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Comparative study of the thermostabilizing properties of mannosylglycerate and other compatible solutes on model enzymes

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Abstract The protection of mannosylglycerate, at 0.5 M concentration, against heat inactivation of the model enzyme lactate dehydrogenase (LDH) was compared to that exerted by other compatible solutes, namely, trehalose, ectoine, hydroxyectoine, di-*myo*-inositol phosphate, diglycerol phosphate, and mannosylglyceramide. Mannosylglycerate and hydroxyectoine were the best stabilizers of the enzyme and showed comparable protective effects. Diglycerol phosphate, trehalose, and mannosylglyceramide protected the enzyme to a lower extent. Ectoine conferred no protection, and di-*myo*-inositol phosphate had a strong destabilizing effect. The superior ability of mannosylglycerate to prevent LDH inactivation was accompanied by a higher efficiency in preventing LDH aggregation induced by heat stress. Moreover, mannosylglycerate induced an increase of 4.5°C in the melting temperature of LDH, whereas the same molar concentration of trehalose caused an increase of only 2.2°C. The effectiveness of mannosylglycerate in protecting LDH was also compared to that of other chemically related compounds: mannose, methyl-mannoside, potassium glycerate, glucosylglycerol, glycerol, and glucose. Mannosylglycerate conferred the highest protection, but glucosylglycerol and potassium glycerate were very efficient; glucose exerted a low degree of protection, glycerol and methyl-mannoside had no significant effect, and mannose caused destabilization. Mannosylglycerate was also a good thermoprotectant of glucose oxidase from *Aspergillus niger*, an enzyme with a net charge opposite to that of LDH under the working conditions. Given the superior performance of mannosylglycerate as a thermoprotectant of enzyme activity in vitro, it is conceivable that it also fulfills a protein thermoprotective function in vivo.

Key words Mannosylglycerate · Compatible solutes · Thermal stabilization · Lactate dehydrogenase

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Introduction

The survival of microorganisms in the natural environment implies their ability to adapt, within intrinsic limits, to parameters such as fluctuations in the external osmotic pressure, temperature, and pH. In particular, variations in the osmotic pressure trigger a series of biochemical responses directed at maintaining a positive cell turgor, required for cell viability. In order to adjust to higher osmolarities of the environment, microorganisms accumulate osmolytes to reestablish the cell turgor pressure and to protect intracellular enzymes and other macromolecules from dehydration. These osmolytes have been designated compatible solutes to convey the idea that they must not disturb the normal activities within the cell (Brown 1990; da Costa et al. 1998).

Most of our knowledge on osmoadaptation in microorganisms derives from research conducted in mesophiles (Brown 1990). A large variety of compatible solutes have been found in these microorganisms, including amino acids, sugars, polyols and their derivatives, betaines, ectoine, and hydroxyectoine. However, the recent growing interest in organisms adapted to extremely high temperatures has shown that thermophiles and hyperthermophiles use several solutes for osmoadaptation, which are uncommon or nonexistent among mesophiles. For example, glycine betaine and ectoines are not found in hyperthermophiles, but mannosylglycerate (MG), mannosylglyceramide (MGA), di-*myo*-inositol-1,1′-phosphate (DIP), diglycerol phosphate (DGP), di-mannosyl-di-*myo*-inositol phosphate, and cyclic 2,3-bisphosphoglycerate (cBPG) seem to play a role not only in the osmotic adaptation of thermophilic and hyperthermophilic organisms, but also in their adaptation to

high temperature (Ciulla et al. 1994; Kanodia and Roberts 1983; Martins and Santos 1995; Martins et al. 1996, 1997; Nunes et al. 1995; Scholz et al. 1992; Silva et al. 1999). Indeed, growth at supraoptimal temperatures induces a considerable increase in the intracellular pools of many of these solutes, leading to the hypothesis that they could contribute as extrinsic stabilizers of enzymes and other cell components in vivo (Santos and da Costa 2001). There is now a growing amount of evidence showing that compatible solutes from thermophiles contribute to the thermostability of enzymes, at least in vitro. The ability of MG to act as an enzyme stabilizer was recently demonstrated in several enzymes from mesophilic, thermophilic, or hyperthermophilic origin (Ramos et al. 1997). The thermostabilizing properties of DGP on several proteins was also shown by Lamosa et al. (2000). Also, the potassium salt of cBPG has a thermostabilizing effect on glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and malate dehydrogenase from *Methanothermus fervidus*; however, cBPG does not have an effect on the thermostabilization of rabbit glyceraldehyde 3-phosphate dehydrogenase (Hensel and König 1988). Recently, the stabilizing effect of cBPG was also demonstrated on two enzymes involved in methane formation in *Methanopyrus kandleri* (Shima et al. 1998).

The role of DIP in the thermostabilization of enzymes remains questionable. This solute had a positive effect on the stabilization of GAPDH (Scholz et al. 1992) but did not enhance the stability of hydrogenase and pyruvate ferredoxin oxidoreductase from *Thermotoga maritima* (Ramakrishnan et al. 1997).

Ectoine and hydroxyectoine, solutes so far found only in mesophiles, were also shown to be protective against thermal stress on several enzymes (Cánovas et al. 1999; Lippert and Galinski 1992). Moreover, many studies have demonstrated the superior ability of trehalose, one of the most widespread compatible solutes, to act as a protein stabilizer as compared to other disaccharides, polyalcohols, proline, and betaine (Hottiger et al. 1994; Paiva and Panek 1996).

