Pure & Appl. Chem., Vol. 63, No. 10, pp. 1367–1380, 1991.
Printed in Great Britain.
© 1991 IUPAC

Stability of proteins at subzero temperatures: thermodynamics and some ecological consequences

Felix Franks* and Ross H.M. Hatley

Pafra Ltd., Biopreservation Division, 150 Cambridge Science Park, Cambridge CB4 4GG, U.K.

Abstract - The thermal stability of proteins is limited by two temperatures at which cooperative transitions are observed which either render the protein inactive or cause it to change its function. The thermodynamic features of the cold-induced transition are analysed in detail and its ecological significance is briefly discussed.

It is shown that the common assumption of the the temperature independence of the heat capacity change associated with the transition, coupled with extrapolations of heat denaturation data to subzero temperatures is unrealistic and does not provide correct estimates of cold-induced transition temperatures.

Now that techniques exist for direct studies of proteins in undercooled solutions, down to -40°C, such methods are to be preferred over the use of cryosolvents, chaotropes and pH-destabilization, as means of bringing the cold-induced transition into a temperature range where it can be more conveniently investigated.

PHENOMENOLOGY

Many forms of life have to cope with a variety of suboptimal conditions, imposed by climatic or manmade changes in the environment. Such conditions are perceived as physiological stresses which an organism has to counter in order to survive. Of all the environmental stresses, cold is by far the most widespread and its injurious effects on many forms of life continue to be extensively catalogued (ref. 1). It has also long been known that the functioning of isolated organelles and individual biomacromolecules can be adversely affected by cold (ref. 2).

Most land-based ectotherms, especially those whose natural habitats are the temperate, Arctic or mountainous regions, possess survival mechanisms which enable them to cope with seasonal cold stress conditions. Their responses to such stresses may take the form of freeze tolerance or freeze avoidance (ref. 3). In all cases the survival strategies involve changes in enzyme structures, functions and, in some cases, cold-induced changes have also been observed in the relative locations of enzymes in the cytoplasm (ref. 4); the *in vitro* cold inactivation of individual proteins is also well established (ref. 5). The following discussion concentrates on the effects of low temperatures on the stabilities and thermodynamics of isolated proteins.

CHILL *versus* FREEZING

A clear distinction must be drawn between cold *per se* and freezing. The latter process is accompanied by increases in the concentrations of all water-soluble substances; most of the observed protein damage during freezing results directly from such concentration effects (ref 6). Freeze denaturation is largely irreversible and is due mainly to rapid aggregation, following an initial unfolding and/or subunit dissociation. Its kinetics are complex and very much dependent on eutectic phase separations and glass/rubber transitions which take place in the freeze-concentrate (ref. 7). Freeze denaturation is of particular importance in the food preservation and lyophilization technologies (ref. 8).

Low temperature *per se* (chill) exerts its influence mainly through changes in the physical properties of the aqueous solvent medium, e.g. acid/base ionization, diffusion and reaction rates and hydrogen bond energies. In this review we are not concerned with the damage, usually irreversible, inflicted on proteins by freeze concentration, but with the reversible stability/instability relationships induced by low temperatures. We shall attempt to draw parallels between direct observations and the documented data for heat destabilization.

Fundamental thermodynamic studies require homogeneous systems (constant composition or constant chemical potential), even at subzero temperatures. This is the reason why most attention is devoted to the effects of *high* temperatures on protein stability. Thus, the phenomenon of heat denaturation has been extensively studied and the thermodynamics of the cooperative transition from a native (N) state to one or several denatured (D) states is well understood. Such transitions, while of importance in protein process technology, e.g. enzyme immobilization and freeze-drying, bear little relevance to ecolocigal situations. Cold-induced changes in protein activity, on the other hand, are of prime importance in cold acclimation and survival mechanisms. Mainly because of experimental problems associated with uncontrolled freezing, they were largely unexplored until quite recently, although the phenomena of cold inactivation of enzymes and cold lability of multi-subunit structures (e.g. microtubules) had been known for a long time (ref. 5). Even since it has been realized that cold-induced transitions are probably a universal feature of protein stability, their thermodynamic characterization has usually been performed by extrapolating heat denaturation data to low (subzero) temperatures which were not accessible to a direct study because freezing intervened.

EXPERIMENTAL TECHNIQUES FOR THE STUDY OF PROTEIN DENATURATION

Suitable experimental techniques for the thermodynamic characterization of protein stability are of two types: "direct" and "indirect". Indirect methods involve the monitoring of an N/D equilibrium process within the protein, for example the exposure of residues during denaturation. An equlibrium constant K is calculated and hence $\triangle G$ obtained. Indirect methods have many advantages: they are experimentally relatively simple, but more importantly, they permit $\triangle G$ to be calculated at temperatures on either side of the transition temperature. This enables a $\triangle G(T)$ profile to be constructed for the protein under a common set of experimental conditions. However, since such techniques are based on the assumption of a specific equilibrium, it is necessary to verify that the observed transition does in fact correspond to a two-state equilibrium. The temperature (and pressure) derivatives of $\triangle G$ are obtained by curve fitting and differentiation.

The "direct" method relies on calorimetry. It has the advantage that it does not require the assumption of the two-state equilibrium model. $\triangle H$ and $\triangle C$ are obtained directly from the instrumental output. Calorimetry has one major disadvantage: the thermodynamic quantities can only be obtained *at* the denaturation temperature. In order to obtain results over even a limited temperature range, it is necessary to destabilise the protein with additives. A favourite device is pH destabilisation; it is claimed that H^+ ions do not themselves have an effect on the the value of $\triangle H$. As will presently be shown, the evidence for and against this assumption is controversial, and until such time as this question can be resolved, the data obtained by such techniques must be treated with caution. The low molar concentrations of proteins that can be used present a second difficulty. Changes in heat capacity of proteins during denaturation are small and instruments of high sensitivity and superior signal-to-noise ratios are required. They are generally expensive and fragile. A disadvantage in the study of cold denaturation is the danger of sudden freezing which can cause damage to the calorimeter. Such problems can be overcome by the inhibition of ice nucleation (undercooling), *vide infra*, but the requirement for the aqueous solution to be dispersed in an organic carrier phase makes the procedure more complex and reduces the effective sample volume. From the experimentally determined $\triangle H$ and $\triangle C$, $\triangle G$ is evaluated by integration:

$$\triangle G(T) = -\triangle H[(T_{H,L} - T)/T_{H,L}] - \int \triangle C dT + T \int (\triangle C/T) dT$$

The denaturation temperatures T_H and T_L are then obtained by solving for $\triangle G = 0$.

The relative merits of the direct and indirect techniques have been discussed by Pfeil and Privalov (ref. 9). In principle, a direct (thermodynamic) method is generally to be preferred for the thermodynamic characterization of protein stability, because successive differentiations of experimetal results introduce appreciable uncertainties into the calculated $\triangle H$ and $\triangle C$ values. Against this must be placed the limitation of calorimetry: $\triangle H$ and $\triangle C$ can only be obtained *at* the transition temperature, so that their study over a temperature range requires the perturbation of the protein to different extents.

The integrations required for the calculation of $\triangle G(T)$ give rise to further problems, as does also the extrapolation of $\triangle G$ to zero perturbant concentrations and to low temperatures, when such measurements cannot be made directly. We shall discuss typical examples in the following sections.

