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Protein Structure and Function at Low Temperatures [and Discussion]

R. Jaenicke, U. Heber, F. Franks, D. Chapman, Mary C. A. Griffin, A. Hvidt and D. A. Cowan

Phil. Trans. R. Soc. Lond. B 1990 **326**, 535-553
doi: 10.1098/rstb.1990.0030

Phil. Trans. R. Soc. Lond. B **326**, 535–553 (1990)

Printed in Great Britain

Protein structure and function at low temperatures†

By R. Jaenicke

Institut für Biophysik und Physikalische Biochemie, Universität Regensburg, D-8400 Regensburg, F.R.G.

Proteins represent the major components in the living cell that provide the whole repertoire of constituents of cellular organization and metabolism. In the process of evolution, adaptation to extreme conditions mainly referred to temperature, pH and low water activity. With respect to life at low temperatures, effects on protein structure, protein stability and protein folding need consideration.

The sequences and topologies of proteins from psychrophilic, mesophilic and thermophilic organisms are found to be highly homologous. Commonly, adaptive changes refer to multiple alterations of the amino acid sequence, which presently cannot be correlated with specific changes of structure and stability; so far it has not been possible to attribute specific increments in the free energy of stabilization to well-defined amino-acid exchanges in an unambiguous way.

The stability of proteins is limited at high *and* low temperatures. Their expression and self-organization may be accomplished under conditions strongly deviating from optimum growth conditions. Molecular adaptation to extremes of temperature seems to be accompanied by a flattening of the temperature profile of the free energy of stabilization. In principle, the free energy of stabilization of proteins is small compared to the total molecular energy. As a consequence, molecular adaptation to extremes of physical conditions only requires marginal alterations of the intermolecular interactions and packing density. Careful statistical and structural analyses indicate that altering the number of ion pairs and hydrophobic interactions allows the flexibility of proteins to be adjusted so that full catalytic function is maintained at varying temperatures.

1. Introduction

Proteins as the major components of the living cell provide the basic elements of cellular organization and metabolism. Their structure–function relation is generally assumed to be optimized with respect to the physical conditions characteristic for the natural biotope. Adaptation to extreme conditions during evolution mainly refers to temperature, pH and low water activity (Jaenicke 1981). Low water activity and extremes of pH do not necessarily require molecular adaptation of the cellular inventory as avoidance may take the place of adaptation; for example high salinity or a pH value less than 1 or greater than 11 may be compensated by compatible solutes or proton pumps. In the case of temperature, it is evident that cells are more or less isothermal with respect to their environment. As a consequence, both psychrophiles and thermophiles have to adapt their cell inventory to their respective set of conditions. Strategies promoting thermal stability of proteins have been investigated for many years. The outcome is that in the native state of functional proteins, stabilizing and destabilizing interactions more or less balance each other so that no general mechanism of temperature adaptation can be put forward. Adaptation at the protein level may be

† Dedicated to Professor Hans Neurath on the occasion of his eightieth birthday.

accomplished by either mutative exchanges of amino acids or extrinsic factors such as polyphosphates (Hensel & König 1988). Low-temperature adaptation is by far more complex as 'low temperatures' in the biosphere refer to the range from −80 °C to the suboptimal temperatures at which a living organism can still function or at least survive in a state of dormancy (Franks 1985). Thus the present discussion has to envisage phenomena under conditions of 'cryptobiosis' and cryobiology, as well as undercooling, hardening, etc.

In the following article, attention will be focused on the stability and structure–function relation of proteins under suboptimal temperature conditions. For studies of enzyme catalysed reactions at subzero temperatures in mixed aqueous solvents, X-ray diffraction of protein crystals and spectral investigations of functionally relevant substates of proteins at low temperatures, reference is made to Douzou (1977), Frauenfelder *et al.* (1979), Petsko & Ringe (1984, 1987), Privalov & Gill (1988) and Griko *et al.* (1988*a*, *b*).

2. Protein structure and protein stability

The three-dimensional structure or proteins is specified by the covalent structure of their polypeptide chains and their native environment. From the ampholytic properties of amino-acid residues and from the amphipathic nature of secondary-structure elements it is evident that the aqueous or non-polar surrounding of the nascent protein must be crucial in determining its final structure. For soluble proteins in their cellular environment three fundamental principles are important: (i) minimization of hydrophobic surface area by segregating non-polar residues from water; (ii) minimum hydration of the interior of the molecule and (iii) maximum packing density. On forming the 'hydrophobic core', the loss in chain entropy must be compensated for by the sum of the intramolecular interactions, on the one hand, and the positive solvation entropy on the other. Both give rise to hydrophobic interactions. As the 'core' cannot accommodate the total number of non-polar residues, merging of domains and assembly of subunits lead to the native tertiary and quaternary structure and, ultimately, to the supermolecular organization of the cell (Jaenicke 1987*a*).

The equilibrium conformation of a given protein is defined by many thermally accessible minima in the vicinity of the potential energy minimum corresponding to the crystallographic structure. In sampling these substates in the neighbourhood of the average structure, the protein may optimize its function (Frauenfelder *et al.* 1979; Huber 1988). Whether the average structure is thermodynamically stable (global minimum of free energy) or metastable (i.e. kinetically determined) is still under dispute. However, there is no evidence to reject the thermodynamic hypothesis (Dill 1987).

The stability of proteins depends on non-covalent electrostatic and hydrophobic interactions with interaction energies of the order of 20–40 kJ mol^{-1}; they are highly sensitive to high and low temperatures, both of which may cause unfolding. Protein denaturation, by definition, leaves the covalent chemical structure untouched. It relates exclusively to alterations in the conformation, extending to the random coil, and derives from intermolecular protein–solvent interactions competing with the intramolecular interactions stabilizing the native state.

The kinetics of the reaction sequences constituting the network of metabolic pathways are also strongly affected by temperature changes. Taking the temperature dependence of two consecutive reactions as an example, it is obvious that the differential retardation of the various

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

TABLE 1. REDUCTION OF REACTION RATES AT VARYING ACTIVATION ENERGIES ($\Delta G^{\neq}$)

(Rates at 20 °C are normalized to 1.)

$\Delta G^{\neq}$/kJ mol^{-1}	20	40	60	80
20 °C	1	1	1	1
0 °C	1.8	3.6	6.7	11.4
−20 °C	4	14	46	120

steps in a series of enzyme catalysed reactions at suboptimal temperature will have a profound effect on physiological function. Table 1 illustrates the effect of temperature on the reaction rate considering the range of activation energies typical for biochemical reactions.