One may question whether compatible solutes that are restricted to hyperthermophiles are better suited to conferring thermoprotection than solutes encountered in mesophiles. Therefore, we deemed it important to perform a comparative study on the effectiveness of solutes derived from hyperthermophiles and from mesophiles as enzyme protectants against heat inactivation. The model enzyme rabbit muscle lactate dehydrogenase (LDH) was selected as the target for these studies. The thermoprotective effect of chemically related compounds was also assessed to obtain information on chemical features relevant to the performance of MG as a protein thermostabilizer.

Materials and methods

Organisms and growth conditions

Pyrococcus furiosus DSM 3638, the source organism of MG and DIP, was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany, and grown on the maltose–peptone–yeast extract medium described previously (Raven and Sharp 1997). *Pseudomonas mendocina* DSM 50017 was grown at 30°C on a malate-casamino acids medium (Smith et al. 1988), with the NaCl concentration increased to 4%. *Rhodothermus marinus* DSM 4252 was grown at 65°C on Degryse 162 medium, as described by Nunes et al. (1995), containing 1.0% NaCl.

Preparation of cells extracts

Ethanol-chloroform extracts of *P. furiosus*, *P. mendocina*, and *R. marinus* were prepared following the method of Reed et al. (1984), modified as previously described (Martins and Santos 1995).

Purification of mannosylglycerate and mannosylglyceramide

MG was purified from *P. furiosus* cells grown at 90°C in the presence of 5.0% NaCl, by anion exchange chromatography on QAE-Sephadex (Pharmacia, Uppsala, Sweden), as described previously (Ramos et al. 1997), with the following modifications: the elution was performed with a linear gradient of 5.0 mM to 1.0 M sodium bicarbonate buffer ($NaHCO_3$), pH 9.8, at a flow rate of 10 ml/min. Fractions were analyzed for carbohydrate content using the method of Dubois et al. (1956), and carbohydrate-containing fractions were pooled and further analyzed with proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy; the fractions which eluted between 200 and 220 mM $NaHCO_3$ contained a mixture of DIP and MG, and the fractions which eluted up to 300 mM $NaHCO_3$ contained pure MG. $NaHCO_3$ was removed by passage through a Dowex 50W-X8 (BioRad, Richmond, CA, USA) column (H^+ form), the carbohydrate-containing fractions were pooled, degassed under vacuum to remove residual carbon dioxide, and titrated with ultra-pure solutions of KOH or NaOH (1.0 M) to obtain the potassium or sodium salts of MG, respectively. The samples were lyophilized and the residues dissolved in water to obtain 2.0 M solutions of MG, which were stored at –20°C until used. MG was quantified by ^{1}H-NMR spectroscopy and the concentration of K^+ or Na^+ was determined by plasma emission spectroscopy using a Jobin Yvon spectrometer (model JY24; Longjumeau, France). The ratio between MG and the counterion was 1. MGA was purified from ethanol-chloroform extracts of *R. marinus* as previously described (Silva et al. 1999).

Preparation of the potassium salt of glyceric acid

A solution of glyceric acid (Ca^{2+} salt; Sigma, St. Louis, MO, USA) was applied onto a Dowex 50W-X8 column (H^+ form) which was eluted with distilled water. The fractions were pooled, neutralized with 1 M KOH, lyophilized, dissolved in ultra-pure water, and the K^+ concentration was determined as described above.

Purification of glucosylglycerol

Glucosylglycerol was purified from ethanol-chloroform extracts of *P. mendocina*. The freeze-dried extract was dissolved in 10 ml of dichloromethane/methanol (1 : 1, v/v), vigorously vortexed, and the precipitate obtained removed by centrifugation (6,000 *g*, 4°C, 10 min); this procedure was repeated twice. The clear supernatants obtained from each extraction were pooled, the solvent was removed by rotary evaporation, and the residue lyophilized and dissolved in ^2H_2O. The sample was analyzed by ^{1}H-NMR spectroscopy, showing that glucosylglycerol was the major component in addition to a small amount of another compound, assigned as *N*-acetylglutaminylglutamine amide, based on chemical shift data (Pocard et al. 1994). Adsorption chromatography was used to further purify glucosylglycerol. For this purpose, a silica gel S column (20 × 2 cm) was packed with a mixture of dichloromethane/methanol (1 : 1, v/v), which was also used to dissolve the sample and for elution of the column. The eluted fractions were analyzed for the presence of carbohydrates, and those giving a positive response were pooled and concentrated by evaporation. ^{1}H-NMR spectroscopy showed that the resulting samples contained pure glucosylglycerol, which was lyophilized and dissolved in water to obtain a 1.0 M solution. This solution was judged free of K^+ and Na^+ using plasma emission spectroscopy.

Purification of di-*myo*-inositol phosphate

DIP was purified from ethanol-chloroform extracts of *P. furiosus* cells grown at 100°C in the presence of 2.8% NaCl by adsorption chromatography. The extract was loaded onto a silica gel S column (20 × 2 cm) prepared in dichloromethane/methanol/25% ammonia (6 : 10 : 5, v/v), and the column was eluted with the same solvent. Carbohydrate-containing fractions were pooled, analyzed by ^{1}H-NMR, and found to contain pure MG. DIP remaining in the column was then eluted with water, and judged pure by ^{1}H-NMR analysis. The sample was dissolved in distilled water and loaded onto a Sephadex G-10 (Pharmacia) column (55 × 1.6 cm). The potassium salt of DIP was obtained following a procedure similar to that used for preparation of potassium mannosylglycerate. The K^+ concentration in this sample was determined by plasma emission spectroscopy, and DIP was quantified by ^{1}H-NMR spectroscopy.