EXPERIMENTAL STUDIES OF COLD DESTABILIZATION

A clear distinction must be drawn between cold inactivation resulting from drastic retardations of reaction rates and lability due to cooperative structural transitions, giving rise to inactive states. Brandts first speculated on the possibility of the *in vitro* cold denaturation of proteins. His very detailed spectrophotometric studies of

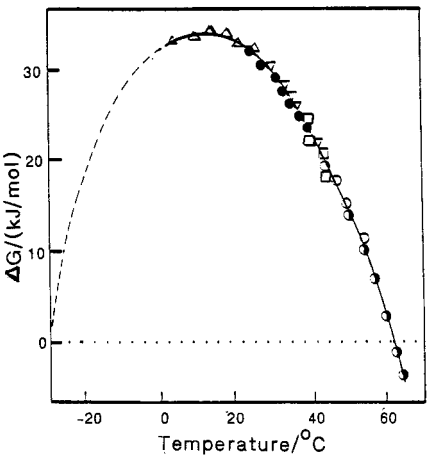

Fig. 1. The △G(T) master curve for the denaturation of chymotrypsinogen under various conditions of pH: 1.11 (●), 1.71 (▽), 2.07 (□), 2.56 (○), 3.00 (◑) and 1.11 in the presence of 2.3 M urea; after Brandts (ref. 10).

the heat and pH-induced destabilization of chymotrypsinogen suggested that the stability profile △G(T) could be fitted adequately only by a third-order temperature function and that the process could be described by a simple two-state equilibrium of the type $N \rightleftharpoons D$. The full description of △G(pH, T) could only be achieved by a very complex empirical function, but Brandts found that at any given temperature, △H was independent of pH, and this enabled him to combine the observed denaturation curves at different pH values and to construct the "master curves" for △G(T), shown in Fig. 1 (ref. 10). At the lowest temperatures the protein had to be further destabilized by 2.3 M urea, to bring the denaturation into a temperature range where freezing could be avoided.

The experimental results in Fig. 1 strongly suggested that a cold-induced denaturation was likely to occur at some subzero temperature T_L, and that for any given pH or solvent medium there should exist a temperature of maximum stability T_{max}. An analysis of the N/D transition also indicated that the stability margin, even at T_{max}, was small, suggesting a close correspondence of △H and T△S. Such entropy/enthalpy "compensation" had already previously been noted for a range of processes in aqueous solution and was subsequently related to the unique structural properties of water (ref. 11).

Since the pioneering work of Brandts, several more △G(T) profiles have been published; for a review, see ref. 12. In the experimentally accessible temperature range they all exhibit parabolic shapes, suggesting again a low temperature T_L at which a cooperative transition to the D-state occurs. Since the thermodynamic treatment is based on the two-state $N \rightleftharpoons D$ equilibrium model, then this transition should be the mirror image of heat denaturation that occurs at T_H, i.e. it should occur with the evolution of heat and a *decrease* in the entropy of the total system.

The △G(T) profiles of all proteins examined in the pH range corresponding to maximum stability indicate that T_L values lie well below the equilibrium freezing point T_f of water, mostly below -15°C. This makes the direct determination of T_L and associated thermodynamic properties difficult, although not impossible, unless experimental devices are used to destabilize the protein and thus to bring T_L into an experimentally accessible temperature range (> -5°C). Alternatively, the freezing point T_f of the aqueous solution can be depressed by the addition of cosolvents to bring it to below T_L. The former alternative is easily achieved by a partial pH destabilization of the protein or by the addition of chaotropes, such as urea or guanidinium hydrochloride (GuHCl). The latter course of action requires the use of cryosolvents, i.e. aqueous mixtures with organic solvents, such as methanol, glycerol or dimethylsulphoxide (DMSO). It must be borne in mind, however, that some cryosolvents actually stabilize native proteins, even to the extent that T_L cannot be reached at all. While the heat stabilizing effects of sugars and polyols have been reported in detail (ref. 13), no published data exist on their possible stabilizing role at T_L. On the other hand, their common occurrence as natural cryoprotectants is currently receiving considerable attention, especially by insect biochemists (ref. 4). Other means of achieving a direct measurement of T_L include the use of cold-sensitive mutants and undercooling (the inhibition of ice nucleation). Both techniques have been used in studies of cold denaturation and will be discussed below.

Cryosolvents were first used to good effect by Douzou and his colleagues in kinetic studies of enzyme-catalysed reactions (ref. 14). By the criteria chosen by these workers, the presence of high concentrations of organic solvents did not materially affect the enzyme kinetics; they concluded that cryosolvents provided a convenient means of making possible the temporal resolution of complex reaction sequences without, at the same time, altering the nature, sequence or pH profiles of the reactions. They did not consider the effects that cryosolvents might have on relative thermal stabilities of proteins.

Fink and Painter (ref. 15) reported on the heat-induced N/D transition T_H of ribonuclease at subzero temperatures, in the presence of H_2O/MeOH/GuHCl cryosolvents as a function of MeOH and GuHCl concentration and pH*. The meaningful interpretation of their results is complicated by the difficulty of expressing the influence of the hydrogen ion activity in an unambiguous manner. Fink and Painter follow the convention established by Douzou for mixed aqueous solvents, whereby a function pH* is defined which becomes equal to pH at zero cosolvent content (ref. 14). The problematic nature of pH* in mechanistic studies where pK_a values of acids have to be defined has been discussed elsewhere (ref. 3). Alcohol/water mixtures present special problems, because their physical properties tend to exhibit a strongly nonmonotonic dependence on concentration (ref. 16) which is also reflected in their effect on T_H (ref. 17). Matters are even more complicated in the presence of GuHCl, and little can be said about the actual nature of the protonated species or the various equilibria which govern the ionization of water, MeOH, GuH^+ or the protein residues.

Fink and Painter were able to identify intermediate folded and unfolded states and to obtain useful information about the mechanism(s) of unfolding and refolding. Interestingly, the "anticipated" cold denaturation, estimated to lie at -45°C, was not detected. The reasons may be twofold: T_L may lie below the lowest temperature investigated, and/or the high enzyme concentrations which had to be used to measure activities at the very low temperatures had a self-stabilizing effect, thus depressing T_L, a well-known effect in the region of T_H.

We have also used aqueous methanol cryosolvents to probe the subzero temperature behaviour of enzymes, specifically lactate dehydrogenase (LDH) (refs. 18, 19). Both T_H and T_L vary in a linear manner with the weight fraction (w) of methanol, at least within the limited measurable solvent composition range. There is of course no reason for a linear relationship between transition temperature and cryosolvent composition. Figure 2 shows the destabilizing effect of MeOH to be more pronounced at T_H than at T_L. The linearity of the $T_L(w)$ relationship could not be rigorously tested, because freezing intervened (but see below). Conversely, in water/glycerol mixtures the *stabilizing* effect of glycerol is much more pronounced at T_L than at T_H (to be published).

