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# Stabilization of Enzymes against Thermal Stress and Freeze-Drying by Mannosylglycerate

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**2-*O*-β-Mannosylglycerate, a solute that accumulates in some (hyper)thermophilic organisms, was purified from *Pyrococcus furiosus* cells, and its effect on enzyme stabilization in vitro was assessed. Enzymes from hyperthermophilic, thermophilic, and mesophilic sources were examined. The thermostabilities of alcohol dehydrogenases from *P. furiosus* and *Bacillus stearothermophilus* and of glutamate dehydrogenases from *Thermotoga maritima* and *Clostridium difficile* were improved to a significant extent when enzyme solutions were incubated at supraoptimal temperatures in the presence of 2-*O*-β-mannosylglycerate, but no effect on the thermostability of glutamate dehydrogenase from *P. furiosus* was detected. On the other hand, there was a remarkable effect on the thermal stabilities of rabbit muscle lactate dehydrogenase, baker's yeast alcohol dehydrogenase, and bovine liver glutamate dehydrogenase, which were used as model systems to evaluate stabilization of enzymes of mesophilic origin. For all of the enzymes examined and at the highest temperatures tested, 2-*O*-β-mannosylglycerate was a better thermoprotectant than trehalose. The stabilizing effect exerted by 2-*O*-β-mannosylglycerate on enzymes suggests a role for this compound as a protein thermostabilizer under physiological conditions. 2-*O*-β-Mannosylglycerate was also effective in the protection of enzymes against stress imposed by freeze-drying, with its protecting effect being similar to or better than that exerted by trehalose. The data show 2-*O*-β-mannosylglycerate to be a potential enzyme stabilizer in biotechnological applications.**

Life under extreme conditions raises interesting questions about the biochemical strategies used for stabilization of cellular components. In the case of proteins, the central issue of adaptation to extreme physicochemical conditions is the conservation of a functional state optimized with respect to stability and flexibility (39). Considering the marginal extra free energy of stabilization of enzymes from thermophiles and hyperthermophiles compared to the ease for their homologous mesophilic counterparts, it is clear that enhanced stability may be achieved by a large number of mechanisms, such as (i) intrinsic stabilization of the whole-cell proteins, (ii) enhanced protein turnover, (iii) action of molecular chaperones, and (iv) extrinsic stabilization by compatible solutes or specific ligands (18, 19).

To keep the appropriate turgor pressure and/or to protect cytoplasmic components when the salt concentration in the growth medium increases, most organisms accumulate low-molecular-weight organic solutes, the so-called compatible solutes, a term which refers to compounds that can accumulate to high intracellular levels without affecting cell metabolism or enzyme activity (6). Compatible solutes found in mesophiles fall into limited categories of compounds, such as polyols, sugars and sugar derivatives, ectoines, amino acids, and amino acid derivatives (11). In addition to their purely osmotic function, compatible solutes have been shown to exert a protective effect against stress on enzymes and other cellular components in several organisms (3, 33).

In thermophilic and hyperthermophilic *Archaea* and *Bacteria*, novel low-molecular-weight organic compounds that may play a role in osmotic and/or thermal adjustment have been identified. Some methanogenic organisms accumulate cyclic-2,3-bisphosphoglycerate (15, 20, 23), a compound which has a thermoprotectant role on glyceraldehyde-3-phosphate dehydrogenases from hyperthermophiles (16). In addition, di-*myo*-inositol-phosphate was reported to accumulate in *Pyrococcus woesei* (38), *Methanococcus igneus* (10), *Pyrococcus furiosus* (29), *Thermotoga neapolitana* (30), and *Thermotoga maritima* (30, 34). Two phosphoinositol derivatives, di-*O*-β-mannosyl-di-*myo*-inositol-phosphate and a new isomer of di-*myo*-inositol-phosphate, were found to accumulate mainly at supraoptimal growth temperatures in *T. maritima* and *T. neapolitana* (30). A novel compound, 2-*O*-β-mannosylglycerate (referred to as mannosylglycerate in the rest of this paper), was identified in thermophilic bacteria such as *Rhodothermus marinus*, "*Thermus thermophilus*" (32), and *Petrotoga miotherma* (30), in the hyperthermophilic archaea *P. furiosus* and *P. woesei* (29), in *Methanothermus sociabilis* and *Methanothermus fervidus* (31), and in several organisms of the genus *Thermococcus* (24). The α-isomer of this solute was identified previously in species of the algal order *Ceramiales*, where its role as a compatible solute was proposed (22). In *P. furiosus*, the total intracellular solute pool increased in response to an increase in either temperature or salinity, but mannosylglycerate accumulated mainly at high salinities, whereas di-*myo*-inositol-1,1′-phosphate increased in response to temperature stress (29).