NMR spectroscopy

^{1}H-NMR spectra were obtained at 300.14 MHz on a Bruker AMX 300 spectrometer (Rheinstetten, Karlsruhe, Germany) with a 5-mm broadband probe head with inverse detection. Spectra were acquired at 27°C with water presaturation, a 5-μs pulse width (corresponding to a 60° flip angle), and a repetition delay of 3 s. For quantification purposes, known amounts of acetate and formate were added to the samples as concentration standards, and a repetition delay of 65 s was used.

Measurement of enzyme activities

For determination of LDH activity, the 1.0 ml standard reaction mixtures contained 0.25 μg of enzyme, 1.6 μmol of pyruvate (sodium salt), and 0.2 μmol of nicotinamide-adenine dinucleotide (NADH) in 80 mM Tris/HCl buffer, pH 7.6 (Vassault 1987). Activity was determined using a spectrophotometer with a cell compartment thermostated at 30°C.

For determination of glucose oxidase (GOD) activity, the assay mixture (1.0 ml) contained 6 μg enzyme, 95 μmol of glucose, and 0.17 μmol of 2,6-dichlorophenolindophenol (DCPIP) in 50 mM sodium acetate buffer, pH 6.0. The reaction was started by addition of the enzyme and monitored at 35°C by measuring the decrease in the absorbance at 600 nm.

Influence of mannosylglycerate and other compounds on the thermal stability of enzymes in vitro

LDH (Sigma Type II, rabbit muscle), purchased as a suspension in ammonium sulfate, was dialyzed at 4°C against 50 mM potassium phosphate buffer, pH 7.6, for 24 h. The enzyme concentration was calculated assuming a value of 1.44 ml mg^{-1} cm^{-1} for the extinction coefficient at 280 nm of LDH solutions (Jaenicke and Knof 1968). The incubation mixtures (200 μl) contained 10 μg of LDH in the absence or presence of one of the following compounds: MG, mannose, glycerol, methyl-α-mannoside, glycerate, glucosylglycerol, MGA, glucose, hydroxyectoine, ectoine, trehalose, DGP, and DIP. The potassium salts of MG, glycerate, DGP, and DIP were used, unless stated otherwise. Since the concentration of MG needed for achieving maximal protection of LDH against thermal inactivation was around 0.5 M (Ramos et al. 1997), all the solutes were added at this final concentration. The potassium phosphate concentration in the incubation mixtures was 2.5 mM (data shown in Fig. 2) or 22.5 mM (data in Fig. 3). We verified that the final pH was approximately 7.6.

GOD (Sigma Type VII-S, *Aspergillus niger*) was purchased in the lyophilized form with phosphate buffer and sodium chloride. Phosphate and sodium chloride were removed by passage through a PD-10 column (Pharmacia), which was equilibrated and eluted with 50 mM sodium acetate buffer, pH 6.0. Protein concentration was determined using the method of Bradford (1976) with bovine serum albumin as a standard. The incubation mixtures were prepared at an enzyme concentration of 0.4 mg/ml in the absence or presence of 0.5 M MG or KCl. The final concentration of acetate buffer pH 6 was 5 mM.

The reaction mixtures were placed in Eppendorf tubes, incubated in a water-bath at the appropriate temperatures (50° or 70°C for LDH or GOD, respectively), withdrawn at time intervals, cooled on ice, and the remaining activity was immediately measured and compared to that of an unheated control (100% activity). The compounds examined did not inhibit appreciably the enzyme activities (not shown).

To study the self-stabilization of LDH against thermal stress, enzyme solutions at several concentrations (50–1,000 µg/ml) were prepared and incubated at 50°C in the absence of solutes for 10 min, after which the residual activity was measured as described above.

Light-scattering measurements

LDH solutions were prepared as described above for the thermostability assays in the absence or in the presence of MG, trehalose, or KCl (final concentration 0.5 M). Due to sensitivity limitations of the spectrofluorometer (SPEX-Fluorolog 1680, Edison, NJ, USA), the LDH concentration was increased to 170 µg/ml. The mixtures were placed in quartz cuvettes, the temperature increased to 50°C at a rate of 5°C/min, and aggregation was monitored by measuring the light scattering as a function of time with excitation and emission at 320 nm. Light scattering is expressed as a percentage of that measured for the LDH in the absence of solutes after incubation at 50°C for 30 min.

Determination of the melting temperature by differential scanning calorimetry

Measurements were carried out with a MicroCal VP differential scanning calorimeter (Northampton, MA, USA). Samples contained LDH (170 µg/ml) in 25 mM potassium phosphate buffer, pH 7.6. MG, trehalose, and KCl were added at a final concentration of 0.5 M. After degassing for 8 min, the sample was heated between 40° and 70°C with a heating rate of 1°C/min. The melting temperature was calculated by a nonlinear fit of raw data to a Gaussian curve using the MicroCal software.

