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Protection of a model enzyme (lactate dehydrogenase) against heat, urea and freeze-thaw treatment by compatible solute additives

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Abstract

In this study on M_4-lactate dehydrogenase (LDH) we were able to show that the addition of compatible solutes (glycine betaine, hydroxyectoine) shifts the enzyme's activity curve towards higher temperature. This increase in temperature stability is gained at the expense of a slightly reduced maximal activity and is also reflected in an increase in activation energy. In addition, tryptophan fluorescence spectroscopy has been used to monitor structural changes of the enzyme under conditions of freeze-thawing and urea treatment in the presence of a number of organic and inorganic solutes. As the data revealed that changes in fluorescence intensity are directly related to changes in enzyme activity, we were able to evolve a method for rapid assessment of enzyme stabilisation on the basis of fluorescence measurements. All organic solutes under investigation displayed remarkable stabilising properties, although the degree of stabilisation depended on both the type of solute and the stress factor chosen. It has to be noted that ammonium sulphate also performed very well as a stabiliser against heat and urea treatment, whereas the addition of inorganic salts during freeze-thawing apparently destabilises protein structure, at least under the test conditions employed. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Lactate dehydrogenase (LDH); Organic osmolyte; Stabilisation; Tryptophan fluorescence

1. Introduction

To cope with the osmotic stress imposed by a saline environment, halotolerant eubacteria as well as some eucaryotic organisms control cytoplasmic osmolality by the accumulation of organic osmolytes which, typically, have a low molecular mass and no net charge. The so-called compatible solutes (Brown [1]) can accumulate to very high levels ($\sim$ 1–2 M) without significant effect on the cell's metabolism and are represented by the following major classes: polyols, disaccharides and sugar derivatives, free amino acids and their derivatives, *N*-acetylated diamino acids, ectoines and methylamines. Apart from their purely osmotic function, compatible solutes are also distinguished by their protective effect on enzymes under conditions of heat and chemical denaturation (Rajendrakumar et al. [2]), freezing and drying (Lippert and Galinski [3], Ramos et al. [4]). Interestingly, many iso-osmotic elasmobranchs (sharks, rays) faced with the problem of retaining water in a marine

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environment accumulate urea in combination with trimethylamine-*N*-oxide (TMAO). Urea is accumulated at concentrations of 0.4–0.6 M, high enough to perturb structure and function of many enzymes. This perturbing effect is apparently counterbalanced by the presence of the co-solute TMAO (Yancey and Somero [5]). A similar situation is encountered in kidney cells where the disturbing effect of urea is relieved by a combination of other solutes (glycerophosphocholine and glycine betaine).

So far, most studies on enzyme stabilisation have been based on activity measurements. In our study we used fluorescence spectroscopy of endogenous tryptophan-residues as a very sensitive technique for monitoring structural changes of an enzyme in addition to activity assays. In the present work, rabbit muscle M_4-lactate dehydrogenase (LDH) was used as a test enzyme to investigate the influence of compatible solutes on the enzyme's structure and function under conditions of elevated temperature, freeze–thawing and urea treatment. Salts were included in this study for reasons of comparison because 'salting-out-salts' such as ammonium sulphate are usually regarded as very effective enzyme stabilisers.

2. Experimental

2.1. Chemicals

Rabbit muscle M_4-LDH, *N*α-acetyllysine, *N*ε-acetyllysine, *N*α-acetylornithine, trehalose and TMAO were purchased from Sigma, Munich, Germany. Sucrose, ammonium chloride, ammonium sulphate, disodium sulphate and urea were obtained from Merck, Darmstadt, Germany. Hydroxyectoine was isolated from *Marinococcus* strain M52 following the procedure described by Frings et al. [6], ectoine was synthesised in our laboratory from L-diaminobutyric acid and ortho acetic acid trimethyl ester and *N*δ-acetylornithine was synthesised according to the method of Leclerc and Benoiton [7]. Chemicals used for fluorescence spectroscopic studies were of highest purity and filtered (Dynagard filter 0.2 μm) before use.

