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Stabilizing effect of sucrose against irreversible denaturation of rabbit muscle lactate dehydrogenase

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Abstract

Measurements of the kinetics of activity loss by rabbit muscle lactate dehydrogenase in acetate–chloride buffer, pH 5.0, I 0.20, have shown that the enzyme exhibits greater stability against irreversible inactivation when the buffer is supplemented with sucrose (0.1 M–0.5 M). On the basis of sedimentation equilibrium distributions obtained for enzyme in the absence and presence of sucrose (0.5 M), the lactate dehydrogenase is essentially dimeric in both environments. The observed stabilization of enzyme activity has therefore been considered in terms of the space-filling effects of sucrose on an isomerization equilibrium between native and unfolded forms of dimeric lactate dehydrogenase, which precedes irreversible inactivation of the unfolded isomer. Interpretation of the kinetic results on that basis has led to the conclusion that the initial stage of enzyme unfolding entails a minor change in volume and/or asymmetry of the lactate dehydrogenase that gives rise to a 4% increase in the second virial coefficient describing excluded volume interactions between dimeric enzyme and sucrose.

Keywords: Excluded volume; Lactate dehydrogenase; Molecular crowding; Protein stabilization; Thermodynamic nonideality

1. Introduction

As part of the search for an explanation of the accumulation of small solutes such as proline [1], glycine betaine [2] and sucrose [3] in plants subjected to drought or other forms of stress, it has been observed that high concentrations of these solutes stabilize enzymes against irreversible denaturation [4–7]. Such stabilization of native protein structure by high concentrations of small inert solutes has usually been attributed to preferential solvation [6,8,9], but has subsequently been considered in terms of thermodynamic nonideality arising from the space-filling effects of the added small solute [10,11] on a reversible isomerization step that precedes irreversible denaturation [12].

That explanation suffices to account for stabilization of proteins either devoid of quaternary structure or in a stable quaternary state on the grounds that the reversible stage of the denaturation should lead to minor unfolding of protein structure, whereupon molecular crowding by the inert solute should displace the isomerization equilibrium in favour of the more compact, native state (N); and thereby decrease the concentration of expanded isomer (E) undergoing irreversible denaturation to inactive state (Eq. 1)

$$N \overset{X}{\rightleftarrows} E \overset{k}{\rightarrow} D \qquad (1)$$

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However, for enzymes that comprise an equilibrium mixture of two states, isomeric or polymeric, the seemingly universal stabilizing effect of inert solutes poses a dilemma if the larger isomer or the monomer of a self-associating system is the active (native) form of the enzyme. Because the space-filling effect of an inert solute is to displace an isomerization equilibrium in favour of the smaller isomeric state [12–14] and a self-association equilibrium in favour of polymeric state [15–17], destabilization rather than stabilization would be the predicted effect on any system conforming with either of these postulated situations.

To comment further on the stabilization of an enzyme whose quaternary state is a function of stress, we have examined the effect of sucrose on the irreversible denaturation of rabbit muscle lactate dehydrogenase at pH 5.0. Under neutral or slightly alkaline conditions the enzyme is tetrameric [18], but exposure to more acidic environments leads to dissociation and irreversible denaturation. At pH 5.0 rabbit muscle lactate dehydrogenase is essentially dimeric in acetate–chloride buffer, pH 5.0, *I* 0.20, and undergoes a gradual, irreversible loss of enzymic activity [19]. This irreversible denaturation is effectively eliminated by 60 mM phosphate, which also suppresses completely the dissociation of enzyme into dimers at pH 5.0. A seemingly reasonable explanation of these findings would be that phosphate binds preferentially to the tetrameric (native) state of lactate dehydrogenase, and thereby decreases the proportion of dimer that is undergoing irreversible denaturation. On the grounds that molecular crowding by sucrose should also displace a dimer–tetramer equilibrium towards tetramer [17], the present study was undertaken in the belief, albeit mistaken in retrospect, that the lactate dehydrogenase–sucrose system would provide an example of a system in which the greater enzyme stability in the presence of inert solute reflected the consequence of thermodynamic nonideality on a self-association equilibrium.