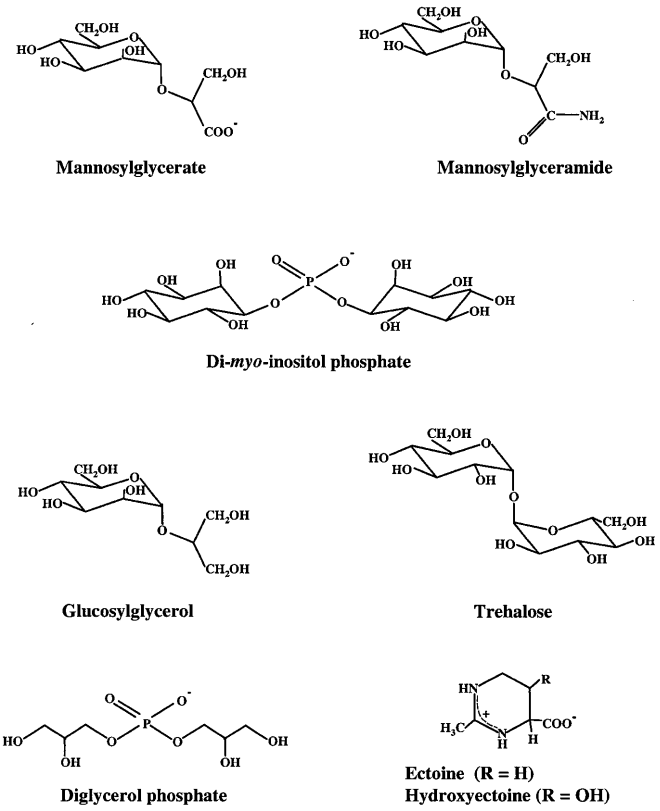

Fig. 1. Chemical structures of some organic compounds used in this study

Results

Effect of different compatible solutes on the stabilization of LDH against thermal stress

The inactivation of rabbit muscle LDH induced by a 10 min incubation at 50°C was examined as a function of the enzyme concentration in the range 50–1,000 µg/ml. As previously reported for freeze–thaw treatment (Lippert and Galinski 1992), the increasing LDH concentration resulted in enhanced resistance against thermal inactivation, the activity being fully retained at an enzyme concentration of 500 µg/ml or higher (data not shown). In all the subsequent experiments an enzyme concentration of 50 µg/ml was used.

Earlier work in our group has shown that the addition of MG enhances the thermal stability of several enzymes isolated from hyperthermophiles, thermophiles, and mesophiles (Ramos et al. 1997). In the present study, the protective effect of several compatible solutes (Fig. 1) was evaluated using rabbit muscle LDH as a model system and compared to that rendered by MG. The effectiveness of MGA, trehalose, DIP, ectoine, hydroxyectoine, and DGP was investigated by measuring the residual activity of LDH after incubation at 50°C for different periods of time (Fig. 2). KCl was included as a control for potassium, since the potassium salts of MG, DIP, and DGP were used throughout the experiments described in this article.

In the absence of solutes, LDH retained only 5.5 ± 1.5% of the initial activity after incubation at 50°C for 30 min; the thermal stability was increased by the addition of some of the compatible solutes examined, such as MG, hydroxyectoine, DGP, and trehalose. The effectiveness of MG and hydroxyectoine was remarkable; in fact, approximately 80% of the activity was retained after 20 min incubation in the presence of these solutes. Interestingly, MGA was much less effective than MG, and only 21.0 ± 1.8% of the initial activity was retained after incubation for 30 min. A similar time of incubation in the presence of DGP, KCl, or trehalose led to 30%–40% recovery of the initial activity. Ectoine showed no significant protection (9.4 ± 0.3% residual activity after 30 min), and DIP caused a strong destabilizing effect on the enzyme. The potassium and sodium salts of MG were equally effective in protecting LDH against thermal stress.

Effect of compounds related to mannosylglycerate on the thermal stability of LDH

In an attempt to obtain information on which chemical features of MG are relevant for the mechanism of LDH

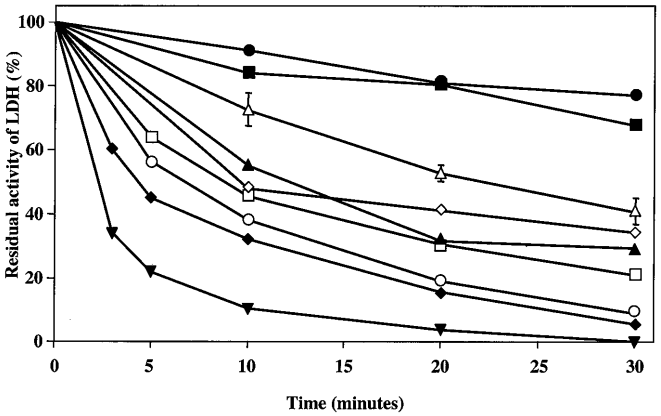

Fig. 2. Effect of compatible solutes on the thermal inactivation of rabbit muscle lactate dehydrogenase (LDH). Aliquots containing the enzyme at a concentration of 50 µg/ml and the solutes (final concentration, 0.5 M) were incubated at 50°C, withdrawn at appropriate time intervals, cooled on an ice-bath, and assayed immediately for enzyme activity. The final potassium phosphate concentration in the mixture was 2.5 mM. The results are averages of three independent experiments; activity is normalized to that of the mixture before heating to 50°C. For the sake of simplicity, representative *error bars* are shown only for one of the solutes. No additions (*solid diamonds*), mannosylglycerate (*solid squares*), hydroxyectoine (*solid circles*), diglycerol phosphate (*open triangles*), ectoine (*open circles*), KCl (*solid triangles*), mannosylglyceramide (*open squares*), trehalose (*open diamonds*), and di-*myo*-inositol phosphate (*solid inverted triangles*)

