Cold Denaturation of Proteins

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ABSTRACT

This article summarizes all experimental facts concerning the cold denaturation of single-domain, multi-domain, and multimeric globular proteins in aqueous solutions with and without urea and guanidine hydrochloride. The facts obtained by various experimental techniques are analyzed thermodynamically and it is shown that the cold denaturation is a general phenomenon caused by the very specific and strongly termperature-dependent interaction of protein nonpolar groups with water. Hydration of these groups, in contrast to expectations, is favorable thermodynamically, i.e., the Gibbs energy of hydration is negative and increases in magnitude at a temperature decrease. As a result, the polypeptide chain, tightly packed in a compact native structure, unfolds at a sufficiently low temperature, exposing internal nonpolar groups to water. The reevaluation of the hydration effect on the base of direct calorimetric studies of protein denaturation and of transfer of non-polar compounds into water leads to revision of the conventional conception on the mechanism of hydrophobic interaction. The last appears to be a complex effect in which the positive contributor is van der Waals interactions between the nonpolar groups and not the hydration of these groups as it was usually supposed.

I. INTRODUCTION

The denaturation of protein upon heating has never been considered as surprising. It always seemed to be a natural phenomenon, even when nothing was known about protein structure, and was only supposed that the structure should be rather sophisticated to fulfill various very delicate functions. According to Le Chatellier's principle, any process which is induced by increasing temperature, should proceed with heat absorption and thus with an increase in the enthalpy and entropy, i.e., with disordering of the considered system. Therefore, disruption of the native protein structure upon heating, the heat denaturation of protein appeared to be an obvious effect. By the same argument, a decrease of temperature should only induce processes leading to increasing order and, since a protein in its native state is believed to be the most ordered, one cannot expect that cooling could lead to any significant change of protein structure and, the more so, to its disruption. Nevertheless, this is just what is found experimentally with many proteins. Cold denaturation appears now as a surprising, but very general property of globular proteins. This paradox has raised great interest. Without solving it one could hardly hope to progress in understanding the mechanism of folding and stabilization of the native protein structures. This interest is also enhanced by the practical importance of cold denaturation as low temperatures are widely used by biochemists in their usual practice.

II. HISTORICAL SURVEY

A. Influence of Temperature on Protein Denaturation by Urea or GuHCl

Interest in the influence of low temperature on the stability of native proteins probably began with a paper published by Hopkins in 1930 in *Nature*.[1] Studying precipitation of ovalbumin, denatured by concentrated urea at various temperatures, he noticed that the rate of denaturation was higher at 0°C than at 23°C. The negative influence of temperature upon the rate was surprising since it had already been established that the rate of heat denaturation of a protein in aqueous solution is characterized by a positive and large temperature coefficient. This finding of Hopkins was confirmed later by Clark,[2] who reported that the temperature coefficient of the rate of ovalbumin denaturation is negative, but only in the presence of a high concentration of urea, while at a low concentration of urea it is positive. Therefore, a decrease in the stability of ovalbumin at low temperature appeared as a specific effect of urea on the protein.

The influence of temperature on urea denaturation of ovalbumin was studied in detail by Simpson and Kauzmann,[3] who used optical rotation for monitoring the process. They found that the temperature coefficient of ovalbumin denaturation rate by urea is negative only at temperatures below 20°C and at high temperatures it is positive, i.e., the rate of denaturation of ovalbumin by urea is minimal at 20°C which means that at this temperature ovalbumin is most stable to urea denaturation.

A similar situation was observed on β-lactoglobulin by Jacobsen and Christensen.[4,5] According to these authors, the rate of β-lactoglobulin denaturation in 6 *M* urea solution, as measured by protein precipitation, is maximal at 0°C, decreases to the minimum value upon increasing the temperature to 30°C, and then increases again with a further rise of temperature. This observation was confirmed by Schellman,[6] who studied protein denaturation by optical rotatory dispersion. Equilibrium studies of β-lactoglobulin denaturation in the presence of urea were carried out later by Pace and Tanford.[7] They showed that the stability of β-lactoglobulin is maximal at about 35°C in

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4.5 to 5.5 *M* urea solution and that GuHCl has a similar effect as urea on the stability of this protein (Figure 1).

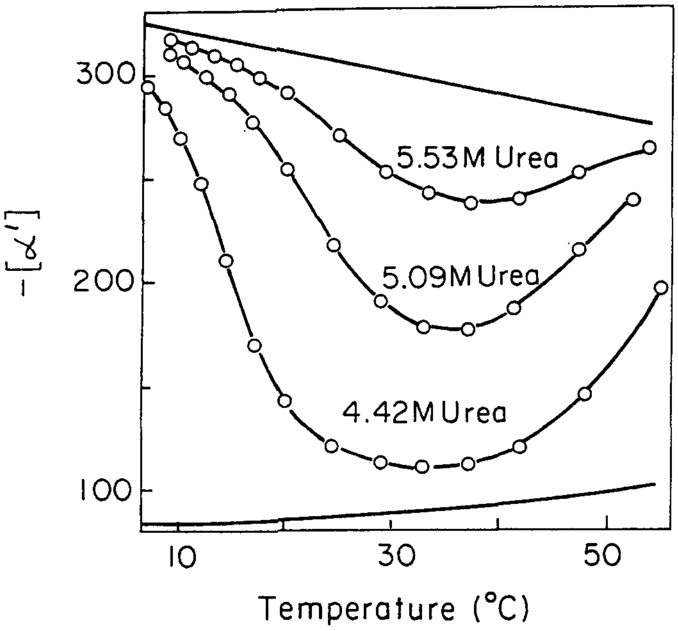

FIGURE 1. Temperature dependence of β-lactoglobulin optical rotation at 365 nm in 0.15 *M* KCl solution, pH 2.5 to 3.5, containing various concentrations of urea (indicated on the curves) according to Pace and Tanford.[7]

The effect of temperature on the GuHCl-induced denaturation was studied extensively on cytochrome c-552 by Nojima et al.[8] They found that this protein is most stable at about 27°C in 5.5 to 6.0 *M* GuHCl solutions in which it denatures not only upon heating from this temperature, but also upon cooling as well (Figure 2). Similar results have been obtained by Chen and Schellman[9] on a mutant of phage T_4 lysozyme in 3 *M* GuHCl solution; the protein was found most stable at about 12.5°C and unfolded at higher and lower temperatures.

Considering the effect of temperature on urea denaturation, one should mention also the early studies of Stanley and Lauffer[10] on tobacco mosaic virus, Deutsch[11] on erythrocyte catalase, Jarabak et al.[12] on hydroxysteroid dehydrogenase, and Feldberg and Datta[13] on threonine deaminase. These proteins, in contrast to the single subunit proteins considered in the preceding paragraph, are multimeric and this complicates somewhat the interpretation of the observed acceleration of their urea denaturation at decreasing temperature.