As indicated by high-resolution X-ray crystallography and calorimetry as well as kinetics, no general mechanism describing the effect of temperature on the structure and function of proteins can be given. The reason is that from the energetic point of view, proteins do not represent a uniform class of chemical entities. As linear polypeptides they are simple polymers; however, the structure and stability of any individual protein with its specific amino-acid sequence and its specific organization depends on the ratio and distribution of polar and non-polar residues. They contribute to the delicate balance of opposing weak intramolecular and intermolecular interactions that may be expressed by the sum of the contributions of the conformational entropy and the net enthalpies for all kinds of local and non-local interactions within and without the inner core of the molecule(s).

For a given protein to fold, the native (N) state must be kinetically accessible and have a lower free energy than the denatured (D) states. These represent an ensemble of readily interconvertible conformers with equal or closely similar energies. It is this greater conformational entropy that drives the N → D transition.

Inspecting the weak intermolecular interactions responsible for the structural integrity of a protein, the effect of temperature cannot be predicted, because there are too many unknown contributions in the hypothetical free-energy balance and its temperature dependence (Finney 1982). In particular, the contribution of hydrophobic interactions is uncertain (Franks 1985; Privalov & Gill 1988). Because of compensatory effects, stability derived from intramolecular hydrogen bonds is only marginal. Internal salt-linkages seem to promote thermal stability; whether destabilization at low temperature is correlated with a corresponding decrease in electrostatic interactions is unknown. Generally, experimental data for psychrophilic adaptation are scarce. As in the case of thermophilism, the range of stability is shifted rather than extended (Jaenicke 1981).

Psychrophilic adaptation at the protein level has been neglected in the past. Growth inhibition at temperatures below 20 °C has been attributed to the inhibition of transcription and translation (Malcolm 1968; Harder & Veldkamp 1968; Jaenicke 1981). Focusing on specific enzymes, aminoacyl tRNA synthetases and polymerases seem to be involved. In this context, psychrophilism may be nothing but the expression of extreme temperature sensitivity of certain proteins. Nothing is known about specific alterations in the amino-acid sequences of psychrophilic proteins compared to mesophilic homologues. The only attempts to analyse the specific structural features of an enzyme from a psychrophilic microorganism was made by H. Zuber (1988 and personal communication). Comparing homologous lactate dehydrogenases from *Bacillus psychrosaccharolyticus* ($T_{max} \approx 40$ °C), *Bacillus megaterium* ($T_{max} \approx 50$ °C)

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

and *Bacillus subtilis* ($T_{max} \approx 60$ °C), he found that the psychrophilic enzyme is closely related to the mesophilic one. The only significant difference seems to be that there are more charged residues resulting from exchanges of hydrophobic to polar or charged residues.

In principle, the free energy of stabilization of soluble globular proteins, $-\Delta G_{stab}$, does not exceed 50–100 kJ mol^{-1} (Hinz 1987). This means that, on balance, the stabilization is based on the equivalent of a few hydrogen bonds, ion pairs or hydrophobic interactions, although numerous intramolecular interactions assist in the stabilization. Considering the many H-bonds that contribute to the stabilization of the secondary structure, and the stabilization of the inner core of a protein by hydrophobic interactions, $-\Delta G_{stab}$ emerges as a small difference between large numbers. From this we may conclude that the structure of native proteins is evidently not optimized for maximum stability. On the contrary, under physiological conditions, proteins are at the margins of their capacity to exist as native species: ΔG_{stab} is frequently not even 10 kT. The optimization in the course of evolution was obviously based on function instead of stability, i.e. on catalysis, regulation, mobility and turnover.

3. Molecular adaptation to high and low temperatures

Comparing the free energy of stabilization for mesophilic and thermophilic proteins, an extension in the temperature range of viability of ≈ 20 °C corresponds to an increase in ΔG_{stab} by no more than *ca.* 40 kJ mol^{-1}. In no case has it yet been possible to clarify the molecular mechanism unequivocally (Jaenicke 1988). By using thermally stable mutants of lysozyme from phage T4, Matthews and co-workers (Matthews 1987) were able to localize structural differences accompanying the increase in thermal stability. Skarzynski *et al.* (1987) confirmed previous findings of Perutz (1978) that indicated that ion pairs contribute significantly to thermal stability. Attempts to define the 'gross traffic' of preferred amino-acid exchanges in temperature adaptation (Argos *et al.* 1979) have been questioned by recent investigations (Hensel *et al.* 1987; Skarzynski *et al.* 1987; Zuber 1988; Fabry *et al.* 1989; Schultes 1989; Wrba 1989). In rare cases, single point mutations or chemical modification could be attributed to altered thermostability; evidently the effects of single point mutations may be additive.

In general, at low temperatures, proteins show several peculiarities that cannot be extrapolated from their properties under standard conditions. In the present context, cold denaturation, exothermic dissociation and accompanying solvation effects will be considered.

In seems to be axiomatic that an ordered structure should become destabilized by an increase in temperature and stabilized at low temperature. However, the temperature profile of the free energy of stabilization of globular proteins is found to be parabolic (figure 1). This proves that there are different regimes of the temperature dependence of the various weak intermolecular interactions contributing to the overall stability of proteins. Temperature adaptation of proteins may be caused by shifts of the range of stability or by a flattening of the temperature profile of ΔG_{stab}. Obviously, there has been a strong tendency during evolution to conserve both the topology and catalytic efficiency of proteins under corresponding physiological conditions. In no case has the increase in thermal stability provided access to cold denaturation at ambient temperature. This is confirmed by the observation that extreme thermophilic enzymes may be expressed in a mesophilic host such as *Escherichia coli* by recombinant-DNA techniques (Fabry & Hensel 1988). Considering the subtle balance of weak intermolecular interactions governing structure formation, this finding is unexpected. In

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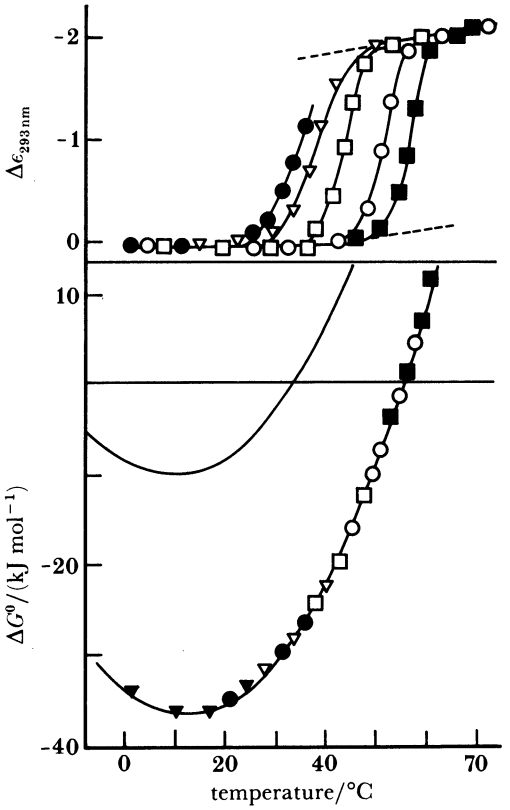