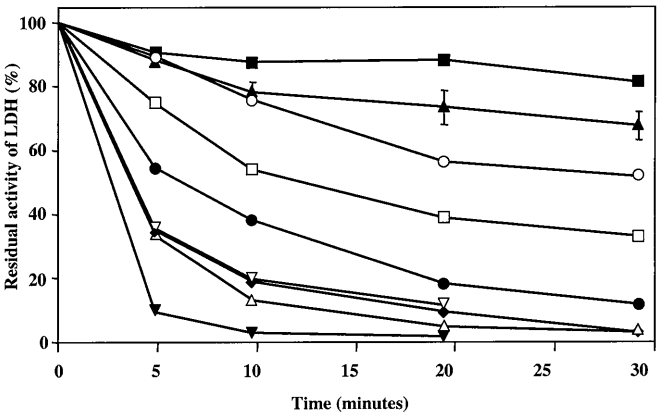

Fig. 3. Effect of mannosylglycerate and chemically related solutes on the protection of rabbit muscle LDH against thermal inactivation. Experimental conditions were the same as described for Fig. 2 except for the final potassium phosphate concentration, which was 22.5 mM. No additions (*solid diamonds*), K-glycerate (*open circles*), glucose (*solid circles*), methyl-mannoside (*open triangles*), mannose (*solid inverted triangles*), mannosylglycerate (MG; *solid squares*), mannosylglyceramide (*open squares*), glycerol (*open inverted triangles*), and glucosylglycerol (*solid triangles*)

thermostabilization, we examined the effect of chemically related compounds at a final concentration of 0.5 M. The results are shown in Fig. 3. When compared to the effect of MG (81 ± 4% residual activity after 30 min incubation at 50°C), glucosylglycerol exerted the highest protective effect, with 68 ± 4% residual activity being measured. In the presence of potassium glycerate or MGA, the enzyme

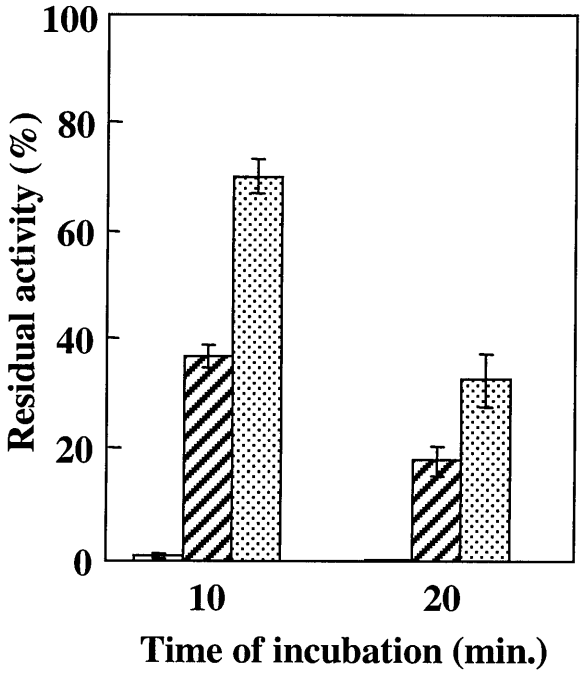

Fig. 4. Effect of MG on the thermal inactivation of glucose oxidase, a protein with a negative global charge at the working pH. Experimental conditions as described in "Materials and methods." Assay mixtures were incubated at 70°C for 10 and 20 min, cooled on an ice-bath, and assayed for enzyme activity. No additions (*open column*), MG (*stippled column*), and KCl (*hatched column*)

retained 52 ± 3% or 34 ± 2% activity, respectively. The addition of glycerol, methyl-mannoside, or glucose did not increase significantly the resistance of LDH against thermal stress (less than 10% activity remained after 30 min), whereas mannose exerted a clear destabilizing effect on the enzyme. Potassium phosphate, at a final concentration of 0.5 M, was a strong protectant of LDH: 100% activity was retained after incubation at 50°C for 30 min (not shown). The small discrepancy between results presented in Figs. 2 and 3 for MG are due to the different concentration of potassium phosphate used in the two sets of experiments (see "Materials and methods").

Effect of mannosylglycerate on the thermal inactivation of glucose oxidase

The isoelectric point of LDH is 8.6 (Susor et al. 1969), and therefore the total net charge of the enzyme was positive in all the experiments reported above. Since MG has a negative charge, it seemed interesting to examine the protecting efficiency on a negatively charged enzyme. GOD has an isoelectric point of 4 (Voet et al. 1981) and was selected for this purpose. After 10 min incubation at 70°C, the GOD activity was negligible; however, in the presence of MG, approximately 70 ± 3% of the initial activity was retained. A considerable, but clearly lower protection, was exerted by KCl (Fig. 4).

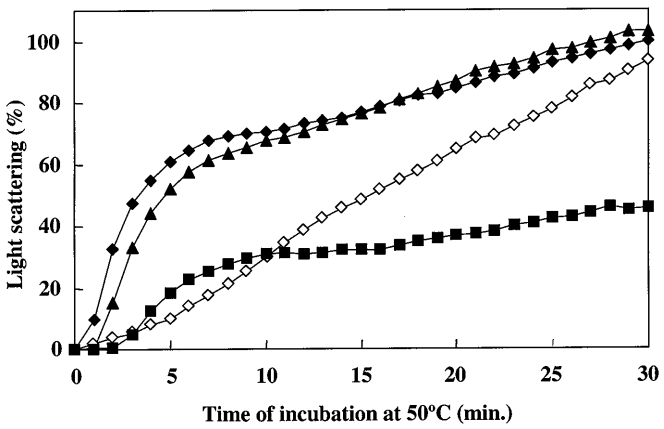

Fig. 5. Effect of MG, trehalose, and KCl on the aggregation of LDH subjected to thermal stress at 50°C. LDH (final concentration, 170 μg/ml) was incubated in the absence or in the presence of solutes (final concentration, 0.5 M). Aggregation was detected from light-scattering measurements with excitation and emission at 320 nm. No additions (*solid diamonds*), MG (*squares*), KCl (*triangles*), and trehalose (*open diamonds*)