2.2. LDH activity assay

The 1 ml reaction mixture contained 50 mM potassium phosphate buffer pH 7.5, 2.3 mM pyruvate, 0.35 mM NADH and 0.525 μg LDH. The reaction was initiated by the addition of 50 μl of the LDH stock solution (10.5 μg/ml) and was followed by measuring the decrease in absorbance at 340 nm (UV-3100 photometer, Shimadzu, Kyoto, Japan). Prior to the addition of enzyme, the reaction mixture was incubated in the temperature controlled cuvette holder at the indicated temperatures for at least 5 min, residual activities (after freeze-thaw and urea treatment) were measured at 30°C.

2.3. Fluorescence studies

A Perkin Elmer spectrofluorometer (type LS 50B) equipped with a temperature controlled cuvette-holder was used for fluorescence measurements. All measurements were carried out at 20°C with an excitation wavelength of 295 nm and a band width of 5 nm. LDH was used at a concentration of 10.5 μg/ml, osmolytes urea were applied at 0.5–2.0 M (final concentrations). All components of the test samples were dissolved in potassium phosphate buffer, pH 7.5. Time resolved fluorescence measurements were recorded over 20 min at the maximum emission wavelength of LDH (344 nm). The samples were stirred magnetically during measurements.

The LDH's maximum emission wavelength (λ_{EMmax}) of 344 nm was determined by recording fluorescence emission spectra (300–400 nm) as average of at least three measurements. λ_{EMmax} was found to be only slightly affected by the solutes under investigation at the concentrations used in denaturation studies. Furthermore, native and denatured enzyme displayed the same λ_{EMmax}. Fluorescence intensities (at

344 nm) of samples containing osmolytes had to be compensated for the impact of impurities. All solutes under investigation did not influence the enzyme's fluorescence intensity (see Section 3), neither in the native nor in the denatured state.

2.4. Freeze-thawing

LDH was dissolved in 50 mM potassium phosphate buffer pH 7.5 and supplemented with solutes at final concentrations of 1 osM/kg water. The enzyme preparations (not more than 8 ml) were transferred into test tubes. After determination of the enzyme's catalytic activity and fluorescence intensity at 344 nm, the LDH preparations were frozen in liquid nitrogen for 60 s and thawed in a 35°C waterbath. Such cycles were repeated five times. After each cycle, aliquots for time-resolved fluorescence measurements and enzyme activity assays were taken.

2.5. Denaturation by urea treatment

The sample containing urea in combination with a given solute at various concentrations (see Section 3) was incubated for 5 min at 20°C in the temperature-controlled cuvette holder of the spectrofluorometer. Enzyme was added without removing the cuvette from the cuvette holder to a final concentration of 10.5 μg/ml. Time resolved fluorescence emission intensity was recorded at 344 nm immediately after addition of the enzyme.

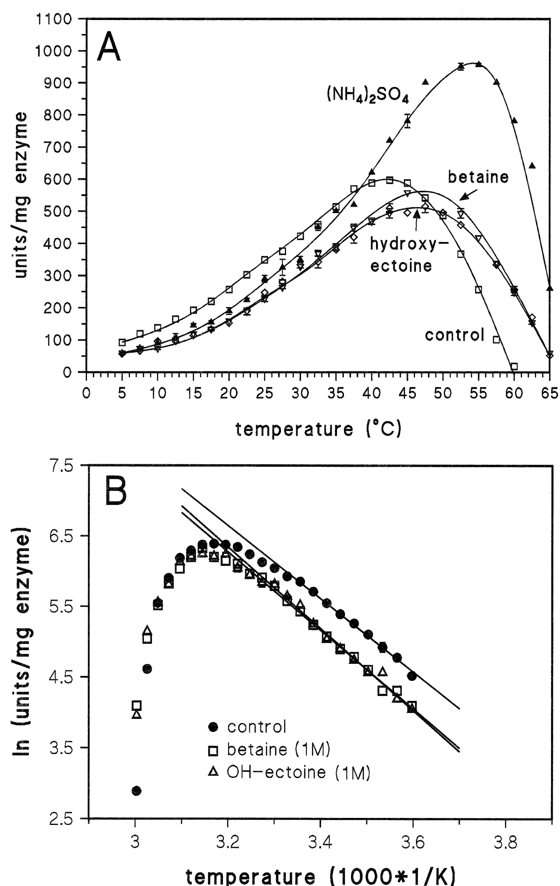

Fig. 1. (A) Temperature activity profiles of LDH. (B) Arrhenius plot of data. All additives were applied at a concentration of approx. 1 osM/kg water (1 M for betaine and hydroxyectoine and 0.33 M for ammonium sulphate).

