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Protein stability: the value of 'old literature'

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Abstract

The concepts of protein structure and function have been subjects of intensive study throughout the 20th century; they continue to fascinate present-day scientists. Our understanding received a major boost when it was realised during the 1960s, that the physical properties of water play a major role in determining the stability of native proteins *in vitro*. This recognition changed the emphasis of physicochemical studies towards 'hydration', i.e. protein–water interactions. A rigorous quantitative description of 'hydration' still escapes us, but several semi-quantitative treatments, some with predictive potential, are now available and can account for the marginal stabilities of native proteins in aqueous solvent environments. This article charts the progress achieved during the latter half of the 20th century, which in present day parlance is termed 'old literature'. The thesis is advanced that the common practice of uncritically equating 'recent literature' with 'progress' is of dubious value. In the general area of in vitro protein stability some recent developments seem questionable and have yet to stand the test of time before their usefulness or validity can be accepted.

1. Neglecting 'old literature' in 'new science'

The teaching of philosophy and social sciences has always made due acknowledgement to the origins and histories of these disciplines, but this is hardly the case with the teaching of physical and, in particular, the life sciences. Students are given the impression that anything printed more than 5 years ago can be considered as 'old literature' and need not figure in today's agenda. Implicit in such an approach is the erroneous assumption that anything published today must be more correct than what was printed in the 'old literature'. One can compare the attitudes of the majority of today's researchers with those of a student of plant physiology, who limits himself to studying the leaves of trees, without realising that there would be no leaves, were it not for the roots. For him, roots are 'old literature'. Another feature of this approach is the common rediscovery of the wheel, approximately once every 7 years. Sometimes these rediscoveries smack of alchemy and metaphysics, because they appear to be incompatible with the basic laws of physics, as we accept them today. It is the chief aim of this article to bring home to an interested reader the value of some 'old literature', especially where it pertains to present-day protein science.

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2. Towards an understanding of protein stability

It is instructive to sketch out the development of protein science and its associated technology. The origins of structure, function and stability first became subjects of active research interest during the 1920s, when it became generally apparent that proteins, in common with other biopolymers, possessed certain, almost magical properties. At that time, the available tools for the study of chemically complex molecules were limited. The advent of X-ray crystallography provided a boost to further investigations, especially when it was realised that proteins could be crystallised and studied by classical diffraction methods. The more recent development of neutron crystallography has give protein structural studies a further boost, limited only by a realisation that few of the available proteins will probably ever be crystallised [1]. Armed with the structural information, i.e. atomic co-ordinates, biochemists developed the hypothesis of 'Structure and Function', i.e. that there exists a relationship between the structure of a protein and the function that it can perform in vivo. It appeared likely, almost certain, that the specific function of a protein could somehow be read from its molecular structure. Even now, despite mounting evidence to the contrary, the obsession with 'Structure and Function' is still firmly embedded in biochemical teaching.

The Golden Period of research into protein physical chemistry began soon after the scientific community had grasped the significance of Walter Kauzmann's seminal publication, where the concept of 'hydrophobicity' was first introduced to explain the configurational complexities of protein folding and unfolding [2]. The important realisation that water is closely involved in directing and controlling these processes was itself based on earlier work of Henry Frank, which had centred on the peculiar thermodynamic properties of aqueous solutions of hydrocarbons [3]. The remarkable finding, which still forms the real basis of much of protein chemistry, was that the low solubility of non-polar species in water is *not* due to unfavourable energetic interactions. On the contrary, despite their low solubilities, the enthalpies of solution of hydrocarbons are *negative*. The immiscibility arises entirely from unfavourable configurational (entropic) factors (see below). Thus, the observed positive free energies of mixing of water with non-polar molecules are due entirely to negative entropies of mixing. This was an entirely new concept in the physical chemistry of solutions, where the classical theories had always accounted for immiscibility in terms of enthalpic repulsions between chemical species. It had always been assumed that, because of the general randomising nature of a mixing process, entropies of mixing had to be positive. Frank realised that the origin of his unique findings arises from the peculiar tetrahedral molecular structure of the water molecule and the directional hydrogen-bonded interactions between water molecules in the liquid. But, since nothing is ever completely new, consequences of the 'water structure' tetrahedrality had already been intensively studied by John Bernal a decade earlier [4]. Fig. 1 represents his proposed disposition of nearest neighbour water molecules, which is compatible with the then available X-ray diffraction data for liquid water.