Determination of the melting temperature and light-scattering measurements of LDH

To evaluate the extent of structural damage occurring during the incubation of LDH at 50°C, the temperature used in the thermal stability assays, the melting temperature (T_m) of the enzyme, was determined with differential scanning calorimetry. The LDH solution was prepared as described for the thermostability assays except for the higher concentration of the enzyme (170 μg/ml). A T_m of 55.9°C was found in the control conditions (no addition of solutes). The melting temperature increased to 60.4°C in the presence of 0.5 M MG; upon addition of the same concentration of KCl and trehalose the T_m increased to 56.8°C and 58.1°C, respectively. Besides these experiments, light-scattering measurements of LDH solutions were carried out to detect enzyme aggregation induced by thermal stress. MG or trehalose clearly reduced LDH aggregation, but MG was much more effective than trehalose in preventing aggregation when the enzyme was heated for prolonged periods of time. KCl had no significant effect (Fig. 5).

Discussion

Research on extremophilic bacteria and archaea has intensified during the last decade primarily because of the industrial importance of their resistant enzymes (Bauer et al. 1996; Ladenstein and Antranikian 1998). In addition to extremozymes, several low-molecular mass compounds, also derived from these organisms, have potential in a wide range of applications involving stabilization of cells or biomaterials against different stresses, such as heating, freezing, or dehydration (Carpenter et al. 1990; Lippert and Galinski 1992; Paiva and Panek 1996). In particular, the ability of solutes to stabilize enzymes is of great importance in biotechnology since enhanced protein stability allows for a better performance under adverse conditions such as those imposed by high temperature, pH extremes, high ionic strength, detergents, and organic solvents. The observation that MG was effective in protecting enzymes from several sources against thermal stress and freeze-drying led to the suggestion that this compound could be used as an enzyme stabilizer (Ramos et al. 1997). In the present study, we compared the ability of MG and other compatible solutes to protect rabbit muscle LDH against thermal inactivation. MG and hydroxyectoine were the most effective solutes. Interestingly, MGA, the amide derivative of MG, and ectoine, which is closely related to hydroxyectoine, did not confer a significant level of thermoprotection. The thermostabilization of LDH and phosphofructokinase by hydroxyectoine, as well as the differential ability of hydroxyectoine and ectoine to act as enzyme thermoprotectors, have been reported previously (Cánovas et al. 1999; Galinski 1993; Lippert and Galinski 1992). Taken together, the data show that even small differences in chemical structure may be crucial for the ability of a certain solute to stabilize a given enzyme. Surprisingly, DIP strongly destabilized LDH. In the literature, there are conflicting reports concerning the effect of this solute on enzyme stabilization. DIP was found to improve the thermal stability of glyceraldehyde 3-phosphate dehydrogenase from *P. woesei* (Scholz et al. 1992); on the other hand, the thermostability of hydrogenase and ferredoxin oxidoreductase from *T. maritima* was not enhanced in the presence of this solute (Ramakrishnan et al. 1997). It is worth pointing out that the conflicting results on enzyme thermoprotection by a given compatible solute are primarily due to the different experimental conditions used in different laboratories; therefore, the establishment of well-defined protocols for these studies is extremely important.

In an attempt to establish which chemical features of MG are determinant in the protection of LDH against thermal inactivation, the effect of several compounds chemically related to MG on LDH thermostability was evaluated. The negative charge in MG seems to be decisive for the degree of enzyme protection, since its noncharged derivative, MGA, had no significant protective effect. In this context, it may be also relevant to stress that most compatible solutes used by hyperthermophiles are negatively charged (Santos and da Costa 2001). Furthermore, the fact that glycerate was a better thermoprotector than glycerol reinforces the view of a charge-dependent mechanism for LDH thermoprotection. However, the molecular basis for the stabilizing effect of MG is not as straightforward, since the neutral compound glucosylglycerol was also rather effective in protecting LDH against thermal inactivation. On the other hand, mannose had a strong destabilizing effect, whereas methyl-mannoside (a nonreducing sugar in which the OH group at the anomeric carbon of mannose is replaced by a methyl group) gave a result similar to the control without solutes. The free aldehyde group in mannose is likely to react with amino groups in the protein (the Maillard reaction), thereby causing inactivation of the enzyme. It has been shown that the extent of these reactions can affect

biological activity even at low temperature (Newman et al. 1993). Glucose, the other reducing sugar examined in this study, did not show a detrimental effect on the stability of LDH, but the nonenzymatic glycosylation of proteins is known to depend on the type of sugar.

The transition temperature of LDH increased by 4.5°C in 0.5 M MG. Although values for the effect of other osmolytes on this enzyme could not be found in the literature, the increase in T_m observed here compares favorably with 2°C and 1.6°C, respectively, for hydroxyectoine and betaine on ribonuclease A, calculated at 0.5 M solute concentration and taking into account that the effect is often approximately linear with the solute concentration (Knapp et al. 1999). Regrettably, in our experimental conditions, LDH precipitation was observed upon heating above the melting temperature, with or without solute. Therefore, the determination of thermodynamic parameters was not attempted due to the irreversibility of the unfolding process. Similar findings have been reported earlier by other authors (Jacobson and Braun 1977).