According to Clark,[2] the degree of experimentally observed precipitation of a protein is the result of two changes caused by urea; the first is the denaturation per se, and the second is the splitting of the molecule into "subparts" which makes it soluble. These two reactions may proceed independently and simultaneously, or the second reaction may follow the first. Both reactions have positive temperature coefficients, but as urea concentration increases, the second reaction is affected more by an increase in temperature, and, consequently, the amount of the precipitated protein decreases with a decrease of temperature. However, this explanation is flawed because not all of the proteins on which the acceleration of urea denaturation at decreasing temperature was observed were multimeric, and moreover, the principal protein that gave the basis for this explanation, namely ovalbumin, was found later to be monomeric and therefore does not split into subparts upon denaturation. Furthermore, similar effects of low temperature were observed where denaturation was detected not by precipitation but by optical rotation.

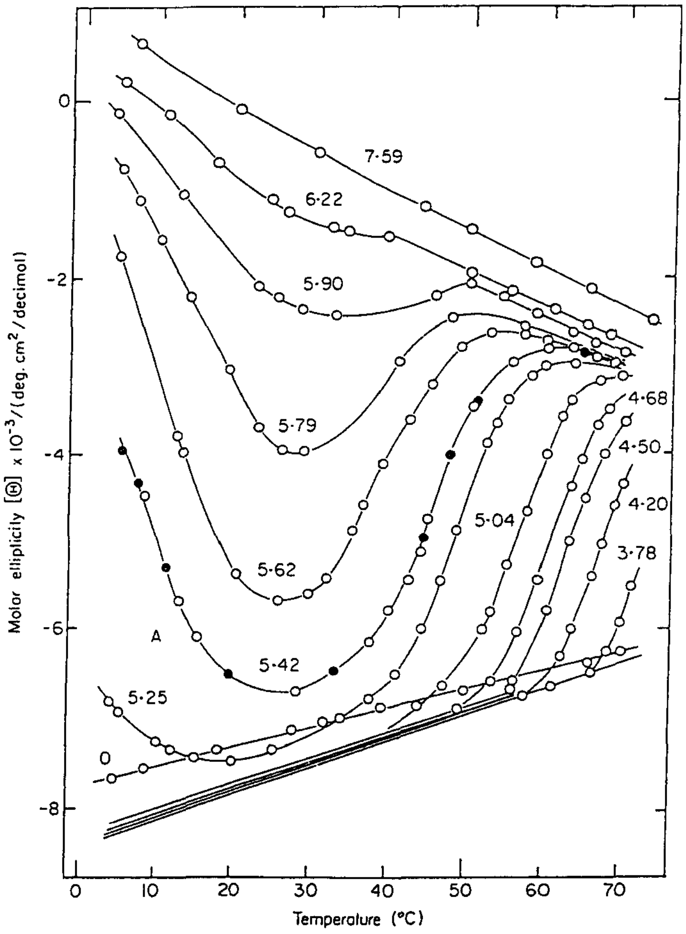

FIGURE 2. Temperature dependence of cytochrome c-552 from *Thermus thermophilus* in 20 m*M* tris-HCl solution, pH 7.5, containing various concentrations of GuHCl (indicated on the curves) according to Nojima et al.[8]

Hopkins[1] explained the apparent negative temperature coefficient of protein denaturation by assuming that the protein denatures in the form of the urea-protein complex:

$$\text{(Native protein)} + \text{urea} \overset{\text{I}}{\rightleftharpoons} \text{(Native protein) urea} \xrightarrow{\text{II}} \text{(denatured protein) urea}$$

If rising temperature shifts the equilibrium I to the left, the temperature coefficient of the total reaction may become negative, even if the rate of the reaction II increases with increasing temperature. Lauffer[14] attempted to give this interpretation a quantitative formulation, but was unable to apply it to his results on tobacco mosaic virus denaturation because of the strong dependence of the rate on pH. This difficulty was overcome by Simpson and Kauzmann,[3] who analyzed their results on ovalbumin urea denaturation and showed that the explanation given by Hopkins for the unusual temperature dependence of the rate could be given quantitative expression. However, they concluded, that there might be a fundamental difference in the heat and urea denaturation, i.e., that an entirely different denaturation mechanism might be involved above and below 20°C.

B. Cold Denaturation of Enzymes in Aqueous Solution

Meanwhile, facts were accumulating which showed that many enzymes in aqueous solution, without urea or GuHCl, were inactivated at low temperature (at about 0°C) but their activity was restored at room temperature. Table 1 summarizes these results, showing that widely practiced isolation and storage of enzymes in the cold is not always justified. It shows also that most, if not all, of the listed enzymes are multimeric and it is likely that their reversible cold inactivation is associated with their reversible dissociation into subunits.

It was found then that cooling leads to dissociation of various supramolecular structures, such as collagen fibriles[70-73] or microtubules,[74-77] which reassociate upon incubation at physiological temperatures.

It is likely that disruption of quaternary and supramolecular structures of proteins at low temperature occurs without a significant change of the tertiary structure of monomeric subunits. Thus, fatty acid synthetase dissociates into subunits upon cooling, but the subunits do not unfold as judged by invariable titration of their SH-groups.[34] Therefore, the observed temperature effects, displayed by multimeric enzymes and supramolecular structures, could hardly be classified as cold denaturation. On the other hand, the results show that a decrease in temperature leads to a decrease of the interaction between monomeric subunits or to the appearance of forces, which destabilize the molecular structure in aqueous solution, not containing urea of GuHCl. The question arises: what are these forces and why don't they destabilize the tertiary structure of proteins? Is this situation due to the tertiary structure being much more stable than the quaternary one and therefore its disruption on cooling could proceed only in the presence of urea or GuHCl, or at much lower temperatures than those used in the experiments on cold inactivation of enzymes?

C. Hypotheses and Their Experimental Verification

The most simple explanation of the observed cold lability

Table 1
Cold Inactivation of Enzymes

Enzyme	Source	Comments	Ref.
Acetyl-coenzyme-A hydrolase	Rat liver	Dimer at 37°C; inactivates reversibly upon cooling dissociating in monomers	15,16
Acetyl-coenzyme-A carboxylase	Rat epididymal adipose tissue	Inactivates reversibly dissociating in subunits upon cooling	17
Acetyle-coenzyme-A carboxylase	Rat epididymal adipose tissue	Inactivates reversibly dissociating in subunits upon cooling	18
Argininosuccinase	Steer liver	Inactivates reversibly dissociating in subunits upon cooling	19—21
Argininosuccinase	Bovine liver	Inactivates reversibly dissociating in subunits upon cooling	22
Aspartic β-semialdehyde dehydrogenase	Yeast	Inactivates reversibly dissociating in two dimers upon cooling to 0°C	23
ATPase	Beef heart mitochondria	Inactivates reversibly dissociating in subunits upon cooling to 0°C	24
ATPase	Rat liver mitochondria	Inactivates reversibly dissociating in subunits upon cooling to 0°C	25
ATPase	Chloroplast	Inactivates reversibly dissociating in subunits upon cooling to 0°C	26