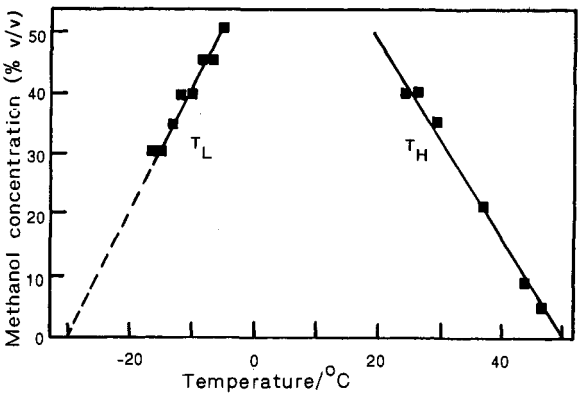

Fig. 2. Cold- and heat-induced denaturation temperatures of LDH in methanol/water cryosolvent mixtures as functions of the solvent composition. Redrawn from refs. 18 and 19.

Surprisingly, the cold denaturation was found to be completely reversible over several cooling/heating cycles, even at very high protein concentrations. Also, the first renaturation resulted in an enzyme with a higher specific activity than the original material, indicating that any partial inactivation during the isolation and purification stages can be reversed by a cooling/heating cycle.

With the aid of CD spectroscopy, Chen and Schellman have studied the thermal stability of a cold-sensitive T4 lysozyme mutant. In order to bring T_L to within a measurable temperature range, they had to perturb the enzyme yet further by high GuHCl concentrations (up to 3 M) (ref. 20). Under these conditions they obtained T_L = -3°C and T_H = 28°C, a very limited temperature range. They evaluated the thermodynamic quantities with the aid of the following regression equation:

$$\ln K \equiv A + B(T_o - T) + D \ln (T_o/T) \qquad (1)$$

so that

$$A = [-\triangle C + \triangle S(T_o)]/R$$

$$B = [\triangle C - \triangle S(T_o)]/R - \triangle G(T_o)/RT_o$$

$$D = -\triangle C/R$$

where T_o is some reference temperature, e.g. T_L or T_H.

Equation (1) implies that $\triangle C$ = constant, independent of T. Equation (1) provides a good fit to the data, except in the region of maximum stability, between 5 and 20°C, where systematic deviations become apparent.

Privalov and his colleagues have chosen the method of pH destabilization (sometimes in conjunction with chaotropic solutes) to bring T_L into an experimentally accessible temperature range. They were thus able to investigate the cold (and heat) denaturation of metmyoglobin and staphylococcal nuclease, although not to completion, a limit being set at -8°C for experimental safety reasons (refs. 21, 22). With the high chaotrope concentrations used, reliable values for the thermodynamic quantities associated with the N/D transition in the absence of chaotrope cannot be obtained by extrapolation. Much of their discussion of cold denaturation is based on heat denaturation data which are more plentiful, easier to obtain and more reliable. We shall presently discuss how far the analogy between T_H and T_L can safely be taken.

Arguably the best means of reaching T_L, but without the need for destabilizing additives, is provided by the inhibition of ice nucleation in undercooled aqueous solution. By suitably dispersing an aqueous solution of a protein in an inert organic carrier fluid in the form of microdroplets, ice nucleation can be inhibited at subzero temperatures, down as far as -40°C; cold denaturation can then be studied directly, the only perturbing factor being the temperature. In this manner, we were able to confirm Brandts' speculation of the existence of a cold-induced transition for chymotrypsinogen (ref. 23). By transposing the data to fit his "master curve", shown in Fig. 1, T_L was estimated to lie near -22°C (at pH 3). Upon warming, the protein was renatured, a surprising result being that renaturation is completely reversible, even at protein concentrations of > 5 mg/ml.

To follow up our earlier studies of LDH in cryosolvents, we also examined this enzyme in undercooled solution and confirmed the T_L value suggested by the extrapolation in Fig. 2 (ref.24).

Now that practical methods exist for the elimination of cryosolvents and chaotropes in studies of protein behaviour at subzero temperatures, let us examine briefly whether, or how cryosolvents affect the shape of the $\triangle G(T)$ profile and/or the nature of the D-state produced at T_L. All that is known is that for the heat denaturation of proteins (specifically α-lactalbumin), different cosolvents produce different D-states, as monitored by the degree of residual secondary structure (ref. 25). For the few systems for which comparative data are available, it has been observed that alcohols affect the $\triangle G(T)$ profiles in a particularly complex manner (ref. 17). The non-monotonic dependence of $\triangle G$ on solvent composition and temperature resembles that found for most physicochemical properties of alcohol/water mixtures (ref. 16).

Comparisons of enzyme reactions in undercooled water and in aqueous cryosolvents have shown that, although the nature of the end product is unaffected, the reaction pathways may differ. Such a difference has been established in the luciferase-catalysed oxidation of reduced flavine mononucleotide (FMN). One of the intermediates consists of the enzyme/substrate complex linked to molecular oxygen: E-FMN-O_2 which, at -20°C, has a half-life of several days and can be purified; its *in vivo* decomposition gives rise to bioluminescence. In aqueous ethane diol cryosolvent the reaction proceeds by a "dark" pathway, whereas in undercooled water the characteristic bioluminescence is observed (ref. 26).

In summary, undercooled water as reaction medium more than doubles the temperature range over which protein mediated processes can be studied. The fact that the aqueous phase is in the form of a fine dispersion in an inert oil does not affect the properties of the protein (ref. 27). Cryosolvents, on the other hand, give rise to interactions which, depending on the nature of the cosolvent, may stabilize or destabilize proteins and which can also affect reaction mechanisms. Although their use allows experiments to be performed over an extended temperature range, their protein-modifying effects should not be overlooked. Any results obtained on systems which contain cryosolvents, except those synthesized by cold adapted organisms themselves, may be of theoretical interest but bear only limited relevance to *in vivo* cold stabilization/resistance phenomena.

THE EVALUATION OF THERMODYNAMIC FUNCTIONS OF COLD DENATURATION

Where enough experimental data exist for the complete characterization of the T_L-transition, it is possible to obtain the thermodynamic quantities associated with cold denaturation directly from such data. Published results which enable such an analysis to be performed are severely limited. Instead, it has become common practice to characterize the cold denaturation process by an extrapolation of the more extensively studied heat denaturation thermodynamics to T_L. Such a procedure requires very reliable data for the temperature derivatives of $\triangle G$, because the extrapolation typically extends

over a temperature range of up to 100 deg. Where calorimetric data form the basis of the calculations, the exact nature of $\triangle H'$ and $\triangle C'$ must be known, where the primes refer to the temperature derivatives. Most denaturation studies in the past have been based on the assumption, implicit or explicit, that $\triangle C' = 0$, see, for instance, eqn. (1). It has also been suggested that, even for a nonzero $\triangle C'$, it is likely to be very small, within the limits of experimental error (ref. 28). On the other hand, it has more recently been claimed that $\triangle C$ is not constant, but that it decreases with increasing temperature, tending to zero at some high limiting temperature, in the neighbourhood of 160°C (ref. 29), hardly a temperature of practical relevance to the study of protein denaturation.