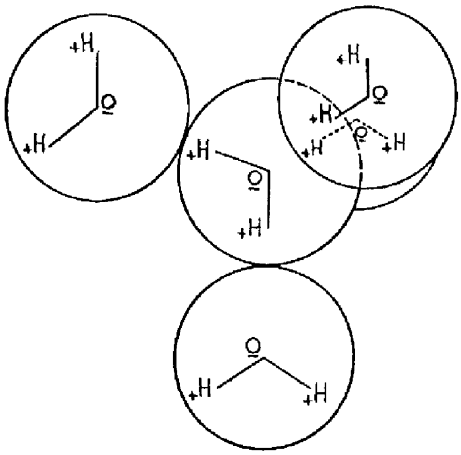

Fig. 1. Tetrahedral arrangement of molecules in ice and liquid water, giving water the unique ability of forming three-dimensional hydrogen-bonded networks. Reproduced from [4].

The realisation of the dominant role of water and hydration phenomena in protein behaviour changed the direction of the emerging discipline of protein science, which reached its zenith, both in innovation and printed output, during the two

decades following the publication of Kauzmann's famous paper. One should now define a protein as a (peptide + water) system, because in the absence of an aqueous medium, polypeptide chains are unable to adopt their biologically significant structures. Anything that perturbs the delicate and labile hydrogen-bonded water structure will equally affect the behaviour of a protein in solution. Perhaps the most remarkable evidence for the finely tuned involvement of $^{1}H_2O$ in protein properties and life processes generally, is provided by its substitution by $^{2}H_2O$. To the physicist, such a replacement is considered almost trivial, just small differences in the moments of inertia and the zero-point energies. A life scientist, on the other hand, is well aware that heavy water is toxic to life processes. Only protozoa and other very low forms of life can tolerate a gradual, but complete isotope substitution, which then enables them to synthesise fully deuterated proteins. Any higher life forms will exhibit all the symptoms of accelerated senescence and will suffer eventual death. The conclusion must be that protein structural and functional behaviour, as well as metabolic rates, are extremely sensitive to the energetic and configurational details of the hydrogen bond, as it exists in $^{1}H_2O$.

The 1960s and 1970s witnessed an intensification in investigations of proteins in solution, mainly from the physico–chemical viewpoint. Accent was placed on the role of water in directing the folding and stability of proteins [5]. Efforts were made to establish a thermodynamic framework which could lead to an understanding of denaturation/renaturation phenomena, i.e. reversible conformational transitions, brought about by a perturbation of the environmental factors that influence protein stability: pH, temperature, pressure, changes in the solvent medium, etc. Alongside the solution thermodynamics, spectroscopic investigations were performed, specifically to obtain an understanding of the weak, non-covalent interactions that govern the stabilities of native (biologically active) protein states. Two important conclusions emerged: (1) the stability margin of a native protein, relative to its inactive form, hardly ever exceeds 50 kJ mol^{-1}, equivalent to approximately three hydrogen bonds and (2) this very marginal stability is brought about by a parallel

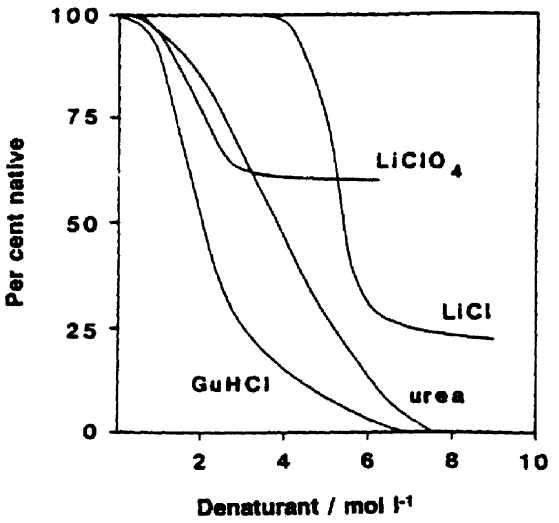

Fig. 2. Effects of different denaturation treatments on the residual 'native character' of ovalbumin, as determined by optical rotatory dispersion; after [7].

temperature dependence of the enthalpic and entropic contributions, which later came to be known as H/S compensation [6].

While X-ray diffraction has helped in the structural elucidation of several native (crystalline) proteins, a similar detailed description of inactive states (denatured) still eludes present day investigators. At one time, it was assumed that a denatured protein could be approximated to a 'random coil', a state much used in polymer science. That this is a gross oversimplification becomes apparent from the realisation that the peptide bond, linking neighbouring amino acid residues, has some double bond character, i.e. the freedom of rotation about the C–N bond is partially inhibited. It has also been established and is illustrated in Fig. 2, that the method by which denaturation is brought about affects some physical properties of the species so obtained [7]. Furthermore, if the denatured state could really be treated as random coil, i.e. completely flexible, then spontaneous refolding and renaturation in real time would become most improbable. Yet the denaturation/renaturation process is not only possible, but is commonly used in the downstream processing and purification of recombinant proteins [8].

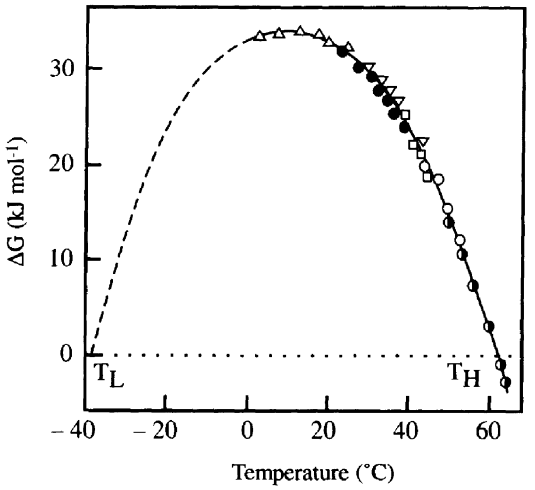

Fig. 3. Stability profile $\Delta G(T)$ of chymotrypsinogen, constructed from measurements at various pH values and the addition of urea at the lowest temperatures (Δ); from [18]. The broken line indicates a parabolic fit to the data. T_H is the thermal denaturation temperature and T_L the cold denaturation temperature, determined in undercooled water; see [19].

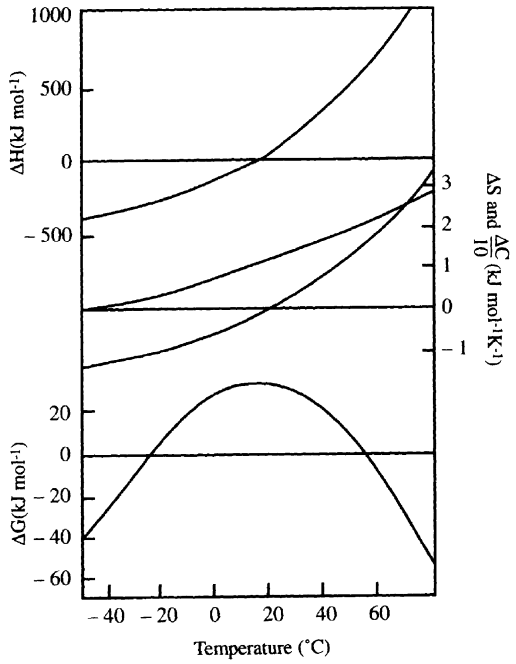

Fig. 4. Thermodynamic characterisation of $\Delta G(T)$ for LDH in solution at pH 7.5 (measured at 25 °C), indicating cold and heat-induced denaturation. Note the skew of the $\Delta G(T)$ parabola at the low temperature side, indicative of a complex ΔC behaviour. Redrawn, with alterations, from [20].

3. Origins of stability — the role of 'hydrophobia'

The almost complete ignorance of the structural and configurational details of unfolded protein states still makes it impossible to provide a full description of a denaturation process. At the most basic level, equilibrium thermodynamics describes changes in various energetic properties of a system, arising from a transition from one well-defined initial state to another, final one, equally well defined, where the transition is independent of the pathway. With protein denaturation, an adequate description of the final state is lacking, although differences can be observed between various denaturation treatments, e.g. heat, urea or pH.

The development of Monte Carlo (MC) and Molecular Dynamics (MD) computer simulation techniques as means of studying structure and dynamics in liquids, especially water [9,10], soon led to extensions of these methods to studies of simple aqueous solutions and then to protein dynamics [11]. Even today, however, a general ignorance of the structural details of the denatured state still limits the value of computer modelling as a means of gaining a good insight into the molecular mechanisms of unfolding and refolding in vitro. Whereas formerly, folded globular proteins had been regarded as more or less rigid spheres, the development of realistic atom–atom potential functions in simulations provided evidence of librational and torsional motions within the molecule. Initially, the MD methods were subject to a serious shortcoming: the known crystal co-ordinates were used as a starting point, but under in vacuo conditions. Water, although it makes up approximately 60% w/w of the crystal, could not be included in the computer 'experiment', because at the time, high power computers were not yet available. The simulation was thus performed on a folded polypeptide chain, placed in a deep potential well from which it could not escape, i.e. unfolding was made impossible, even at very high (unrealistic) temperatures. Yet, it was well known at the time that unfolding could easily be achieved

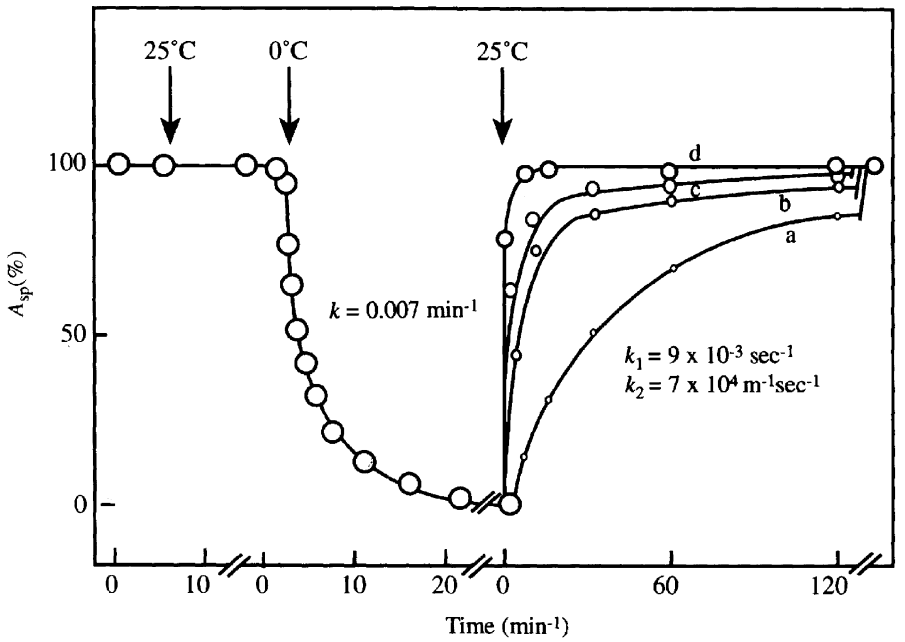

Fig. 5. Kinetic representation of the cold inactivation and reactivation of glyceraldehyde-3-phosphate dehydrogenase, subjected to two temperature jumps, as indicated; for experimental details, see [21]. The molecular weight M and the corresponding specific activity A indicate a reversible dissociation, and subsequent reassociation of the tetrameric enzyme, where the former process follows first order kinetics, and the consecutive reassociation indicates three sequential second order processes, the first one fast, and the other slow (rate constants indicated). The four reassociation profiles refer to different GPDH concentrations, from top to bottom: 70, 27, 13 and 6.5 nM. Reproduced, with permission, from Phil. Trans. Roy. Soc. B326 (1990) 535.

by even a moderate rise in the temperature of a protein solution or by a moderate concentration of urea, even at room temperature.

With the advent of ever more powerful computers, and the parallel development of n.m.r. and neutron scattering methods for the investigation of aqueous protein solutions, the scope of MD simulations was extended to include water [11]. It is, however, questionable whether a simple additive sum of atomic pair interactions, including a mixed bag of covalent bonds and bond angles, torsional potentials, hydrogen bonds, van der Waals interactions, etc. can provide a realistic picture of a folded protein in its aqueous medium. Even now, a hypercomputer is in process of being constructed, with the sole purpose of helping with modelling the folding of a simple enzyme, surely indicating that presently available hardware is insufficiently powerful for that purpose. Another severe problem was posed by the dawning realisation that energy minimisation alone could not possibly provide a correct description of protein stability, which is measured as a *free* energy. The reason was that the important entropic contribution of hydrophobic effects could not readily be accommodated in the simulations. The entropy problem has now been partially solved, but it is still impossible to compute a protein-folding process, i.e. a disorder→order transition, which is routine under in vivo conditions and is also easily achievable in vitro. In principle, a truly 'knowledge based' approach should enable the protein researcher to predict a peptide sequence, able to fold spontaneously into a tertiary structure and allow him/her to make a good guess about its possible biochemical function. Conversely, being presented with a sequence, he/she should be able to calculate or compute conditions under which it could fold into a native structure. Such an ability would truly be a great step forward in the search for 'rational' drug

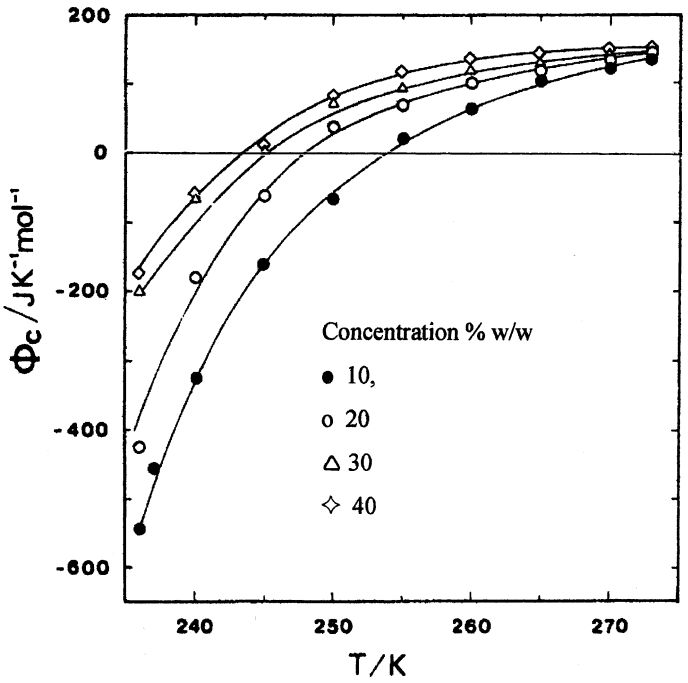

Fig. 6. Apparent heat capacities ϕ_c of polyvinyl pyrrolidone in undercooled water. Redrawn, with changes, from [22].

design, which, at present, still relies entirely on sequence database searching, hardly a 'rational' or 'knowledge-based' procedure.

Towards the end of the 1970s, and as a result of an enormous research effort, a consensus had been reached about the interactions, which determine the stability/instability balance of native proteins. They were said to include three major effects: hydrophobic interactions, intrapeptide hydrogen bonds and the loss of configurational freedom by the (previously hydrated) peptide chain upon folding. Minor contributing effects include electrostatic interactions, peptide–water hydrogen bonds and van der Waals interactions. Various attempts, some more realistic than others, are on record to estimate the relative magnitudes of these contributions [12]. Such an enterprise to dissect $\Delta G(T)$ into various contributions resembles the drawing up of a profit-and-loss balance sheet. The only experimentally accessible range is centred on $\Delta G = 0$ at the denaturation temperature and usually extends to ± 5 kJ mol^{-1} on either side of this value.

The largest uncertainty attaches to the magnitude of the hydrophobic contribution, as it affects $\Delta G(T)$. Even if it were possible, and attempts have been made, to estimate the change in free energy upon the transfer of an isolated amino acid residue from the unfolded (solvent accessible) to the interior (solvent inaccessible) state, there still remained the problem of the range of a hydrophobic hydration shell. That problem still exists today, although X-ray and neutron scattering data suggest that the hydration shell is not confined to the nearest neighbour water molecules but decays exponentially, with a characteristic correlation length, over several layers of water molecules.

The importance of hydrophobic hydration/interaction phenomena in affecting protein stability cannot be overstated. This claim is supported by two observations: for a polypeptide chain to be able to fold into a stable globular configuration, the amino acid sequence must contain at least 50% of apolar residues and the apolar amino acids tend to be the most conserved residues of globular proteins. Thus, the apolar residues of all globins,

whatever their functions, are completely conserved, whereas ionic and polar residues exhibit many mutations. During the 1980s, a general agreement existed on how hydrophobic hydration and its reversal can be treated in physico–chemical terms. The thermodynamics of clathrate hydrate formation by apolar molecules shows remarkable similarities to those of apolar amino acids. The main features are a rearrangement of water molecules, so as to form polyhedral cages, able to accommodate the apolar species either partially or completely. The results of this rotational rearrangement are changes in the free volume distribution of the system and a reduction of the permitted configurational degrees of freedom of water molecules. In the hydrate structure, –OH vectors may not point towards the centre of the cavity, but are allowed to point only along edges of the polyhedral hydration structure, thus giving rise to a corresponding reduction of the entropy. The actual number of water–water hydrogen bonds in the clathrate hydrate crystal, their lengths, directionalities and energies are almost identical to those of ice. As regards the properties of solutions, they closely resemble those of the crystalline state, after corrections for latent heats of fusion. The ice lattice is marginally more stable than a hypothetical empty clathrate hydrate lattice, but the latter becomes stabilised by dispersion interactions between the guest molecule, with the surrounding water molecules forming the cavity. A thorough physicochemical analysis of the hydrophobic effects is part of the 'old literature' [13] and it was hoped at the time of a Faraday Symposium on the subject [14] that here was a problem solved to everybody's satisfaction. But this was not to be and we have witnessed one of the common examples of rediscovering the wheel, only in this case the rediscovered wheel is not round. The tenet developed jointly by physicists, computer simulators, chemists and biochemists over 30 years was stood on its head, based on claims about current confusion, due to the '…recent shift in understanding of the hydrophobic interactions' [15].

As a postscript to the debate on hydrophobic phenomena, now unfortunately reignited, mention is made of a more recent neutron scattering study of aqueous solutions of argon, which shows that the co-ordination number of the argon atom in solution, (i.e. the number of nearest neighbour water molecules) is 16 ± 2, in the range of 0.28–0.54 nm, almost exactly the dimensions of the well-known crystalline type II clathrate hydrate structure [16]. The validity of the crystal as a model for the time-averaged aqueous solution seems to be well founded and can account for the observed solution behaviour of hydrocarbons, apolar amino acids and globular proteins.

4. The binding myth of protein denaturation

The marginal stability of native proteins is easily upset, or enhanced, by a variety of physical or chemical means. Urea and guanidinium hydrochloride (GuHCl) are favourite denaturing agents. Polyhydroxy compounds (PHCs), on the other hand, but also $(GuH)_2SO_4$, act in the opposite sense, i.e. they are known to stabilise native structures. For many years, the denaturation potential of urea was ascribed to its binding to the peptide backbone. Even today, this mechanism is still invoked and hypothetical binding constants are calculated from denaturation isotherms. Such interpretations are, however, not supported by any *direct* evidence in support of binding. A more realistic mechanism was proposed but now lies buried in the 'old literature.' It relies on the observed physical properties of urea–water mixtures. The stability of a native protein relies to a large extent on the hydrophobic effect which, in turn, arises from the peculiar features of the so-called 'water structure.' Hydrogen bonding patterns in ice and water rely on the sp^3 hybridised orbitals of the water molecule and its ability to participate in four such interactions, two by accepting, and two by donating protons. Urea is also a molecule capable of participating in multiple hydrogen bonding, via the carbonyl $>C{=}O$ and the two $-NH_2$ groups. It is, however, a planar molecule and any hydrogen bonding topology is incompatible with the 4-coordinated tetrahedral arrangement of water molecules. Energetically, on the other hand, urea–water hydrogen bonds closely resemble those between water molecules, i.e. the enthalpy of mixing is close to zero. Despite earlier claims for urea aggregation or preferential water–

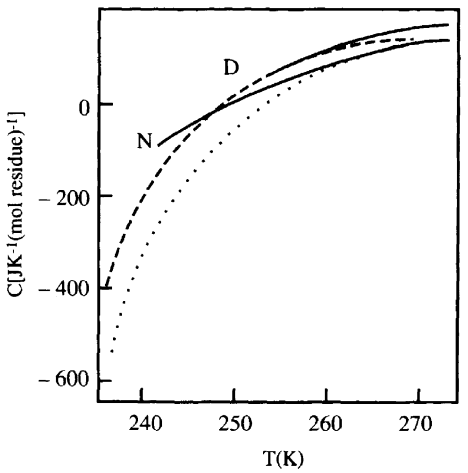

Fig. 7. Heat capacities per mol amino acid residue of native (N) and denatured (D) chymotrypsinogen in undercooled aqueous solution. Broken and dotted curves refer to PVP data at 20 and 10 mol residue concentrations, respectively. The cold denaturation T_L is indicated. Reproduced, with alterations, from [25].

urea interactions, no evidence for such effects could be found. On the contrary, the rotational tumbling times of ^{14}N-enriched urea in an aqueous solution are remarkably 'normal' [17]. The fast rotation, increasing monotonically with urea concentration (viscosity), is evidence against specific effects of larger urea oligomers. All the experimental findings favour ideal mixing of urea and water. Configurationally, therefore, urea appears to destroy the favoured tetrahedral arrangement of water molecules. Urea thus robs water of the single most important feature that promotes the stability of native proteins. The urea molecule has therefore been described as a 'statistical water structure breaker.' The consequences of this type of structural perturbation, as regards the weakening of hydrophobic interactions have been described by a consideration of the thermodynamics of ternary water–urea–hydrocarbon mixtures. The above interpretation is fully compatible with the observed solubilisng and denaturing effects of urea. The involvement of hydrophobic, rather than binding, phenomena are demonstrated by similar influences of urea on hydrocarbon solubility, where binding can hardly be invoked [18].

5. Water, temperature and protein stability

Evolutionally, protein architecture developed so that the diverse functions of these remarkable polymers could be optimally exercised in vivo within the physiological temperature range of a host organism's habitat. The slender stability margins ensure that proteins maintain enough flexibility to fulfil their diverse roles. Since water existed on this planet long before the evolution of proteins, it is likely that any process that affects the structure or other physical properties of water will also influence the structural and functional integrity of a native protein. The multitude of thermal denaturation studies bears witness to the strong coupling of the intermolecular energetics between water and proteins.

Perhaps, for reasons of experimental convenience, heat denaturation still receives an inordinately large amount of attention. Yet, it is hardly surprising that the slender stability margin of a native protein in solution is easily upset by a moderate increase in its kinetic energy and this is indeed observed. Under optimum conditions of pH, denaturation temperatures of small globular proteins lie in the range of 50–65 °C. John Brandts first commented on the apparent parabolic shape of the $\Delta G(T)$ profile of chymotrypsinogen, as shown in Fig. 3, and speculated about the possibility of a 'cold denaturation' phenomenon [19]. Cold inactivation as a real process was subsequently demonstrated by Hatley and Franks, based on experiments performed in undercooled water. They also found that the actual cold denaturation temperature $T_{L,}$ also shown in Fig. 3, lies close to the value estimated from an extrapolation of Brandts' data at 'ordinary' temperatures [20].

The view has been expressed for many years that denaturation is of necessity characterised by a positive change in the heat capacity ΔC of the solution. For an order→disorder transition this seems a reasonable assumption. It has also been repeatedly claimed that ΔC is constant and independent of temperature. The solution thermodynamics of lactate dehydrogenase (LDH), shown in Fig. 4, and covering an extended temperature range, are probably the most reliable data available for a complete denaturation and/or dissociation

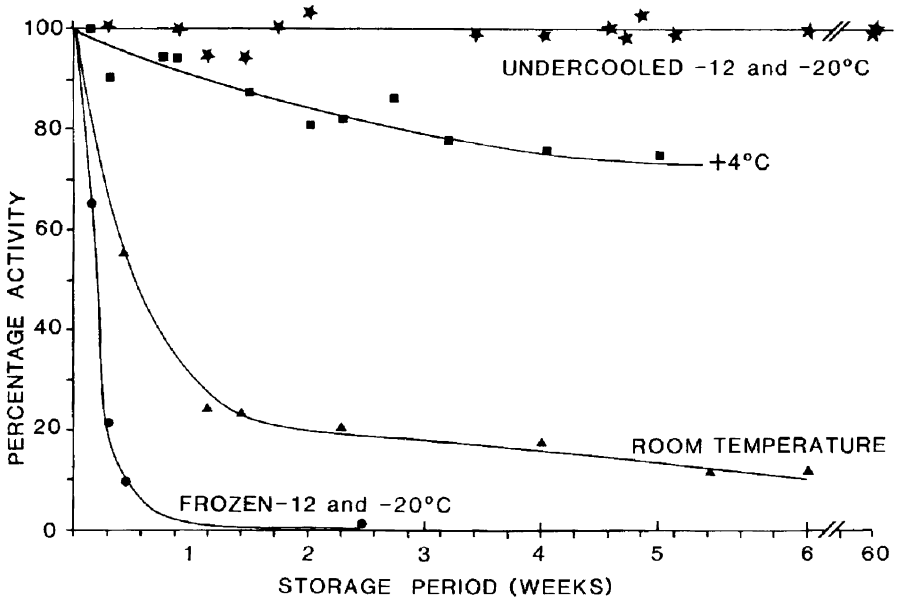

Fig. 8. Retained enzyme activity of lactate dehydrogenase (10 μg ml^{-1} in phosphate buffer, pH 7), stored under different conditions. Assays performed over a storage period of 6 years in undercooled solution at −20 °C (unpublished) indicated a 100% retention of enzyme activity. Reproduced, with permission, from [26].

profile of a native globular protein [21]. In the case of glyceraldehyde-3-phosphate dehydrogenase, the mechanism has unambiguously been shown to involve a fully reversible dissociation of the tetrameric enzyme into its four constituent subunits, see Fig. 5; [22]. Unpublished LDH data suggest a similar mechanism. In Fig. 5, $\Delta C(T)$ is seen to exhibit a very definite trend, i.e. *not* constant, and also a very definite non-linearity. It should here be emphasised that the experiments described in Fig. 4 were performed under optimum pH conditions (measured at 25 °C) and that for subzero temperatures the process was studied in undercooled, (i.e. unfrozen) water, in the absence of lyoprotectants. In other words, temperature was the only perturbant of the native protein in a homogeneous solution.

Previous heat capacity studies of a model polymer, polyvinyl pyrrolidone (PVP), in undercooled water had revealed two striking results [23]: with decreasing temperature, the apparent heat capacity ϕ_C changes sign and assumes large negative values, and it also assumes an increasing concentration dependence, which is not observed at 'ordinary' temperatures. Fig. 6 shows that, on approaching −50 °C, ϕ_C tends to −600 J mol^{-1}, a magnitude characteristic of the strongly hydrated SO_4^{2-} ion, in solution at 25 °C [24]. In the terminology of polymer science, undercooled water can therefore be classified as a 'good' solvent. These results are compatible with the phenomenon of lower critical demixing, as observed for many organic molecules containing both polar and apolar groups, e.g. tetrahydrofuran, pyridine, polyethylene glycol and polyvinyl alcohol.

The PVP studies were later extended to chymotrypsinogen, which made possible the independent determination of the heat capacities of the native and denatured states. The results are shown in Fig. 7[25]. The qualitative similarities with PVP are interesting, but the apparent convergence or intersection of the two curves is, if substantiated by further experiments, of fundamental significance. A revision of currently held views on denaturation mechanisms is overdue and any such revised interpretation must in due course be able

to account for the observed low temperature behaviour of proteins.

On a qualitative basis, the temperature dependence of protein stability can be explained by the competing influences of enthalpically and entropically driven interactions, and their respective temperature dependences. The upper and lower critical demixing phenomena (closed solubility loops) observed for simpler molecules serve as good models. Mechanistically, cold denaturation arises from the progressive weakening of hydrophobic interactions and the simultaneous strengthening of intrapeptide, and direct hydration interactions between water and polar and ionised sites. The slender stability balance of approximately 50 kJ mol^{-1} is thus easily upset. Cold denaturation is completely reversible, even at high protein concentrations, i.e. unlike heat denaturation, it does not give rise to random aggregation of apolar sites or random *S-S* rearrangements [26].

In conclusion, it is to be emphasised that cold inactivation in undercooled water is not just a scientific curiosity but plays an important role in the natural cold survival of many species. It is also of immediate practical use as a means of achieving long-term storage stability of labile molecules in aqueous solution. The contrast with the irreversibly damaging effects of freezing is strikingly illustrated in Fig. 8 for LDH in dilute solution [27].

6. Conclusions and advice

Although protein science is a relative newcomer to the scientific scene, the discipline has generated a large volume of literature. The realisation that protein structures and functions are strongly coupled to the unique features of 'water structure' was slow in coming and now lies buried in the 'old literature.' Any newcomer to the field should consider carefully whether a recently published article actually constitutes an advance of our understanding, or whether, at best, it rehashes earlier work or, at worst, actually obscures older insights into complex problems. To be able to make such a judgement requires a familiarity with the highlights of 'old literature.' There can be no excuse for anybody claiming to study protein stability not to have read, and struggled with, the Kauzmann article [2]. It is as important and relevant today as it was in 1959; perhaps it is even more important today.

For reasons that are fairly obvious, the current trend to rush into print is a sign of the times, especially so in the life sciences. Even so, it takes a while for the new literature to prove its worth. It is well to distrust the value of scientific articles, published today, that include only citations going back 2 years. It is also well to reflect on the proportions of medical breakthroughs, reported every week in the popular newspapers, which actually see the light of day; most of them will be buried and never heard of again.

In this article, I have tried to demonstrate the truth of *Ecclesiastes i.2*: 'There is no new thing under the sun.' The wisdom also pertains to protein science, as illustrated by the 'Old Literature'. A brief survey of the Science indices will soon enable the newcomer to differentiate between those who may once have had their day of glory, but have since been forgotten, and those who have left their enduring mark on this exciting and still growing discipline.

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