Table 1 (continued)
Cold Inactivation of Enzymes

Enzyme	Source	Comments	Ref.
ATPase	*Escherichia coli*	Inactivates reversibly dissociating in subunits upon cooling to 0°C	27
F_1-ATPase	Pea cotyledon mitochondria	Inactivates reversibly dissociating in subunits upon cooling to 0°C	28
ATPase	Beef heart mitochondria	Inactivates reversibly dissociating in subunits upon cooling to 0°C	29
Carbamoylphosphate synthase	Rat liver	Inactivates reversibly dissociating in two subunits upon cooling	30
Carbamylphosphate synthetase	Frog	Inactivates at 4°C; active at 30°C	31
Fatty acid synthetase	Pigeon liver	Inactivates reversibly dissociating in two subunits upon cooling to 0°C	32,33
Fatty acid synthetase	Rat mammary gland	Inactivates reversibly dissociating in two subunits upon cooling to 0°C	34
D-Fructose 1,6-diphosphatase	*Candida utilis*	Dimer at 22°C; inactivates reversibly in monomers upon cooling to 4°C	35
Glucose 6-phosphate dehydrogenase	Human erythrocytes	Dimer at 37°C; inactivates reversibly dissociating in two subunits upon cooling to 0°C	36
Glucose 6-phosphate dehydrogenase	Mammary gland	Dimer at 37°C; inactivates reversibly dissociating in two subunits upon cooling to 0°C	37
Glutamate decarboxylase	*Escherichia coli*	Inactivates at 0°C more rapidly than at 25°C	38
Glutamate dehydrogenase	*Neurospora crassa*	Inactivates reversibly upon cooling without dissociation in hexamers	39
Glutamate dehydrogenase	*Candida utilis*	Inactivates reversibly upon cooling without dissociation in hexamers	40
Glutaminase B	*Escherichia coli*	Inactivates reversibly without any change of molecular weight	41
Glyceraldehyde-3-phosphate dehydrogenase	Rabbit skeletal muscle	Inactivates reversibly upon cooling to 0°C; dissociates in monomers	42
Glyceraldehyde-3-phosphate dehydrogenase	Rat skeletal muscle	Inactivates reversibly upon cooling to 0°C; dissociates in monomers	43
Glyceraldehyde-3-phosphate dehydrogenase	Yeast	Inactivates reversibly upon cooling to 0°C; dissociates in monomers	44
Glycogen phosphorylase B	Rabbit muscle	Inactivates reversibly upon cooling to 0°C; dissociates in monomers	45
Glycogen synthetase B	Rat liver	Inactivates reversibly dissociating in monomers upon cooling to 0°C	46
Homoserine dehydrogenase	*Escherichia coli*	Inactivates reversibly dissociating in subunits upon cooling	47

Table 1 (continued)
Cold Inactivation of Enzymes

Enzyme	Source	Comments	Ref.
D(−)-β-Hydroxybutyrate dehydrogenase	*Rhodospirillum rubrum*	Inactivates reversibly upon cooling to 0°C	48
3-Hydroxy-3-methylglutaryl coenzyme A reductase	Rat liver mitochondria	Dimer at 37°C; inactivates reversibly dissociating in monomers upon cooling	49
Isocytrate dehydrogenase	Bovine heart mitochondria	Inactivates reversibly dissociating in monomers upon cooling to 0°C	50
Nitrogen fixing enzyme	*Clostridium pasteurianum*	Inactivates reversibly upon cooling to 0°C	51
Peroxidase	Turnip	Inactivates reversibly upon cooling to 0°C	52
Phosphatase	Bovine intestine	Inactivates reversibly upon cooling to 0°C	52
Phosphofructokinase	Rabbit muscle	Inactivates reversibly dissociating in monomers upon cooling to 0°C	53—55
Phosphofructokinase	Chicken liver	Inactivates reversibly dissociating in monomers upon cooling to 0°C	56
Phosphofructokinase	*Bacillus licheniformis*	Inactivates reversibly dissociating in two subunits upon cooling to 0°C	57
Pyruvate carboxylase	Chicken liver mitochondria	Inactivates reversibly dissociating in monomers upon cooling to 0°C	58
Pyruvate carboxylase	Rat liver	Inactivates reversibly dissociating in monomers upon cooling to 0°C	59
Pyruvate carboxylase	*Arthrobacter globiformis*	Inactivates reversibly dissociating in monomers upon cooling to 0°C	60
Pyruvate carboxylase	Sheep kidney	Inactivates reversibly dissociating in monomers upon cooling to 0°C	61
Pyruvate carboxylase	Chicken liver mitochondria		62,63
Pyruvate kinase	Yeast	Inactivates reversibly dissociating in dimers upon cooling to 0°C	64
Pyruvate kinase	Rabbit muscle	Inactivates reversibly dissociating in dimers upon cooling to 0°C	65
Pyruvate kinase	Ehrlich ascite tumor	Inactivates reversibly dissociating in dimers upon cooling to 0°C	66
Ribulose phosphate carboxylase	Tobacco leaves	Inactivates reversibly upon cooling to 0°C without changing the molecular weight	67
Tryptophanase	*Escherichia coli*	Inactivates reversibly dissociating in monomers upon cooling to 5°C	68
Threonine deaminase	*Rhodospirillum rubrum*	Inactivates reversibly dissociating in dimers at 0°C	13
Urease	Jack bean		69
17-β-hydroxy-steroid dehydrogenase	Human placenta	Inactivates reversibly dissociating in subunits, probably with their unfolding, upon cooling below 10°C	12

of enzymes in aqueous solutions, not containing denaturants, is based on the assumption that it is caused by the ionization of some essential groups of protein molecules with decreasing temperature.[53,78] Since the enthalpy of protonation of the protein groups is negative and for some of the groups is rather large (e.g., for histidine it is -28.8 kJ mol^{-1} and for the α-amino group -43.8 kJ mol^{-1}; see, e.g., References 79 and 80), one can expect that at decreasing temperatures the protonation of these groups increases. This, in principle, could not only switch off the active site in an enzyme, but could also induce dissociation of the subunits, if the enzyme is multimeric, or even unfold the compact structure of a globular molecule, if the number of charges provided by cooling would be sufficient. The temperature region, in which these changes could occur, depends on the enthalpy of ionization. However, using the van't Hoff equation, one can easily calculate that even 43.8 kJ mol^{-1} is not a sufficient value to explain the observed sharpness of the process by the ionization of individual groups. On the other hand, if one supposes that the ionizable groups cooperate, it is necessary to suggest the mechanism of their cooperation. It is even more difficult to explain why the enthalpies of the cold and heat denaturation have the opposite sign. Is this because these two processes are absolutely different?

Much wider popularity has been gained by another explanation of cold-induced changes in a protein based on the very unusual feature of hydrophobic interactions. These are the forces postulated to explain the low solubility of nonpolar substances in water, and their tendency to associate into clusters. Since there are many nonpolar groups in proteins and most of them are not exposed to water in the native protein, one can suppose that hydrophobic forces are important for stabilization of compact protein structure and for assembly of subunits in multimeric proteins.