FIGURE 1. Temperature dependence of the extinction coefficient at 293 nm (above) and the standard free energy of denaturation (below) of chymotrypsinogen at various pH values: (●), pH 1.1; (▽), pH 1.7; (□), pH 2.1; (○), pH 2.6, (▮), pH 3.0; (▼), pH 1.6 plus 2.3 M urea. $\Delta G/T$ profile at pH1 and pH3 corrected according to Brandts (1964).

refolding experiments, the pyruvate dehydrogenase multienzyme complex from *Bacillus stearothermophilus* has been shown to require physiological temperature (*ca.* 55 °C) to allow *in vitro* reconstitution; at low temperature either cold deactivation or 'wrong aggregation' prevail (Jaenicke 1987*a*).

4. THERMODYNAMICS V. KINETICS

Intermolecular interactions

In discussing the intermolecular forces determining the stability of proteins, emphasis has been shifting between hydrogen bonding, charge effects and hydrophobic interactions. Since Kauzmann's classical review (1959), the latter were commonly considered dominant. However, any detailed calculation suffers from the fact that ill-defined solvent effects are of crucial importance. In addition, the contribution of each type of bond exceeds the total free energy of folding by at least one order of magnitude so that the tentative balance sheet summarizing the various contributions to the free energy of folding is rudimentary. This holds especially for the hydrophobic free energy change and entropic changes from both chain folding and water release (Finney *et al.* 1980). Bearing in mind that ΔG_{stab} represents a marginal difference of potentially very large contributions, the estimated magnitudes of the various increments suggest that no one effect dominates.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Electrostatic energies exceed the other weak intermolecular interactions by an order of magnitude. At ambient temperature, kT is small compared to the electrostatic interaction energy so that ion pairs do not show significant temperature dependence. Hydrogen bonds, because of their low bond energy, are strengthened at low temperature, whereas hydrophobic interactions have been commonly assumed to be weakened because of their entropic origin. Based on these considerations, extremes of temperature are expected to cause divergent effects depending on the relative weight of the various intermolecular forces contributing to the stabilization of the molecule. General predictions cannot be made.

Calorimetric and kinetic results

Calorimetric measurements have shown that the enthalpy change on unfolding depends on temperature. At room temperature, ΔH is small, but becomes large at high temperature, where ΔH is positive for unfolding; ΔC_p, the difference in partial molal heat capacity between the native and unfolded states, has been generally assumed to be positive and independent of temperature. However, this implies that decreasing the temperature below ΔG_{min} (compare with figure 1) results in a positive ΔC_p; this seems phenomenologically unreasonable.

To overcome this problem, Franks *et al.* (1988) explored a model allowing for a temperature dependent ΔC_p, which changes sign at some temperature within the range of stability of the native protein. Based on the assumption that ΔC_p for an order–disorder transition is always positive, the enthalpy change ΔH is expected to decrease with decreasing temperature, becoming negative at low temperature. For chymotrypsinogen, the respective data for 40, 10 and -33 °C are 502, 0 and -284 kJ mol^{-1}, respectively. Taking the temperature dependence of ΔC_p into consideration, the temperature profile of the free energy of the N$\rightleftharpoons$D transition gains the form of a skewed parabola, which has actually been observed for a variety of proteins (Privalov 1979).

In quantifying thermodynamic stability, the temperature dependence of ΔH and ΔC_p has to be considered. By using the 'melting temperature' (T_m) and the corresponding enthalpy (ΔH_m) as references, the temperature dependence of the free energy may be written as the following (from Baldwin & Eisenberg (1987)):

$$\Delta G^\circ = \Delta H_m(1 - T/T_m) - \Delta C_p(T_m - T) + T\ln(T/T_m).$$

The origin of the enthalpy change on folding may be explained in terms of both improved van der Waals contacts and hydrogen bonding accompanying the packing of the polypeptide chain. As indicated by the association of small model compounds (Schellman 1955; Gill & Noll 1972) hydrogen bonds make a significant contribution in spite of compensatory effects in an aqueous environment. The hydrophobic interaction switches from entropy-driven at low temperature to enthalpy-driven at high temperature (Baldwin 1986; Privalov & Gill 1988).

Calorimetric and solubility measurements of the transfer of hydrocarbons to water reflect this transition exhibiting a common extrapolated value of the temperature (T_s) at which the entropy of transfer reaches zero. At this temperature water does not solvate the non-polar solute. For hexane, benzene and other model hydrocarbons, T_s is found to be 113 ± 2 °C (Baldwin 1986), whereas proteins seem to extrapolate to a somewhat higher value (figure 2*a*). The corresponding temperature dependence of the difference between the enthalpy and entropy of the native and denatured states of model proteins and the entropy of transfer of benzene from the pure liquid phase to water is illustrated in figure 2*b*.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