The following re-examination of the heat and cold denaturation of metmyoglobin will serve to demonstrate the reliability of extrapolations of T_H data to the subzero temperature range, coupled with assumptions about the nature of $\triangle C'$ (ref. 21). Metmyoglobin was heat-denatured in a range of sodium acetate buffers, and the transition enthalpies were measured. Additionally, the cold denaturation was observed under certain pH conditions, but enthalpies could not be measured, as freezing intervened before the N -> D transition reached completion . If $\triangle H$ was assumed to be independent of pH, then a $\triangle H(T)$ plot for the high temperature data could be fitted with a straight line. The authors claimed, without giving supporting details, that if $\triangle G(T)$ was fitted to the high temperature data, with $\triangle G = 0$ at T_H for a given pH, then on extrapolation to low temperatures, $\triangle G$ again reached zero at a temperature that coincided with the experimentally observed T_L. We performed a linear regression fit to the data. $\triangle H = -3146.325 + 10.13639T$ was the best fit ($r^2 = 0.964$); i.e. $\triangle C = 10.13639$ kJ/(K mol). This value lies in the middle of the experimentally determined $\triangle C$ values which range from 9.8 to 11 kJ/(mol K). It thus appears to be a suitable value for testing the constant $\triangle C$ model.

Considering denaturation at a range of T_H values, it should be possible to construct $\triangle G(T)$ profiles to predict T_L under the same conditions as those that lead to heat denaturation. According to the model, $\triangle H' =$ constant for all pH values. $\triangle S$ must, however, depend on pH, since it must be the pH-induced change in $\triangle S$ that causes the change in T_H. Since $\triangle C = 10.1363$, then $\triangle S^* = 10.1363 \ln(T) + D^*$, where the asterisk signifies a dependence on pH. We then obtain

$$\triangle G^* = -3146.325 + 10.136339T - 10.136339T\ln T - D^*T \quad (2)$$

Hence the $\triangle G^*$ profiles can be obtained under any condition, provided that T_H^*, the thermal denaturation temperature under the pH conditions for which the profile is to be fitted, is known. First, the constant D^* needs to be evaluated for the given conditions. At T_H^*, $\triangle G^* = 0$, hence, substituting this value along with T_H^* and rearranging eqn. (2), D^* is given by:

$$D^* = (-3146.325 + 10.136339T_H^* - 10.136339T_H^*\ln T_H^*)/T_H^* \quad (3)$$

$\triangle G^*$ can now be calculated at any temperature under the given pH conditions for which D^* was calculated. Considering denaturation under three sets of denaturation conditions: pH 3.83, 3.95 and 4.08, for which $T_H^* = 57.5$, 63.2 and 69.2 °C respectively, and substituting these value into eqns. (2) and (3), D^* and $\triangle G^*(T)$ profiles are obtained, as shown in Fig. 3. Table 1 summarises the transition temperatures thus obtained.

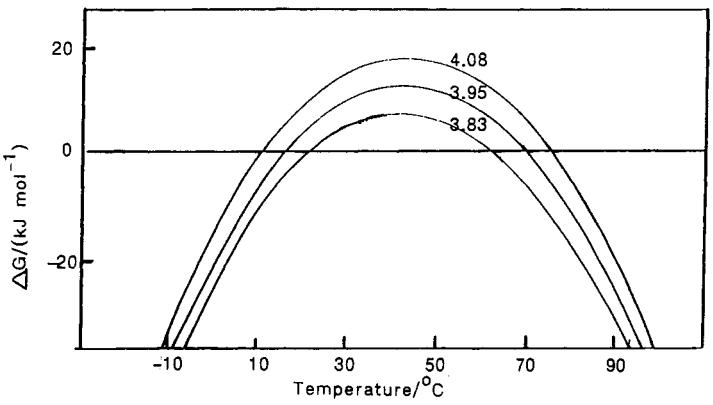

Fig. 3. The temperature dependence of $\triangle G$ for metmyoglobin in 10 mM sodium acetate buffer at the pH values indicated. $\triangle G(T)$ has been derived by substitution experimental T_H data into eqn. (2), where the (constant) $\triangle C$ was obtained from the line of best fit to the $\triangle H$ data (ref. 21). The T_L values, so calculated, do not agree with those reported by Privalov et al., although based on the same experimental data; see Table 1 and text.

TABLE 1. Analysis of the metmyoglobin cold denaturation and calculated T_L^* values (°C), based on heat denaturation data and values reported in ref. 21. For details see text; see also Fig. 3.

pH	T_H^*	T_L^*	T_L^* (Privalov) experimental	T_L^* (Privalov) calculated
3.83	57.5	17	9	5
3.95	63.2	14	0	0
4.08	69.2	12	< 0	< 0

The calculated T_L^* values (column 3) differ significantly from the experimentally determined values (column 4). Column 5 shows the values of T_L^* calculated by Privalov et al. by fitting a regression line to the calorimetric $\triangle H$ data. Since we used the identical method and the same data, the difference requires an explanation. It turns out that the regression line used by Privalov et al. is *not* the line of best fit. Further examination indicates that its slope, i.e. $\triangle C$, is 8 kJ/(mol K). Inserting this value into our calculations, rather than $\triangle C$ obtained from the line of best fit, we do indeed find that the calculated T_L^* values agree with those in column 5. This $\triangle C$ value is however, well outside the range of the experimental values. The claimed experimental accuracy of $\triangle C$ is ± 10%, so that this difference cannot be considered as being within experimental error. It is also apparent that the derived T_L^*, with $\triangle C$ = 8, only provides a good agreement with the experimental T_L^* for pH 3.95. It was from the results at this single pH value that the conclusion was reached that an extrapolation of T_H data, together with the constant $\triangle C$ model, reproduces the correct T_L^*. It is clear that for other pH values, T_L^*(calc) deviates from T_L^*(experimental).

We have dealt in great detail with the analysis favoured by Privalov et al. in order to demonstrate that a description of *cold* denaturation, based only on measurements in the neighbourhood of T_H, coupled with simplifying assumptions about the temperature dependence of thermodynamic quantities, fails to account for the actual behaviour near T_L.

Chen and Schellman were able to observe the cold-induced denaturation of a cold-labile T4 lysozyme mutant, following further destabilisation by GuHCl (ref. 20). Their experiments have already been described. The data were fitted to a constant $\triangle C$ model, eqn. (1). The authors considered this fit of the data to be a confirmation of the correctness of this model, although they did acknowledge the possibility that $\triangle C$ might be temperature dependent, because of systematic deviations of the fitted curve from the true data, particularly in the region of T_{max}.

We have reanalysed the original experimental data by a different curve fitting procedure. A $\triangle G(T)$ profile, derived from a temperature-independent $\triangle C$ model yields r^2 = 0.985 and shows the poor fit in the maximum-stability region, as described by Chen and Schellman. Fitting a curve, allowing for a temperature-dependent $\triangle C$ gives r^2 = 0.995 and, while maintaining a good fit for the values on either side of the thermal and cold-induced denaturation regions, also provides a better fit throughout the stability region (Fig. 4).