Studies on the dissolution of nonpolar hydrophobic substances in water at room temperature show that the large increase in Gibbs energy, required for their transfer into water, originates from the large decrease in entropy due to the ordering of water around the nonpolar molecule.[81,82] The repelling action of water, as a consequence of the ordering around a nonpolar solute, was imagined as a hydrophobic force or as a hydrophobic interaction between the associated nonpolar molecules.[83] However, being entropy driven, these interactions should decrease with decreasing temperature, as the entropy contribution to the Gibbs energy, $T\Delta S$, is proportional to the absolute temperature.[83,84] This might explain the dissociation of multimeric enzymes into subunits and disruption of a compact structure of globular proteins as the temperature is decreased (see, e.g., Feldberg and Datta[13]).

However, the above conclusion on the temperature dependence of hydrophobic interactions is too simplified since it is based on the assumption that the enthalpy and entropy of transfer of a nonpolar substance to water do not depend on temperature. However, it is known from earlier studies on dissolving such substances in water that this process is accompanied by a surprisingly large heat capacity increment, i.e., the enthalpy and entropy of transfer of nonpolar molecules to water are strongly temperature dependent.[85,86] This circumstance must be taken into account when evaluating the enthalpy/entropy balance in the Gibbs energy function and determining the hydrophobic force.

One should expect that protein denaturation, which consists of disruption of a compact structure and results in the exposure of the internal nonpolar groups to water, should be accompanied also by a significant heat capacity increment and, therefore, the enthalpy and entropy of protein denaturation should be strongly dependent on temperature. This was found indeed from the calorimetric titration of serum albumin[87] and ferrihemoglobin,[88] carried out at different temperatures. They showed that the enthalpy of denaturation increases with increasing temperature, as if denaturation proceeds with a heat capacity increment of 25 kJ K^{-1}mol^{-1} for both proteins.

The denaturation heat capacity increment was measured directly in the first study of heat denaturation of ovalbumin by an absolute heat capacity calorimeter.[89] It was found that the partial molar heat capacity of this protein increases upon denaturation by 20 kJ K^{-1}mol^{-1} and the denaturational enthalpy decreases with a decrease of denaturation temperature induced by pH variation.

A similar conclusion was reached in analyzing the asymmetry in the optically observed melting profiles of chymotrypsinogen[90,91] and ribonuclease A by Brandts with co-workers.[92-94] Assuming that denaturation of these proteins is a two-state transition and treating it by the van't Hoff equation, they found that the enthalpy of denaturation should be supposed to be temperature dependent, as if denaturation is associated with a heat capacity increment. If the heat capacity increment does not depend on temperature, then the Gibbs energy difference between the native and denatured states should be expressed by a parabolic function of temperature with the extremum at about 10 and 0°C for chymotrypsinogen and ribonuclease, respectively. Thus, at these temperatures, the considered proteins should be expected to be most stable and their stability should decrease at lower temperatures. Then one can imagine that, upon cooling, these proteins might denature at a low enough temperature, releasing heat and decreasing their entropy.

The conclusion that protein denaturation, which is usually associated with disordering of the structure, could proceed with an entropy decrease, i.e., an increase of order, seemed to be a paradox and induced great doubt. It was based on a too extensive extrapolation of the thermodynamic properties of protein, which in themselves required a serious substantiation; at that time it was not evident that the protein molecule could be considered as a macroscopic system, that this system has only two macroscopic states, and that denaturation is a transition between these states. A certain scepticism concerning the applicability of thermodynamics for proteins in general could be overcome only by the demonstration of its predictive ability and, namely, by direct demonstration that (1) proteins in aqueous solutions in the absence of denaturants can indeed denature upon cooling, and (2) the thermodynamic characteristics of cold denaturation are close to those expected from the heat denaturation studies. Therefore, the studies of cold denaturation have become of paramount importance for understanding the general principles of protein structure.

However, progress in this field was not particularly easy and fast since, according to the prediction, cold denaturation of all known proteins in pure aqueous solutions should occur at temperatures below the freezing point of water. It was somewhat easier to show that the stability of protein decreases indeed as the temperature decreases, even in the absence of urea or GuHCl in solution. The only problem was to choose a proper test for the stability. One such test was pressure.

Hawley,[95] in his study of chymotrypsinogen denaturation by pressure at various temperatures, found that its stability against pressure decreases at a temperature decrease below room temperature. A similar study was carried out by Zipp and Kauzmann[96] on metmyoglobin at different pHs, varying the pressure from 1 to 6000 atm and the temperature from 5 to 80°C. The combination of pressure, temperature, and pH for the midpoint of the denaturation transition, which was observed optically by light absorption, led to a surface in (p, T, pH) space, from which it was clear that protein stability against pressure was maximal at room temperature and decreased both on cooling or heating (Figure 3). However, they could not observe cold denaturation at normal pressure since it should occur at temperatures below 5°C, and that was the lower limit in their study.

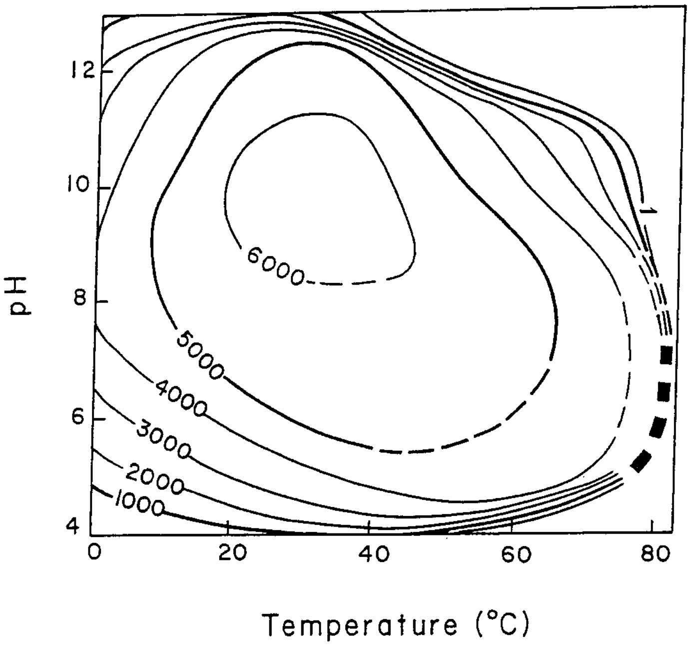

FIGURE 3. Contours of the constant pressure in the pH-temperature plane at which $\Delta_N^D G = 0$ for denaturation of metmyoglobin. The native state is more stable than the denatured one inside each contour.[96]

All attempts to find an antifreeze which would depress efficiently the freezing point of water without changing the thermodynamic properties of a protein in solution, i.e., could provide an ideal "cryosolvent" for proteins (see, e.g., Reference 97), were unsuccessful. According to our experience, alcohols and glycerol depress the temperature of cold denaturation as well and do not help in its observation, but only complicate the situation. The protective influence of glycerol and other polyhydroxylic compounds against the cold inactivation of enzymes has been observed by many authors.[12,98-100] According to Graves et al.,[45] addition of 10% methanol, propylenglycol, or dimethylsulfoxide protects phosphorylase β against cold inactivation completely. Hatley and Franks,[101] using a solution of 40% aqueous methanol as a solvent, observed significant changes in the optical properties of lactate dehydrogenase in the temperature range from − 10 to − 20°C upon cooling the solution, which appeared as denaturation. However, according to King and Weber,[102] lactate dehydrogenase in a methanol-free aqueous solution inactivates reversibly at 4°C. It is unclear whether this wide difference found in temperatures is caused by the presence of methanol or, in these two cases, are considered the disruptions of two different levels of organization of this multimeric protein — of its quaternary and tertiary structures. In any case, the presence of methanol in the solution raises the same problems as the presence of urea or GuHCl since one should discriminate between the contribution of water solvation and of the second component solvation by protein upon its cooling. An attempt has been made to depress the freezing point of water by high pressure.[103] At 1370 atm, water freezes at − 14°C. Under this pressure, the denaturation of chymotrypsinogen was observed optically upon cooling below 20°C. However, the high temperature per se influences the stability of protein. Therefore, we face here the same problem as in the case of using antifreezes.

In fact, there are only two possibilities for an unequivocal direct observation of cold denaturation of protein in a cooled aqueous solution: either to find protein and solvent conditions where cold denaturation takes place above 0°C, or to work with supercooled solutions.

Aqueous solutions of proteins can be easily supercooled to a rather low temperature, but the lower the temperature, the higher the probability of the spontaneous freezing of the solution, which can damage the experimental device. To extend the cooling range, Franks and Hatley[104] studied an aqueous protein solution emulsified in oil. In such a system, droplets of the solution, enclosed in micelles, are isolated and cannot be all involved into the freezing process, if it is initiated in some of droplets. Therefore, the emulsified solution can be supercooled to a very low temperature. However, the turbidity of the emulsion would appear to present a serious obstacle to optical observations of changes occurring with the proteins within the micelles, and consequently in the quantitative treatment of the obtained results. In their cooling and heating experiments with chymotrypsinogen, Franks and Hatley[104] observed some changes in light absorption at −12 and 0°C, respectively, which they interpreted as due to denaturation and renaturation of the protein. The largest hysteresis in the temperature profiles of the changes observed upon cooling and heating indicated that the temperature-induced process is far from equilibrium. Therefore, one can hardly gain from this experiment any quantitative thermodynamic information specifying the process.

The experimental complications of using emulsions for supercooling protein solutions prevent their wide use in studying the cold denaturation of proteins. A more practical solution has been found by supercooling dust-free solutions. After thorough centrifugation, an aqueous solution of a protein can be supercooled down to at least −10°C. This allows one to observe cold denaturation on some globular proteins with properly chosen solvent conditions, using various physical methods, not only optical ones, for the observation of the process.[105] For studying the thermodynamics of cold denaturation, scanning microcalorimetry has been found to be of paramount importance since it provides direct information on the functional dependence of the two basic conjugate thermodynamic variables, the enthalpy and temperature without which one cannot describe a temperature-induced process.

III. THERMODYNAMIC DESCRIPTION OF A PROTEIN

A. Temperature-Induced Changes

A protein molecule, consisting of many thousands of atoms, represents a macroscopic system, and as such it should be specified thermodynamically. A thermodynamic description is the most general physical specification of any macroscopic system, without which one cannot judge its stability and behavior when the external conditions are varied.

A macroscopic object is specified thermodynamically if the functional dependences between its basic extensive thermodynamic parameters and their conjugate intensive variables are known, i.e., the dependences between the volume and pressure, the enthalpy and temperature, the amount of bound ligands and ligand concentration, etc.[106] The particular importance of any of the above-mentioned conjugate pairs depends on the considered process, i.e., on the variable, inducing the process. For example, if one is interested in processes that are induced by temperature variation, it is necessary, first of all, to know the dependence of the enthalpy on temperature, as this function includes all the thermodynamic information on the macroscopic states of the system, which are realized in the considered temperature range.[106-108] If these states are known and specified, then, by studying their equilibrium within a given temperature range, one can deduce the enthalpy function of the system. However, if nothing is known on the macroscopic states, then the enthalpy function cannot be found from the equilibrium studies and it can be determined only by direct calorimetric measurements.

In the case of proteins, we face just this situation since usually nothing is known about the macroscopic states of these macromolecules, while any assumption about these states only opens the prospect of speculations. That is why scanning microcalorimetry, which permits direct measurements of the enthalpy dependence on temperature, has become such an important tool in thermodynamic studies of the temperature-induced processes in protein molecules. (For the method, see reviews by Privalov,[109] Privalov and Potekhin,[108] and Privalov and Plotnikov[110]).

Calorimetric studies of the heat denaturation of small monomeric globular proteins and of individual domains of large proteins have led to the establishment of the two general features of these molecules: (1) their denaturation is an almost all-or-none process, which can be approximated rather well by a two-state transition, and (2) this process results in an essential increase in heat capacity by a value that does not depend significantly either on temperature or on the environmental conditions (Figure 4) and is specific for the given protein.[112,113]

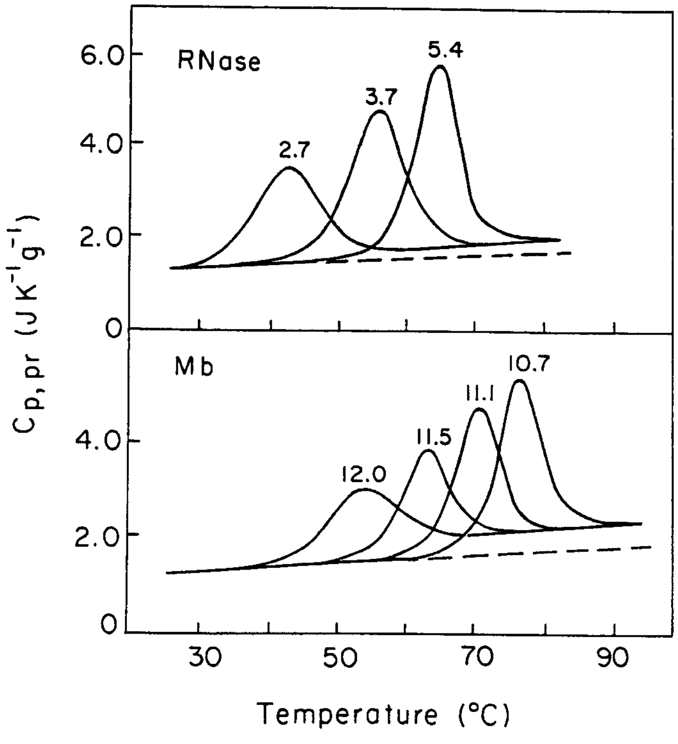

FIGURE 4. Temperature dependence of partial heat capacity of pancreatic ribonuclease A (RNase) and sperm whale metmyoglobin (Mb) in solutions with various pH, indicated on the curves according to Privalov and Khechinashvili.[111]

This leads to the conclusion that a small protein or a single domain of a large protein represents a cooperative system with only two stable macroscopic states, the native (N) and the denatured (D), and that the differences between the thermodynamic parameters of these states are temperature-dependent functions. Indeed, since

$$\frac{\partial \Delta H}{\partial T} = \Delta C_p \quad \text{and} \quad \frac{\partial \Delta S}{\partial T} = \frac{\Delta C_p}{T}$$

we have for the enthalpy and entropy differences between the native and denatured states:

$$\Delta_N^D H(T) = H^D(T) - H^N(T) = \Delta_N^D H(T_G) + \int_{T_G}^{T} \Delta_N^D C_p \, dT \qquad (1)$$

$$\Delta_N^D S(T) = S^D(T) - S^N(T) = \Delta_N^D S(T_G) + \int_{T_G}^{T} \frac{\Delta_N^D C_p}{T} \, dT \qquad (2)$$

where $\Delta_N^D C_p$ is the heat capacity difference between the native and denatured states and T_G is the transition temperature, at which the Gibbs energy difference between these states is equal to zero, i.e.,

$$\Delta_N^D G(T_G) = \Delta_N^D H(T_G) - T_G \Delta_N^D S(T_G) = 0$$

and thus

$$\Delta_N^D S(T_G) = \frac{\Delta_N^D H(T_G)}{T_G} \qquad (3)$$

As is evident,

$$\Delta_N^D H(T_G) \equiv \Delta_d H \quad \text{and} \quad T_G = T_d$$

where $\Delta_d H$ is the enthalpy of protein heat denaturation, which is determined experimentally from the calorimetrically observed denaturation heat absorption peak area and T_d is the temperature of the heat denaturation midpoint which is close to the temperature of the heat absorption peak (for details, see Privalov and Potekhin[108]).

In the temperature range from 20 to 80°C, the denaturation heat capacity increment seems to be temperature independent, i.e., the heat capacities of the native and denatured states appear to change in parallel with a temperature increase.[111] However, more detailed studies, carried out recently over a broader temperature range, showed a definite deviation in the course of these functions (Figure 5); while the heat capacity of the native state increases linearly with temperature, in any case, in the range from 0 to 80°C, where it can actually be measured, the heat capacity of the denatured state is a nonlinear function of temperature in the range from 0 to 140°C.[114] Thus, linear extrapolation of the heat capacity of the native state above 80°C leads to the conclusion that $\Delta_N^D C_p$ should decrease to zero at about 140°C. It is likely that it decreases also at temperatures below 20°C.

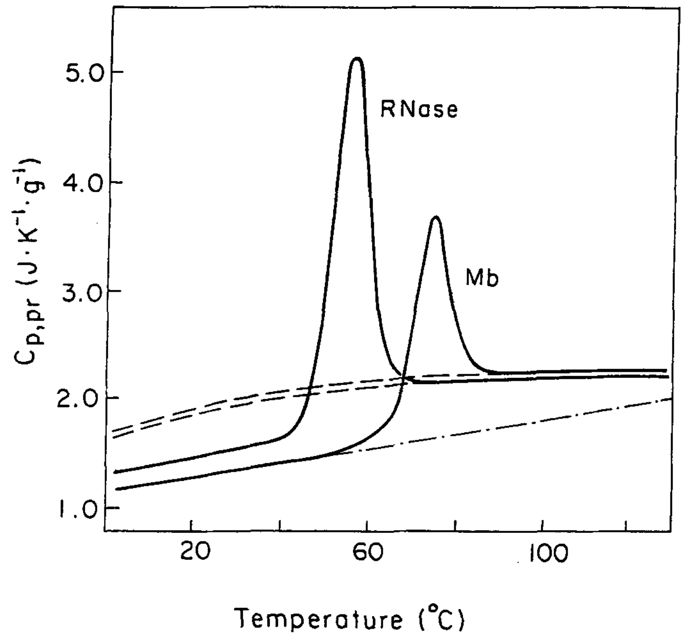

FIGURE 5. Partial heat capacity of ribonuclease A (RNase) and metmyoglobin (Mb) in the temperature range from −5 to 130°C. The dashed line shows heat capacity functions for RNase with the disrupted SS crosslinks and apomyoglobin in an acidic solution (pH 2.5) where the polypeptide chains are completely unfolded.[114]

In the approximation that $\Delta_N^D C_p$ is constant, Equations 1 and 2 can be simplified to

$$\Delta_N^D H(T) \cong \Delta_N^D H(T_G) - (T_G - T)\, \Delta_N^D C_p \qquad (4)$$

$$\Delta_N^D S(T) \cong \frac{\Delta_N^D H(T_G)}{T_G} - \Delta_N^D C_p \ln(T_G/T) \qquad (5)$$

The $\Delta_N^D H$ and $\Delta_N^D S$ functions are presented in Figure 6 for ribonuclease A and myoglobin which differ significantly in $\Delta_N^D C_p$ values. The dot-dash line shows the approximation in which $\Delta_N^D C_p$ is assumed to be temperature independent and equal to the value measured at about 50°C in the denaturation experiment. This approximation is rather good in the temperature range from 0 to 80°C in which the enthalpy difference between the native and denatured states decreases almost linearly with a temperature decrease. So, one can expect that at a sufficiently low temperature, T_H, the enthalpy should become zero and then invert its sign

$$T_H = T_G - \frac{\Delta_N^D H(T_G)}{\Delta C_p} \qquad (6)$$

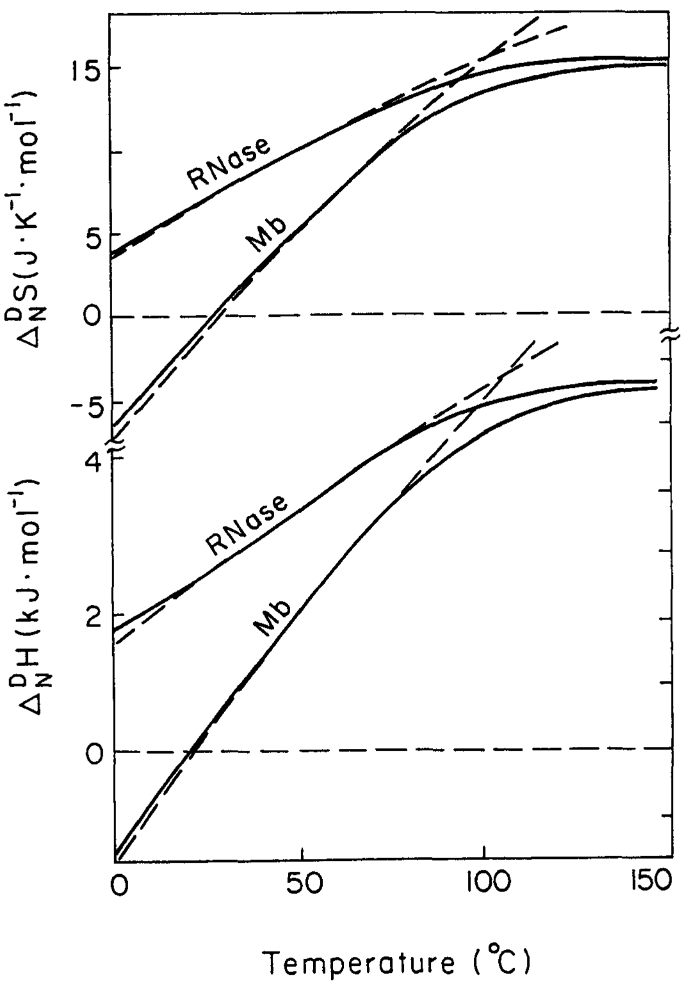

FIGURE 6. The $\Delta_N^D H$ and $\Delta_N^D S$ functions for RNase and Mb at pHs close to neutral, calculated from the assumption that $\Delta_N^D C_p$ is temperature independent (dashed line) and temperature dependent (solid line).[114]

Correspondingly, one can rewrite Equation 4 in the following way

$$\Delta_N^D H(T) = (T - T_H)\Delta_N^D C_p \tag{7}$$

The entropy difference should also decrease with the temperature decrease, but nonlinearly, and reach zero at a somewhat higher temperature T_S than does the enthalpy function[105]

$$T_S \cong \frac{T_G}{\dfrac{\Delta_N^D H(T_G)}{\Delta_N^D C_p \cdot T_G} + 1}$$

$$\cong \frac{T_G^2}{2T_G - T_H} = T_H + \frac{(T_G - T_H)^2}{2T_G - T_H} \tag{8}$$

In contrast to the enthalpy and entropy functions, the Gibbs energy difference, which determines the relative probabilities of the native and denatured states and can be actually considered as a measure of stability of the native protein structure (on this aspect see also Bectel and Schellman[115]) is represented by the function with the extremum

$$\Delta_N^D G(T) = \Delta_N^D H(T) - T\Delta_N^D S(T)$$

$$= \Delta_N^D H(T_G)\,\frac{T_G - T}{T_G} + \int_{T_G}^{T} \Delta_N^D C_p \, dT - T\int_{T_G}^{T} \frac{\Delta_N^D C_p \, dT}{T} \tag{9}$$

or, in the approximation that $\Delta_N^D C_p$ is a constant

$$\Delta_N^D G(T) \cong \Delta_N^D H(T_G)\,\frac{T_G - T}{T_G} + (T - T_G)\Delta_N^D C_p$$

$$- T\Delta_N^D C_p \ln(T/T_G) \tag{10}$$

$$= \left[\frac{T \cdot T_H}{T_G} - T_H + T \ln T_G/T\right] \Delta_N^D C_p$$

As seen from Figure 7, this function is insensitive to the assumption as to whether $\Delta_N^D C_p$ is temperature dependent or temperature independent (for the discussion of various hypothetical dependences of $\Delta_N^D C_p$ on temperature, see Franks et al.[116]).

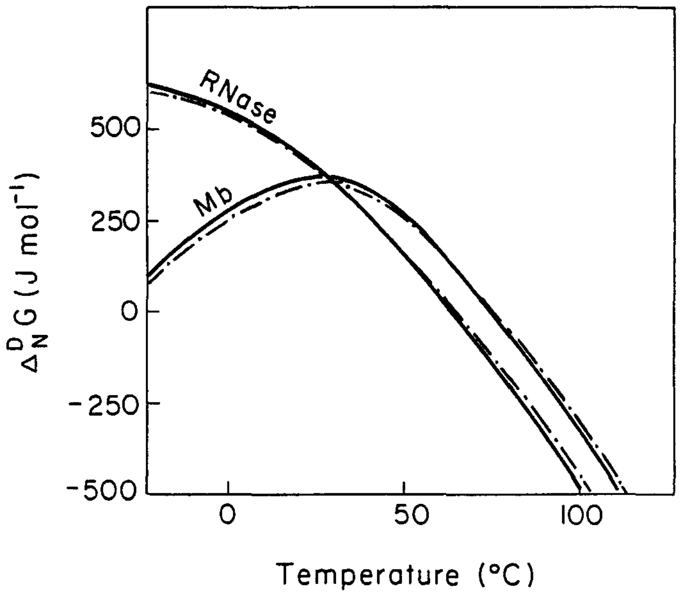

FIGURE 7. The $\Delta_N^D G$ function for RNase and Mb for the same conditions as in Figure 6 calculated from the assumption that $\Delta_N^D C_p$ is temperature independent (dot-dash line) and temperature dependent (dashed line).[114]

The maximum value of this function is reached at a temperature T_{max}, which can be determined from the condition

$$\left(\frac{\partial \Delta_N^D G}{T}\right)_{T_{max}} = -\Delta_N^D S(T_{max}) = 0$$

from which it follows that

$$T_{max} \equiv T_S \tag{11}$$

i.e., the stability of globular protein is maximal at the temperature at which the entropies of the native and denatured states are equal and the structure is stabilized only by the enthalpy difference between these states. At temperatures above and below T_{max}, the protein stability decreases: upon heating, it reaches zero at temperature T_G, and upon cooling, zero is reached at temperature T'_G.[105]

$$T'_G \cong \frac{T_G^2}{2\left[\frac{\Delta_N^D H(T_G)}{\Delta_N^D C_p}\right] + T_G} = \frac{T_G^2}{3T_G - 2T_H} \tag{12}$$

In the vicinity of these two temperatures, one should expect a breakdown of the native protein structure. Its disruption on heating is usually called heat denaturation since it proceeds with heat absorption and, consequently, with increases in the molecular enthalpy and entropy. As for the disruption of the native structure upon cooling, which could be called, by analogy, cold denaturation, it should proceed with the release of heat and, hence, with enthalpy and entropy decreases since these functions reverse their signs at temperatures T_H and T_S, both of which are always between T_G and T'_G.

Assuming that protein denaturation represents a transition between two macroscopic states, i.e.,

$$N \overset{K}{\rightleftharpoons} D$$

where K is the equilibrium constant

$$K = \frac{[N]}{[D]} = \exp(-\Delta_N^D G/RT) \tag{13}$$

one can calculate the excess heat capacity of the protein over the studied temperature range

$$\langle \Delta C_p \rangle = \frac{d\langle \Delta_N^D H \rangle}{dT} = \frac{d(\Delta_N^D HF)}{dT}$$
$$= \Delta_N^D H \frac{dF}{dT} + \Delta_N^D C_p F \tag{14}$$

where $F = K/(1 + K)$ is the fraction of the protein in the denatured state.[108]

The results of computer calculations of the expected excess heat capacity of an abstract globular protein of the size of myoglobin with different stabilities are presented in Figure 8. At the lowest temperature, the protein is supposed to be in the cold denatured state. As the temperature is increased, it should fold, absorbing heat into the compact native structure with a lower heat capacity; then, at a higher temperature, it should denature, again absorbing heat. Thus, if one heats from low temperatures, both the low temperature renaturation and the high temperature denaturation proceed with the absorption of heat and, inversely, if one cools from high temperatures, the renaturation at high temperatures and the denaturation at low temperatures should both proceed with the release of heat. As protein stability is increased (e.g., by varying pH), the high temperature heat absorption peak shifts to higher temperatures, while the low temperature peak shifts to lower temperatures. For practical observation of cold denaturation, however, it is essential that this should occur at temperatures above the freezing of the solution.