Light-scattering measurements showed the ability of MG to prevent aggregation of LDH at 50°C, its effectiveness surpassing that of trehalose. This observation correlates with the results from the thermal stability assays, where the ability of MG to protect LDH against heat was much higher than that of trehalose, suggesting the implication of protein aggregation in the process of thermal inactivation. The superiority of MG compared to trehalose in hindering LDH aggregation may be well worth further attention. In fact, recent reports on the ability of trehalose and glycine betaine to prevent protein aggregation and to influence folding pathways have opened new fields of applications of compatible solutes as inhibitors of the formation of inclusion bodies during heterologous overexpression, and as therapeutic agents in the treatment of diseases caused by protein aggregation or misfolding (Bourot et al. 2000; Singer and Lindquist 1998; Yang et al. 1999).

The principles underlying the stabilizing action of compatible solutes are still a matter of debate, but it seems obvious that a given solute interacts with water and proteins at different levels, and that the outcome reflects a balance of those interactions (Brown 1990). It has been proposed that the effect of solutes is due to a preferential exclusion mechanism, which leads to solute exclusion from the solvation layer of the protein (Arakawa and Timasheff 1983). However, the occurrence of more specific solute/protein interactions may be also involved in the stabilization mechanism. For example, diglycerol phosphate exerts very different thermostabilizing effects on highly homologous rubredoxins from *Desulfovibrio gigas* and *Clostridium pasteurianum*; furthermore, DGP was very efficient in conferring thermoprotection at concentrations (100 mM) for which glycerol, a canonical protein protectant, was completely ineffective (Lamosa et al. 2000). Also significant is the ability of MG to stabilize two enzymes, LDH and GOD, with opposite net charges under the experimental conditions used in this work. Surprising examples of proteins bearing a negative net charge that bind to polyanions, and of proteins bearing a positive charge that may bind to polycations, have been reported (cited in Andersson et al. 2000). The existence of clusters of positive and negative charges on the protein surface may explain these observations, and the same explanation may also hold true in the case of a small organic anion, such as MG

This work shows the high performance of MG as an enzyme stabilizer when compared to other solutes from mesophilic and thermophilic sources, foreseeing its application as an enzyme stabilizer, either alone or as a component of complex mixtures. Further insight into the molecular basis of the protective effect exerted by MG on proteins is essential to achieve the desired rational design of chemical derivatives especially suited for a specific biotechnological application.

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References

Andersson MM, Hatti-Kaul R (2000) Dynamic and static light scattering and fluorescence studies of the interactions between lactate dehydrogenase and poly(ethyleneimine). J Phys Chem B 104:3660–3667

Arakawa T, Timasheff SN (1983) Preferential interactions of proteins with solvent components in aqueous amino acid solutions. Arch Biochem Biophys 224:169–177

Bauer MW, Halio SB, Kelly RM (1996) Proteases and glycosyl hydrolases from hyperthermophilic microorganisms. Adv Protein Chem 48:271–305

Bourot S, Sire O, Trautwetter A, Touzé T, Wu LF, Blanco C, Bernard T (2000) Glycine betaine-assisted protein folding in a *lysA* mutant of *Escherichia coli*. J Biol Chem 275:1050–1056

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilising the principle of protein dye-binding. Anal Biochem 72:248–254

Brown AD (1990) Compatible solutes. In: Microbial water stress physiology. Principles and perspectives. John Wiley and Sons, Chichester, pp 241–275

Cánovas D, Borges N, Vargas C, Ventosa A, Nieto JJ, Santos H (1999) Role of Nγ-acetyldiaminobutyrate as an enzyme stabilizer and an intermediate in the biosynthesis of hydroxyectoine. Appl Environ Microbiol 65:3774–3779

Carpenter JF, Crowe JH, Arakawa T (1990) Comparison of solute-induced protein stabilization in aqueous solution and in the frozen and dried states. J Dairy Sci 73:3627–3636

Ciulla RA, Burggraf S, Stetter KO, Roberts MF (1994) Occurrence and role of di-*myo*-inositol-1,1′-phosphate in *Methanococcus igneus*. Appl Environ Microbiol 60:3660–3664

da Costa MS, Santos H, Galinski EA (1998) An overview of the role and diversity of compatible solutes in *Bacteria* and *Archaea*. Adv Biochem Eng Biotechnol 61:117–153

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for determination of sugars and related substances. Anal Chem 28:350–356

Galinski EA (1993) Compatible solutes of halophilic eubacteria: molecular principles, water-solute interaction, stress protection. Experientia 49:487–496

Hensel R, König H (1988) Thermoadaptation of methanogenic bacteria by intracellular ion concentration. FEMS Microbiol Lett 49:75–79

Hottiger T, De Virgilio C, Hall MN, Boller T, Wiemken A (1994) The role of trehalose synthesis for the acquisition of thermotolerance in yeast. II. Physiological concentrations of trehalose increase the thermal stability of proteins in vitro. Eur J Biochem 219:187–193

Jacobson AL, Braun H (1977) Differential scanning calorimetry of the thermal denaturation of lactate dehydrogenase. Biochim Biophys Acta 493:142–153

Jaenicke R, Knof S (1968) Molecular weight and quaternary structure of lactic dehydrogenase. 3. Comparative determination by sedimentation analysis, light scattering and osmosis. Eur J Biochem 4:157–163

