3.7 (betaine) and 4.8 (ectoine) mol water/mol

solute (Galinski, unpublished). They are comparable to those of the "unfreezable water" recently reported for trehalose[35] and markedly higher than those of well-known denaturants like for example urea and KCl (2.3 and 2.5 mol/mol solute, respectively). From this it seems justified to conclude that compatible solutes have relatively large hydration shells. More information concerning the quality of these hydration shells is obtained by difference spectroscopy (fig. 3B). The difference spectra of all compatible solutes are characterized by absorption bands above 1500 nm (designated bands D and E) and, in that respect, are clearly distinguished from those of denaturing agents (fig. 3C). These findings provide further evidence that compatible solutes are not only strong water-structure formers but also aggregate a hydration shell which, at least in part, bears resemblance to typical ice-like structures.

With the help of a recently published model by P. Wiggins[78] describing structural differences between hydration water and bulk water in the cytoplasm we now have a much better understanding of the function of compatible solutes. According to her model, two water populations of different physical properties have to be considered: dense (or weakly bonded) water in the hydration shell of proteins and less dense water (or structured water) in bulk. Given these structural differences it follows that different solutes show preferences for one or the other of both water populations: small highly charged molecules preferentially dissolve in hydration water, whereas large molecules of low charge density are better dissolved in bulk water. This holds especially true for compatible solutes, which due to their strongly bonded hydration shell will fit much better into the wider, more structured form of water. The observation of a preferential exclusion of compatible solutes from the hydration shell of proteins[1] can thus be explained by a preference for less dense water. The resulting protective effect of compatible solutes may, therefore, be seen as a consequence of surface minimization (comparable to the phenomenon observed with two immiscible solvents). On the basis of the above concept one would predict that compatible solutes counteract all kinds of denaturing effects and stabilize the native structure against both high and low temperatures. Similarly, one would assume that compatible solutes – at least partially – relieve the deleterious (denaturing) effects of inorganic salts and, thus, also protect against freezing. Removal of the so-called unfreezable water however, as experienced during the process of drying, seems to be a different matter as successful protection would require a solute able to replace hydration water at critical regions of the protein[18]. Compatible solutes have with time found their way into the literature and are, in recognition of their biotechnological potential, currently labelled for example protein stabilizers, association promotors, enzyme activators, salt antagonists, heat and freeze protectants. Examples of possible applications as stress protectants will be given below.

Stress protection

Stabilizing labile macromolecular structures like proteins has always attracted scientific interest. Consequently, typical Hofmeister salts (e.g. $[NH_4]_2SO_4$), polyols (e.g. glycerol) and sugars (e.g. sucrose, trehalose) have – empirically – been found to exhibit remarkable protection, long before the term "compatible solute" was coined. With the discovery of a whole range of new compatible solutes and recent proposals as to their molecular function (see above) research on stress protection and stabilization has gained new impetus. Most investigations have so far dealt with enzyme protection against salt[52,51,42,73,44], heating, freezing and drying[16,34,1,8,9,10,18,40]. From our present knowledge of the function of compatible solutes one may propose that all modes of stress protection in the liquid state are possibly based on a common physical phenomenon (i.e. preference of compatible solutes for non-hydration water). In the case of drying, however, the protectant would need to exhibit additional functions enabling at least partial replacement of water in the hydration shell[17,12,13,18]. As generally accepted test systems two labile enzymes, lactate dehydrogenase and phosphofructokinase, have often been the object of stability research[8,11]. Both enzymes are very sensitive towards elevated temperatures and also rapidy inactivated by freeze-thaw treatment. Using lactate dehydrogenase (LDH) as a test enzyme the following set of figures provides examples of the stress protection obtained by the addition of compatible solutes (figs 4–6, data are taken from Lippert & Galinski[40,41]).

Figure 4 illustrates the remarkable freeze-stabilizing effect of a whole range of compatible solutes (including sugars) with betaine as an exception. A similar experiment performed on phosphofructokinase, however, revealed a slightly different order of efficacy: ectoine (100%), followed by hydroxyectoine, betaine and trehalose (90–80%) and finally sucrose (60%)[40,41]. As all tests were performed at a solute concentration where stabilization was maximal, they clearly reflect differences on the molecular level. Hence the degree of stabilization by a particular solute apparently also depends on the enzyme in question. This is especially pronounced in the case of betaine, which is a good protectant for phosphofructokinase, but displays almost no effect on lactate dehydrogenase.