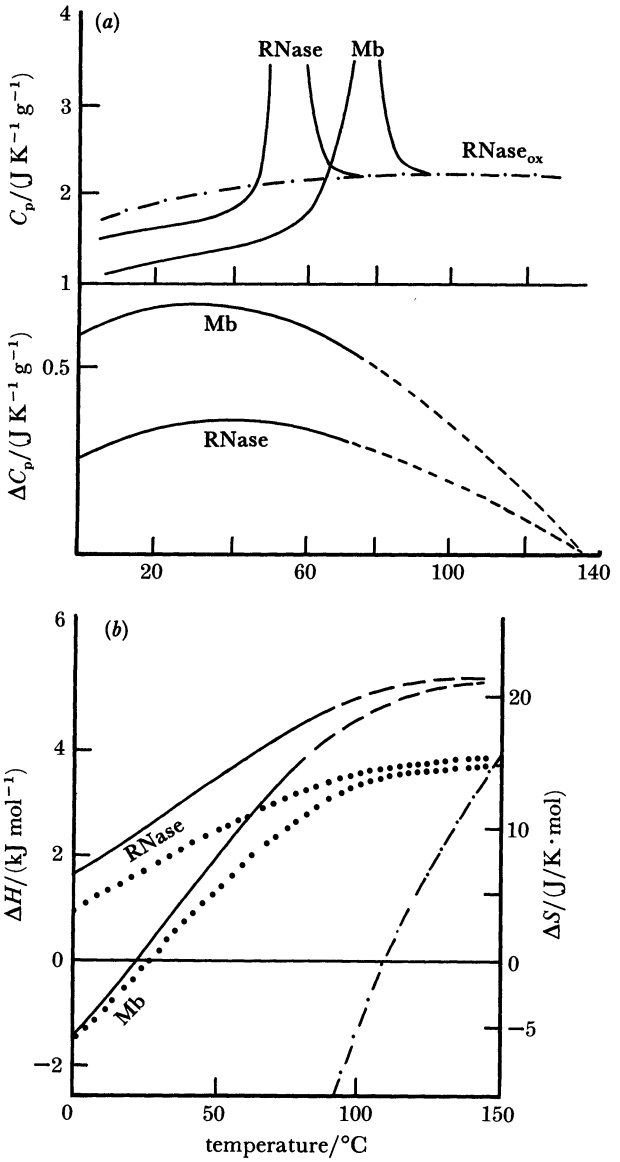

FIGURE 2. (*a*) Temperature dependence of the partial specific heat capacity (top), and the denaturational increment of C_p (bottom) of pancratic ribonuclease (RNase A) and sperm whale myoglobin (Mb). The smooth curve (–·–·) depicts the Cp/T profile for the denatured proteins. Extrapolating ΔC_p in the lower frame beyond 80 °C (– – –), the profiles converge at *ca.* 140 °C (Privalov & Gill 1988). (*b*) Temperature dependence of the difference enthalpy (full lines) and entropy (dotted lines) of the native and denatured states for bovine ribonuclease A (RNase) and sperm whale myoglobin (Mb); (–·–·) illustrates the temperature dependence of the entropy of transfer of benzene from the pure liquid to water (compare with Privalov & Gill 1988).

For the denaturation of proteins, a significant heat capacity increment is observed. Its value exceeds that expected for the increase in vibrational freedom of a polypeptide chain at the breakdown of its native structure. The effect is caused mainly by the hydration of the non-polar groups that are exposed to the aqueous medium upon unfolding (Kauzmann 1959; Privalov & Gill 1988). The shape of the ΔC_p versus T profile clearly shows that above and below a certain temperature the heat capacity increment of protein denaturation (as previously described for the transfer of non-polar solutes to water) decreases. At high temperatures, the

enthalpy and entropy of denaturation increase, approaching a constant value in exactly the same temperature range (*ca.* 110 °C) where water does not solvate non-polar groups anymore (Baldwin 1986). Thus, at high temperatures, ΔH_{N-D} and ΔS_{N-D} are determined by the order–disorder transition of the polypeptide chain rather than hydration effects.

The fact that the temperature profile of ΔG_{N-D} of proteins is parabolic clearly proves that hydration of non-polar groups is involved in protein denaturation. ΔG_{hydr} is always negative and increases in its absolute value with decreasing temperature. As a result, at a certain limiting low temperature value the stability of the native protein decreases to zero, giving rise to 'cold denaturation' (Brandts 1964; Privalov & Gill 1988).

Schellman and co-workers, in analysing the low temperature unfolding of a mutant of phage T4 lysozyme, were able to fit the entire stability curve of the protein for both high and low temperature denaturation, assuming a linear change in heat capacity over the whole temperature range from −10 to +40 °C (figure 3) (Schellman 1987; Chen & Schellman 1989).

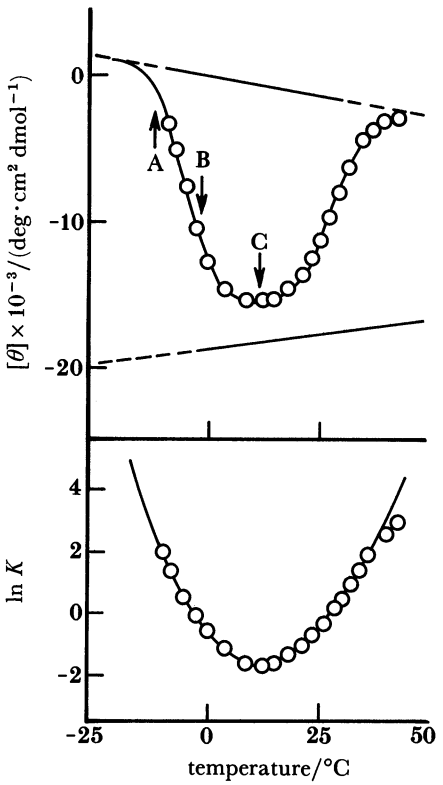

FIGURE 3. Thermal stability of phage T4 lysozyme (I3C-C97/C54T). Top: molar ellipticity at 223 nm; 23 μg ml^{-1} protein in 3 M guanidine·HCl at pH 5. Final values after up to 10 h. Bottom: stability curve calculated from the above melting data (Chen & Schellman 1989). The first-order kinetics of the A → C and B → C transitions yield pairwise identical relaxations, proving the two-state model to hold (Chen *et al.* 1989).

In determining the rates of unfolding and refolding, Chen *et al.* (1989) attempted to explore the chemical nature of cold denaturation. As one would expect for a simple isomerization reaction, the kinetics are independent of protein concentration. The relaxation times are identical for the forward and backward reaction, irrespective of the initial conditions. This proves that the transition may be described by the two-state model without a significant population of intermediates.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Applying the transition state theory to the unfolding–refolding data, the transition state is found to be compact and relatively close to the native state with regard to changes in heat capacity and exposure of groups to the solvent. It has a high energy relative to both the native and unfolded states. The energy barrier for refolding comes largely from the formation of hydrophobic interactions. This is consistent with the observed enthalpy–entropy compensation. For the unfolding reaction, the positive enthalpy change in going from the native to the transition state supports the idea that hydrogen bonds involved in secondary structure formation rather than hydrophobic interactions are important.

The structural interpretation of the 'compactness' of the transition state does not favour any of the current models of protein folding. Both the framework model and the sequential model satisfy the observed data (Jaenicke 1987*a*). Whether the shuffling of intramolecular interactions keeps part of the native secondary and tertiary structure, or generates a state comparable to the molten globule (Ptitsyn 1987) remains open.