2. Experimental

Crystalline preparations of rabbit muscle lactate dehydrogenase were obtained as ammonium sulphate suspensions from Sigma Chemical Co., who also supplied pyruvate and NADH. Because of the instability of lactate dehydrogenase in acetate–chloride buffer, pH 5.0, *I* 0.20 (the conditions of interest), the enzyme was first dialyzed exhaustively against unbuffered NaCl (0.2 M, pH 6–7) to remove the ammonium sulphate. For the series of kinetic experiments the dialyzed enzyme solution was then diluted with more of the unbuffered saline to give a lactate dehydrogenase solution that could be stored at 4°C for several days without loss of activity. Concentrations of these stock enzyme solutions were in the range 0.7–0.8 mg/ml, based on spectrophotometric measurements at 280 nm and an absorption coefficient ($A_{1\,cm}^{1\%}$) of 14.4 for rabbit muscle lactate dehydrogenase [18].

2.1. Enzymic assay procedure

Lactate dehydrogenase activity was assayed by monitoring the rate of oxidation of NADH at 340 nm in a Hitachi U2000 double-beam spectrophotometer, the cell compartment of which was maintained thermostatically at 25°C. For these assays, performed in 0.32 M Tris–HCl, pH 8.0 [19], the reaction mixtures routinely comprised assay buffer (2.8 ml) supplemented with bovine serum albumin (20 μg/ml) and sodium pyruvate (2.15 mM), NADH solution (0.2 ml, 2.25 mM in assay buffer) and enzyme solution (5 μl, 18–180 μg/ml). Activities, determined by linear regression analysis of absorbance measurements recorded at 20-s intervals for 2 min, exhibited linear dependence upon enzyme concentration.

2.2. Kinetics of acid-induced inactivation

In preparation for these experiments enzyme solution (72–720 μg/ml) in unbuffered saline and also some acetate–chloride buffer (0.04 M sodium acetate, 0.016 M acetic acid, 0.16 M NaCl), pH 5.0, *I* 0.20, were equilibrated separately at 1°C, the temperature to be used for the inactivation studies. Experiments were initiated by rapidly mixing equal volumes (2 ml) of the two solutions, after which the reaction mixture was maintained at 1°C. At specified time intervals aliquots (100 μl) of reaction mixture were withdrawn and added to an equal volume of phosphate buffer (0.015 M NaH_2PO_4, 0.062 M Na_2HPO_4), pH 7.4, to terminate irreversible enzyme

inactivation [19]. Aliquots (5 μl) of these terminated reaction mixtures were then subjected to enzymic assay to determine the residual activity.

A parallel series of experiments designed to test the stabilizing effect of sucrose was conducted in accordance with the same protocol except that the acetate–chloride buffer (pH 5.0, *I* 0.20) was supplemented with sucrose (0.2 M, 0.5 M or 1 M) to provide reaction mixtures in which the concentration of inert solute was 0.1 M, 0.25 M or 0.5 M.

2.3. Sedimentation equilibrium studies

The effect of molecular crowding by sucrose on the molecular weight of rabbit muscle lactate dehydrogenase in acetate–chloride buffer, pH 5.0, *I* 0.20, was examined by sedimentation equilibrium studies in a Beckman XL-A analytical ultracentrifuge operated at 20°C and 12 000 rpm. Because of the need for dialysis equilibrium to be established between sample and solvent in these experiments [20], solutions were prepared by zonal gel chromatography of unbuffered enzyme solution (0.25 ml) on a column (0.9 × 2.8 cm) of Sephadex G-25 preequilibrated with either acetate–chloride buffer (pH 5.0, *I* 0.20) or with the same buffer containing 0.5 M sucrose. In these experiments on 1-mm columns of 0.8 mg/ml enzyme the sedimentation equilibrium distributions were recorded spectrophotometrically at 280 nm; and then analyzed in terms of the basic sedimentation equilibrium expression for a single macromolecular solute (A) to obtain the buoyant molecular weight [21], $M_A(1 - \phi'_A \rho)$, where ρ is the density of the diffusate with which the enzyme is in dialysis equilibrium [20,22,23] and ϕ'_A is the apparent partial specific volume of rabbit muscle lactate dehydrogenase. The magnitudes assigned to ϕ'_A are discussed in Section 3.1.