In addition, it had been claimed that polymeric forms (e.g. polyethylene glycol) provide a better freeze protection than the monomers[11]. This has been confirmed, in principle, by our own observations using polyvinylpyrrolidone (PVP) and a commercially available pro-

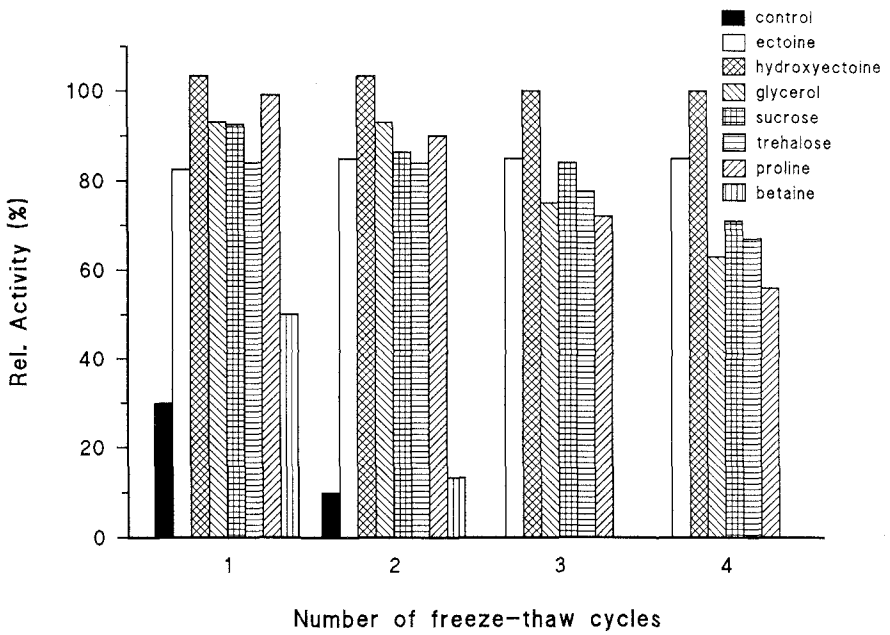

Figure 4. Protection of lactate dehydrogenase (LDH) against freeze-thaw treatment. The unprotected enzyme is totally inactivated after three freeze-thaw cycles. Note that betaine provides almost no protection whereas others (e.g. hydroxyectoine) provide 100% stability under the conditions employed. Concentration of protectants: 1 mol/kg water. Each bar represents the average of at least 3 experiments, standard deviation: 5–10%.

duct based on collagen (Prionex, Merck). However, the protection of polymers against freezing did not exceed that obtained by the ectoines. And contrary to natural compatible solutes the polymeric forms under investigation displayed no protection against elevated temperatures (Galinski & Lippert, unpublished).

Similarly, previous investigations concerned with heat stabilization were unable to present a consistent picture due to largely different effects with different enzymes[2, 50, 39]. Our own findings – again using lactate dehydrogenase and phosphofructokinase as test enzymes – are equally inconclusive as for example ectoine, a good stabilizer against freezing, is less suitable as a heat protectant for lactate dehydrogenase (fig. 5) and shows almost no effect on phosphofructokinase (not shown). On the contrary, the protection by hydroxyectoine against both stress factors and both enzymes is remarkable (90–100%) and proves the point that some solutes may have a much wider range of possible applications than others.

The obvious but unpleasing result that compatible solutes vary tremendously in their degree of heat and freeze protection, depending on the nature of the solute and the enzyme under investigation, shows that our picture of compatible solute action is still far from being clear. According to the preferential exclusion model, stabilization by compatible solutes is explained as an entropically unfavourable exclusion from the enzyme's hydration shell. As one could suppose differences in the degree of exclusion (possibly depending on the temperature), variations in the degree of protection should not be surprising as such and may well be consistent with the above mentioned model. However, the controversial effects observed with the same solute and different enzymes suggest that specific solute-enzyme interactions also have to be considered. In this context, it seems important to recall that, on a molecular level, compatible solutes typically combine polar and hydrophobic regions. Therefore, depending on the surface characteristics of the protein, one could envisage a competitive and counteracting effect of hydrophobic interactions with unpolar protein residues.