Cryoenzymology and high-resolution structural analysis

Under physiological conditions, enzyme catalysis normally runs too fast to allow a detailed analysis of intermediate states on the reaction pathway. Low temperatures seem promising to slow down the reaction (table 1). However, freezing (in aqueous solution), altered structure and catalytic properties (in cryosolvents), and non-linear Arrhenius behaviour set limits to the apparently simple approach (Jaenicke 1981). Replacing organic cosolvents by water-soluble polyelectrolytes or amphiphilic compounds in non-polar organic solvents, or applying water-in-oil emulsions minimizes perturbations (Franks 1985; Hatley & Franks 1989). The general conceptual problem of whether cryo-conditions may affect the reaction mechanism, remains unsolved by the given approaches. This has to be borne in mind in all attempts to investigate biological mechanisms in atomic detail.

Myoglobin has been thoroughly investigated at temperatures from −193 to +27 °C; X-ray data and spectral analysis unveiled a whole sequence of functionally important substates involved in ligand binding (Frauenfelder *et al.* 1979; Parak *et al.* 1987). Kinetic and structural information relating to trapped intermediates have been accumulated for a variety of proteins including single-chain and oligometric enzymes, as well as complex structures such as bacteriorhodopsin, light-harvesting systems and photosynthetic membranes. In the given context, new horizons have been opened by the time-resolved X-ray analysis by using synchrotron radiation (Hajdu *et al.* 1986, 1987).

5. Low temperature dissociation and deactivation

The significance of hydrophobic interactions in protein structure and stability is reflected in the efficiency of residue packing in the inner core of a protein (Richards 1977). In the case of oligomeric proteins, subunit association has been generally assumed to be entropy driven as a consequence of the minimization of hydrophobic hydration. The corresponding endothermic character of the assembly reaction would then provide a simple explanation for the low-temperature deactivation of oligomeric enzymes in terms of low-temperature dissociation. The same mechanism may hold for growth inhibition, hypothermia and hibernation.

Considering effects of pH and ionic strength, it is evident that the positive temperature coefficient of hydrophobic interactions is not sufficient to fully explain low-temperature

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

deactivation and dissociation; temperature effects on the heat of ionization of dissociable amino-acid residues must also be involved. This is clearly shown by the fact that for both the rate and extent of deactivation of several enzymes, lowering the temperature at a given pH is equivalent to lowering the pH at the given temperature. From chemical modification studies it is evident that ionizable groups are involved in the formation of the native quaternary structure (Bock & Frieden 1978; Gerl *et al.* 1988).

Examples that allow the low temperature dissociation of multimeric systems to be quantitatively determined with respect to their thermodynamic and kinetic parameters are the reversible disassembly–assembly of tobacco mosaic virus protein and flagellin, on the one hand, and the ATP-dependent cold deactivation of glyceraldehyde-3-phosphate dehydrogenase, on the other.

Reversible assembly of tobacco mosaic virus protein and flagellin

Tobacco mosaic virus protein as a model to illustrate the autonomous self-assembly of proteins has been investigated in detail. Because of their specific binding properties and geometry, the protein subunits and the viral RNA are able to form assembly intermediates that may combine to helical rods or to the virion. The kinetics of the process are complicated by a slow nucleation reaction that may cause non-equilibrium states (Potschka *et al.* 1988). However, as a first approximation, the assembly may be described by an isodesmic condensation polymerization model. The reaction is endothermic under all conditions that favour association (Sturtevant *et al.* 1981). The corresponding increase in entropy is caused by water release (table 2). On the other hand, low temperature shifts the equilibrium toward the monomer; as shown by buoyancy measurements, in this case the driving force of the reaction is the exothermic hydrophobic hydration (Jaenicke & Lauffer 1969).

In contrast to the endothermic polymerization of tobacco mosaic virus protein, the aggregation of flagellin (to form helical flagella) shows a temperature-dependent change in sign of ΔH_{ass} (figure 4). Thus the driving force of the reaction changes from entropy at low temperature (< 14 °C) to enthalpy (and entropy) at room temperature. Strongly temperature-dependent enthalpies have been frequently observed in protein–protein and protein–ligand interactions (Bode *et al.* 1974; Hinz 1986). The high negative heat-capacity change accompanying the polymerization reaction reflects the decrease in water accessible surface area. As in the case of the endothermic polymerization of tobacco mosaic virus protein, desolvation must be involved, in accordance with the common concept of hydrophobic interactions. This hypothesis is supported by the observation that both tobacco mosaic virus protein and flagellin assembly are associated with a significant volume increase, clearly indicated by the inhibition of the polymerization reaction at elevated hydrostatic pressure (Jaenicke 1987*b*).

One further contribution to the large negative heat-capacity change during flagellin polymerization may arise from tightening of the structure with concomitant loss of excitable internal degrees of freedom (Sturtevant 1977). The large increase in negative ellipticity at 220 nm points in this direction, suggesting that the isolated 'native' flagellin monomer represents an intermediate state between a thermally disordered and a compact structure, and that polymerization is only a continuation of the folding reaction. Kinetic results (Asakura 1970; Kuroda 1972), which prove at least two main association steps to be involved in flagella formation, may be interpreted as a consecutive desolvation–transconformation reaction. The example of flagellin shows that assembly reactions involving proteins may exhibit positive and

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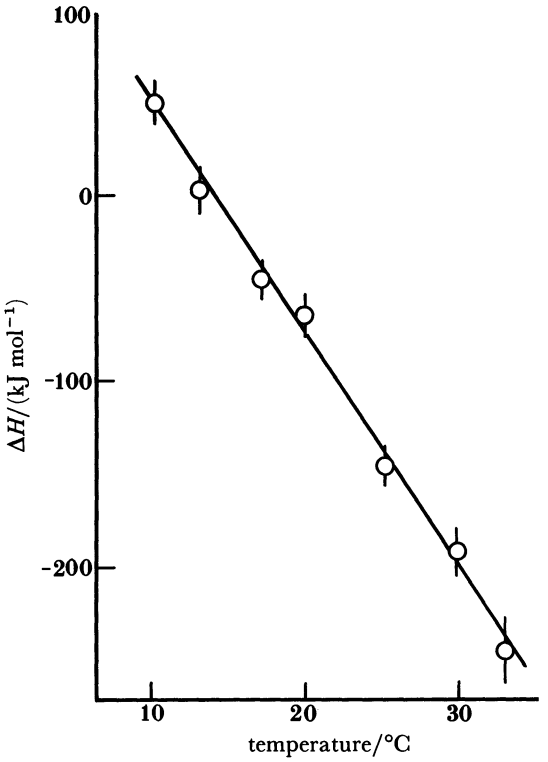

FIGURE 4. Temperature dependence of the molar enthalpy of polymerization of flagellin at 4–12 mg ml^{-1} concentration, 10 mM sodium phosphate pH 7, 0.2 M NaCl.