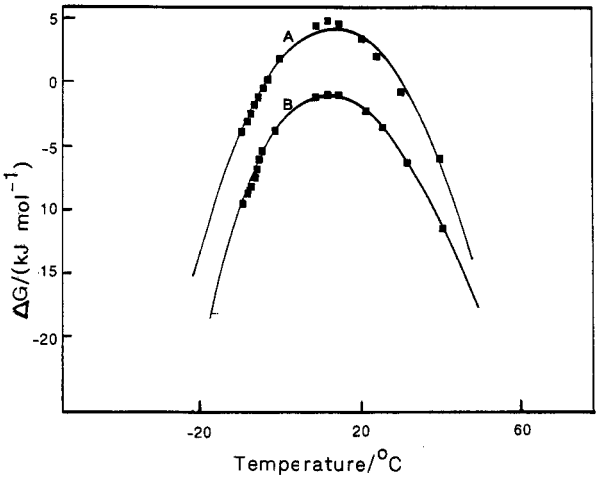

Fig. 4. Reanalysis of the denaturation of a cold-sensitive T4 lysozyme mutant. Curve A allows for a temperature-dependent $\triangle C$; curve B is the original fit discussed in ref. 20. All data in curve A have been offset by 5 kJ/mol.

Of even greater significance to an understanding of cold and heat stability of proteins would be the thermodynamic functions in the absence of GuHCl. Chen and Schellman employed a range of GuHCl concentrations and performed a linear extrapolation to zero concentration in order to obtain the intrinsic stability profile. Such a device is open to question. Where protein stability measurements have been performed as function of additive concentration, non-linear relationships, especially in the low concentration range, have been the rule (ref. 30), although at higher concentrations the additive concentration dependence of $\triangle G$ approaches linearity.

Apart from the measurements at the lowest temperatures, the experiments performed by Brandts of the thermal denaturation of chymotrypsinogen lend themselves well to a test of the various assumptions and extrapolation procedures. HCl was used as the perturbant and corrections were made for the chloride ion activities at different pH values. The following equation provided the best fit to the data (see Fig. 1):

$$\triangle G = 509192.8 - 9405.6T + 48.409T^2 - 0.0746T^3$$

from which the temperature derivatives were obtained as

$$\triangle = 9405.6 - 96.818T + 0.2238T^2$$

$$\triangle C = -96.818T + 0.4476T^2$$

$$\triangle H = 509192.8 -48.409T^2 + 0.1492T^3$$

These functions are plotted in Fig. 5.

The same analysis was applied to the denaturation data for LDH (ref. 18). A simple polynominal was fitted to the calculated $\triangle G$ values. The curve of best fit ($r^2 > 0.99$) is shown in Fig. 6 and takes the following form:

$$\triangle G(T)= 600826.2 - 11761.22T + 60.901453T^2 - 0.09362T^3$$

As was earlier found by Brandts, at least four terms are required to produce an adequate fit to the data. $\triangle C$ is then a function of temperature, expressed in terms of T^2, that is, $\triangle C$ will be non-linearly temperature dependent:

$$\triangle S = 11761.22 - 121.8029T + 0.28086T^2$$

and, hence,

$$\triangle C = -121.8029T + 0.56172T^2$$

Finally,

$$\triangle H = 600826.2 - 60.901453T^2 + 0.18714T^3$$

The temperature profiles of these three functions are also shown in Fig. 6. The resemblance to the chymotrypsinogen data (Fig. 5) is striking. As the temperature decreases, so does $\triangle C$: $\triangle C$ = 18 kJ/(mol K) at T_H and 3 kJ/(mol K) at T_L. Although extrapolations beyond the experimental temperatures are always uncertain, it appears that at low temperatures $\triangle C$ becomes negative while at high temperatures it continues to increase. The details of $\triangle G'$ and $\triangle G''$ are at odds with those reported for the denaturation of metmyoglobin (ref. 21). We have discussed the reasons for these discrepancies elsewhere (ref. 32). The shape of the $\triangle H(T)$ profile in the high temperature region is such that it could appear that $\triangle H$ is linear with temperature, i.e. $\triangle C' = 0$. However, the greatly extended temperature range over which our measurements were made illustrates that, with a reduction in temperature, $\triangle H$ becomes negative at 16°C but its rate of descent rapidly decreases. We also notice, as would be expected, that $\triangle S$ displays a similar trend. At low temperatures $\triangle H$ and $\triangle S$ are both negative.

A GENERALIZED PROTEIN STABILITY PROFILE

In order to test the universality of cold denaturation and the dependence of the $\triangle G(T)$ profile on second-order effects, such as $\triangle C(T)$, Franks et al. performed a thermodynamic analysis of protein stability based only on the assumption of the two-state N/D model (ref. 33). The following identity provided the starting point:

$$G(T) \equiv G(T_o) - (T - T_o)S(T_o) - \int_{T_o}^{T} dt \int_{T_o}^{t} C(u)du/u \qquad (4)$$

where T_o is some reference temperature. By introducing the reduced temperature

$$\theta = (T - T_o)/T_o$$

and a simplifying expression for C(T), eqn. (4) becomes

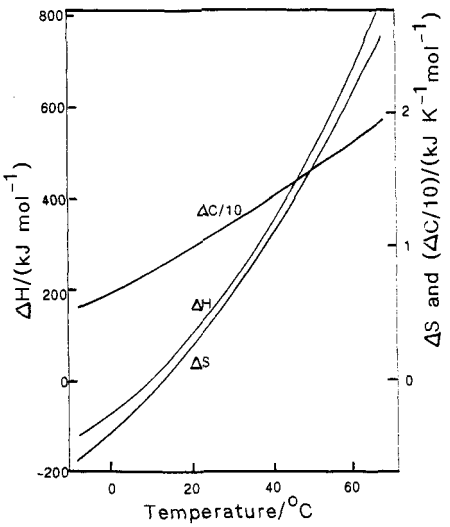

Fig. 5. Thermodynamic functions for the denaturation of chymotrypsinogen (ref. 10), derived from the best fit to $\triangle G(T)$, shown in Fig. 1. Note the resemblance to Fig. 6.

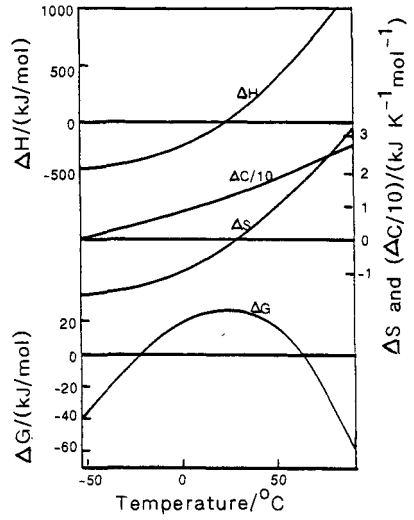

Fig. 6. Thermodynamic functions for the denaturation of LDH (ref. 24), derived from the best fit to $\triangle G(T)$.

$$G[\theta] \equiv G[0] - \theta T_o S[0] - T_o s(\theta) C[0] - (1/6) T_o^2 \theta^3 C'[0] \quad (5)$$

where $G[\theta] = G(T)$ etc, and

$$s(\theta) \equiv (1 + \theta)\ln(1 + \theta) - \theta$$

which has the shape of a skewed parabola in the range $-1 < \theta < 1$.

By taking the difference $G^N - G^D$, i.e. considering the folding process, eqn. (5) becomes

$$\triangle G[\theta] = \triangle G[0] - \triangle C[0] s(\theta) T_o - (1/6) T_o^2 \theta^3 \triangle C'[0] \quad (6)$$

where T_o has been chosen such that $\triangle S[0] = 0$, which corresponds to the temperature of maximum stability T_{max}. Also, within the range $-0.2 < \theta < 0.2$, $\theta_L = (T_L - T_o)/T_o$ etc., and this is the θ-range which is of practical significance to protein denaturation.