3. Results

3.1. Protection at high temperatures

To assess enzyme stabilisation under stress conditions, enzyme activity assays were performed over a temperature range of 5–65°C. As depicted in Fig. 1A the addition of hydroxyectoine and glycine betaine shifted the activity profiles towards higher temperatures in comparison to control measurements without additives. The increased thermotolerance was gained at the cost of a slightly lowered maximum activity (95% for glycine betaine, 85% for hydroxyectoine). Ammonium sulphate obviously exerted an activating as well as a stabilising effect. Compared to control measurements, maximum activity was increased to 165%. This combined effect of ammonium sulphate has, to our knowledge, not been described so far, possibly because it is only noticed at temperatures above those usually applied in LDH test assays (35°C). Arrhenius plot of the data (Fig. 1B) revealed a markedly increased activation energy of the en-

zyme for all three solutes (107%, 112% and 121% for betaine, hydroxyectoine and ammonium sulphate, respectively).

3.2. Cryoprotection

3.2.1. Correlation of fluorescence intensity and enzyme activity

It is known that fluorescence of tryptophan-residues is sensitive to the microenvironment. In general, intensity of emission will decrease as the polarity of the environment increases (Tomiuchi et al. [8]). Unfolding of the enzyme leading to an exposure of the tryptophan-residues to the aqueous solvent of the test system consequently causes a decrease in fluorescence intensity.

Time resolved fluorescence measurements were used to follow the structural changes of the enzyme imposed by freezing and thawing in the presence of solutes. In general, native enzyme displayed maximal, completely denatured enzyme, a markedly reduced fluorescence intensity. Consequently, changes in fluorescence were referenced to Δ fluorescence observed for maximal denaturation (Δ fluorescence$_{max}$ = 100%).

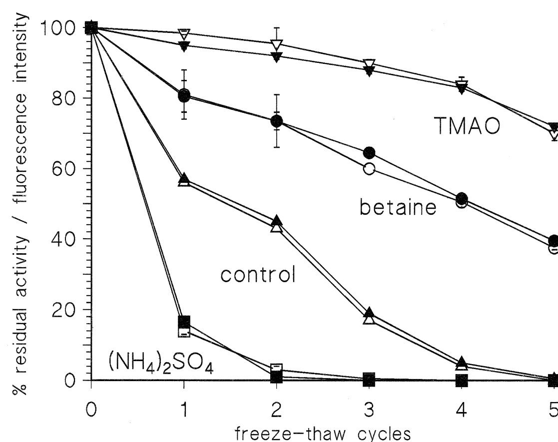

Fig. 2. Relative fluorescence intensity (344 nm) and residual activity (%) of LDH after freeze-thawing in the presence of solutes. Fluorescence intensity (%) was referenced to Δ fluorescence observed for maximal denaturation. All solutes were applied at a concentration of approx. 1 osM/kg water, control measurements were performed without solutes.

Table 1
Percent fluorescence intensity[a] (residual activity) of LDH after being freeze-thawed in the presence of solutes (1 osM/kg water)

Solute	Number of freeze-thaw-cycles	
	1	5
	Percent intensity (= residual activity)	
Control	56	0
Glycine betaine	81	38
TMAO	99	70
Ectoine	92	62
Hydroxyectoine	94	70
$N\alpha$-acetyllysine	94	67
$N\alpha$-acetylornithine	96	74
$N\varepsilon$-acetyllysine	97	85
$N\delta$-acetylornithine	96	78
Sucrose	96	73
Trehalose	96	73
$(NH_4)_2SO_4$	14	0
NH_4Cl	3	0
NaCl	1	0

[a]Percent fluorescence intensity was referenced to Δ fluorescence observed for maximal denaturation.

Prior to normalisation, fluorescence measurements were corrected for fluorescence background caused by impurities of the chemicals. Fig. 2 depicts normalised residual fluorescence intensity and residual activity of LDH with respect to the number of freeze-thaw cycles.

Our results reveal that residual fluorescence intensity correlates well with the residual activity determined by activity assays. It is therefore justified to conclude that the observed residual fluorescence intensity reflects the proportion of native enzyme and to assign a specific fluorescence measurement a defined catalytic activity.