3. Results and discussion

In view of the earlier demonstration [19] that phosphate prevents dissociation of tetrameric rabbit muscle lactate dehydrogenase into dimers at pH 5.0, the first point to be established is whether a high concentration of sucrose can also stabilize the tetrameric enzyme state against dissociation under these conditions. For that purpose sedimentation equilibrium studies have been conducted on the enzyme in acetate–chloride buffer (pH 5.0, *I* 0.20) and in the same medium supplemented with 0.5 M sucrose. However, a value of ϕ'_A, the apparent partial specific volume of enzyme, is clearly a prerequisite of molecular weight (M_A) determination from the buoyant molecular weight that emanates from analysis of a sedimentation equilibrium distribution.

3.1. Values of ϕ'_A for rabbit muscle lactate dehydrogenase

For the calculation of enzyme molecular weight in the absence of sucrose, ϕ'_A has been taken as the reported partial specific volume, $\bar{v}_A$, of 0.740 ml/g for rabbit muscle lactate dehydrogenase [18]; but this value is totally inappropriate for evaluating the buoyancy term in the experiment with inert solute present. Ideally, $(1 - \phi'_A \rho)$ should be obtained experimentally from the difference between the densities of a dialyzed enzyme solution and diffusate with which it is in dialysis equilibrium [20]. However, because insufficient rabbit muscle lactate dehydrogenase was available for pursuit of that approach, an alternative means has been sought to estimate the magnitude of $(1 - \phi'_A \rho)$ in the presence of sucrose.

The theoretical calculation of a magnitude for ϕ'_A in three-component systems has, of course, already been addressed in the context of determining apparent partial specific volumes of proteins in concentrated solutions of guanidine hydrochloride [24,25] and urea [26,27]. We adopt a similar approach, except that allowance for the effects of protein–sucrose interaction is made on the statistical-mechanical basis of excluded volume [11,23] rather than in terms of the preferential solvation concept [20,28]. As noted previously [11,29], these two treatments of the thermodynamic nonideality of a protein in the presence of an inert solute are mathematically equivalent.

The buoyancy correction factor, $(1 - \phi'_A \rho)$, has been calculated from the expression [11,23]

$$(1 - \phi'_A \rho) = (1 - \bar{v}_A \rho_s) - (1 - \bar{v}_M \rho_s)(M_M C_M)(B_{AM}/M_A) \quad (2)$$

where ρ is the density of the diffusate (buffer containing 0.5 M sucrose) and ρ_s is the density of the

acetate–chloride buffer (pH 5.0, I 0.2): $\bar{v}_M$, the partial specific volume of inert solute (M) has been taken as 0.619 ml/g, the value inferred from Lee and Timasheff [9] for 0.5 M sucrose. Because density measurements are invariably expressed on the weight-concentration scale, the concentration of sucrose is expressed as the product of its molar concentration (C_M) and molecular weight (M_M). In similar vein the second virial coefficient describing excluded volume interactions between enzyme and inert solute (B_{AM}) is divided by the molecular weight of enzyme (M_A) to conform with the requirement of Eq. 2 that excluded volumes be expressed in l/g protein. For the excluded volume interaction of a protein with a small uncharged solute such as sucrose, B_{AM} is effectively the solvated volume of A, $4\pi NR_h^3/3$, where R_h is the Stokes radius [10,11,23]. Provided that spherical geometry is assumed to describe all polymeric states of lactate dehydrogenase, the value of the ratio B_{AM}/M_A then becomes independent of the actual molecular weight being determined under the conditions of the experiment. On the basis that tetrameric lactate dehydrogenase has a molecular weight of 140000 and a Stokes radius of 4.22 nm [18], $B_{AM}/M_A \approx 4\pi NR_h^3/3M_A$ is calculated to be 1.36 ml/g. Incorporation of this value and the acetate–chloride buffer density (1.0053 g/ml) into Eq. 2 then yields a buoyancy correction factor, $(1 - \phi'_A \rho)$, of 0.168.

3.2. Quaternary state of lactate dehydrogenase at pH 5.0

The effect of including sucrose (0.5 M) in the acetate–chloride buffer (pH 5.0, *I* 0.2) on the solute distributions obtained in sedimentation equilibrium experiments on rabbit muscle lactate dehydrogenase is summarized in Fig. 1. Combination of the buoyant molecular weight, 18200 (±700) obtained by nonlinear regression analysis of the results for enzyme in the absence of sucrose (Fig. 1a) with values of 1.0053 g/ml and 0.740 ml/g for the solvent density and partial specific volume yields a molecular weight of 71000 (±3000). This finding that rabbit muscle lactate dehydrogenase is effectively dimeric in acetate–chloride buffer (pH 5.0, *I* 0.20) confirms an earlier conclusion based on a z-average molecular weight of 74000 under the same conditions [19].

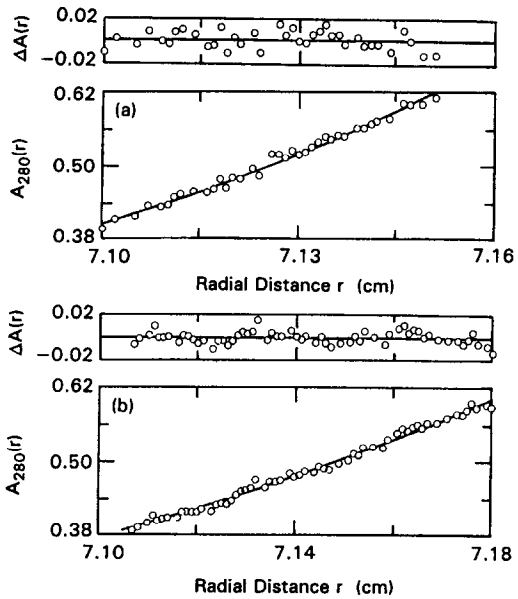

Fig. 1. Analysis of sedimentation equilibrium distributions for rabbit muscle lactate dehydrogenase in (a) acetate–chloride buffer, pH 5.0, *I* 0.20, and (b) the same medium supplemented with 0.5 M sucrose. The lines denote the best-fit relationships obtained by nonlinear regression analysis of the results in terms of the basic sedimentation equilibrium expression for the buoyant molecular weight of a single solute species. Upper patterns display the disparity between theoretical and experimental values of the absorbance at each radial distance *r*.

Corresponding analysis of results obtained in the presence of 0.5 M sucrose (Fig. 1b) yields a buoyant molecular weight of 12600 (±700) and hence a molecular weight of 75000 (±4000) on substituting the buoyancy correction factor of 0.168 obtained from Eq. 2. Inasmuch as the proportion of tetramer coexisting in equilibrium with dimer should have been enhanced substantially by the presence of 0.5 M sucrose [17], any stabilization of rabbit muscle lactate dehydrogenase against irreversible denaturation at pH 5 that is effected by sucrose cannot reflect the displacement of a dimer–tetramer equilibrium as the result of thermodynamic nonideality arising from molecular crowding.

3.3. Kinetics of irreversible enzyme inactivation at pH 5

The time-course of irreversible inactivation of a preparation of rabbit muscle lactate dehydrogenase

in acetate–chloride buffer, pH 5.0, I 0.20, is summarized by the open symbols in Fig. 2a, which presents the results in first-order kinetic format. In that regard the essential linearity of the plot, and also its independence upon a 10-fold variation in enzyme concentration, reinforce the above conclusion (Section 3.2) that rabbit muscle lactate dehydrogenase is not an equilibrium mixture of dimeric and tetrameric states under these conditions. From the results presented in Fig. 2a for the time-course of inactivation in the presence of 0.1 M sucrose (●) it is evident that the inclusion of this inert solute leads to marked stabilization of the enzyme against irreversible denaturation, the effect becoming progressively more pronounced on increasing the sucrose concentration to 0.25 M (▲) and 0.5 M (■). The dependence of the rate of inactivation upon sucrose concentration is not described by the change in relative viscosity (---), the situation that would pertain if the decreased velocity were merely reflecting viscosity dependence of a reaction subject to diffusion control. In any event such an explanation of the effect is rendered most unlikely by the time-scale of the inactivation, which seems far too slow for diffusion control to be the rate-limiting factor. Because the molecular weight studies (Section 3.2) and the first-order, concentration-independent denaturation kinetics (Fig. 2) provide two independent lines of evidence that sucrose is not displacing a dimer–tetramer equilibrium, we need to consider the results obtained with this inert solute in terms of its space-filling properties on an isomerization equilibrium (Eq. 1).