TABLE 2. ENDOTHERMIC POLYMERIZATION OF TOBACCO MOSAIC VIRUS PROTEIN IN 0.05–0.1 M PHOSPHATE BUFFER, 2 mM EDTA, PROTEIN CONCENTRATION 2–56 mg ml^{-1}

(Data taken from Jaenicke & Lauffer (1969) and Sturtevant *et al.* (1981).)

type of assembly	pH	T_m [a]	ΔH^0/(kJ mol^{-1})	ΔC_p/(kJ K^{-1} mol^{-1})	ΔS^0 *e.u.*	water release (mol H_2O/protomer)
helical rod	6.45	12.0	53	−1.5	—	—
	6.75	17.9	53	−1.5	170	32
	7.00	21.0	41	−0.3	—	—
double disk	7.50	24.4	25	+0.6	—	—

[a] T_m, the temperature of maximal excess heat capacity equals the turbidity-determined transition temperature.

negative enthalpy values, depending on temperature. Therefore, no general predictions with respect to the effect of low temperatures can be made.

Cold dissociation of oligomeric enzymes

Temperature effects on the dissociation–association of subunits have been shown for many oligomeric enzymes. For the majority of 'cold sensitive enzymes' involved in the regulation of metabolic pathways, low temperature deactivation is known to be accompanied by subunit dissociation (Jaenicke 1981). The physiological significance of the phenomenon is not fully established as cold susceptibility of enzymes may not always be true under cellular conditions. An alternative mechanism would be temperature-dependent shifts of conformational equilibria. There is no elaborate experimental proof for this concept apart from the well-established fact

that $R \rightleftharpoons T$ equilibria usually exhibit a significant temperature-dependence. To give an example, the cooperative conformational transition of yeast glyceraldehyde-3-phosphate dehydrogenase only occurs at elevated temperature; below 20 °C cooperativity vanishes (Jaenicke & Gratzer 1969). Cold inactivation of the enzyme at 0 °C in the presence of ATP and mercaptoethanol is found to be accompanied by dissociation to inactive dimers and monomers (figure 5). The final product of low temperature dissociation is the 'structured

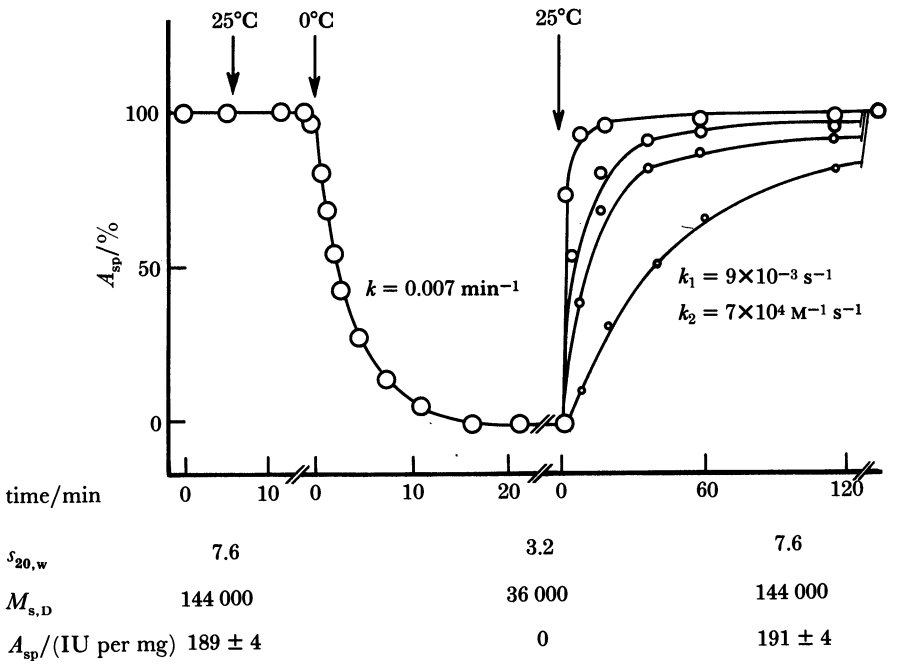

FIGURE 5. Cold inactivation of yeast glyceraldehyde-3-phosphate dehydrogenase in the presence of 5 mM ATP, 0.1 M Tris pH 8.5 plus 0.1 M 2-mercaptoethanol. Dissociation at 0 °C, (c_{GAPDH} = 1 mg ml^{-1}; k, first-order rate constant. The monomer is capable of binding 1 mol NAD^+ (K_d = 0.1 mM). Reconstitution at 25 °C and varying concentrations: (○) 70 nM, (○) 27 nM, (○) 13 nM, (∘) 6.5 nM (based on relative molecular mass of 144000). Solid lines calculated according to a consecutive folding association mechanism with k_1 and k_2 as first- and second-order rate constants.

monomer', which is still capable of binding one molecule of NAD^+ per subunit with high affinity (K_{diss} = 0.1 mM). Deactivation–dissociation at 0 °C is a slow first-order reaction with a half-life of 180 min, whereas reactivation at 25 °C and low enzyme concentration is found to obey a consecutive uni-bimolecular folding-association mechanism:

$$4M \underset{\text{fast}}{\rightarrow} 2M_2 \overset{k_1}{\underset{\text{slow}}{\rightarrow}} 2M_2^* \overset{k_2}{\underset{\text{slow}}{\rightarrow}} M_4^*,$$

where M, M* code for the monomer in different conformational states, M_4^* is the reconstituted (native) tetramer, and $k_1 = 9 \times 10^{-3}$ s and $k_2 = 7 \times 10^4$ M^{-1} s^{-1} are first- and second-order rate constants that quantitatively describe the concentration dependent reconstitution reaction. The second-order process proves clearly that dissociation is involved in the cold deactivation of the enzyme (Bartholmes & Jaenicke 1978).

Cold deactivation as a consequence of subunit dissociation involving 'structured monomers'

is common to oligomeric enzymes and other proteins that are composed of several polypeptide chains (Bock & Frieden 1978; Jaenicke 1981). As with glyceraldehyde-3-phosphate dehydrogenase, under appropriate experimental conditions the deactivation is fully reversible. There are exceptions where deactivation has been found to be accompanied by irreversible low temperture aggregation rather than reversible dissociation. Obviously, in this case, subunit dissociation (as a primary reaction) uncovers additional hydrophobic surface area that is subsequently buried again by irregular aggregation. This mechanism is well-known from the kinetic competition of folding and association in the in vitro reconstitution of subunit proteins (Jaenicke 1987*a*).