A role of low-molecular-weight organic compounds in ther-

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mostabilization, based on the increase in their intracellular concentration in response to increased growth temperature and on in vitro assays with purified enzymes, has been proposed. Halophilic organisms accumulate ectoine-type compounds which were shown to be effective protectants against thermal and osmotic stress imposed on several enzymes, such as phosphofructokinase and lactate dehydrogenase (LDH) isolated from mammalian sources (27), and an effect of di-*myo*-inositol-1,1′-phosphate as an enzyme thermostabilizer in vitro was reported (38). In contrast, the effect of di-*myo*-inositol-1,1′-phosphate on protein stability was investigated recently, and it was found that the thermostability of hydrogenase or pyruvate ferredoxin oxidoreductase from *T. maritima* was not improved (34).

In this work, we have examined the effect of mannosylglycerate on thermoprotection and protection against freeze-drying of enzymes isolated from mesophilic, thermophilic, and hyperthermophilic sources. The results indicate that mannosylglycerate effectively stabilizes enzymes, indicating a potential role in future biotechnological applications.

(Part of the data presented here are included in European patent application no. 97670002.1.)

## MATERIALS AND METHODS

**Organism and growth conditions.** *P. furiosus* was grown in continuous culture at 90°C on the maltose-peptone-yeast extract medium described by Raven and Sharp (35), adjusted to 5% NaCl.

**Preparation of cell extracts.** Ethanol-chloroform extracts were obtained by the method of Reed et al. (36) with slight modifications as previously described (29).

**Purification of mannosylglycerate.** Mannosylglycerate was purified from ethanol-chloroform extracts by anion-exchange chromatography on QAE-Sephadex A25. The extract, corresponding to approximately 100 g of cells (wet weight), was loaded onto a QAE-Sephadex column (5 by 20 cm) equilibrated with 5 mM ammonium bicarbonate, pH 8.0. The elution was performed with a linear gradient of 5 mM to 1 M $NH_4HCO_3$ at a flow rate of 1 ml · $min^{-1}$. Aliquots of each fraction were analyzed for the content of carbohydrates by the method of Dubois et al. (13) and for total phosphorus by the method described by Ames (1). Fractions containing carbohydrates or phosphorus were pooled separately, concentrated by lyophilization, and analyzed by $^1$H nuclear magnetic resonance ($^1$H-NMR) spectroscopy. Three main carbohydrate-containing fractions were eluted: the first fraction eluted at 5 mM $NH_4HCO_3$ and contained a nonidentified polysaccharide, the second fraction eluted at between 0.6 and 0.7 M $NH_4HCO_3$ and contained a mixture of di-*myo*-inositol-phosphate and mannosylglycerate, and the third fraction eluted at 0.8 M $NH_4HCO_3$ and contained pure mannosylglycerate as judged by $^1$H-NMR spectroscopy. The fractions containing a mixture of di-*myo*-inositol-phosphate and mannosylglycerate were concentrated by lyophilization, loaded onto a QAE-Sephadex column (2.6 by 20 cm), and eluted as described above; the fractions containing pure mannosylglycerate were pooled, and $NH_4HCO_3$ was partially removed by passage through the ion retardation resin AG 11-A8 (2 by 40 cm). Residual $NH_4^+$ was exchanged with $H^+$ by passage through a Dowex AG 50W-X8 column (2.5 by 10 cm). The $K^+$ salt of mannosylglycerate was obtained by neutralization with KOH, and the sample was lyophilized and resuspended in ultrapure water in order to obtain a 1 M solution of mannosylglycerate (potassium salt), pH 7.0. The amount of $NH_4^+$ in this solution was measured by a colorimetric method as previously described (28), and $K^+$ was determined by plasma emission spectroscopy with a Jobin Yvon spectrometer (model JY24).

The yield of mannosylglycerate was approximately 2.5 g per 100 g of *P. furiosus* cells paste (wet weight). No residual $NH_4^+$ was detected in the solution of mannosylglycerate utilized for evaluation of protection against stress factors. The potassium concentration in the final solution of mannosylglycerate (prepared to a final concentration of 1 M in the organic solute) was 0.96 M. The mannosylglycerate solution (potassium salt) was aliquoted and stored at −20°C until used. The thermal stability of mannosylglycerate was monitored by incubating the pure compound (pH 7.0, 300 mM) at 95°C in an oil bath and recording $^1$H-NMR spectra at intervals over a period of 180 min. No modifications in the spectrum were detected throughout the experiment, nor were extra resonances observed (data not shown).

**NMR spectroscopy.** $^1$H-NMR spectra were recorded at 300.14 MHz on a Bruker AMX 300 spectrometer with a 5-mm probe head. For quantification purposes, spectra were acquired with water presaturation, a 6-μs pulse width (corresponding to a 60° flip angle), and a repetition delay of 10 s. Formate was added as a concentration standard. Chemical shifts were referenced to the resonance of 3-(metylsilyl)propanesulfonic acid (sodium salt), which was designated as being at 0 ppm.

**Enzyme purification and activity measurements.** *P. furiosus* and *Clostridium difficile* glutamate dehydrogenases (GDHs) were produced and purified from *Escherichia coli* as described previously (25); *P. furiosus* alcohol dehydrogenase (ADH) was purified as described by Voorhorst et al. (41). *Bacillus stearothermophilus* ADH was purified as described by Cannio et al. (8). *T. maritima* GDH was obtained as follows. A cell extract from *E. coli* XLI-Blue containing the enzyme was prepared and heat incubated as described by Kort et al. (21) with 10 mM potassium phosphate (pH 7.2)–1 mM EDTA. The extract was loaded onto a Q-Sepharose column equilibrated with the same buffer, and GDH was eluted with a linear gradient of NaCl (0 to 1 M). Pure fractions were pooled and dialyzed against 0.1 M sodium phosphate buffer, pH 7.0 LDH (Sigma type III, rabbit muscle), purchased as a suspension in ammonium sulfate, was centrifuged, the supernatant was discarded, and the enzyme was suspended in 50 mM potassium phosphate, pH 7.5. ADH (Sigma, baker's yeast) and GDH (Sigma type III, bovine liver) were obtained in the lyophilized form and used without further purification. Enzymes were assayed by using an Olis spectrophotometer with a thermostated cell compartment at 30°C, unless stated otherwise. The 1.0-ml standard reaction mixtures contained the following: for *P. furiosus* GDH, 100 mM potassium phosphate buffer (pH 7.6), 75 mM ammonia, 0.75 mM α-ketoglutarate (potassium salt), 0.1 mM NADPH, and 0.5 μg of enzyme; for *T. maritima* GDH, 100 mM potassium phosphate buffer (pH 7.6), 300 mM ammonia, 5 mM α-ketoglutarate (potassium salt), 0.2 mM NADH, and 0.5 μg of enzyme (21); for *C. difficile* GDH, 100 mM potassium phosphate buffer (pH 7.6), 600 mM ammonia, 4 mM α-ketoglutarate (potassium salt), 0.2 mM NADH, and 0.25 μg of enzyme (25); for bovine liver GDH, 100 mM potassium phosphate buffer (pH 7.5), 75 mM ammonia, 7.0 mM α-ketoglutarate (potassium salt), 0.2 mM NADH, and 0.5 μg of enzyme (37); for *B. stearothermophilus* ADH, 20 mM sodium phosphate buffer (pH 8.0), 20 mM ethanol, 1 mM $NAD^+$, and 0.25 μg of enzyme (8); for rabbit muscle LDH, 80 mM Tris-HCl buffer (pH 7.6), 1.6 mM pyruvate (sodium salt), 0.2 mM NADH, and 0.25 μg of enzyme (40); for baker's yeast ADH, 100 mM EPPS (*N*-2-hydroxyethylpiperazine-*N*′-3-propanesulfonic acid) buffer (pH 8.4), 60 mM ethanol, 0.4 mM $NAD^+$, and 0.25 μg of enzyme; and for *P. furiosus* ADH (the activity was measured at 70°C), 100 mM glycine buffer (pH 10.0), 90 mM 2-pentanol, 0.28 mM NADPH, and 2.0 μg of enzyme (41). One unit of enzyme activity was defined as the amount of enzyme catalyzing the conversion of 1 μmol of NAD(P)H or $NAD(P)^+$ per min under the experimental conditions used. Specific activity was expressed as units per milligram of protein. The protein concentration was determined by the method of Bradford (5) with bovine serum albumin as a standard.

**Thermal stability assays.** The assay mixtures for experiments in which thermal stress was imposed were prepared at an enzyme concentration of 50 μg · $ml^{-1}$, unless stated otherwise, in 20 mM potassium phosphate buffer at the appropriate pH, in the presence or absence of mannosylglycerate (potassium salt), trehalose, potassium chloride, or other solutes (all at a final concentration of 500 mM). For the enzymes isolated from thermophilic or hyperthermophilic organisms, aliquots were incubated in sealed glass capillaries in an oil bath at the temperatures indicated in Results. Samples were withdrawn at appropriate time intervals and cooled in an ice bath, and the activity was immediately assayed as described above. The results are presented as percent activities with respect to those assayed for aliquots kept at room temperature. It was verified that the pHs of the incubation mixtures did not change with the increasing temperature, since the $pK_a$ of phosphate buffer is much less dependent on temperature than those of many other buffers (14).

In the experiments in which thermal stress was imposed on enzymes obtained from mesophilic sources, the reaction mixtures were prepared as described above, placed in Eppendorf tubes, incubated in a water bath at the appropriate temperatures, withdrawn at appropriate time intervals, cooled in an ice bath, and assayed immediately. All solutes were used at a final concentration of 500 mM, except for those in the experiments in which the effect of solute concentration on the thermostability of rabbit muscle LDH was evaluated; in this case, assay mixtures were prepared at solute concentrations of 50, 100, 300, and 500 mM.

**Freeze-drying experiments.** The assay mixtures for freeze-drying experiments were prepared at an enzyme concentration of 50 μg · $ml^{-1}$ in 20 mM potassium phosphate buffer at the appropriate pH. Mannosylglycerate (potassium salt), KCl, trehalose, or other solutes were added to obtain a final concentration of 500 mM. Enzyme activity was measured as described above, the volume of each sample was determined, and all samples in a given experiment were frozen simultaneously by immersion in liquid nitrogen for 60 s. The frozen samples were lyophilized for 24 h on an Edwards Modulyo lyophilizer and rehydrated in water to the same volume. The residual activity was immediately determined and expressed as percentage of the activity obtained before freeze-drying.

**Chemicals.** QAE-Sephadex A25 and Q-Sepharose were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden); Dowex AG 50W-X8 and the ion retardation resin AG 11 A8 were obtained from Bio-Rad Laboratories (Richmond, Calif.). LDH (type III, rabbit muscle), ADH (baker's yeast), GDH (type III, bovine liver), and EPPS buffer were supplied by Sigma. All other chemicals were reagent grade.

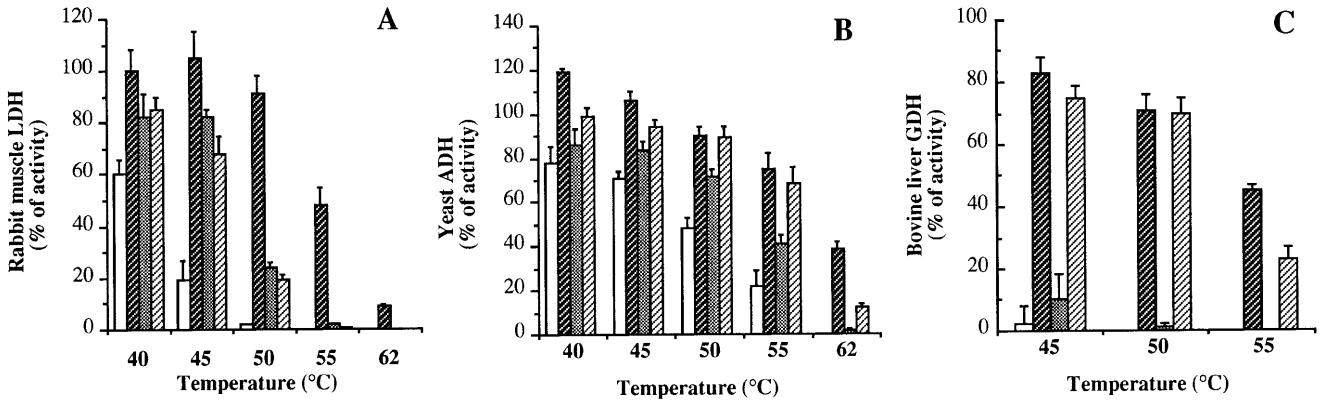


FIG. 1. Effect of mannosylglycerate, trehalose, and KCl on the thermostability of rabbit muscle LDH (A), baker's yeast ADH (B), and bovine liver GDH (C). Aliquots containing the enzymes, all at a concentration of 50 $\mu g \cdot ml^{-1}$, and the solutes (final concentration, 500 mM) were incubated for 10 min at the indicated temperatures, cooled on an ice bath, and assayed immediately for residual activity. ▭, no additions; ▨, mannosylglycerate; ▥, trehalose; ▨, KCl. The results are averages and standard deviations from at least four independent experiments.

## RESULTS

**Effect of mannosylglycerate on enzyme thermostability. (i) Enzymes from mesophilic sources.** The effect of mannosylglycerate on the thermostability of enzymes obtained from mesophilic sources was evaluated by using the model systems rabbit muscle LDH, baker's yeast ADH, and bovine liver GDH (Fig. 1). The effect of mannosylglycerate was compared to those of known thermostabilizers, such as trehalose and sucrose; potassium chloride was always added as a control, since the potassium salt of mannosylglycerate was used throughout the experiments described in this paper. Mannosylglycerate exerted a protective effect on LDH at all temperatures tested, but its effectiveness was remarkable at the higher temperatures tested: in fact, after 10 min of incubation at 50°C in the absence of mannosylglycerate, LDH retained only 5% of activity, whereas in its presence 90% activity was recovered; the corresponding values found after incubation at 62°C were 0 and 10%, respectively. Furthermore, a comparison of the effect exerted by mannosylglycerate with those of sucrose (not shown) and trehalose showed that neither of these other solutes was as efficient as mannosylglycerate as a thermoprotector of LDH, with the effect of sucrose being similar to that of trehalose. ADH was intrinsically more stable than LDH. Furthermore, the activities retained after 10 min of incubation at temperatures ranging from 40 to 55°C were similar in the presence of mannosylglycerate or KCl. However, after incubation at 62°C, the percentage of activity determined was significantly higher in the presence of the former compound: 40 and 10% activity was measured after incubation with mannosylglycerate and KCl, respectively. The thermal stability of bovine liver GDH was also studied, since GDHs isolated from thermophilic or hyperthermophilic sources were used in this work. The enzyme was extremely thermolabile: activity was almost completely lost after incubation for 10 min at 45°C, and mannosylglycerate was an effective protectant against thermal inactivation after incubation of the enzyme at supraoptimal temperatures; for example, incubation at 55°C resulted in a residual activity of 45 or 23% in the presence of mannosylglycerate or KCl, respectively. No activity was found after incubation at 62°C under the experimental conditions used.

The effect of the concentration of mannosylglycerate on the thermal stability of LDH was also monitored (Fig. 2). Increased concentrations of mannosylglycerate were increasingly effective in protecting LDH against thermal stress. Concentrations of mannosylglycerate higher than 500 mM were not tested. However, the increase in protection was much more pronounced when the concentration of mannosylglycerate was increased from 100 to 300 mM than when the increase was from 300 to 500 mM. This suggests a trend for saturation of the effect on LDH for a concentration of mannosylglycerate of around 500 mM. In accordance with the above-mentioned experiments, trehalose and KCl were much less effective than mannosylglycerate in protecting LDH against thermal stress; in fact, at temperatures above 50°C, no protection was provided by any of the concentrations of trehalose or KCl tested (data not shown).

**(ii) Enzymes from thermophiles and hyperthermophiles.** The effect of mannosylglycerate on the thermal stabilities of GDHs from *P. furiosus*, *T. maritima*, and *C. difficile* and of ADHs from *B. stearothermophilus* and *P. furiosus* was investigated. *P. furiosus* GDH was incubated in the presence and in the absence of mannosylglycerate, and aliquots of the incubation mixtures were withdrawn at time intervals up to 120 min in order to determined the remaining activity. Mannosylglycerate exerted no detectable effect on the thermostability of *P. furiosus* GDH at either 90 or 105°C, compared to incubation of

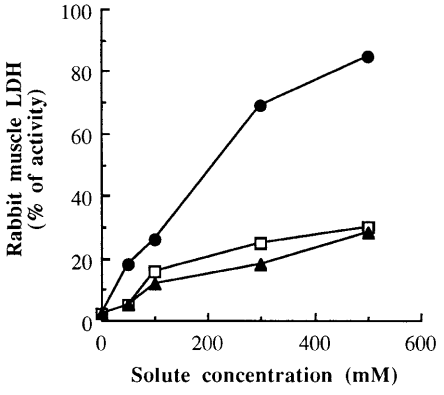


FIG. 2. Evaluation of the effect of the concentration of mannosylglycerate (●), trehalose (▲), and KCl (□) on the thermal stability of rabbit muscle LDH incubated for 10 min at 50°C. The assay mixtures contained LDH at a concentration of 50 $\mu g \cdot ml^{-1}$ in the absence or in the presence of solutes at concentrations of 50, 100, 300, and 500 mM. The experiments were performed as described for Fig. 1.

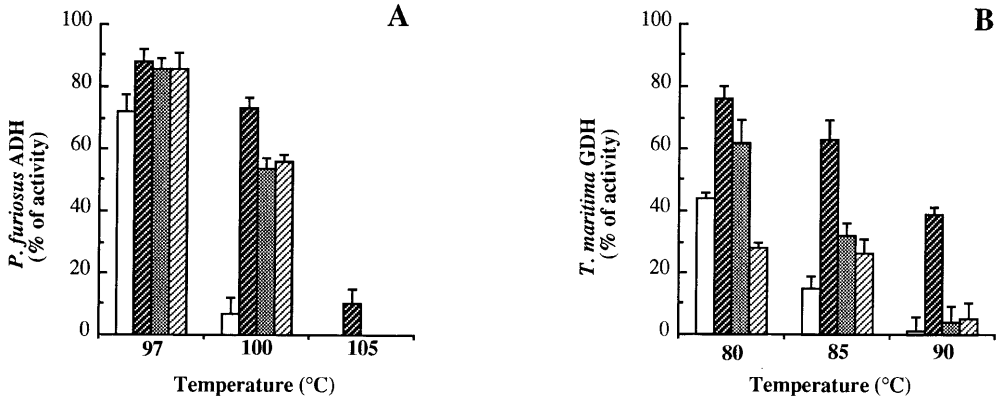


FIG. 3. Effect of mannosylglycerate, trehalose, and KCl on the thermal stability of *P. furiosus* ADH (A) and *T. maritima* GDH (B). *P. furiosus* ADH and *T. maritima* GDH were prepared at concentrations of 200 and 50 $\mu g \cdot ml^{-1}$, respectively, in the absence or in the presence of solutes (final concentration, 500 mM). Aliquots containing the assay mixtures were placed in glass capillaries which were sealed and incubated for 15 min at the indicated temperatures, cooled in an ice bath, and assayed immediately for activity. ▭, no additions; ▨, mannosylglycerate; ▦, trehalose; ▨, KCl. The results are averages and standard deviations from three independent determinations.

the enzyme in the absence of added solutes (data not shown). In contrast to this result, mannosylglycerate was an effective thermoprotectant of ADH from *P. furiosus* and of GDH from *T. maritima* (Fig. 3). After incubation of *P. furiosus* ADH at 100°C for 15 min with mannosylglycerate or trehalose, 75 and 53% activity, respectively, was retained. Incubation at 105°C for the same period of time totally inactivated the enzyme, except when mannosylglycerate was added; in that case, significant activity (15%) was recovered. Following incubation of *T. maritima* GDH at 80°C, the activity retained in the presence of mannosylglycerate, trehalose, or KCl was 76, 62, and 28% respectively, and the activity in the absence of added solutes was 42%; after incubation for 15 min at 90°C, the activity measured in the absence of solutes or in the presence of trehalose or KCl was negligible, whereas the enzyme retained 40% activity when incubated with mannosylglycerate. *C. difficile* GDH and *B. stearothermophilus* ADH were also stabilized by mannosylglycerate when thermal stress was imposed, although to a lesser extent than for the enzymes isolated from hyperthermophilic sources (data not shown).

**Effect of mannosylglycerate on enzymatic activity after freeze-drying.** The effect of mannosylglycerate on protection of mesophilic and hyperthermophilic enzymes against freeze-drying was investigated, and the results obtained for LDH (rabbit muscle) and GDH (*T. maritima*) are illustrated in Fig. 4. LDH was very sensitive to freeze-drying; only 12% of the initial activity was retained after freeze-drying in the absence of added solutes. In contrast, when mannosylglycerate or trehalose was added, the enzyme retained 85 and 54% of the initial activity, respectively. Furthermore, the enzyme was completely inactivated after freeze-drying in the presence of KCl. Mannosylglycerate was also an effective protectant of *T. maritima* GDH, which retained 91% activity when lyophilized in the presence of that solute; 55, 86, and 28% activity was measured in the absence of solutes or in the presence of trehalose or KCl, respectively.

The effect of solutes on the resistance to freeze-drying of all of the other enzymes studied in this work was also evaluated (Table 1). The addition of mannosylglycerate or trehalose increased the activity of all enzymes tested after freeze-drying, with the exception of GDH from *P. furiosus*, for which no protecting effect by either of these solutes was detected. The lowest recovery after freeze-drying was always found in the presence of KCl. Furthermore, the degrees of protection supplied by mannosylglycerate and trehalose were similar in most cases, except for LDH, for which the addition of mannosylglycerate provided a significantly higher recovery (approximately 30%) compared to that with addition of trehalose. The other enzymes used as model systems from mesophilic organisms were intrinsically less sensitive to freeze-drying than

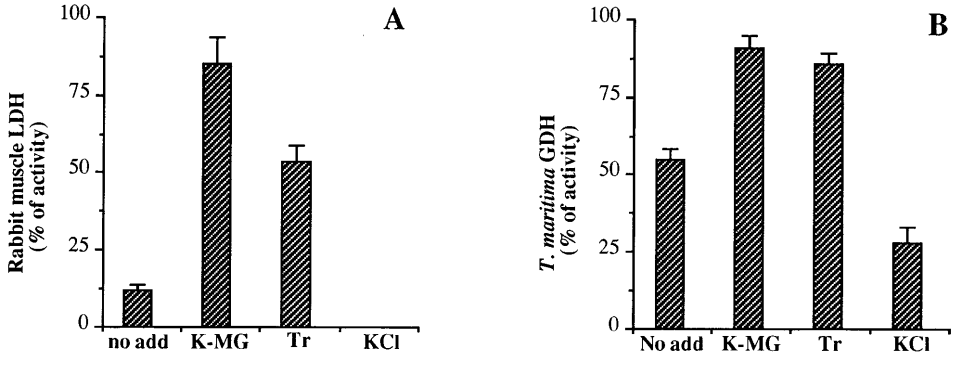


FIG. 4. Effect of mannosylglycerate (K-MG), trehalose (Tr), and KCl on the resistance against freeze-drying of rabbit muscle LDH (A) and *T. maritima* GDH (B). Enzyme solutions (50 $\mu g \cdot ml^{-1}$) were prepared in the absence (no add) or presence of solutes (500 mM). Experimental details are given in Materials and Methods. The results are averages and standard deviations from at least three independent determinations.

TABLE 1. Comparison of the effects of mannosylglycerate, trehalose, and KCl on the recovery of enzymatic activity after freeze-drying[a]

| Enzyme | % of activity recovered[b] after freeze-drying with: | | | |
|---|---|---|---|---|
| | No additions | Mannosyl-glycerate | Trehalose | KCl |
| *P. furiosus* GDH | 89 | 87 | 79 | 57 |
| *P. furiosus* ADH | 78 | 82 | 86 | 62 |
| *T. maritima* GDH | 55 | 91 | 86 | 28 |
| *B. stearothermophilus* ADH | 50 | 65 | 64 | 14 |
| *C. difficile* GDH | 47 | 80 | 77 | 23 |
| Rabbit muscle LDH | 12 | 85 | 54 | 0 |
| Yeast ADH | 55 | 89 | 98 | 34 |
| Bovine liver GDH | 65 | 95 | 86 | 32 |

[a] Assay mixtures were prepared at an enzyme concentration of 50 $\mu g \cdot ml^{-1}$, and the solutes were added to obtain a final concentration of 500 mM.

[b] The results are averages from at least three independent determinations, with standard deviations lower than 8%.

LDH; baker's yeast ADH retained 55% activity in the absence of solutes, and this value was increased to 89 and 98% in the presence of mannosylglycerate or trehalose, respectively. The behavior of bovine liver GDH was similar to that of ADH.

## DISCUSSION

The observation that the level of mannosylglycerate in *P. furiosus* increased considerably in response to stress, notably in response to an osmotic stress, led to the suggestion that this solute could play a role in the protection of intracellular components against heat or freeze-drying (29). The present work shows that mannosylglycerate is actually effective in the protection of isolated enzymes against stress imposed by supraoptimal temperatures and freeze-drying. The stabilizing effect of mannosylglycerate against heat stress was observed with enzymes isolated from thermophilic or hyperthermophilic organisms, such as *P. furiosus* ADH, *T. maritima* GDH, *C. difficile* GDH, and *B. stearothermophilus* ADH. In contrast to these results, the thermostability of *P. furiosus* GDH, a very stable enzyme, was not improved by mannosylglycerate; the enzyme shows an initial fast inactivation of approximately 50% when incubated for a few minutes at 105°C followed by a second phase of much slower inactivation (25), and the addition of mannosylglycerate did not change this behavior. Mannosylglycerate also exerted a strong protective effect on the activity of all enzymes isolated from mesophilic sources when thermal stress was imposed, and it should be stressed that its effectiveness was remarkable at the higher temperatures tested, i.e., well above the optimal temperature for activity. Moreover, under all circumstances, the addition of mannosylglycerate resulted in a more effective thermoprotection than addition of trehalose. These results indicate that mannosylglycerate can be a general protector of enzymes against thermal stress and suggest a potential application of this compound as an enzyme stabilizer in biotechnological processes.

The data also suggest that extrinsic factors, such as compatible solutes, may play an important role in thermal stabilization of proteins in (hyper)thermophiles in vivo. In fact, it is well known that many intracellular proteins originating from hyper-(thermophiles) are actually rather thermolabile in vitro. Moreover, although enzymes from hyperthermophiles may exhibit maximal catalytic activity at high temperatures, the activity measurements are in general of short duration, and it has been observed that many enzymes are not stable over prolonged periods of time (4, 38).

Mannosylglycerate also exerted a strong protective effect when enzymes from both mesophilic and hyperthermophilic sources were subjected to freeze-drying. A comparison of the activities retained after freeze-drying in the presence of mannosylglycerate and trehalose showed that the degrees of protection provided by these solutes were in general similar, except in the case of muscle LDH, for which the effect of mannosylglycerate was much higher than that of trehalose (Table 1). The role of trehalose in the stabilization of cellular components against thermal and osmotic stress is well documented. In fact, massive synthesis of trehalose is induced by heat shock in *Saccharomyces cerevisiae*, and the thermotolerance of this organism is correlated with the concentration of trehalose (17). The preservation of enzymes under conditions where total dehydration occurs requires an effective mechanism of stabilization. It has been proposed that the mechanism by which trehalose (and other sugars) protects enzymes against freeze-drying consists of maintaining the dry proteins in their hydrated conformations, possibly by binding, via −OH groups, to the hydrophilic domains of the proteins and preventing inter- and intraprotein hydrogen bonding during drying and rehydration (9, 26). Recent studies have shown that the presence of compatible solutes in the cytoplasm influences water structure, water activity, and the solution properties of the environment, exerting a pronounced effect on the thermodynamic parameters of enzyme stabilization (for a review, see reference 12). Furthermore, it has become clear that the effect of compatible solutes implies both the preferential exclusion mechanism and the tendency of the solutes for binding the exposed protein surface (2, 9).

The search for the unifying principles of the stabilizing action of compatible solutes is far from completion, but it seems likely that a given solute interacts with water and proteins at different levels and that the outcome reflects a balance of those interactions (7). The results presented in this paper support the occurrence of direct interactions between compatible solutes and protein surfaces, since the extent of stabilization provided by mannosylglycerate depends on the enzyme and on the type of stress imposed. For example, the thermostability of ADH from *P. furiosus* was clearly increased by the compound (Fig. 4), whereas the protection provided against freeze-drying was considerably smaller (Table 1). Since mannosylglycerate is a negatively charged solute, the occurrence of electrostatic interactions with charged amino acid residues at the protein surfaces may be relevant. It has been observed that the majority of organic compounds that accumulate in thermophilic *Bacteria* and *Archaea* are charged, while mesophilic bacteria accumulate primarily neutral solutes (12). A putative relationship between the accumulation of negatively charged solutes and growth at high temperatures could be rationalized by the superiority of this type of solute in protecting cellular components in hot environments (30).

The results presented in this work indicate that mannosylglycerate has a potential application as a protectant of proteins in biotechnological processes, and they support a role for mannosylglycerate in protein stabilization in vivo, assisting organisms to cope with temperature and saline stress. This solute accumulates in *P. furiosus* up to intracellular concentrations of 250 mM when 5% NaCl is added to the growth medium (29). Despite the fact that compatible solutes are generally effective at higher concentrations, one should keep in mind that in vivo, stabilization may involve a diversity of other factors.

Further research will aim at the evaluation of the effective-

ness of mannosylglycerate in the stabilization of other cellular components, such as membrane structures and nucleic acids.

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