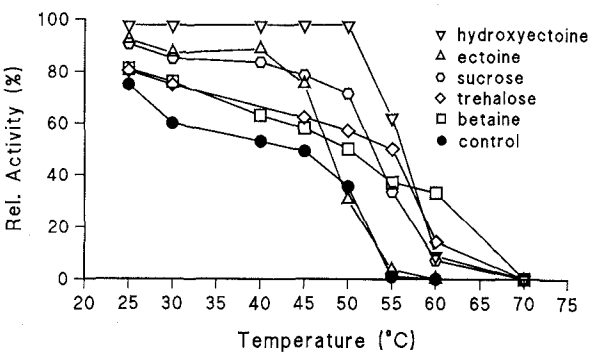

Figure 5. Protection of lactate dehydrogenase (LDH) against heat stress (10 min exposure). All solutes markedly increase the stability against heat denaturation. The protecting effect is especially pronounced in the case of hydroxyectoine, which provided 100% stabilization up to 50 °C. Average of 3 experiments, standard deviation: 5–10%.

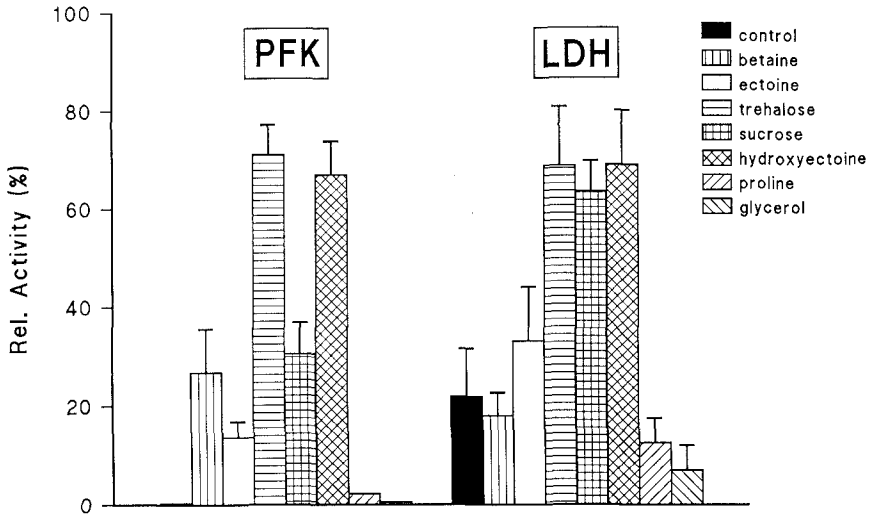

Figure 6. Protective effect of compatible solutes against freeze-drying of phosphofructokinase (PFK) and lactate dehydrogenase (LDH). Average of at least four experiments. Of all eubacterial amino-acid type compatible solutes only hydroxyectoine proved an effective stabilizer against total dehydration of enzymes. These findings support the view that OH-groups may possibly serve as water replacement in the hydration shell. However, other hydroxyl carriers like glycerol (and also sucrose in the case of PFK) were unsuitable protectants against drying.

The view of Crowe et al.[18] that freezing and drying represent fundamentally different stress factors is based on IR-spectroscopy investigations indicating that, in the dried state, OH-groups of the protectant interact with polar groups on the surface and partially replace hydration water, thus preserving the native conformation[59,12,13]. Therefore, the presence of hydroxyl groups should be regarded as an important prerequisite for suitable drying protectants. Unsurprisingly, only sugars and hydroxyectoine proved to be good protectants against drying (fig. 6). Although these observations seem to stress the importance of hydroxyl groups for drying protection, one should be aware of the fact that sucrose proved an unsuitable stabilizer for phosphofructokinase (PFK) and that glycerol also failed to protect either enzyme. Therefore, additional properties seem to be required for optimal stabilization, possibly a steric conformation suitable to function as a spacer at critical regions of the dried protein.

It seems clear from the above that the present working model ("preferential exclusion" in the liquid state and "water replacement" for dehydration) has to be taken with caution, as additional effects caused by for example hydrophobic interactions and steric conformation may also determine the suitability of particular compatible solutes. In order to establish a useful correlation between classes of enzymes (to be protected) and optimal groups of protectants to be used in a particular stress situation much more research is required on both physical properties of solutes as well as surface characteristics of protectable enzymes.

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