Cold denaturation

Cold denaturation of proteins has been observed for a variety of globular proteins. As shown in figure 6, the transition proceeds with a release of heat, i.e. with a decrease in ΔH and ΔS, which suggests that the hydration of non-polar groups is the driving force of the reaction. There

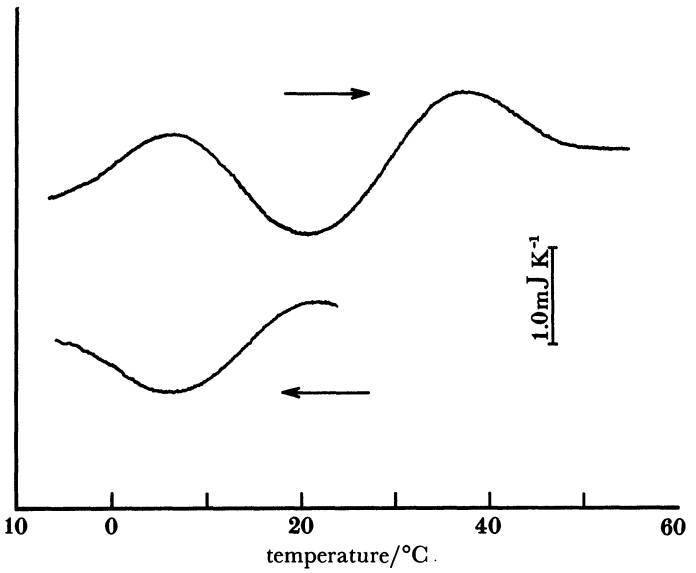

FIGURE 6. Microcalorimetric recording of cooling and subsequent heating of staphylococcal nuclease (c = 3.9 mg ml^{-1}) in 2 M urea (pH 6.5) (Griko *et al.* 1988*a*).

is ample evidence that both the high and low temperature transition involves significant conformational changes. In their first report, Brandts *et al.* (1970) concluded from difference spectra that the overall conformational changes in both temperature ranges are similar. This observation has been confirmed by a variety of techniques including far-UV and near-UV circular dichroism (CD), nuclear magnetic resonance, viscometry and calorimetric titration (Privalov *et al.* 1986; Griko *et al.* 1988*a*, *b*). Figure 7 illustrates the result of CD measurements using staphylococcal nuclease as an example. As indicated by the flattening of the far-UV profile at −7 and +55 °C, both high and low temperature cause a significant loss of secondary structure. In the case of myoglobin and apomyoglobin, spectral changes and alterations of the hydrodynamic properties have been shown to parallel each other. Obviously, the final states at high and low temperatures show a certain degree of similarity with regard to their physical characteristics. In both cases, the proteins show substantial flexibility with a considerable freedom of rotation about the dihedral angles. This means that all of the residues have changed

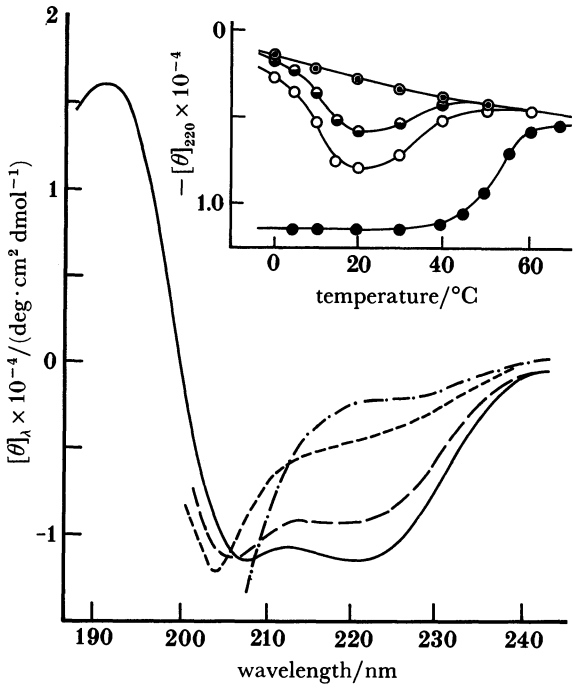

FIGURE 7. Far-UV circular dichroism of staphylococcal nuclease in 2 M urea (pH 6.5) at different temperatures (–·–·) −7 °C, (——) +20 °C, (---) +55 °C, (—) +20 °C in the absence of urea. Inset: temperature dependence of $[\theta]_{220}$ at various pH values: (⊙), pH 3.0; (⊖), pH 6.0; (○), pH 6.5; (●), pH 6.5 in the absence of urea.

from the narrow structural probability distribution characterizing the native protein to broad distributions in the unfolded state, where the majority of the residues are widely distributed over the Ramachandran plane. Local short-range correlations may still be preserved, but long-range correlations are small. It may be assumed that high-temperature denatured protein differs in its structural distribution from low-temperature denatured protein. Qualitatively, however, they are considered to be similar, because low-temperature melting can be inferred from the extrapolation of data from high-temperature melting (compare with results from Schellman (1987) and Chen & Schellman (1989)). Under physiological conditions, the problem arises that cold denaturation only occurs to a very limited extent at temperatures above the equilibrium freezing point of water. Therefore, in most experiments, perturbations of the native state by either zymogen activation (Brandts 1964), or by changes in pressure, pH or solvent composition (moderate denaturant concentrations, cryosolvents, etc.) have been utilized (Brandts *et al.* 1970; Nojima *et al.* 1977). Additives such as urea or guanidine not only lower the stability curve so that low-temperature destabilization is shifted to the range of experimental accessibility, but also accelerate the unfolding reaction and lower the freezing temperature of water. The water-in-oil droplet emulsion technique to inhibit heterogeneous nucleation of ice in the undercooled solution extends the temperature range to *ca.* −40 °C. Attempts to shift the range of cold denaturation by choosing proteins from extreme thermophiles failed; obviously the increased rigidity of thermophilic proteins does not allow the detection of anomalous low-temperature effects (Wrba 1989).

Low temperature effects on proteins in frozen solutions (in contrast to the liquid state) present additional problems resulting from the existence of multiple phases in the frozen state.

In addition to pure temperature effects, they lead to changes in the concentration of solutes, alterations in pH, separation of protein into multiple phases, etc. Therefore, the temperature dependence of denaturation is complex and not accessible to a quantitative explanation (Brandts *et al.* 1970; Franks 1985). The same holds for the protection of proteins against denaturation. The phenomenology of preferential solvation and salt binding is well-established; however, the lyotropic series and the correlation of structure and solvation are still mysterious.