Kanodia S, Roberts MF (1983) Methanophosphagen: unique cyclic pyrophosphate isolated from *Methanobacterium thermoautotrophicum*. Proc Natl Acad Sci USA 80:5217–5221

Knapp S, Landenstein R, Galinski EA (1999) Extrinsic protein stabilization by naturally occurring osmolytes β-hydroxyectoine and betaine. Extremophiles 3:191–198

Ladenstein R, Antranikian G (1998) Proteins from hyperthermophiles: stability and enzymatic catalysis close to the boiling point of water. Adv Biochem Eng Biotechnol 61:37–85

Lamosa P, Burke A, Peist R, Huber R, Liu M, Silva G, Rodrigues-Pousada C, LeGall J, Maycock C, Santos H (2000) Thermostabilization of proteins by diglycerol phosphate, a new compatible solute from the hyperthermophile *Archaeoglobus fulgidus*. Appl Environ Microbiol 66:1974–1979

Lippert K, Galinski EA (1992) Enzyme stabilization by ectoine-type compatible solutes: protection against heating, freezing and drying. Appl Microbiol Biotechnol 37:61–65

Martins LO, Santos H (1995) Accumulation of mannosylglycerate and di-*myo*-inositol-phosphate by *Pyrococcus furiosus* in response to salinity and temperature. Appl Environ Microbiol 61:3299–3303

Martins LO, Carreto LS, da Costa MS, Santos H (1996) New compatible solutes related to di-*myo*-inositol-phosphate in members of the order *Thermotogales*. J Bacteriol 178:5644–5651

Martins LO, Huber R, Huber H, Stetter KO, da Costa MS, Santos H (1997) Organic solutes in hyperthermophilic *Archaea*. Appl Environ Microbiol 63:896–902

Newman YM, Ring SG, Colaco C (1993) The role of trehalose and other carbohydrates in biopreservation. Biotechnol Genet Eng Rev 11:263–264

Nunes OC, Manaia CM, da Costa MS, Santos H (1995) Compatible solutes in the thermophilic bacteria *Rhodothermus marinus* and "*Thermus thermophilus*". Appl Environ Microbiol 61:2351–2357

Paiva CLA, Panek AD (1996) Biotechnological applications of the disaccharide trehalose. Biotechnol Ann Rev 2:293–314

Pocard JA, Smith LT, Smith GM, Le Rudulier D (1994) A prominent role for glucosylglycerol in the adaptation of *Pseudomonas mendocina* SKB70 to osmotic stress. J Bacteriol 176:6877–6884

Ramakrishnan V, Verhagen MFJM, Adams MWW (1997) Characterization of di-*myo*-inositol-1,1′-phosphate in the hyperthermophilic bacterium *Thermotoga maritima*. Appl Environ Microbiol 63:347–350

Ramos A, Raven NDH, Sharp RJ, Bartolucci S, Rossi M, Cannio R, Lebbink J, van der Oost J, de Vos WM, Santos H (1997) Stabilization of enzymes against thermal stress and freeze-drying by mannosylglycerate. Appl Environ Microbiol 63:4020–4025

Raven NDH, Sharp RJ (1997) Development of a defined and minimal media for the growth of the hyperthermophilic archaeon *Pyrococcus furiosus* Vc1. FEMS Microbiol Lett 146:135–141

Reed RH, Richardson DL, Warr SRC, Stewart WDP (1984) Carbohydrate accumulation and osmotic stress in cyanobacteria. J Gen Microbiol 130:1–4

Santos H, da Costa MS (2001) Organic solutes from thermophiles and hyperthermophiles. Methods Enzymol 336:302–315

Scholz S, Sonnenbichler J, Schäfer W, Hensel R (1992) Di-*myo*-inositol-1,1′-phosphate: a new inositol phosphate isolated from *Pyrococcus woesei*. FEBS Lett 306:239–242

Shima S, Hérault DA, Berkessel A, Thauer RK (1998) Activation and thermostabilization effects of cyclic 2,3-diphosphoglycerate on enzymes from the hyperthermophilic *Methanopyrus kandleri*. Arch Microbiol 170:469–472

Silva Z, Borges N, Martins LO, Wait R, da Costa MS, Santos H (1999) Combined effect of the growth temperature and salinity of the medium on the accumulation of compatible solutes by *Rhodothermus marinus* and *Rhodothermus obamensis*. Extremophiles 3:163–172

Singer MA, Lindquist S (1998) Multiple effects of trehalose on protein folding in vitro and in vivo. Mol Cell 1:639–648

Smith LT, Pocard JA, Bernard T, Le Rudulier D (1988) Osmotic control of glycine betaine biosynthesis and degradation in *Rhizobium meliloti*. J Bacteriol 170:3142–3149

Susor WA, Kochman M, Rutter WJ (1969) Heterogeneity of presumably homogeneous protein preparations. Science 165:1260–1262

Vassault A (1987) Lactate dehydrogenase. UV method with pyruvate and NADH. In: Bergmeyer HU (ed) Methods of enzymatic analysis, vol 3, 3rd edn. VCH, Weinheim, pp 118–126

Voet JG, Coe J, Epstein J, Matossian V, Shipley T (1981) Electrostatic control of enzyme reactions: effect of ionic strength on the pK_a of an essential acidic group on glucose oxidase. Biochemistry 20:7182–7185

Yang DS, Yip CM, Huang THJ, Chakrabartty A, Fraser PE (1999) Manipulating the amyloid-β aggregation pathway with chemical chaperones. J Biol Chem 274:32970–32974