From eqn. (6) and $s(\theta)$ it becomes apparent that $\triangle G[\theta]$ is a skewed parabola, where the inclusion of a small $\theta^3 \triangle C'$ term only affects the degree of the skew. Thus, with or without a $\triangle C$ term, the two-state model predicts that $\triangle G[\theta]$ has the form of a *distorted* parabola, consistent with the results of Brandts (ref. 10). Depending on $\triangle G[0]$ and the details of $\triangle C(T)$, this parabola has zero values at two temperatures, T_L and T_H.

The $\triangle G(T)$ profile has been explored for the three cases: 1) $\triangle C' \equiv 0$, 2) $\triangle C'$ = constant and 3) $\triangle C'$ is a function of temperature, i.e. the full eqn. (6). According to Privalov et al., $C^N(T)$ appears to be linear, (ref. 31) and this approximation has been adopted in Fig. 7, although the same conclusions would apply for a more complex temperature dependence.

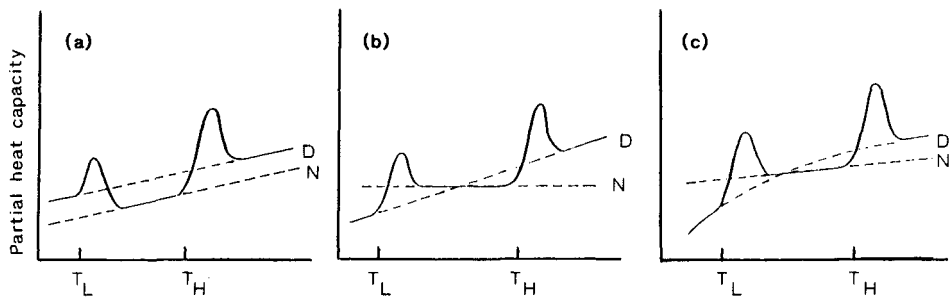

Fig. 7. Schematic C(T) curves for protein folding at T_L and unfolding at T_H: (a) according to the assumption of $\triangle C' = 0$; (b) according to eqn. (6) with $\triangle C'$ = constant; (c) a general representation, based on the full eqn. (6) with $\triangle C'(T)$. Redrawn from ref. 33.

The inadequacy of $\triangle C' = 0$ was demonstrated in the previous section; it is illustrated in Fig. 7a. Putting $\triangle C' =$ constant was also discussed in the previous section, see also Fig. 7b. The available experimental data are probably not good enough to allow a reliable distinction to be made between this model and the $\triangle C$ behaviour according to the full eqn. (6) which yields

$$\triangle C[\theta_L] = \triangle C[0] + T_o\theta_L\triangle C'[0]$$

and a similar expression for $\triangle C[\theta_H]$. Bearing in mind that eqn. (6) refers to the folding process D -> N, both at T_L and T_H, then eqn. (6) shows that heating the protein through T_L (renaturation) *increases* its partial heat capacity, just as does heating through T_H (denaturation); C(T) is sketched in Fig. 7c. A comparison of the three alternatives for $\triangle C$ demonstrates that the extrapolation of results in the neighbourhood of T_H can give a misleading picture of cold denaturation, but Fig. 7c also shows how easy it is to reach the conclusion that $\triangle C' = 0$, because in the region of T_H this may well be a reasonable approximation.

There are as yet not enough experimental data against which the models can be rigorously tested. It does seem to be physically realistic that a transition which is brought about by an increase in kinetic energy, such as during the D -> N transition at T_L, should be accompanied by an increase in C, according to Figs. 7b or 7c. Recent heat capacity studies by Privalov et al. (see Fig. 10 in ref. 31) on a variety of proteins provide clear indications that $\triangle C$ will indeed change sign at some subzero temperature. It is curious, therefore, that the authors state that "...the cold denaturation phenomenon...induces a similar heat capacity *increase* as heat denaturation" (our italics).

In summary, the available evidence suggests that for any system which, in aqueous solution, can undergo order/disorder transitions that can be described by the two-state approximation, then $\triangle G(T)$ takes the form of a skewed parabola with two characteristic temperatures, and $\triangle C(T)$ is a complex function which probably changes sign at some temperature within the stability range. Similar conclusions have been reached more recently by Blandamer et al. (ref. 34). Other systems which exhibit the above behaviour include surfactants (micellization) and synthetic polymers, e.g. polyvinyl alcohol, polyethylene glycols (upper and lower cloud point phenomena). Just as in proteins, T_H and T_L can be controlled by "mutations", i.e. the HLB-values (hydrophil/lipophil balance) and/or the ionic charge density. We would speculate that the thermotropism and lyotropism of polar lipids might also be subject to cold-induced transitions, although this phenomenon remains to be studied in detail.

HEAT CAPACITIES OF POLYMERS AT SUBZERO TEMPERATURES

For reasons discussed earlier, published heat capacity data for proteins at subzero temperatures are non-existent, so that the predicted change in sign of $\triangle C$ has not yet been observed experimentally. However, calorimetric studies on the water soluble polymer polyvinylpyrrolidone (PVP) have revealed a behaviour which lends strong support to the predicted $\triangle C$ behaviour. Franks and Wakabayashi used the droplet emulsion technique to study the partial heat capacity ϕ_C of PVP in undercooled aqueous solutions, down to -50°C (ref. 35). PVP is a flexible homopolymer and is completely miscible with water. It exhibits the lower critical demixing phenomenon, characteristic of molecules the hydration interactions of which are dominated by hydrophobic effects. The $\phi_C(T)$ curves, shown in Fig. 8, were found to be of a surprising nature, because hydrophobic interactions are usually associated with $\phi_C > 0$. Large negative ϕ_C values are, however, common for ions in aqueous solutions. We shall presently discuss the remarkable $\phi_C(T)$ behaviour. At this stage we emphasize that, with decreasing temperature, ϕ_C decreases, changes sign and becomes extremely concentration and temperature-sensitive. The available calorimeter lacked the necessary sensitivity for a reliable determination of the limiting ϕ_C^o value (infinite dilution) to be performed. At elevated temperatures, > 10°C, C'(T) is seen to decrease and it may eventually approach zero, in line with recent results for heat-denatured proteins (ref. 31).

It has been suggested that PVP can serve as a model for a denatured globular protein. The pyrrolidone side chains bear a strong chemical resemblance to prolyl residues. Privalov et al. have estimated that the prolyl group contribution to C^D amounts to 175 J/(K mol residue) at 25° (ref. 31) which agrees well with the value per base mol of PVP.

The very large negative C values in undercooled water at -50° suggest strong interactions between water and polar residues. It is thus reasonable to put $C'^D > 0$ and $C'^D < 0$, tending possibly to zero at high temperatures. The question then remains whether $C'^D = C'^N$, as is almost universally claimed in the protein literature (refs. 21, 28, 36, 37). Once again little reliable information exists about the temperature dependence of C^N because of the limited temperature range over which measurements can be made.