In conclusion, it is interesting to note that low-temperature denaturation and dissociation are not confined to proteins. Similar effects have been reported for micellar solutions of detergents where critical micelle concentrations exhibit minima at certain temperatures. Demixing phenomena encountered in aqueous polyethyleneglycol or polyvinylpyrolidon solutions illustrate that the balance between hydrophobic and polar hydration interactions determines structural changes in synthetic polymers as it determines the high-temperature and low-temperature behaviour of proteins (Franks *et al.* 1988).

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Discussion

U. Heber (*Institute of Botany and Pharmaceutical Biology, University of Würzburg, F.R.G.*). Low temperature denaturation of proteins is readily reversible, whereas damage suffered by cells during exposure to low temperatures is not (or at least not easily) reversible. In which instances can we be certain that low temperature denaturation of proteins is the cause of cellular damage?

R. Jaenicke. One has to be extremely cautious when trying to apply conclusions from *in vitro* studies (involving isolated proteins in dilute aqueous buffer solutions) to the *in vivo* situation. This is simply because the cell is more than a 'bag' full of macromolecules. The network of cellular processes at low temperatures is expected to undergo 'metabolic dislocation' caused by the combined effects of activation energies plus cold deactivation, etc. At the same time, various kinds of stress obviously lead to the expression of shock proteins (heat, cold, pressure). All these effects must be involved in the balance of responses that finally cause tolerance, repair or damage. I am convinced that there is not just one cause of low temperature damage at the cellular level. There is no instance where I would dare to correlate low temperature denaturation with cellular damage in terms of a clearcut one-to-one relation.

F. Franks (*Pafra Ltd., Cambridge, U.K.*). In comparisons of cold and heat denaturation, some degree of confusion arises because the choice of the native state as reference, and the adoption of ΔG (N $\rightarrow$ D) for both processes. In the case of cold denaturation this involves cooling, whereas in the more commonly studied heat denaturation, the N $\rightarrow$ D transition is effected by heating.

A thermodynamically more useful reference state might the cold-denatured state so that both transitions would then result from supplying heat to the protein. Both transitions would then appear endothermic ($\Delta H > 0$).

ΔC accompanying an N $\rightarrow$ D transition is always claimed to be positive and independent of temperature. On the premise that $C(T)$ is probably always positive and that $(\mathrm{d}C^{\mathrm{N}}/\mathrm{d}T) < (\mathrm{d}C^{\mathrm{D}}/\mathrm{d}T)$, especially at low temperatures Franks & Wakabayashi (1987) we have concluded that ΔC changes sign at some temperature between T_{H} and T_{L}, where T_{H} and T_{L} are the heat and cold-induced transition temperatures (Franks *et al.* 1988).

Despite the surprising conclusion that at T_{L}, ΔC is then negative, this representation of the temperature dependence of C seems to be physically more satisfying.

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R. Jaenicke. I fully agree. However, to give justice to the experts in the field, in all treatments of cold denaturation that have recently been published authors did consider the temperature

dependence of ΔC_p. In this context attempts to get access to sound experimental data deserve mentioning, namely Privalov & Gill (1988) and Privalov *et al.* (1989).

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D. CHAPMAN (*Department of Protein and Molecular Biology, Royal Free Hospital School of Medicine, London, U.K.*). Professor Jaenicke has indicated that water-soluble proteins can undergo 'denaturation' at low temperatures, mirroring, to some extent, what happens at high temperatures. Does he think that behaviour of this type also occurs with membrane proteins such as bacteriorhodopsin?

R. JAENICKE. To my knowledge no detailed thermodynamic studies have been performed dealing with the cold denaturation of membrane proteins. However, considering R. L. Baldwin's and P. Privalov's recent work, it seems clear that hydrophobic solvation vanishes at extremes of temperature. From this one would predict that cold denaturation also occurs in membrane proteins. Needless to say that in this case all three components: water, lipid and protein have to be considered in the energy and entropy balance.

DR MARY C. A. GRIFFIN (*AFRC Institute of Food Research, Reading, U.K.*). Professor Jaenicke has shown us curves of reactivation of pyruvate dehydrogenase multienzyme complex at low and high temperatures after dissociation and denaturation by chemical denaturants, followed by transfer to solvent conditions favouring the native state of the enzyme. At 0 °C no reactivation of the total complex activity was observed over the time course of the experiment, whereas at 53 °C significant recovery was seen. Is there a critical temperature for the onset of recovery of full activity of the complex?

R. JAENICKE. To accomplish the reconstitution of the PDH complex from *Bacillus stearothermophilus*, about half a dozen parameters had to be optimized apart from temperature (see, for example, Jaenicke & Perham (1982)). Therefore, we did not follow the temperature dependence in great detail. What we know from oligomeric enzymes or multimeric protein assemblies shows that there exists a limiting temperature where the entropy term just compensates the positive ΔH_{ass}. Obviously, the situation is complicated by the fact that 'wrong aggregation' or similar side reactions compete with proper folding and association.

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Jaenicke, R. & Perham, R. N. 1982 Reconstitution of the PDH multienzyme complex from *Bacillus sterothermophilus*. *Biochemistry* **21**, 3378–3385.

A. HVIDT (*University of Copenhagen, Denmark*). At some given temperature, a maximum of denaturation of globular proteins means that the entropy of denaturation is negative at lower temperatures. Does Professor Jaenicke have any comments to make on the molecular nature of the background to this phenomenon?

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

R. Jaenicke. Dr Hvidt will agree that the free energy balance may equally well be governed by the exothermic nature of the reaction or by the decrease in entropy, or both. This may be interpreted in terms of an increase in hydrophobic hydration upon subunit dissociation or unfolding (exposure of the hydrophobic core of the protein to the solvent).

D. A. Cowan (*Department of Biochemistry, University College London, U.K.*). Could Professor Jaenicke comment on the differences in molecular flexibility between similar enzymes from mesophiles and extreme thermophiles and on the consequences with respect to maximum catalytic activity?

R. Jaenicke. Not much has been done in this area in terms of sound experiments. The shift in optimum temperature clearly reflects the high relative rigidity of thermophilic enzymes as compared to their mesophilic counterparts. Experiments that corroborate this idea include limited proteolysis, accessibility of titratable groups, H–D exchange, determination of B-values or ΔC_p (differential scanning calorimetry), nuclear magnetic resonance, etc. To give an example, the H–D exchange in glyceraldehyde-3-phosphate dehydrogenase from *Thermotoga maritima* at ambient temperature is drastically reduced compared to the enzyme from lobster or yeast (A. Wrba, A. Schweiger, V. Schultes, R. Jaenicke & P. Závodszky, unpublished results). Obviously, homologous enzymes occupy 'corresponding states' under their respective physiological conditions.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES