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Design of new enzyme stabilizers inspired by glycosides of hyperthermophilic microorganisms

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ABSTRACT

In response to stressful conditions like supra-optimal salinity in the growth medium or temperature, many microorganisms accumulate low-molecular-mass organic compounds known as compatible solutes. In contrast with mesophiles that accumulate neutral or zwitterionic compounds, the solutes of hyperthermophiles are typically negatively charged. (2*R*)-2-(α-D-Mannopyranosyl)glycerate (herein abbreviated as mannosylglycerate) is one of the most widespread solutes among thermophilic and hyperthermophilic prokaryotes. In this work, several molecules chemically related to mannosylglycerate were synthesized, namely (2*S*)-2-(1-*O*-α-D-mannopyranosyl)propionate, 2-(1-*O*-α-D-mannopyranosyl)acetate, (2*R*)-2-(1-*O*-α-D-glucopyranosyl)glycerate and 1-*O*-(2-glyceryl)-α-D-mannopyranoside. The effectiveness of the newly synthesized compounds for the protection of model enzymes against heat-induced denaturation, aggregation and inactivation was evaluated, using differential scanning calorimetry, light scattering and measurements of residual activity. For comparison, the protection induced by natural compatible solutes, either neutral (e.g., trehalose, glycerol, ectoine) or negatively charged (di-*myo*-inositol-1,3′-phosphate and diglycerol phosphate), was assessed. Phosphate, sulfate, acetate and KCl were also included in the assays to rank the solutes and new compounds in the Hofmeister series. The data demonstrate the superiority of charged organic solutes as thermo-stabilizers of enzymes and strongly support the view that the extent of protein stabilization rendered by those solutes depends clearly on the specific solute/enzyme examined. The relevance of these findings to our knowledge on the mode of action of charged solutes is discussed.

1. Introduction

Enzymes and other proteins are used in many industrial and pharmaceutical processes; they are also active components of numerous food and clinical preparations. Therefore, the preservation of their native structure is a prerequisite for the usefulness of the specific industrial process or preparation. Furthermore, the association of structural instability, and consequent protein aggregation, with a number of afflicting human pathologies has been widely documented in recent years.[1–3] In this context, the development of reliable strategies to improve protein stability is of great importance.[3–6]

Compatible solutes are low-molecular-mass organic compounds that accumulate in the cytoplasm of many halophilic or halotolerant organisms to counterbalance the osmotic pressure of the external medium.[7–9] They must be highly soluble and usually belong to one of the following classes of compounds: amino acids, carbohydrates, polyols, betaines and ectoines. Trehalose, glycerol, glycine-betaine and ectoine are typical solutes of mesophiles. The discovery of extreme thermophilic and hyperthermophilic (hereafter designated hyper/thermophilic) microorganisms in the early 1970s and 1980s, respectively, led to the identification of new compatible solutes, highly restricted to organisms isolated from hot habitats.[10–12] In contrast with the neutral or zwitterionic nature of the solutes commonly found in mesophiles, the organisms with optimal growth temperature above 60 °C accumulate mainly negatively charged compounds, such as mannosylglycerate and di-*myo*-inositol-1,3′-phosphate. Furthermore, the concentration of

Abbreviations: MG or mannosylglycerate, (2*R*)-2-(α-D-mannopyranosyl)glyceric acid (potassium salt); ML or mannosyl-lactate, (2*S*)-2-(1-*O*-α-D-mannopyranosyl)propionic acid (potassium salt); MGlyc or mannosylglycolate, 2-(1-*O*-α-D-mannopyranosyl)acetic acid (potassium salt); GG or glucosylglycerate, (2*R*)-2-(α-D-glucopyranosyl)glyceric acid (potassium salt); MGOH or mannosylglycerol, 1-*O*-(2-glyceryl)-α-D-mannopyranoside; MGA or mannosylglyceramide, (2*R*)-2-(α-D-mannopyranosyl)glyceramide; DIP, di-*myo*-inositol-1,3′-phosphate (potassium salt); DGP, diglycerol phosphate (potassium salt); HEct, hydroxyectoine; Ect, ectoine; SNase, recombinant nuclease A from *Staphylococcus aureus*; MDH, mitochondrial malate dehydrogenase from pig heart; LDH, L-lactate dehydrogenase from rabbit muscle; DSC, differential scanning calorimetry; T_m, melting temperature.

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these solutes increases not only with the NaCl concentration of the medium but also with the growth temperature, leading to the view that they play a role in the protection of proteins and other cellular components against heat denaturation. In agreement with this hypothesis, it has been shown that solutes from hyper/thermophiles exert superior thermoprotection on a variety of model enzymes.[13–19]

Mannosylglycerate (MG) is one of the most widespread solutes in hyper/thermophilic organisms.[10] The efficacy of this natural solute in the protection of protein structures has been amply illustrated.[15,17,18,20,21] In this work, several compounds structurally related to MG were tested for their ability to stabilize model proteins against thermal stress. The glyceric acid group in MG was replaced by lactic acid, glycolic acid and glycerol groups; in addition, mannose was replaced by glucose to yield glucosylglycerate. The protecting effect of the synthetic compounds was compared with that rendered by a number of charged and uncharged natural organic solutes as well as inorganic salts. Different aspects of the protein stabilization process, that is, the thermodynamic stability, inhibition of aggregation and retention of the catalytic activity, were assessed by using differential scanning calorimetry, light scattering and residual activity measurements. The motivation behind this study was two-fold: to obtain new compounds with improved protecting properties and to extend our knowledge on the structural features that determine the degree of protein stabilization rendered by a specific compound.

2. Results

2.1. Synthesis of compounds related to (2*R*)-2-(α-D-mannopyranosyl)glycerate

(2*S*)-2-(1-*O*-α-D-Mannopyranosyl)propionate (ML), 2-(1-*O*-α-D-mannopyranosyl)acetate (MGlyc) and 1-*O*-(2-glyceryl)-α-D-mannopyranoside (MGOH) are structural variations of MG (Fig. 1). In comparison with MG, the non-glycosidic group of ML lacks the primary hydroxyl group and the lactate has the opposite configuration (*S*) to the glycerate (*R*); MGlyc does not possess the CH_2OH group and has lost the asymmetric centre; finally, the carboxylic group in MG has been replaced by CH_2OH to yield a neutral molecule, MGOH.

For the synthesis of (2*S*)-2-(1-*O*-α-D-mannopyranosyl)propionate (**4,** Scheme 1), the α-mannose trichloroacetimidate derivative **2** was used as the glycosyl donor (Scheme 1). The glycosylation reaction with (*S*)-methyl lactate was successfully accomplished using $BF_3{\cdot}OEt_2$ in dichloromethane, and as expected only the α-anomer was obtained. Successive removal of the protecting groups afforded **4** in good overall yield (53%).

The same strategy was employed to synthesize 2-(1-*O*-α-D-mannopyranosyl)acetate (**6,** Scheme 2) and 1-*O*-(2-glyceryl)-α-D-mannopyranoside (**8,** Scheme 3). Methyl glycolate was the glycosyl acceptor in the synthesis of **6** and 1,3-di-*tert*-butyldiphenylsilylglycerol was the glycosyl acceptor in the case of **8**.

For the synthesis of (2*R*)-2-(1-*O*-α-D-glucopyranosyl)glycerate (**12**, Scheme 4), a different approach was used since the beta isomer is formed if participating groups, such as acetate, are present in the 2-position. The thioglycoside derivative **9** was used as the glucosyl donor and it was obtained using a procedure described previously.[22] The glycosylation reaction was successfully accomplished employing Crich and Smith conditions.[23] Successive removal of protecting groups afforded the α-anomer **12** in 36% overall yield.

2.2. Effect of solutes on protein structural stability as monitored by DSC

The stabilizing properties of different natural solutes and synthetic compounds on the protein structural stability were assessed for three model enzymes. At the working pH, malate dehydro-

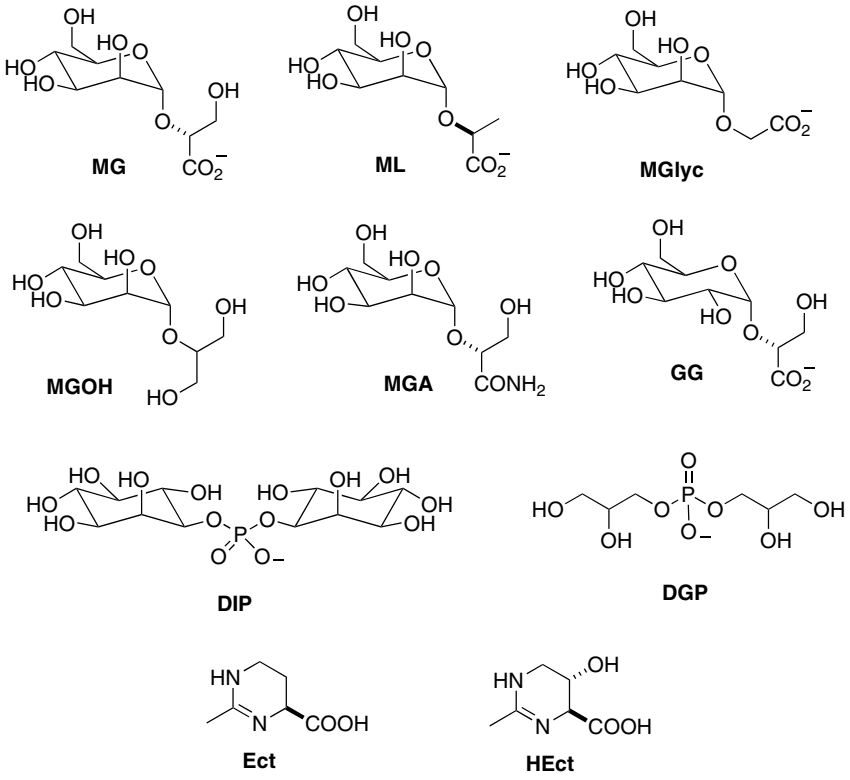
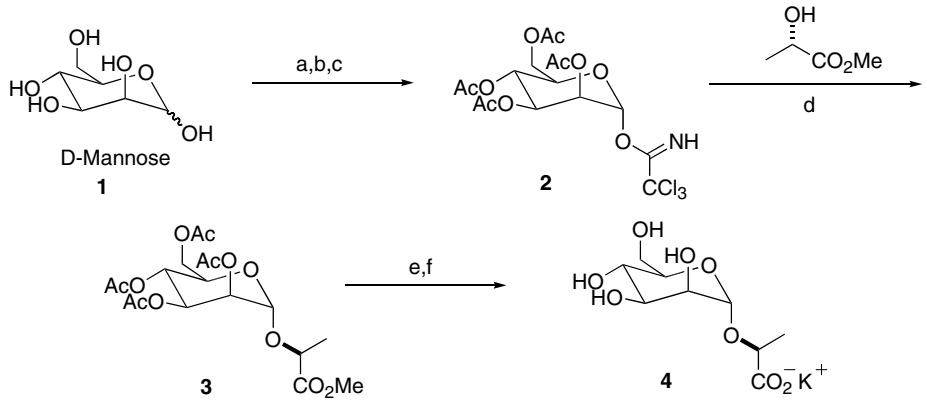
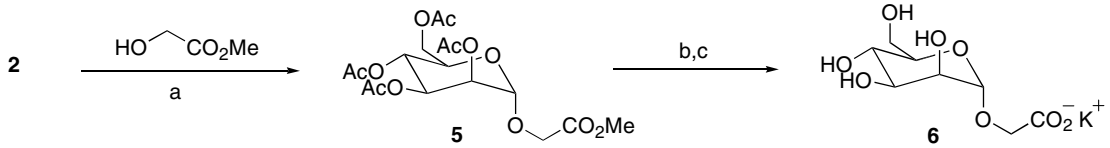


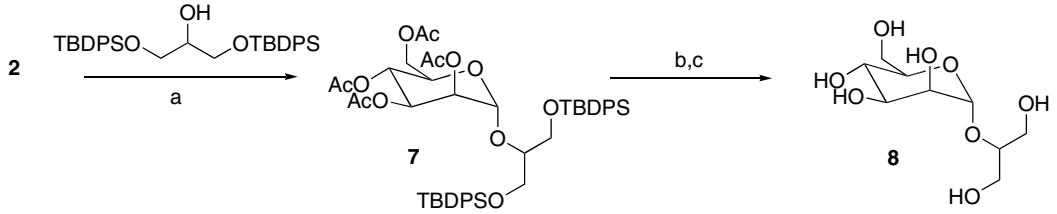
Figure 1. Chemical structures of the organic solutes whose stabilizing effect was studied. MG, mannosylglycerate; ML, mannosyl-lactate; MGlyc, mannosylglycolate; MGOH, mannosylglycerol; MGA, mannosylglyceramide; GG, glucosylglycerate; DIP, di-*myo*-inositol-1,3′-phosphate; DGP, diglycerol phosphate; Ect, ectoine; HEct, hydroxyectoine.



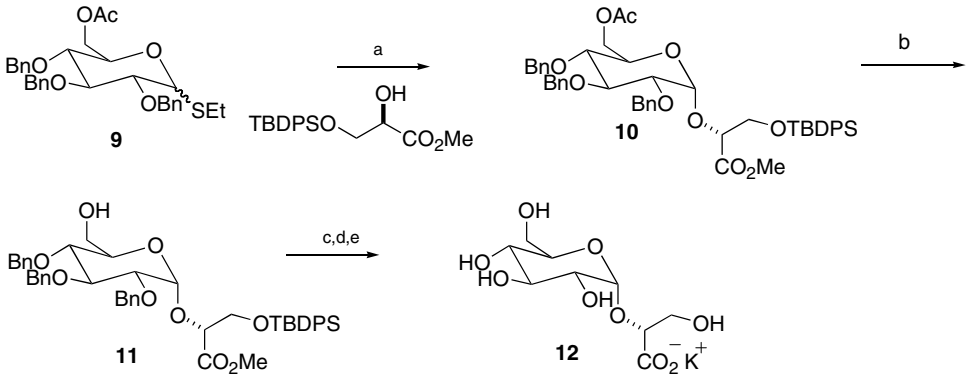
Scheme 1. Synthesis of (2*S*)-*S*-(1-*O*-α-D-mannopyranosyl)propionate **4** from mannose. Reagents and conditions: (a) Ac_2O, pyr, DMAP, 0 °C/rt, 14 h, 99%; (b) $H_2NNH_2{\cdot}CH_3COOH$, DMF, rt, 3 h, 96%; (c) CCl_3CN, CH_2Cl_2, DBU, rt, 2 h, 86%; (d) $BF_3{\cdot}OEt_2$, CH_2Cl_2, −78 °C/rt, 74%; (e) MeOH, MeONa 1 N, rt, 88%; (f) KOH 2 N, H_2O.



Scheme 2. Synthesis of 2-(1-*O*-α-D-mannopyranosyl)acetate **6** from mannose. Reagents and conditions: (a) $BF_3{\cdot}OEt_2$, CH_2Cl_2, −78 °C/rt, 69%; (b) MeOH, MeONa 1 M, rt, 85%; (c) KOH 2 M, H_2O.



Scheme 3. Synthesis of 1-*O*-(2-glyceryl)-α-D-mannopyranoside **8** from mannose. Reagents and conditions: (a) $BF_3{\cdot}OEt_2$, CH_2Cl_2, −78 °C/rt, 85%; (b) TBAF, THF, rt, 96%; (c) MeOH, MeONa 1 M, rt, 90%.



Scheme 4. Synthesis of (2*R*)-2-(α-D-glucopyranosyl)glycerate **12** from glucose. Reagents and conditions: (a) $PhSONC_5H_{10}$, DTBMP, Tf_2O, MS, CH_2Cl_2, 76%; (b) Na, MeOH, 0 °C, 94%; (c) TBAF, THF, 90%; (d) H_2, Pd, 35 psi, 99%; (e) KOH 2 M, H_2O rt.

genase, SNase and lysozyme have net charges of +3.9, +8.1 and +8.8, and melting temperature (T_m) values in the absence of solutes of 48.1, 53.9 and 72.9 °C, respectively. The increment in T_m induced by the presence of the synthesis compounds and several natural solutes of hyper/thermophiles and mesophiles is depicted in Figure 2; the T_m values are presented in Table S1 (Supplementary data).

Glycerol, ectoine, KCl and hydroxyectoine were poor stabilizers of the enzymes examined; in contrast, DIP, ML, MG, GG and MGlyc were highly effective. In particular, DIP and ML were excellent, inducing increases in T_m greater than 10 °C per 0.5 M solute on MDH and SNase, respectively. In comparison with MG, the synthesis solute ML was clearly superior in the stabilization of SNase and lysozyme and had similar effect on MDH. GG was better than MG

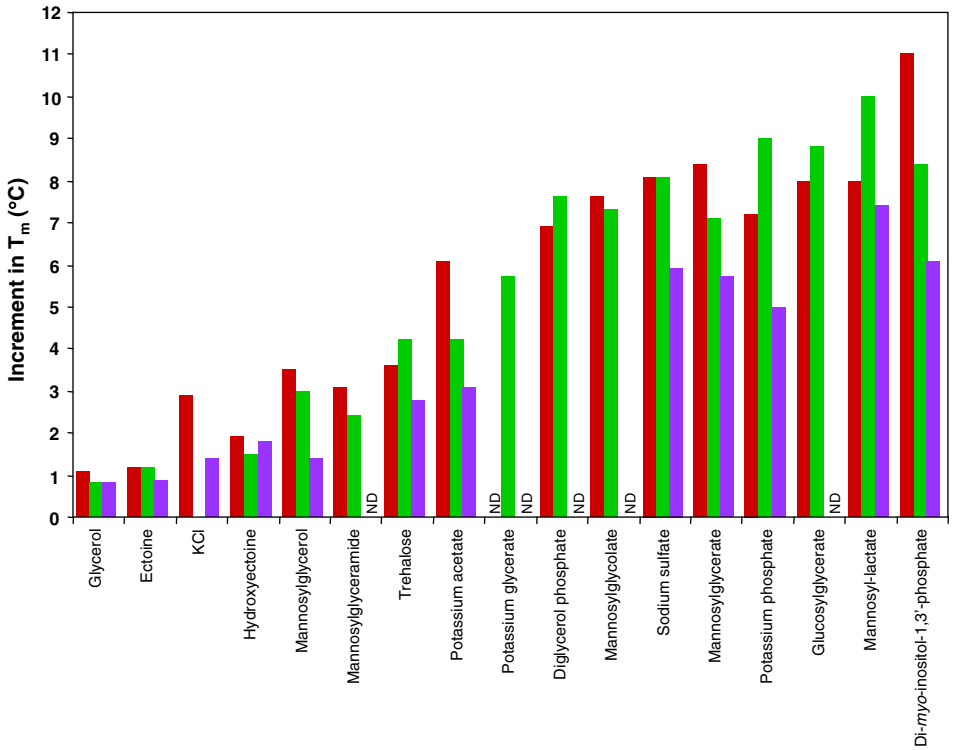


Figure 2. Increment in the melting temperature of malate dehydrogenase, MDH (grey bars, red bars in online), staphylococcal nuclease, SNase (upward diagonal pattern, green bars in online) and lysozyme (black bars, violet bars in online) in the presence of 0.5 M of different compounds. The melting temperature in the absence of solutes was 48.1 °C for MDH, 53.9 °C for SNase and 72.9 °C for lysozyme. ND, not determined.

for the stabilization of SNase, while MGlyc was similar to MG. However, MG was slightly better than GG and MGlyc for the stabilization of SNase. On the other hand, the neutral derivative, MGOH, was substantially less efficient for all the enzymes examined.

The dependence of the increment of T_m (stabilization) on the concentration of solute was studied using SNase and glycerol, KCl, acetate, glycerate, sulfate, phosphate, MG and ML. The two latter solutes were excellent stabilizers of this enzyme, comparable to the well-known kosmotropic agents, sulfate and phosphate; glycerate and acetate showed a moderate beneficial effect, whereas glycerol and KCl were poor stabilizers. In fact, while the concentration of glycerol required to increase the T_m by 3 °C was approximately 2.5 M, the same increment was produced by 0.15 M MG or ML (Fig. 3).

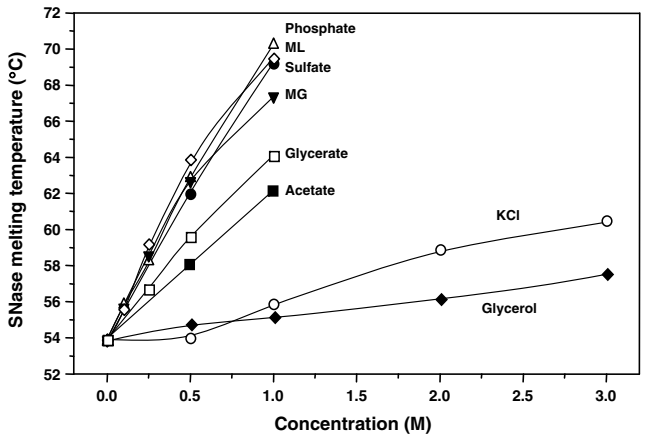


Figure 3. Dependence of staphylococcal nuclease melting temperature on the concentration of glycerol (–♦–), KCl (–○–), acetate (–■–), glycerate (–□–), MG (–▼–), sulfate (–●–), ML (–◇–) and phosphate (–△–). Abbreviations as in the legend of Figure 1.

2.3. Effect on protein aggregation as monitored by light scattering

Thermodynamic data obtained by calorimetry were complemented with light scattering measurements to assess the effective-

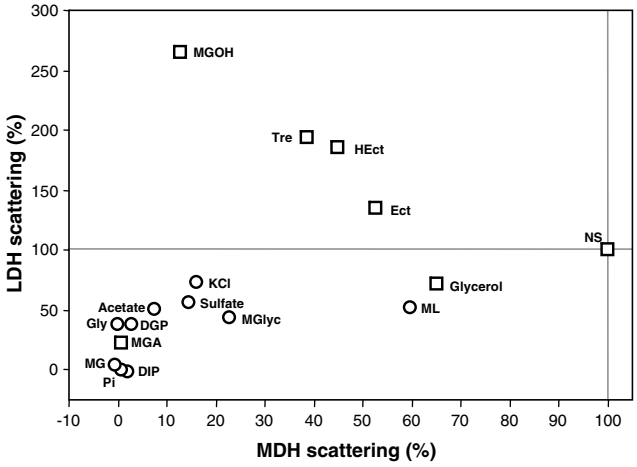


Figure 4. Effect of different compounds at 0.5 M concentration upon malate dehydrogenase (MDH) and lactate dehydrogenase (LDH) light scattering. 100% is defined as the value measured in the absence of solutes after incubation of MDH (0.2 mg/mL) or LDH (0.2 mg/mL) at the final temperature for 50 or 30 min, respectively. Ionic compounds are represented by open circles (○) and non-charged compounds are shown as open squares (□). Abbreviations: Pi, potassium phosphate; Gly, potassium glycerate; Tre, trehalose; other abbreviations as in the legend of Figure 1.

ness of solutes to prevent protein aggregation. In this case, LDH and MDH were used as model enzymes. All the compounds examined reduced the aggregation of MDH but to different extents (Fig. 4 and Table S2). The most efficient compounds, inducing a decrease of light scattering over 85%, were MG, MGA, DGP, DIP, MGOH, phosphate, glycerate and acetate. In the case of LDH, a few compounds, trehalose, ectoine, hydroxyectoine and MGOH, appeared to promote aggregation (increased the light scattered), while all the others reduced protein aggregation. DIP, MG and phosphate were the best inhibitors of LDH aggregation and also exerted excellent protection on MDH. Curiously, a few solutes had opposite effects upon MDH or LDH; for example, MGOH was very effective in reducing MDH aggregation, but increased considerably the scattering observed with LDH. On the other hand, ML and glycerol prevented aggregation of both enzymes but to a modest extent. The effect of MG concentration on LDH aggregation was also studied. In the presence of 0.1 M MG, the scattering was only slightly lower than in the assay without solutes (84% vs 100%). However, with 0.25 M MG the light scattering was reduced to 37%, and 0.5 M MG led to a strong suppression of scattering (4%).

2.4. Effect of solutes on the protection of MDH against thermal inactivation

When a protein is subjected to heat stress, processes like denaturation, aggregation, oxidation of some amino acid residues, reduction of disulfide bonds, deamidation of glutamine or asparagine residues and hydrolysis of peptide bonds may occur, which lead to the irreversible loss of function.[24] The time-course of MDH inactivation was studied to evaluate the inhibitory effect of the different compounds on those irreversible processes (Table 1). Sulfate and MG exerted a high protective effect on MDH, increasing the half-life for inactivation more than 24- and 18-fold, respectively. In contrast, DIP and hydroxyectoine had a deleterious effect, inducing a decrease of approximately 50% in the half-life for MDH inactivation. Unexpectedly, ML, MGlyc and glycerate totally inhibited MDH activity, thereby precluding further investigation on their effect on the thermal inactivation profile.

3. Discussion

In the search for better protein stabilizers, mannosylglycerate, a very efficient protector widely distributed in organisms thriving in hot environments, was used as a lead compound for the design of new molecules. The effectiveness of the newly synthesized compounds in the protection of model enzymes against heat-induced denaturation, aggregation and inactivation was evaluated. The greater stabilization rendered by charged compounds is a clear trend emerging from the large data set presented. This trend is even more explicit in a plot of the increment in the T_m of MDH versus those of SNase and lysozyme, which shows that ionic compounds (except for KCl) cluster in the upper-right corner of the graph, while non-charged compounds appear in the lower-left corner (Fig. 5). This general pattern is also corroborated by the light scattering experiments that reveal a good correlation between charge and the ability to prevent protein aggregation (Fig. 4); curiously, the uncharged solute, mannosylglyceramide, is a notable exception, inhibiting protein aggregation comparable to mannosylglycerate, a closely related charged solute, and conferring a much greater protection than mannosylglycerol, a related uncharged compound. On the other hand, in respect to structural stabilization, mannosylglyceramide falls well in the low performance region, typical of neutral solutes (Fig. 5).

Considering the simpler phenomenon of structural stabilization and the subset of charged compounds, it is clear that the extent of stabilization upon a given protein varies with the structure of the solute, but the scatter observed is difficult to rationalize in molecular terms. Potassium glycerate is significantly less efficient upon SNase than the related compounds mannosylglycerate and glucosylglycerate, suggesting that the presence of the sugar moiety plays an important role. However, the discrepancy between the increment in T_m induced by mannosylglycerate and glucosylglycerate is difficult to explain, given the high similarity of the mannose and glucose groups (Fig. 2). In another example, the replacement of a CH_2OH group in mannosylglycerate by a CH_3 as in mannosyllactate had a considerable beneficial impact on the stabilization of SNase and lysozyme, but not of MDH. These findings also indicate that attempts to correlate the stabilizing effect with the polarity of the solute are probably too simplistic.

The Hofmeister series has been used as a ranking of ions based on their 'salting-out' ability. Initially, this series was based on the minimum concentration of salts required to precipitate egg white proteins.[25–27] However, the accumulation of data on other fields of protein science has broadened the scope of application;[28] for example, the influence of ions on protein thermal unfolding appears to follow the Hofmeister series.[29,30] Our results show that chloride < acetate < sulfate ≈ phosphate in respect to their efficiency to increase the T_m of SNase, and this order agrees well

Table 1
Half-life for thermal inactivation of pig heart mitochondrial malate dehydrogenase in the presence of different compounds (at a final concentration of 0.5 M)

	Half-life (min)
Hydroxyectoine	1.6 ± 0.3
Di-*myo*-inositol-1,3′-phosphate	1.6 ± 0.6
Trehalose	2.6 ± 0.2
No solute	3.2 ± 0.6
Glycerol	3.9 ± 0.3
Ectoine	4.3 ± 0.3
KCl	8.7 ± 0.1
Mannosylglyceramide	13.2 ± 0.3
Potassium phosphate	32.5 ± 5.0
Diglycerol phosphate	50.2 ± 8.2
Potassium acetate	53.0 ± 1.7
Mannosylglycerate	56.5 ± 4.0
Sodium sulfate	72.8 ± 11.3

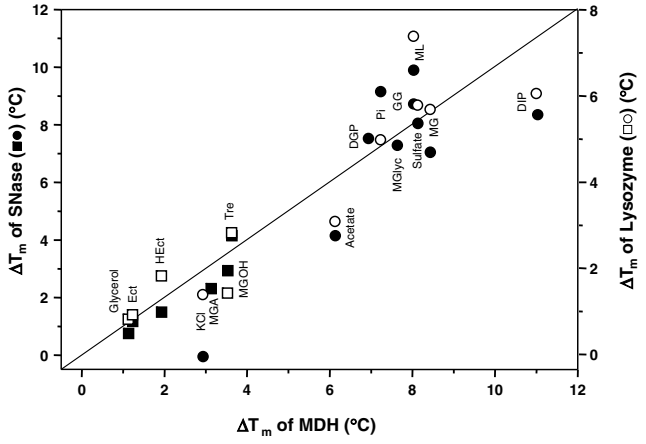


Figure 5. Stabilizing effect of different compounds against thermal denaturation of MDH, SNase and lysozyme. In the abscissa axis, the increment in the melting temperature of MDH induced by 0.5 M of the various compounds, and in the ordinates axis the increment in the melting temperature of SNase (solid symbols) and lysozyme (open symbols) are plotted. The increment value is defined as the difference between the melting temperature in the presence of compound and the value without addition. Ionic compounds are represented by circles (●,○) and non-charged compounds by squares (■,□). Abbreviations as in the legend of Figures 1 and 4.

with the Hofmeister series, suggesting that the increment in the T_m of SNase might be useful to infer the position of the hyper/thermophiles solutes and of the synthetic compounds in the Hofmeister series. In this context, the order of efficiency found for SNase, Cl^- < acetate < glycerate < MG ≈ MGlyc < DGP < sulfate ≈ DIP < GG ≈ phosphate < ML illustrates the point that the solutes from hyper/thermophiles appear close to the best stabilizing ions of the Hofmeister series, that is, sulfate and phosphate; in addition, the synthetic compounds ML and GG are placed at the head of the series, being better or comparable to phosphate. It should be pointed out, however, that the order of efficiency depends on the enzyme studied, and for MDH the following series was found: Cl^- < acetate < DGP < phosphate < MGlyc < GG ≈ ML ≈ sulfate < MG < DIP. In this case, phosphate is significantly worse than sulfate, and MG and DIP are better than the synthetic compounds.

If this analysis is extended to include the data on the prevention of protein aggregation, the salts commonly represented in the Hofmeister series are ordered as Cl^- < sulfate < acetate < phosphate, with the remarkable inversion of positions between sulfate and acetate. In regard to the solutes of hyper/thermophiles, DIP and MG fall into the front region regardless of the enzyme examined, but the synthetic compounds had very distinct effects upon MDH and LDH, thus precluding their positioning in the effectiveness ranking. As a general conclusion of the calorimetric and light scattering experiments, the natural solutes of hyper/thermophiles (MG, DIP and DGP) had stabilizing abilities similar to those of the most efficient inorganic ions, while the ranking of the newly synthesized compounds depends on the assay examined: from the melting temperature experiments ML can be placed among the best stabilizers while it has a poor performance as inhibitor of protein aggregation. It seems as though an increment in structural stability does not translate necessarily into a decrease in protein aggregation, probably due to the distinct nature of the two processes: protein aggregation is controlled at the kinetic level, while T_m is a thermodynamic property.

In practical terms, enzyme thermostabilization should be primarily assessed from the ability to preserve activity, that is, from the inactivation profiles at the operational temperature. Therefore, we deemed it interesting to correlate structural stabilization (T_m values), inhibition of protein aggregation and half-life values for inactivation (Fig. 6). If the abnormal behaviour of DIP is ignored for the moment (see below), there is a good correlation between the increment of T_m and the operational stabilization. In fact, the extra structural stabilization rendered by a solute (T_m increment) is clearly more important than its ability to prevent aggregation. The patterns of MGA and KCl are good illustrations of this view: both compounds inhibited MDH aggregation to a large extent, yet their protection against thermal inactivation was modest. On the other hand, the natural solutes MG and DGP were excellent activity protectors, contrasting with the peculiar destabilizing effect of DIP, the solute that induced the greatest increase in T_m. In fact, a similar destabilizing effect has been observed for DIP on a different enzyme, LDH.[17] A possible explanation could be the ability of DIP to stabilize non-productive oligomeric forms of the enzymes. The reasons for the unexpected inhibition of MDH by the synthetic compounds ML, MGlyc and GG (results not shown) remain elusive. A possible negative effect of a contaminant is ruled out by the high purity of the compounds used in the assay.

In conclusion, besides a sound demonstration of the superiority of charged organic solutes to act as thermo-stabilizers of enzymes, this work strongly supports the view that the extent of protein stabilization rendered by organic solutes depends clearly on the specific solute/enzyme examined. Moreover, the magnitude of the stabilizing effect also depends on the particular step of the inactivation process that is targeted in the assays, for example, denaturation, as assessed by T_m measurements, or aggregation, as assessed by light scattering. These data reinforce the concept that the mechanisms underlying stabilization by charged solutes may depend to a greater extent on specific protein/solute interactions than on general alterations of the water properties that seem to characterize the mode of action of neutral solutes.[31–33]

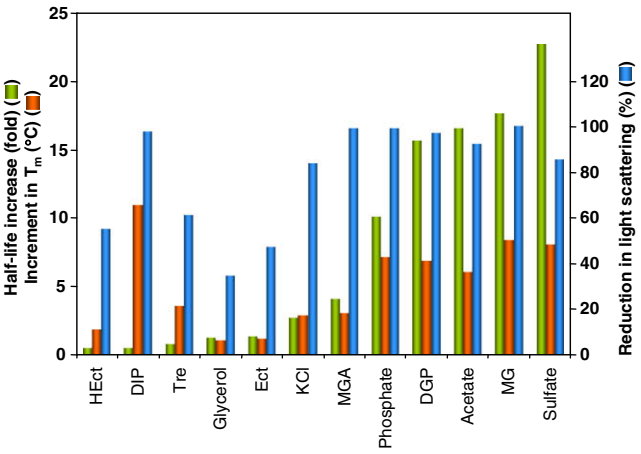


Figure 6. Comparison between the effect of several compounds on MDH half-life for thermal inactivation (upward diagonal pattern, green bars in online), increment in the melting temperature (black bars, orange bars in online) and aggregation inhibition (grey bars, blue bars in online). Thermal inactivation values are depicted as the ratio between the half-life value in the presence of solutes and that without additions. The increment in T_m is defined as the difference between the T_m values with and without solutes. HEct, hydroxyectoine; DIP, di-*myo*-inositol-1,3′-phosphate; Tre, trehalose; Ect, ectoine; MGA, mannosylglyceramide; DGP, diglycerol phosphate; MG, mannosylglycerate.

Our efforts to produce better solutes were reasonably successful, if we consider the complexity of the mechanisms involved in enzyme protection by organic solutes. In fact, mannosyl-lactate was the best stabilizer of SNase and lysosyme in regard to thermal unfolding. Interestingly, the best wide-ranging protector was mannosylglycerate, a canonical solute among hyper/thermophiles. On a physiological point of view, it is notable that hyper/thermophilic cells have evolved to hold high levels of negatively charged solutes, a feature that enables them to benefit from the most effective stabilizers against heat stress.

4. Experimental

4.1. Materials

Mannosylglycerate (MG), mannosylglyceramide (MGA), diglycerol phosphate (DGP), di-*myo*-inositol-1,3′-phosphate (DIP), hydroxyectoine (HEct) and ectoine (Ect) were obtained from bitop AG, Witten, Germany. Potassium glycerate was prepared from hemicalcium glycerate from Sigma. Calcium was removed on a H^+-activated Dowex column (Bio-Rad) and ultra-pure potassium hydroxide (FIXANAL from Riedel-de Haën AG) was used to titrate the glyceric acid solution. Purity and concentration of the compounds was assessed by ^1H NMR spectra recorded on a Bruker AMX300 spectrometer with a 5 mm inverse probe head. For quantification purposes, spectra were acquired with a repetition delay of 60 s with formate as concentration standard. Only samples with purity higher than 98% were used. Trehalose, potassium chloride, glycerol, sodium and potassium phosphate, sodium sulfate and potassium acetate were of the highest purity available. Mitochondrial malate dehydrogenase from pig heart (MDH) and rabbit muscle L-lactate dehydrogenase (LDH) were purchased from Roche; hen egg white lysozyme was from Fluka. These enzymes were used without further purification. Recombinant staphylococcal nuclease

A (SNase) was produced and purified from *Escherichia coli* cells as described by Faria et al.[21] Protein concentration was determined from UV absorbance at 280 nm, using 0.28 $(mg/mL)^{-1}\,cm^{-1}$ for the extinction coefficient of MDH,[34] 1.49 $(mg/mL)^{-1}\,cm^{-1}$ for LDH,[35] 2.64 $(mg/mL)^{-1}\,cm^{-1}$ for lysozyme[36] and 0.93 $(mg/mL)^{-1}\,cm^{-1}$ for SNase.[37]

4.2. Chemical synthesis

Mannosyl-lactate (ML), mannosylglycerol (MGOH), mannosylglycolate (MGlyc) and glucosylglycerate (GG) were obtained by chemical synthesis. All the reactions were carried out under argon and stirring, unless otherwise stated. Preparative TLC was run on silica gel Merck 60 GF254 plates and analytical TLC on aluminium-backed silica gel Merck 60 GF254 plates. Silica Gel 60H was used for the medium pressure column chromatography. Tetrahydrofuran (THF) was dried by distillation over Na/benzophenone under argon and dry CH_2Cl_2 was obtained by distillation over phosphorus pentoxide. Other solvents and reagents were distilled as required. The reaction products were analyzed by NMR; spectra were recorded in $CDCl_3$ and D_2O depending on the solubility of the products, at 300 MHz (^1H NMR) and 75 MHz (^{13}C NMR). The desired compounds were purified by anion exchange chromatography on a QAE-Sephadex A25 column (Amersham-Pharmacia Biotech) eluted with sodium carbonate/bicarbonate buffer, pH 9.8 (from 5 mM to 1 M concentration). The fractions containing the pure compounds were pooled, lyophilized and loaded onto a H^+-activated DOWEX 50W-X8 column (Bio-Rad) eluted with water. The fractions containing the compounds were pooled, degassed under vacuum and the pH was adjusted to 4.5–5.0 with ultra-pure potassium hydroxide (FIXANAL from Riedel-de Haën AG). Purity and quantification were evaluated as described above.

4.2.1. Methyl (2*S*)-2-(2,3,4,6-tetra-*O*-acetyl-1-*O*-α-D-mannopyranosyl)propionate 3

To a solution of trichloroacetimidate **2**[38] (15 g, 30 mmol) in CH_2Cl_2 (60 mL) was added (*S*)-methyl lactate (3.49 mL, 36 mmol). The solution was cooled to −20 °C and $BF_3{\cdot}OEt_2$ (3.90 mL, 30 mmol) was slowly added. The reaction mixture was stirred, while the temperature was allowed to rise to 0 °C, for 2 h (no starting material was detected by analytical TLC). Saturated aqueous $NaHCO_3$ solution (50 mL) was added followed by extractions with CH_2Cl_2 (3 × 60 mL). The combined organic phases were dried ($MgSO_4$) and concentrated. The residue was purified by column chromatography (AcOEt/hexane 2:8) to afford **3** (9.80 g, 74%) as a colourless viscous foam. $[\alpha]_D^{20}$ +22.8 (c 1.91, CH_2Cl_2). ^1H NMR ($CDCl_3$): δ 5.35–5.26 (3H, m), 4.90 (1H, d, J = 1.3 Hz), 4.33–4.24 (2H, m), 4.17 (1H, q, J = 6.8 Hz), 4.00 (1H, dd, J = 12.1 Hz, J = 1.8 Hz), 3.75 (3H, s), 2.16 (3H, s), 2.10 (3H, s), 2.05 (3H, s), 2.00 (3H, s), 1.44 (3H, d, J = 6.8 Hz). ^{13}C NMR ($CDCl_3$): δ 172.4, 170.6, 170.0, 169.8, 169.7, 98.3, 74.6, 69.7, 69.0, 68.9, 65.8, 62.0, 52.1, 20.8, 20.7, 20.6, 20.5, 18.2. FT-IR 1746.6 cm^{-1} (C=O).

4.2.2. (2*S*)-2-(1-*O*-α-D-Mannopyranosyl)propionic acid, potassium salt 4

To a solution of **3** (8.00 g, 18 mmol) in MeOH (60 mL) was added MeONa (1 N in MeOH, 12.4 mL) at rt. After 3 h, all the starting material had been consumed. The solution was diluted with MeOH, and Dowex 50 WX8 resin was added until pH 6. Filtration and evaporation of the solvents afforded a viscous residue, which was diluted with water (10 mL) and washed with CH_2Cl_2 twice (2 × 10 mL). The aqueous phase was concentrated to afford the crude methyl (2*S*)-2-(1-*O*-α-D-mannopyranosyl)propionate as a very colourless viscous foam. To a solution of this compound (4.27 g, 15.4 mmol) in H_2O (16 mL) was added an aqueous solution of KOH 2 M (8.0 mL). The reaction was stirred for 4 h. The pH was adjusted to neutral with HCl 10% and the solvent evaporated to afford **4** (4.68 g, potassium salt) as a colourless viscous foam, which was purified as described above. $[\alpha]_D^{20}$ +30.5 (*c* 2.2, H_2O). ^1H NMR (D_2O): δ 4.86 (1H, d, J = 1.5 Hz), 4.06 (1H, q, J = 6.8 Hz), 3.89 (1H, dd, J = 3.4 Hz, J = 1.7 Hz), 3.81 (1H, dd, J = 8.6 Hz, J = 3.2 Hz), 3.70–3.57 (4H, m), 1.29 (3H, d, J = 6.8 Hz). ^{13}C NMR (D_2O): δ 182.2, 101.9, 77.0, 75.7, 73.1, 72.9, 69.1, 63.2, 20.0. FT-IR 1587.5 cm^{-1} (C=O).

4.2.3. Methyl 2-(2,3,4,6-tetra-*O*-acetyl-1-*O*-α-D-mannopyranosyl)acetate 5

To a solution of **2** (4.18 g, 8.5 mmol) in CH_2Cl_2 (20 mL) was added methyl glycolate (0.741 g, 10.2 mmol). The solution was cooled to −20 °C and $BF_3{\cdot}OEt_2$ (1.0 mL, 8.5 mmol) was slowly added. The reaction mixture was stirred, while the temperature was allowed to rise, until no starting material was detected by TLC. Saturated aqueous $NaHCO_3$ solution (20 mL) was added and the aqueous phase was extracted with CH_2Cl_2 (3 × 20 mL). The combined organic phases were dried ($MgSO_4$) and concentrated. The residue was purified by column chromatography (AcOEt/hexane 2:8 to 4:6) to afford **5** (2.33 g, 69%) as a colourless viscous foam. $[\alpha]_D^{20}$ +55.1 (*c* 0.82, CH_2Cl_2). ^1H NMR ($CDCl_3$): δ 5.40–5.27 (3H, m), 4.95 (1H, s), 4.31–4.26 (2H, m), 4.20–4.13 (2H, m), 4.10 (1H, dd, J = 12.1 Hz, J = 2.2 Hz), 3.77 (3H, s), 2.16 (3H, s), 2.10 (3H, s), 2.05 (3H, s), 2.00 (3H, s). ^{13}C NMR ($CDCl_3$): δ 170.6, 170.0, 169.8, 169.7, 169.6, 169.5, 97.9, 69.2, 69.1, 68.8, 65.9, 64.6, 62.3, 52.0, 20.8, 20.7, 20.6, 20.5. FT-IR 1750.9 cm^{-1} (C=O).

4.2.4. 2-(1-*O*-α-D-Mannopyranosyl)acetic acid, potassium salt 6

To a solution of **5** (4.7 g, 11 mmol) in dried MeOH (40 mL) was added a solution of MeONa in MeOH (1 N, 7.6 mL) at rt. After 3 h, the solution was diluted with MeOH, and Dowex 50 WX8 resin was added until neutral pH. Filtration of the resulting suspension and evaporation of the solvents afforded a viscous residue, which was diluted with water (10 mL) and washed with CH_2Cl_2 twice (2 × 5 mL). The aqueous phase was concentrated under vacuum to furnish methyl 2-(1-*O*-α-D-mannopyranosyl) acetate (2.22 g, 80%) as a colourless viscous foam. To a solution of this intermediate (2.22 g, 8.8 mmol) in H_2O (10.2 mL), was added a 2 M KOH aqueous solution (4.5 mL). The pH was adjusted to 7 with HCl 1 M and the solvent evaporated under vacuum to afford **6** (2.07 g) as a colourless viscous foam, which was purified as described above. $[\alpha]_D^{20}$ +60.4 (*c* 1.88, H_2O). ^{1} H NMR (D_2O): δ 4.81 (1H, d, J = 1.6 Hz), 4.07 (1H, d, J = 16 Hz), 3.99 (1H, d, J = 16 Hz), 3.97–3.95 (1H, m), 3.82–3.79 (3H, m), 3.70–3.66 (2H, m), 3.59–3.57 (3H, m). ^{13}C NMR (D_2O): δ 176.4, 99.6, 72.9, 70.4, 69.9, 66.7, 65.2, 60.8. FT-IR 1594.1 cm^{-1} (C=O).

4.2.5. 2,3,4,6-Tetra-*O*-acetyl-1-*O*-(1,3-*O*-di-*tert*-butyldiphenylsilyl-2-glyceryl)-α-D-mannopyranoside 7

To a solution of **2** (25 g, 0.051 mmol) in CH_2Cl_2 (100 mL) was added 1,3-di-*O*-*tert*-butyldiphenylsilylglycerol (34.2 g, 0.061 mol). The solution was cooled to −20 °C and $BF_3{\cdot}OEt_2$ (6.7 mL, 0.051 mol) was slowly added. The reaction mixture was stirred, while the temperature was allowed to rise to 0 °C, for 2 h. Saturated aqueous $NaHCO_3$ solution (60 mL) was added followed by extractions with CH_2Cl_2 (3 × 80 mL). The combined organic phases were dried ($MgSO_4$) and concentrated. The residue was purified by column chromatography (AcOEt/hexane 1:9 to 2:8) to afford **7** (38.8 g, 85%) as a colourless viscous foam. $[\alpha]_D^{20}$ +26.1 (*c* 2.99, CH_2Cl_2). ^1H NMR ($CDCl_3$): δ 7.65–7.59 (8H, m), 7.44–7.32 (12H, m), 5.40 (1H, dd, J = 10.0 Hz, J = 3.4 Hz), 5.35–5.25 (2H, m), 5.11 (1H, s), 4.21–4.11 (2H, m), 4.01 (1H, m), 3.89 (1H, dd, J = 12.2 Hz, J = 1.8 Hz), 3.72 (4H, t, J = 5.9 Hz), 2.12 (3H, s), 2.04 (3H, s), 1.99 (3H, s), 1.96 (3H, s), 1.02 (9H, s), 0.99 (9H, s). ^{13}C NMR ($CDCl_3$): δ

170.7, 169.8, 169.7, 169.6, 135.5, 135.4, 133.1, 133.0, 132.9, 129.8, 129.7, 127.8, 127.8, 127.7, 96.6, 77.7, 69.5, 69.3, 68.2, 65.9, 63.8, 63.1, 62.2, 26.8, 26.7, 20.8, 20.7, 20.6, 19.1. FT-IR 1752.6 cm^{-1} (C=O).

4.2.6. 1-*O*-(2-Glyceryl)-α-D-mannopyranoside 8

To a solution of **7** (35 g, 0.039 mol) in THF (120 mL) was added TBAF (25 g, 0.098 mol) at rt. After all the starting material had been consumed, H_2O (60 mL) was added. The aqueous phase was extracted with AcOEt (3 × 80 mL), the combined organic phases were dried ($MgSO_4$) and then concentrated. The obtained residue was used in the next reaction without further purification. In order to characterize this compound, only a small sample was purified by preparative TLC (50:50 AcOEt/hexane), and 2,3,4,6-tetra-*O*-acetyl-1-*O*-(2-glyceryl)-α-D-mannopyranoside (96%) was isolated as a colourless viscous foam. $[\alpha]_D^{20}$ +33.8 (*c* 0.66, CH_2Cl_2). ^1H NMR ($CDCl_3$): δ 5.35 (1H, dd, *J* = 10.0 Hz, *J* = 3.3 Hz), 5.31–5.26 (2H, m), 5.05 (1H, d, *J* = 1.6 Hz), 4.25–4.15 (3H, m), 3.82–3.73 (5H, m) 2.16 (3H, s), 2.11 (3H, s), 2.06 (3H, s), 2.01 (3H, s). ^{13}C NMR ($CDCl_3$): δ 170.7, 170.2, 170.1, 169.7, 96.5, 81.2, 69.7, 69.0, 66.2, 63.0, 62.7, 62.1, 20.8, 20.7. FT-IR 1745.6 cm^{-1} (C=O). To a solution of the previous compound (16 g, 0.038 mol) in MeOH (50 mL) was added MeONa (1 N in MeOH, 24.7 mL) at rt. After 3 h, all the starting material had been consumed. The solution was diluted with MeOH, and Dowex 50 WX8 resin was added until pH 6. Filtration and evaporation of the solvents afforded a viscous residue, which was diluted with water (20 mL) and washed with CH_2Cl_2 twice (2 × 10 mL). The aqueous phase was concentrated and furnished **8** (9.6 g, 90%) as a colourless viscous foam, which was purified as described above. $[\alpha]_D^{20}$ +30.3 (*c* 1.5, H_2O). ^1H NMR (D_2O): δ 4.89 (1H, d, *J* = 1.6 Hz), 3.84 (1H, q, *J* = 1.8 Hz), 3.74–3.48 (10H, m). ^{13}C NMR (D_2O): δ 99.3, 78.0, 72.9, 70.4, 70.2, 66.7, 61.3, 60.9, 60.1. FT-IR 3413 cm^{-1} (O–H).

4.2.7. Methyl 3-*tert*-butyldiphenylsilyl-(2*R*)-2-(6-*O*-acetyl-2,3,4-tri-*O*-benzyl-α-D-glucopyranosyl)glycerate 10

To a stirred solution containing the thioglycoside **9**[22] (2.86 g, 5.3 mmol), 1-benzenesulfinyl piperidine (BSP, 1.12 g, 5.3 mmol), 2,6-di-*tert*-butyl-4-methylpyridine (DTBMP, 2.14 g, 10.6 mmol) and powdered 4 Å sieves in CH_2Cl_2 (25 mL) at −78 °C was added Tf_2O (1.00 mL, 5.8 mmol). After 5 min, a solution of methyl (2R)-3-*tert*-butyldiphenylsilylglycerate (2.86 g, 7.9 mmol) in CH_2Cl_2 (10 mL) was added. The reaction mixture was stirred 10 min at −78 °C and 10 min at 0 °C and quenched with saturated aqueous $NaHCO_3$ (40 mL). The aqueous phase was extracted with CH_2Cl_2 (3 × 25 mL), the combined organic phases were dried ($MgSO_4$), filtered and the solvent removed under vacuum. Purification by medium pressure column chromatography (5:95 to 10:90 AcOEt/hexane) afforded the product **10** as colourless viscous foam (4.44 g, 76%). $[\alpha]_D^{20}$ +45.2 (*c* 1.45, CH_2Cl_2). FT-IR (film) 1744.3 cm^{-1} (C=O). ^1H NMR ($CDCl_3$): δ 7.38–7.37 (5H, m), 7.37–7.22 (25H, m), 5.16 (1H, d, *J* = 3.3 Hz), 5.02 (1H, d, *J* = 10.6 Hz), 4.89 (2H, dd, *J* = 11.9 Hz, *J* = 3.1 Hz), 4.76 (1H, d, *J* = 10.5 Hz), 4.70 (1H, d, *J* = 11.8 Hz), 4.54 (1H, d, *J* = 11.2 Hz), 4.49 (1H, dd, *J* = 6.3 Hz; *J* = 4.3 Hz), 4.15–3.94 (6H, m), 3.73 (3H, s), 3.60 (1H, dd, *J* = 9.7 Hz, *J* = 3.5 Hz), 3.48 (1H, t, *J* = 9.5 Hz), 1.99 (3H, s), 1.03 (9H, s). ^{13}C NMR ($CDCl_3$): δ 170.6, 170.2, 138.7, 138.1, 138.0, 135.6, 135.5, 133.0, 132.8, 129.8, 129.8, 128.4, 128.3, 128.2, 128.2, 128.1, 127.8, 127.7, 127.6, 94.8, 81.6, 75.8, 74.8, 72.0, 71.9, 69.0, 65.8, 64.6, 62.9, 51.9, 26.7, 20.8, 19.1. HR-MS: Calcd for $C_{49}H_{56}O_{10}Si$ $[M]^+$: 559.2130; found: 559.2125.

4.2.8. Methyl 3-*tert*-butyldiphenylsilyl-(2*R*)-2-(2,3,4-tri-*O*-benzyl-α-D-glucopyranosyl)glycerate 11

A solution (7.6 mL) of Na (0.046 g) in MeOH (10 mL) was added to a stirred solution of the acetate **10** (2.0 g, 2.4 mmol) in MeOH (7.5 mL) at 0 °C. After 30 min, saturated aqueous NH_4Cl (15 mL) was added. The aqueous phase was extracted with AcOEt (3 × 20 mL), and the combined organic extracts were dried ($MgSO_4$), filtered and the solvent removed. Purification by medium pressure column chromatography (10:90 to 20:80 AcOEt/hexane) afforded the product **11** as very colourless viscous foam (1.79 g, 94%). $[\alpha]_D^{20}$ +17.2 (*c* 1.49, CH_2Cl_2). ^1H NMR ($CDCl_3$): δ 7.70–7.67 (5H, m), 7.44–7.24 (25H, m), 5.14 (1H, d, *J* = 3.5 Hz), 4.99 (1H, d, *J* = 10.8 Hz), 4.90 (1H, d, *J* = 11.5 Hz), 4.88 (1H, d, *J* = 11.2 Hz), 4.78 (1H, d, *J* = 10.1 Hz), 4.71 (1H, d, *J* = 11.7 Hz), 4.62 (1H, d, *J* = 11.3 Hz), 4.46 (1H, dd, *J* = 6.1 Hz, *J* = 4.3 Hz), 4.13–3.95 (3H, m), 3.35–3.79 (1H, m), 3.74 (3H, s), 3.70–3.45 (5H, m), 1.03 (9H, s). ^{13}C NMR ($CDCl_3$): δ 170.3, 138.8, 138.4, 138.2, 135.6, 135.5, 133.0, 132.8, 129.7, 128.3, 128.1, 128.0, 127.7, 127.6, 127.5, 94.9, 81.5, 79.3, 76.9, 75.7, 74.8, 74.7, 72.1, 71.2, 64.6, 61.6, 51.9, 26.7, 19.1. FT-IR 1752.8 cm^{-1} (C=O). HR-MS: Calcd for $C_{47}H_{54}O_9Si$ $[M]^+$: 790.3531; found: 790.3500.

4.2.9. (2*R*)-2-(α-D-Glucopyranosyl)glycerate, potassium salt 12

To a solution of **11** (3.00 g, 3.8 mmol) in THF (15 mL) at rt was added Bu_4NF (1.20 g, 4.6 mmol). The reaction mixture was stirred for 3 h and then water was added. The mixture was extracted with AcOEt (3 × 20 mL), dried ($MgSO_4$) and concentrated to furnish a yellow viscous residue. Purification by medium pressure column chromatography (50:50 to 100% AcOEt) afforded the product methyl (2*R*)-2-(2,3,4-tri-*O*-benzyl-α-D-glucopyranosyl)glycerate as a very colourless viscous foam (1.89 g, 90%). $[\alpha]_D^{20}$ +49.5 (*c* 1.16, CH_2Cl_2). ^1H NMR ($CDCl_3$): δ 7.43–7.26 (18H, m), 5.20 (1H, d, *J* = 3.6 Hz), 5.00 (1H, d, *J* = 10.8 Hz), 4.92–4.80 (3H, m), 4.70 (1H, d, *J* = 11.4 Hz), 4.62 (1H, d, *J* = 10.8 Hz), 4.36 (1H, t, *J* = 4.3 Hz), 4.03 (1H, t, *J* = 9.3 Hz), 3.93 (2H, d, *J* = 4.5 Hz), 3.76 (3H, s), 3.75–3.52 (4H, m), 2.09 (2H, br s). ^{13}C NMR ($CDCl_3$): δ 170.1, 138.6, 137.9, 137.7, 128.5, 128.4, 128.3, 127.9, 94.9, 81.3, 79.4, 77.1, 75.7, 75.1, 74.6, 72.4, 71.6, 63.3, 61.7, 52.1. HR-MS: Calcd for $C_{31}H_{36}O_9$ $[M]^+$: 552.2354; found: 552.2333. Methyl (2*R*)-2-(2,3,4-tri-*O*-benzyl-α-D-glucopyranosyl)glycerate (1.89 g, 3.4 mmol) in AcOEt/EtOH (20 mL/10 mL) was hydrogenated at 35 psi in the presence of a catalytic amount of Pd/C 10% (0.05 equiv). After 3 h, the reaction mixture was filtrated, the solvent was evaporated and the residue dried under vacuum to afford methyl (2*R*)-2-(α-D-glucopyranosyl)glycerate as very colourless viscous foam (0.95 g, 99%). No further purification was needed and we proceeded to the next step. To a solution of methyl (2*R*)-2-(α-D-glucopyranosyl)glycerate (0.95 g, 3.4 mmol) in H_2O (10 mL) was added a 2 M KOH aqueous solution (1.65 mL). The reaction was stirred overnight at rt. The pH was neutralized with HCl 10% and the solvent evaporated to afford **12** as a very colourless viscous foam in the form of its potassium salt. Its NMR data were identical to those of the natural sample. $[\alpha]_D^{20}$ +97.6 (*c* 2.08, H_2O). ^1H NMR (D_2O): δ 5.00 (1H, d, *J* = 3.7 Hz), 4.39 (1H, t, *J* = 3.5 Hz), 3.89–3.87 (2H, m), 3.78–3.62 (4H, m), 3.49 (1H, dd, *J* = 9.8 Hz, *J* = 3.9 Hz), 3.34 (1H, t, *J* = 9.1 Hz). ^{13}C NMR (D_2O): δ 176.2 (C=O), 100.1 (C-1), 78.1, 75.3, 75.0, 73.9, 72.0, 65.1, 63.0. FT-IR

4.3. Differential scanning calorimetry

Differential scanning calorimetry (DSC) was performed on a MicroCal VP-DSC MicroCalorimeter controlled by the VP-viewer program and equipped with 0.51 mL cells. Calibration of temperature and heat-flow was carried out according to MicroCal instructions. Protein stock solutions of nuclease or MDH were prepared in phosphate buffer (10 mM of sodium phosphate, pH 7.5), and lysozyme was prepared in citrate buffer (20 mM sodium citrate, 55 mM NaCl, pH 6.0). These stock solutions were extensively dialyzed against the same buffer before the assays. A volume of 2 mL of buffer with or without solutes was prepared and dispensed

into two Eppendorf tubes, 1 mL each. An aliquot of the concentrated protein solution, or of buffer, was added to each Eppendorf, and these solutions were used to fill up the sample and reference cells, respectively. Both solutions were degassed for 8 min under vacuum, prior to the calorimetric experiments. DSC scans were run at a constant heating rate of 1 °C/min with an overpressure of about 30 psi. The initial temperature of the DSC scans was 20 °C but the final temperature depended on the protein/solute pair under study. For example, in the case of SNase in the presence of KCl or glycerol the sample was heated up to 80 °C, while in the presence of MG, DGP or DIP scans were run up to 90 °C. Protein concentrations approximately 5 μM were typically used for MDH and approximately 20 μM for SNase and lysozyme. Unless otherwise stated, solute concentration was 0.5 M. The dependency of the melting temperature of SNase on the concentration of MG, ML, phosphate, sulfate, acetate, glycerate (up to 1 M), KCl and glycerol (up to 3 M) was studied. Sodium sulfate was used due to the low solubility of the potassium salt. Potassium was the counterion in all other salts examined.

A single endothermic peak was detected in the thermograms of the three proteins studied, indicating that thermal unfolding of these proteins occurred in a single step. Very good reproducibility of the baselines, determined under identical conditions to those of the assay, was always observed. Raw calorimetric data were converted to heat capacity by subtracting the buffer baseline and by dividing the scan rate and the protein concentration in the sample. The protein melting temperature (the temperature at which equal amounts of native and denatured forms are present) was determined using the software supplied with the instrument.

4.4. Light scattering experiments

Protein aggregation was monitored by measuring the light scattering in a Spectrofluorometer Spex-Fluorolog 1680, with excitation and emission at 320 nm. Assays were performed with MDH (0.2 mg/mL) and LDH (0.2 mg/mL) in 50 mM phosphate buffer, pH 7.6, in the absence and presence of solutes. All solutes were used at a concentration of 0.5 M; in the case of LDH, concentrations of 0.1 M and 0.25 M MG were also examined. The protein solutions (with or without solutes) were filtered (0.2 μm pore size) and placed in a quartz cuvette thermostated at 25 °C. Temperature was increased rapidly (heating rate approximately 10 °C/min) to 40 °C for MDH and 50 °C for LDH, and light scattering was measured as a function of time. Results are expressed as percentages of the value measured in the absence of solutes after incubation at the final temperature for 50 or 30 min for MDH or LDH, respectively.

4.5. Activity assays

To evaluate the long-term thermostability of MDH, an enzyme solution (1 U/mL in 20 mM sodium phosphate buffer, pH 7.6) was incubated at 45 °C with or without solutes, and the residual activity was measured at different time intervals as described below. The solutes were used at a final concentration of 0.5 M. MDH activity was assessed by following the conversion of NADH to NAD^+ at 340 nm with a Beckman spectrophotometer DU 70. A cuvette containing 1 mL of reaction mixture (100 mM sodium phosphate buffer, pH 7.6, 0.12 mM NADH and 0.25 mM oxaloacetate) was thermostated at 25 °C for 2 min and the reaction started by the addition of 0.025 U of MDH. The results are presented as half-life times, that is, as the incubation period during which MDH looses 50% of the initial activity. Results are averages of at least two independent experiments.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2008.08.030.

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