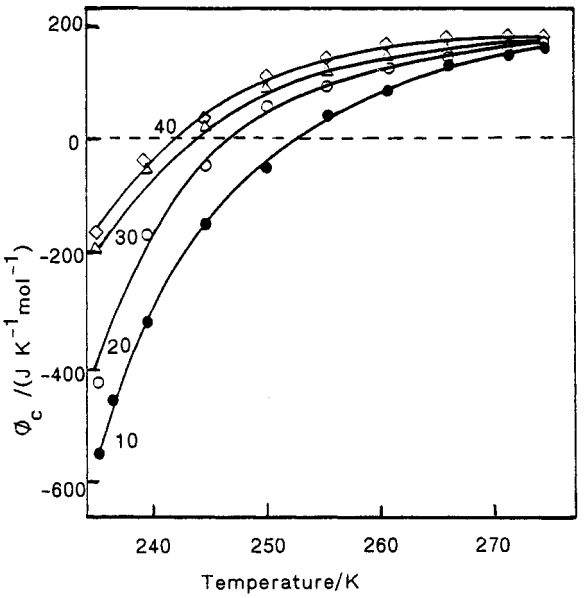

Fig. 8. Partial molar heat capacities of PVP in undercooled solutions as functions of temperature. PVP concentrations (weight per cent) are indicated. Redrawn from ref. 35.

MECHANISM OF COLD DENATURATION

As mentioned earlier, most discussions of protein stability thermodynamics and mechanisms are based on unfolding/denaturation data at T_H, with rarely even a passing reference to cold destabilization (ref. 38). However, simple extrapolations of T_H data to explain processes at subzero temperatures are unwarranted. In any case, they can only be valid when the constant $\triangle C$ model correctly describes the experimental data. We have explained the shortcomings of this approach and demonstrated that it fails to account for the experimental $\triangle G(T)$ profiles of the only two proteins for which adequate T_L data are available, obtained without recourse to chemical and/or pH perturbation. We have discussed elsewhere that such perturbants (cryosolvents) affect protein stabilities and reaction mechanisms, and probably also the conformational details of D-states (ref. 32).

As has frequently been stated, proteins in dilute solutions, even under optimum pH and ionic strength conditions, possess only very marginal stabilities, of the order of $\triangle G[T_{max}] \leq 50$ kJ/mol. Since $\triangle G$ is thus equivalent to the free energy of only very few hydrogen bonds, it is evident that $\triangle G$ must be the resultant of at least two, but probably more contributions of opposite signs.

Leaving aside the many subtleties which might influence protein stability, the major contributors to $\triangle G(T)$ are probably hydrophobic interactions, intrapeptide attractive effects (hydrogen bonding, salt bridges, van der Waals interactions), core repulsion, configurational entropy and solvation effects. Of these, the first two effects are of a general stabilizing nature, while the three latter effects promote the destabilization of the N-state. Table 2 summarizes the major interactions and their (probable) temperature coefficients. The importance of hydrophobic effects becomes evident from the general observation that approx. 50% of the residues of globular proteins are apolar and that apolar residues tend to be much more highly conserved than polar residues.

TABLE 2. Contributions to *in vitro* protein stability and temperature coefficients of the contributing interactions.

Interaction	Contribution to stability	Temperature coefficient
Hydrophobic effects	+++	+
Salt bridges	++	+
Cofigurational free energy	--	-
Intrapeptide hydrogen bonds	++	-
Water-peptide hydrogen bonds	--	-
van der Waals interactions	+	-
Polyelectrolyte effect	-	?

Despite some dissenting views, e.g. ref. 29, there now exists a consensus that the origin of the observed hydrophobic effects reflects a configurational redistribution of water molecules in the proximity of nonpolar atoms, molecules or residues. This occurs as a result of an effort by liquid water to maintain its hydrogen-bonded network, and it takes place with a reduction in the number of degrees of orientational freedom.

In the context of the thermal stability of proteins an important attribute of hydrophobic interactions between alkyl groups is their temperature dependence. It is generally accepted that $\triangle G$ becomes more negative at higher temperature and, conversely, less negative at low temperatures, taking the physiological temperature as reference point. Also of relevance to the present discussion are the well-documented positive partial heat capacities ϕ_C of hydrocarbons and alkyl derivaties which are said to reflect the thermolabile hydrogen-bonded hydration structures. Available experimental results indicate, however, that at high temperatures $\phi'_C < 0$ (ref. 39). Nothing is known about the trends in ϕ_C at subzero temperatures but it has been suggested that ϕ_C goes through a maximum value at some temperature and falls with decreasing and increasing temperature (ref. 40). In any case, ϕ_C is very temperature sensitive.

Turning now to the other factors which play a part in determining the stability of a protein in solution, the potentials of mean force of peptide hydrogen bonds are expected to narrow and deepen with decreasing temperature, as would also direct (via hydrogen bonds) interactions between water and polar groups. This is also reflected in ϕ_C values of ions and polar species in aqueous solutions which are large and negative. Electrostatic interactions (e.g. salt bridges), on the other hand, are of a mainly entropic origin and, like hydrophobic interactions, they would tend to become stronger at higher temperatures.

As shown in Table 2, at low temperatures, some of the N-state stabilizing effects become weaker, whereas at least one of the prime destabilizing factors (direct polar group hydration) becomes stronger. In terms of the language of polymer chemistry, water becomes a "good" solvent at low temperature and causes the macromolecule to swell (denature). The stability margin depends on the balance of the competing effects and their temperature coefficients, but cold denaturation is indicated whenever the stability is governed by two (or more) types of interactions with opposite signs and with temperature coefficients of opposite signs.

SOME ECOLOGICAL CONSEQUENCES

The T_L-values of many proteins lie well within the temperature range which is associated with life on this planet. It is not certain, however, that all enzymes which exhibit cold unfolding/dissociation *in vitro*, also do so *in vivo*. For instance, pyruvate phosphate dikinase, an enzyme involved photosynthesis, is known to be cold labile and to undergo dissociation from its native tetrameric state to dimers and monomers (ref. 41). It can be protected by its substrates phosphoenolpyruvate and pyruvate and by Mg^{2+}, glycerol and sorbitol, all of which do occur in cold hardened plants. It is not certain whether such protection figures in chill resistance *in vivo*.

The situation is more clear-cut for insects, microorganisms and those fish species whose natural habitats are the Arctic and Antarctic oceans. Antifreeze proteins, as well as ice-nucleating proteins are well known, and their synthesis, metabolism and mode of action are still receiving study.

Both freeze-tolerant and freeze-resistant insects rely for their survival on the generation of high concentrations of so-called compatible solutes which serve as cryoprotectants. The favoured chemical species are low molecular weight carbohydrates or amino acids. The physicochemical origin of solute compatibility is obscure, although it is well documented that such solutes also raise T_H values of proteins (ref. 13). We have been able to demonstrate that T_L-values are lowered by such compatible solutes and that the degree of cold stabilization (per mol of solute) greatly exceeds that of heat stabilization [to be published]. The thermodynamic manifestation of such effect is an alteration in the characteristics of the stability parabolas, as shown in Figs. 1 and 6. If it is simply a matter of increasing $\triangle G_{max}$, then the increase in heat stability must be accompanied by a similar increase in cold stability, i.e. a mutation (or chemical additive) which imparts to a protein a degree of thermophilicity will also render it more psychrophilic. If, on the other hand, the skew of the parabola is affected in a major manner, as reflected by changes in $\triangle C$, then a high degree of cold resistance can be induced, without a correspondingly large heat stabilization. This appears to be the role of sugars and sugar alcohols in protecting isolated proteins and intact organisms against cold and freezing damage.