3.2.2. Influence of solutes

As depicted in Fig. 2 and Table 1, the addition of organic solutes led to enhanced enzyme protection against freeze-thaw treatment. With the exception of glycine betaine, which was less effective than the other organic solutes, the stabilising properties of the organic osmolytes examined were very similar: after one freeze-thaw cycle, only little loss of fluorescence intensity (= enzyme activity) was observed (< 10%). Sugars (trehalose, sucrose), hydroxyec-

toine, TMAO, $N\varepsilon$-acetyllysine, $N\alpha$- and $N\delta$-acetylornithine enabled at least 70% enzyme stabilisation after five freeze-thaw cycles. ω-Acetylated diaminoacids ($N\varepsilon$-acetyllysine, $N\delta$-acetylornithine) proved to be especially excellent stabilisers. Compared with control measurements, the addition of inorganic ions (NaCl, NH_4Cl, $(NH_4)_2SO_4$) led to facilitated protein unfolding. In samples containing NaCl or NH_4Cl, almost a complete loss of enzyme activity was observed after one single freeze-thaw cycle, only 15% activity was recovered in the presence of $(NH_4)_2SO_4$.

3.3. Protection against urea treatment and study of LDH denaturation kinetics

3.3.1. Influence of urea

To investigate protein denaturation under urea-treatment, LDH was incubated at urea concentrations ranging from 1–2 M (see Fig. 3A). Time resolved measurements of fluorescence intensity revealed that urea caused protein-unfolding, as displayed by a decrease in fluorescence intensity. Extent and velocity of this unfolding/dissociation process but also the shape (denaturation kinetics) depended on the urea concentration applied.

Curve fitting experiments using the 'Fig P' software (Biosoft, Milltown, NJ, USA) revealed that the data gave best fits for an equation of two exponential functions (components A and B), superimposed by a linear term representing a slow 'drift' in fluorescence intensity of the native enzyme under non-denaturing conditions (see control measurements in Figs. 3A and 4A).

$$y = a_1 e^{m_1 x} + a_2 e^{m_2 x} + (-m_3)x + b \qquad (1)$$

Eq. (1) proved to fit all LDH denaturation kinetics, independent of the presence of solutes (Fig. 3B and 4B). On the basis of this equation it was possible to calculate the initial fluorescence intensity ($t = 0$) and the final equilibrium intensity (end-line) before steady state was actually reached. After 15 min incubation in 2 M urea, no catalytic activity of the enzyme could

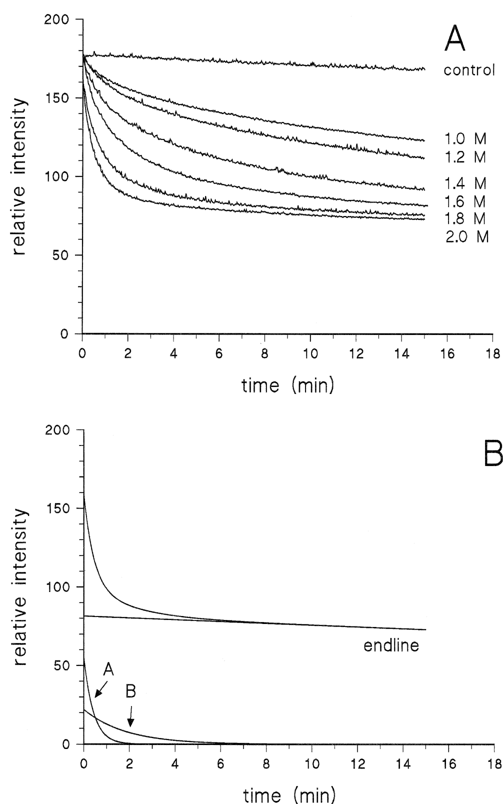

Fig. 3. (A) Time resolved decrease in fluorescence intensity (344 nm) of LDH as a function of urea concentration. Control: native enzyme in buffer. (B) Mathematical curves fitted to denaturation kinetics of LDH in the presence of 2 M urea, the intensity curve is resolved into two exponential functions (A, B) according to Eq. (1).

be detected. On the basis of these results subsequent stabilisation experiments were performed with 2 M urea as denaturant.

3.3.2. Influence of urea in combination with solutes

To assess the influence of glycine betaine on the enzyme's denaturation kinetic, LDH was incubated at 2 M urea in combination with increasing concentrations of glycine betaine. As depicted in Fig. 4A, the intensity curves (= time resolved fluorescence intensity at 344 nm) levelled off with increasing solute concentrations. These results demonstrated that glycine betaine is able to counterbalance the denaturing influence of urea in a concentration dependent manner.

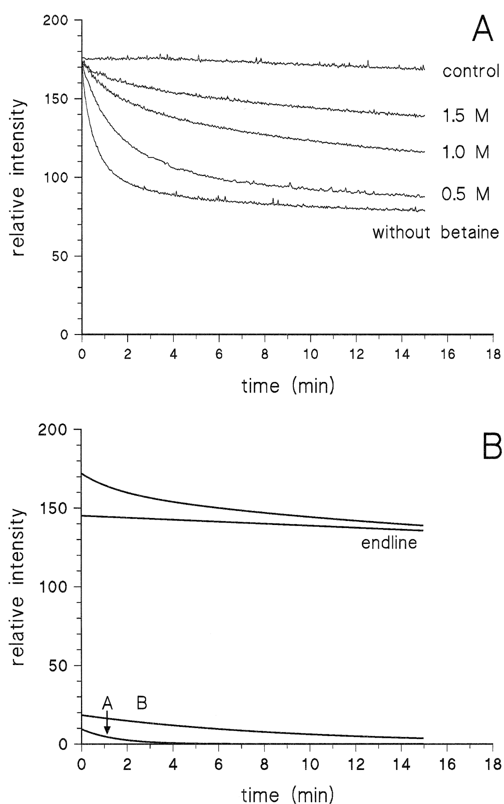

Fig. 4. (A) Time resolved decrease in fluorescence intensity (344 nm) of LDH in the presence of 2 M urea in combination with glycine betaine. (B) Mathematical curves describing denaturation kinetics of LDH in the presence of 2 M urea in combination with 1.5 M glycine betaine. The intensity curve is resolved into two exponential functions (A, B) according to Eq. (1).

Following mathematical fitting of the denaturation kinetics depicted in Fig. 4 we were able to show that fluorescence intensity approached a defined final 'equilibrium intensity' which depended on the concentration of the stabilising solute glycine betaine. As the positions of the 'end-lines' depended on solute concentration and type of solute under investigation, we could not exclude the possibility that unfolding in the presence of different solutes gave different denatured forms of the enzyme with different spectroscopic characteristics. It was therefore necessary to correlate fluorescence intensity and enzyme activity prior to further considerations. Activity measurements were performed to find out whether varying 'steady-state' fluorescence intensities reflected different forms of the fully denatured enzyme (in this case, depending on the solute environment, different structural conformations of the enzyme would result in different intensity levels of the fully denatured form) or an 'equilibrium-line' representing the relative proportion of native and denatured enzyme. Activity control measurements (data not shown) confirmed that steady-state fluorescence intensities reflect the relative proportion of the native enzyme and, therefore, residual enzyme activity. These results made it possible to calculate the final equilibrium residual activity at any time (in our experiments usually 15 min) on the basis of fluorescence intensity measurements.

In order to compare the effectiveness of compatible solutes or salts as enzyme stabilisers, all substances were used at concentrations of approx. 1 osM in combination with 2 M urea. The results of these experiments are presented in Fig. 5. TMAO displayed a higher stabilising property than glycine betaine. At equilibrium, 35% activity were retained. Surprisingly, however, the best protection was obtained by the use of ammonium sulphate (66% residual activity). A comparison of the stabilising properties of disodium sulphate and ammonium chloride suggests that, in particular, the sulphate ion is responsible for the protecting effect.

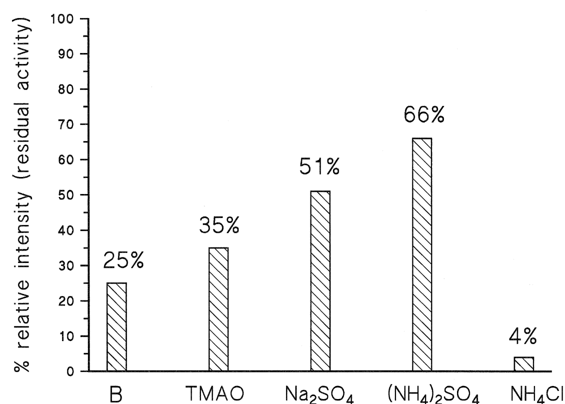

Fig. 5. Residual activity (%) as deduced from fluorescence measurements after 15 min incubation of LDH in 2 M urea in the presence of compatible solutes and salts. All solutes were applied at concentrations of approx. 1 osM/kg water. B: glycine betaine; TMAO: trimethylamine-*N*-oxide.

4. Discussion

Our study revealed that fluorescence spectroscopy is a sensitive technique for monitoring structural alterations of LDH under conditions of freeze-thawing and urea treatment. A comparison of fluorescence intensity and activity measurements showed that the decrease in fluorescence intensity relative to maximal Δ fluorescence is directly related to a loss of enzyme activity. These results agree well with those of Rajendrakumar et al. [2] who monitored fluorescence emission spectra of LDH under similar stress conditions.

Using tryptophan fluorescence, we followed the LDH denaturation kinetics in the presence of organic and inorganic solutes and were able to evolve a method which allows rapid assessment of a solute's stabilising properties. The use of solutes at relatively high concentrations of 1 M made correction of fluorescence intensity for the effect of impurities and/or solute–tryptophan fluorescence interactions an essential part of this method. As fluorescence spectroscopy is very sensitive to perturbations due to turbid samples, the reproducibility of experiments is also markedly increased by using chemicals of highest purity. On the basis of our results we can conclude that fluorescence intensity (at steady state) and residual enzyme activity are proportional, at least for the enzyme and solutes examined. For rapid assessment of the stabilising properties of a given solute, mathematical fitting according to Eq. (1) is used to extrapolate fluorescence intensity for equilibrium between native and denatured state of the enzyme. The method described in this study may be applied to other tryptophan containing biomolecules and seems especially useful for proteins without catalytic activity.

The results presented in this paper showed that all organic solutes tested were able to protect LDH against heat, freeze-thawing as well as urea treatment. The urea counteractive effect of TMAO is well known and is employed in nature where elasmobranchs use a combination of TMAO and urea for osmoadaptation [5]. However, urea counteraction is not a specific feature of TMAO and glycine betaine (present in kidney cells). Using chymotrypsin as a model it has been shown that a number of solutes of the glycine series (glycine, sarcosine, dimethylglycine and betaine) were equally counteractive while, in this case, TMAO was less stabilising at low concentrations (< 3 M) [9]. At the whole cell level (using *Escherichia coli* K12 as a test system) even synthetic aromatic betaine structures such as pyridinium betaine proved effective counteractants and partially relieved growth inhibition by urea [10].

Temperature activity profiles showed that the addition of glycine betaine and hydroxyectoine led to enhanced enzyme stability as revealed by a shift of the profiles towards higher temperatures. In the presence of $(NH_4)_2SO_4$, enzyme activation as well as stabilisation has been observed, resulting not only in a shift of the activity profile but also in a different shape of the curve. In all cases, the Arrhenius plot revealed a distinct increase in the activation energy in the presence of the stabilising solutes (betaine, hydroxyectoine, ammonium sulphate). This observation is in agreement with the view that the enzyme takes a more compact form and consequently tolerates higher temperatures.

The stabilising properties of inorganic ammonium sulphate (at a concentration of 1 osM) against both high temperature and urea denaturation were most remarkable. A comparison with other salts revealed that the sulphate ion is mostly responsible for this effect. Dötsch et al. [11] examined salt-stabilisation of a globular protein structure in very concentrated aqueous urea solution (6–7 M) and reported 100% stabilisation with the addition of 2 M Na_2SO_4 or 2.5 M NaCl. In this study we did not investigate the possibility that NaCl may also be stabilising against urea denaturation but the bad performance of NH_4Cl in our stability tests renders this possibility rather unlikely.

It must, however, be noted that the application of salts (including ammonium sulphate) in

freeze-thawing experiments led to facilitated enzyme denaturation in comparison with control measurements (no additives). Similarly, Carpenter and Crowe [12] reported a destabilising influence of 'salting-out' salts like ammonium sulphate when applied at concentrations lower than 0.5 M (equivalent to approximately 1.5 osM/kg water). At higher concentrations, however, all tested salts, even NaCl, which is not regarded a 'salting-out' salt, displayed enzyme stabilising properties. Apparently, in the case of salts the degree of stabilisation/destabilisation strongly depends on the protein:solute ratio and it remains to be seen if similar concentration dependent effects apply to the test conditions used in our experiments.

Freeze-thawing represents a complex combination of several stress factors. It is not only low temperature and the formation of ice that account for freezing-induced damage of a protein. As water forms ice in course of the freezing process, the proteins which partition into the non-ice phase are exposed to increasing concentrations of buffer salts and co-solutes, possibly accompanied by subsequent pH changes. As our test system contained potassium phosphate buffer, a slight pH change might have occurred during freezing. Consequently, we considered the possibility that the stabilising effect of compatible solutes may be partly based on their buffering capacity. TMAO ($pK_a = 4.65$) for example proved to be a very effective freeze-protectant whereas the stabilising properties of glycine betaine ($pK_a = 1.84$) were less pronounced. However, ectoine ($pK_a = 2.4$) was also a good protectant and $N\varepsilon$-acetyllysine displayed better stabilising properties than $N\alpha$-acetyllysine, although it had a lower pK_a. Freeze-thawing experiments performed on M_4-LDH by Lippert and Galinski [3] showed a similar ranking of solutes with respect to the enzyme stabilising properties. As these authors used Tris–HCl as a buffer, which is known to maintain pH during freezing, results presented here indicate that the buffering capacity of compatible solutes (in our potassium-phosphate containing system) was probably not the main factor responsible for freeze stabilisation. Other potential modes of action may be connected with salt counteraction at increasing salinity and the modulation of the ice forming process.

Underlying principles of solute-induced enzyme stabilisation are not fully explained at present. The theory of Arakawa and Timasheff [13]—'preferential exclusion of solutes from the protein surface'—has gained so far most support. The authors have shown that compatible solutes are excluded from the enzyme's hydration shell, which in turn is preferentially hydrated in the presence of solutes. Stabilisation, therefore, has to be seen as the result of a modulation of the properties of the solvent water. Mashino and Fridovich [14] extended this view by describing the opposing effects of urea and TMAO in such a way that urea loosens and TMAO promotes the structural compactness of proteins. As neither the more rigid nor the more flexible form are expected to represent the most active state (Basakov et al. [15]), the addition of solutes (be they denaturants or stabilisers) will always influence enzyme activity and/or stability. From this one would expect that all compatible solutes behave in a similar manner (i.e., be equally effective as stabilisers). It is, however, a well known fact that the stabilising properties of solutes strongly depend on the enzyme under investigation (Pollard and Wyn Jones [16], Lippert and Galinski [3], Kühn-Velten [17]). Glycine betaine for example has been shown to promote a more compact structure in the polar domains of ATPase but to decrease the overall compaction of the enzyme under the applied conditions (Coelho-Sampaio et al. [18]).

Based on the results presented in this study, we would like to propose the following view concerning the action of compatible solute additives on proteins.

(1) The presence of compatible solutes or other preferentially excluded solutes (by means of modulation of the solvent properties) leads to a more compact conformation of the enzyme. This is reflected in an increased activation en-

ergy and a shift of the temperature activity profile towards higher temperatures.

(2) Urea counteraction and enzyme stabilisation are not separate features but governed by the same principle. Solutes and urea have opposing effects as proposed by Basakov and Bolen [19]. While urea, by loosening the structural conformation, facilitates unfolding, any stabilising solute which increases compactness will (to a certain degree) relieve denaturing effects caused, for example, by urea, chaotropic salts, high (and low) temperature, etc.

(3) The stabilising properties of compatible solutes are not only concentration dependent but also depend on the nature of the enzyme. This can possibly be explained by the fact that solutes (although usually excluded from the hydration shell) also display a low affinity towards certain regions of the protein surface, an effect which becomes increasingly dominant with increasing concentration of the solute. Hence, some stabilising solutes (e.g., betaine, ammonium sulphate) are less effective during the freeze-thawing process where they are concentrated to saturation.

In summary, we believe that there are at least two effects which determine the stabilisation properties of a given solute: (a) the degree of preferential exclusion determining the solvent modulation properties and (b) the concentration dependent affinity properties towards the enzyme in question. From the above it is apparent that the efficiency of stabilising solutes (be they typical organic osmolytes of inorganic 'salting-out' salts) can only be assessed in combination with the target, i.e., the biomolecule in need of stabilisation. We are therefore far from offering the ideal solution for all labile proteins but much closer to understanding the underlying phenomenon. This will hopefully in the future enable us to provide researchers and/or application scientists with a set of guidelines for choosing the right solute additive for their specific stabilisation problems.

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