Because the chemical potentials of the native (N) and unfolded (E) isomeric forms of dimeric lactate dehydrogenase are being defined with constant pressure (rather than constant chemical potential of solvent) as the constraint, the equilibrium constant X in Eq. 1 is most appropriately defined in terms of concentrations and thermodynamic activities defined on the molal scale [11,22,23]. However, as noted previously [12], X is reasonably described in terms of molar concentrations, C_i (i = E or N), by the approximate relationship

$$X \approx (C_E/C_N)\exp[(B_{EM} - B_{NM})C_M + \ldots] \qquad (3)$$

where, as in Eq. 2, B_{iM} (i = E or N) are second virial coefficients describing excluded volume interactions between sucrose and the relevant isomeric forms of the dimeric lactate dehydrogenase: these coefficients were incorrectly identified as $2B_{iM}$ in the earlier publication [12]; but, because sucrose is uncharged, correctly equated with the corresponding covolumes [11].

On the basis that the rate of irreversible inactiva-

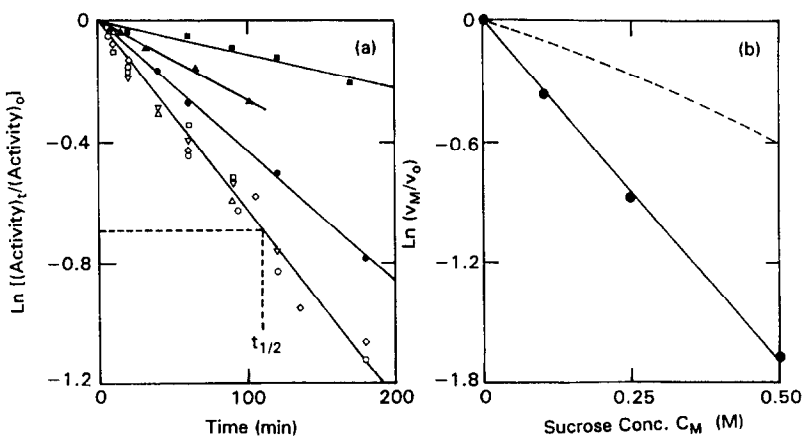

Fig. 2. Kinetics of the irreversible inactivation of rabbit muscle lactate dehydrogenase in acetate–chloride buffer, pH 5.0, I 0.20. (a) Time dependence of enzymic activity (expressed relative to that at zero time) for 36 (○), 72 (◇), 144 (▽), 216 (□) and 360 (△) μg/ml enzyme in the absence of inert solute; and for enzyme (72 μg/ml) in the presence of 0.1 M (●), 0.25 M (▲) and 0.5 M (■) sucrose. (b) Dependence of the ratio of inactivation rates in the presence and absence of inert solute (v_M/v_0) upon sucrose concentration, together with the relationship (---) predicted on the basis of changes in relative viscosity.

tion is proportional to the concentration of expanded isomer E (Eq. 1), it follows that the ratio of the rates of inactivation in the presence and absence of sucrose, v_M/v_0, is given [12] by

$$v_M/v_0 = X'(1+X)/[X(1+X')] \quad (4a)$$

$$= (X'/X)[1+(X-X')/(1+X')] \quad (4b)$$

where $X' = C_E/C_N$ is the apparent isomerization constant relating the molar concentrations of the two isomers. Clearly, there is no exact, simple dependence of v_M/v_0 upon C_M, the molar concentration of sucrose.

At this stage we draw attention to the approximately linear form of the relationship between ln (v_M/v_0) and C_M (Fig. 2b), a dependence that is predicted (Eq. 3) if

$$v_M/v_0 \approx X'/X \approx \exp[(B_{NM} - B_{EM})C_M] \quad (5)$$

In that regard the first approximation in Eq. 5 is certainly valid if $X \ll 1$ (Eq. 4a); and also, from Eq. 4b, if $(X - X') \ll (1 + X')$. It should also be noted that ln (v_M/v_0) becomes independent of C_M if $X' \ll 1$ and $X \ll X'$.

We therefore adopt the viewpoint that the effect of sucrose on the rate of inactivation is, indeed, described by Eq. 5, in which case the slope of Fig. 2b defines the magnitude of $(B_{NM} - B_{EM})$, the difference in covolumes for the native and unfolded isomeric enzyme states with sucrose. As noted in relation to Eq. 2, this difference in second virial coefficients should approximate closely the corresponding difference in solvated volumes of the two isomers. The change in covolume, 3.6 (±0.2) l/mol, obtained from the kinetic measurements in the presence of sucrose (slope from Fig. 2b) thus represents only a 4% expansion of dimeric lactate dehydrogenase (hydrated volume of 95 l/mol) to the putative unfolded isomer. A comparable extent of expansion (5%) has been inferred from corresponding studies on the effect of sucrose on the acid-induced unfolding of ribonuclease A [14]; and also on the isomeric expansion of bovine serum albumin (2%) inferred from the effects of inert solutes on its irreversible coagulation at elevated temperature [12].

The conclusion that the kinetics of inactivation of rabbit muscle lactate dehydrogenase at pH 5 is governed by a half-life of 110 (±6) min (Fig. 2a)

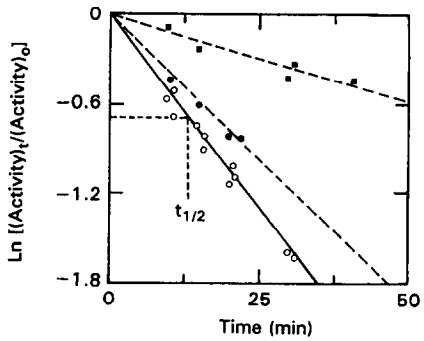

Fig. 3. Kinetics of the irreversible inactivation of a second preparation of rabbit muscle lactate dehydrogenase in acetate–chloride buffer, pH 5.0, I 0.20. Results are plotted in the same format as in Fig. 2a from experiments conducted on enzyme (72 μg/ml) alone (○), and in the presence of 0.1 M (●) and 0.5 M (■) sucrose. The broken lines are theoretical relationships predicted on the basis of Eq. 5 and a covolume difference, $B_{EM} - B_{NM}$, of 3 l/mol.

signifies that the present enzyme preparation is far less stable that investigated twenty years ago [19], for which 70% of the activity remained after exposure of the enzyme to acetate–chloride buffer (pH 5.0, I 0.2) for a period of 4 h at 4°C. That different batches of rabbit muscle lactate dehydrogenase can exhibit considerable variability in their resistance to acid inactivation is evident from results (○) presented in Fig. 3, which refers to the time-course of inactivation of a second lactate dehydrogenase preparation. Nevertheless, despite the fact that inactivation of this batch is governed by a half-life of 13.3 (±0.5) min, the stabilizing effects of 0.1 M (●) and 0.5 M sucrose (■) are again consistent with a difference in covolume, $B_{EM} - B_{NM}$, of approximately 3 l/mol (broken lines in Fig. 3). On the grounds that the extent of reversible unfolding has not changed, the variable stability against acid-induced inactivation must reside either in the value of the isomerization constant (X), or in that of the rate constant (k) governing the irreversible inactivation step.

4. Concluding remarks

This investigation of the effect of sucrose on the irreversible inactivation of rabbit muscle lactate de-

hydrogenase at pH 5 has unequivocally refuted the envisaged situation in which the stabilization would possibly reflect displacement of a dimer–tetramer equilibrium towards the tetrameric state as the result of molecular crowding by the inert solute. Instead, the space-filling effect of the sucrose is exerted on an isomerization equilibrium between native and expanded states of dimeric enzyme. In that regard an interesting side issue of the sedimentation equilibrium study used to characterize the macromolecular state of the enzyme in the presence of sucrose has been the demonstration of an alternative method for estimating the magnitude of the buoyancy correction term Eq. 2.

Although it is too early for any conclusion to be reached about the origin of the seemingly general protective effect of sucrose and other small solutes against enzyme denaturation, it is interesting to note that both systems investigated have found quantitative rationalization in terms of a common model wherein the inert solute acts on an isomerization equilibrium between two states of the enzyme/protein that differ only slightly in volume and/or asymmetry. Furthermore, the existence of this very minor effective expansion of the protein/enzyme is reminiscent of the 'molten globule' intermediate that has been invoked as the first step in the denaturation of proteins by chemical agents such as urea and guanidine hydrochloride [30–32]. Consequently, the 'molten globule' may not merely be an oddity arising from scientific endeavours to examine in detail the process of protein denaturation in vitro: it may well be a protective adaptation of protein conformational structure that has been developed by plants and other biological organisms as a means of surviving stress factors such as drought, heat and excess salinity. We hope that the present investigation may prompt similar studies of the effects of small inert solutes on a wider range of enzyme systems to enable this interesting but speculative possibility to be appraised more critically.

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