The synthesis of sugar-type cryoprotectants results from a cold-induced glycogen phosphorylase activity. Storey has recently reviewed the metabolic regulation of cryoprotectant metabolism in cold hardy insects, where during the acclimation period (typically September - December) almost the total glycogen reserve is converted into sugar alcohols (ref. 4). This requires the inhibition of some glycolytic enzymes, typically pyruvate kinase and phosphofructokinase, coupled with a reorganized enzyme activity which promotes the synthesis of sugar alcohols from the glycolytic intermediates. A major factor in the control of sugar alcohol synthesis is the spatial reorganization of glycogenolytic, glycolytic and hexose monophosphate shunt enzymes in order to maintain the required rates of conversion of glycogen into polyol products.

CONCLUSIONS

Cold inactivation (or activation) of proteins is a universal phenomenon of great ecological importance (ref. 32). The process is implicated in cold acclimation and cold tolerance/resistance of species that are exposed seasonally to periods at suboptimal temperatures or daily to major temperature fluctuations. As regards its thermodynamic features, the cold-induced transition is the approximate mirror image of the well-studied heat denaturation, i.e. it is cooperative and characterized by $\triangle H < 0$ and $\triangle S < 0$. The exact molecular nature of an exothermic order/disorder transition is still something of a mystery, but it must be intimately related to the role of hydration effects in promoting the stabilization of unique folded protein states.

Direct studies of cold-induced protein transitions are of recent origin, because such transitions usually occur at subzero temperatures. However, now that experimental methods exist for the study of undercooled aqueous solutions, such direct approaches are to be preferred to the partial destabilization of the protein by chemical means and/or the extrapolation of heat denaturation data into the subzero temperature range, especially when such long extrapolations involve unwarranted simplifying assumptions.

REFERENCES

1. J. Levitt, Responses of Plants to Environmental Stresses, Vol. 1: Chilling, Freezing and High Temperatures, 2nd edn., Academic Press, New York (1980).
2. O Fennema, (ed.), Proteins at Low Temperatures, Adv. Chem. Ser. 180 (1979).
3. F. Franks, Biophysics and Biochemistry at Low Temperatures, Cambridge University Press, Cambridge (1985).
4. K.B. Storey, Phil. Trans. Roy. Soc. B 326, 635-649 (1990).
5. R. Jaenicke, Phil. Trans. Roy. Soc. B 326, 535-553 (1990)
6. I.V. Sochava, T.V. Belopolskaya, and O.I. Smirnova, Biophys. Chem. 22, 323-336 (1985).
7. J.F. Brandts, J. Fu. and J.H. Nordin, in G.E.W. Wolstenholme and M. O'Connor (eds.) The Frozen Cell, p. 189, J. & A. Churchill, London (1970).
8. F. Franks, Cryo-Letters 11 93-110 (1990).
9. W. Pfeil and P.L. Privalov, in M.N. Jones (ed.) Biochemical Thermodynamics, p. 75, Elsevier, Amsterdam (1979).
10. J.F. Brandts, J. Amer. Chem. Soc. 86, 4291-4303 (1964).
11. R. Lumry and S. Rajender, Biopolymers 9, 1125-1157 (1970).
12. P.L. Privalov, Adv. Protein Chem. 33, 167-241 (1979).
13. T. Arakawa and S.N. Timasheff, Biochemistry 21, 6536-6544 (1982).
14. P. Douzou, Crybiochemistry, Academic Press, New York (1977).
15. A.L. Fink and B. Painter, Biochemistry 26, 1665-1671 (1987).
16. F. Franks and J.E. Desnoyers, Water Science Revs. 1, 171-232 (1985).
17. J.F. Brandts and L. Hunt, J. Amer. Chem. Soc. 89, 4826-4838 (1967).
18. R.H.M. Hatley and F. Franks, Cryo-Letters 7, 226-233 (1986).
19. R.H.M. Hatley and F. Franks, Eur. J. Biochem. 184, 237-240 (1989).
20. B. Chen and J.A. Schellman, Biochemistry 28, 685-691 (1989).
21. P.L. Privalov, Yu.V. Griko, S.Yu. Venyaminov and V.P. Kutyshenko, J. Mol. Biol. 190, 487-498 (1986).
22. Yu.V. Griko, P.L. Privalov, J.M. Sturtevant and S.Yu. Venyaminov, Proc. Natl. Acad. Sci. U.S.A. 85, 3343-3347 (1988).
23. F. Franks and R.H.M. Hatley, Cryo-Letters 6, 171-180 (1985).
24. R.H.M. Hatley and F. Franks, FEBS Letters 257, 171-173 (1989).
25. R.N. Sharma and C.C. Bigelow, J. Mol. Biol. 88, 247-257 (1974).
26. P. Douzou, C. Balny and F. Franks, Biochimie 60, 151-158 (1978).
27. J.S. Thompson, H. Gehring and B.L. Vallee, Proc. Natl. Acad. Sci. U.S.A. 77, 132-136 (1980).

28. W.J. Becktel and J.A. Schellman, Biopolymers 26, 1859-1877 (1987).
29. P.L. Privalov and S.J. Gill, Adv. Protein Chem. 39, 191-235 (1989).
30. D. Shortle, A.K. Meeker and S.L. Gerring, Arch. Biophys. Biochem. 272, 103-113 (1989).
31. P.L. Privalov, E.I. Tiktopulo, S.Yu. Venyaminov, Yu.V. Griko, G.I. Makhatadze and N.N. Khechinashvili, J. Mol. Biol. 205, 737-750 (1989).
32. F. Franks and R.H.M. Hatley, in P.L. Steponkus (ed.), Advances in Low Temperature Biology, Vol. 1, JAI Press, London (in press).
33. F. Franks, R.H.M. Hatley and H.L. Friedman, Biophys. Chem. 31, 307-315 (1988).
34. M.J. Blandamer, B. Briggs, J. Burgess and P.M. cullis, J. Chem. Soc. Faraday Trans. 86, 1437-1441 (1990).
35. F. Franks and T. Wakabayashi, Z. phys. Chem. (N.F.) 155, 171-180 (1987).
36. R.L. Baldwin, Proc. Natl. Acad. Sci. U.S.A. 83, 8069-8072 (1986).
37. R.S. Spolar, J.-H. Ha and M.T. Record, Proc. Natl. Acad. Sci. U.S.A. 86, 8382-8385 (1989).
38. R.L. Baldwin and T.E. Creighton, in R. Jaenicke Protein Folding, p. 217, Elsevier, Amsterdam (1980).
39. S.J. Gill, S.F. Dec, G. Olofsson and I. Wadsö, J. Phys. Chem. 89, 3758-3761 (1985).
40. H. Naghibi, S.F. Dec and S.J. Gill, J. Phys. Chem. 90, 4621-4623 (1986).
41. J.P. Krall, G.E. Edwards and C.S. Andreo, Plant Physiol. 89, 280-285 (1989).