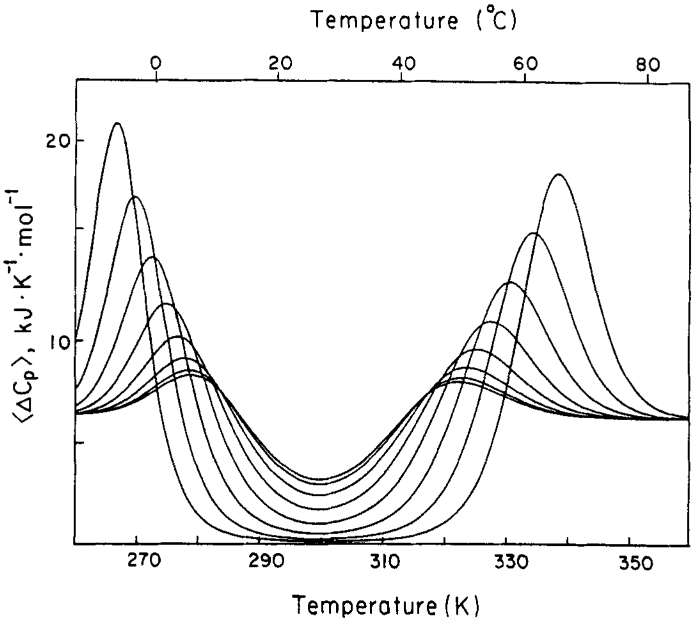

FIGURE 8. Excess heat capacity of an abstract average-size globular protein with variable stability calculated from the assumption that its denaturation represents a two-state transition and that denaturation heat capacity increment is temperature independent and equals 10 kJ $K^{-1}mol^{-1}$.

According to Equation 12, the temperature of cold denaturation, T'_G, is expected to be the largest for proteins with the largest $\Delta_N^D C_p$ and smallest $\Delta_N^D H$ values, i.e., with the highest temperature T_H at which the denaturation enthalpy becomes zero.

Among the calorimetrically studied proteins, myoglobin has the largest $\Delta_N^D C_p$ value[112] and that is why this protein has attracted the greatest attention as a potential object for cold denaturation studies. As for ribonuclease A, it has the smallest $\Delta_N^D C_p$ and this explains the failure of all the attempts to observe its cold denaturation even in the presence of high concentrations of urea.[117]

B. Influence of pH and Buffer

Since there are many ionizable groups in a protein, its stability and temperature of denaturation should greatly depend on pH. This is illustrated in Figure 9 for metmyoglobin. The dependence of T_G on pH is determined by the number of groups $\Delta_N^D\gamma$ ionized upon denaturation[118]

$$\Delta_N^D\gamma = -\frac{\Delta_N^D H(T_d)}{2.3\ T^2} \cdot \frac{dT_G}{dpH} \qquad (15)$$

Therefore, the greater dependence of myoglobin stability on pH in acidic solutions, shown in Figure 9, means that protein denaturation is accompanied by the ionization of more groups in acidic solutions than in alkaline ones.[105,119,120]

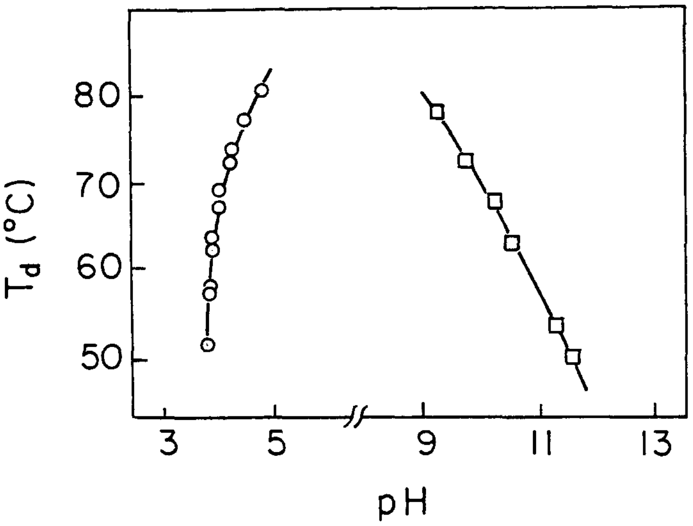

FIGURE 9. pH dependence of the denaturation temperature of metmyoglobin according to Privalov et al.[105]

The calorimetrically measured total enthalpy of protein denaturation in a buffered aqueous solution includes the enthalpy of the conformational transition and the enthalpy of ionization of both the protein and the buffer

$$\Delta_N^D H^{tot} = \Delta_N^D H_{pr}^{conf} + \sum \Delta H_{pr,i}^{ion} + \Delta_N^D\gamma\ \Delta H_{buf}^{ion} \qquad (16)$$

where $\Delta H_{pr,i}^{ion}$ and ΔH_{buf}^{ion} are the enthalpies of protonation of a single group in the protein and buffer, respectively.

If the enthalpies of protonation of protein groups and of the buffer are close, then the heat effects of protonation and deprotonation of the protein and buffer compensate each other and the calorimetrically measured total enthalpy of denaturation corresponds to the enthalpy of the conformational transition of the protein upon denaturation. In particular, this occurs when glycine is used as buffer since the enthalpies of protonation of its carboxylic and amino groups in the acidic and alkaline solutions are rather close to the enthalpies of protonation of most of the ionizable groups in proteins.[111] However, if one uses an acetate buffer, which has a very low protonation enthalpy, the buffer does not compensate for the heat of ionization of protein groups. The sign of this noncompensated heat effect is opposite that of the conformational transition enthalpy. Therefore, the calorimetrically measured total enthalpy of protein denaturation in an acetate buffer is lower than the enthalpy of the conformational transition (Figure 10). This decrease of the enthalpy is particularly significant in the case of myoglobin which has many buried histidines that are exposed upon denaturation, the more so that histidines are characterized by a particularly large enthalpy of protonation, −29 kJ mol^{-1}.[105]

It is remarkable that the slopes of the temperature dependences of the calorimetrically measured total denaturation enthalpy are almost identical in all the solutions studied and are close to that of the enthalpy of the conformational transition, which, in turn, is equal to the heat capacity change at the transition (see Figure 10). This is understandable, since the heats of ionization of both the protein and the buffer do not depend significantly on temperature, while $\Delta_N^D\gamma$ is practically constant in the pH region in which protein denaturation is usually studied

$$\begin{aligned}
\frac{d\Delta_N^D H^{tot}}{dT} &= \left(\frac{\partial \Delta_N^D H^{conf}}{\partial T}\right)_{pH} + \left(\frac{\partial \Delta_N^D H^{ion}}{\partial T}\right)_{pH} \\
&\times \left(\frac{\partial \Delta_N^D H^{ion}}{\partial pH}\right)_T \frac{dpH}{dT} \\
&= \Delta_N^D C_p + \left[\left(\frac{\partial \Delta_N^D\gamma}{\partial T}\right)_{pH} + \left(\frac{\partial \Delta_N^D\gamma}{\partial pH}\right)_T \cdot \frac{d\ pH}{dT}\right] \\
&\times [\Delta H_{pr,i}^{ion} - \Delta H_{buf}^{ion}] \\
&\cong \Delta_N^D C_{p,pr} \qquad (17)
\end{aligned}$$

Thus, the dependence of the total denaturation enthalpy measured calorimetrically in solutions of different pH values on the denaturation temperature, T_G, given in Figure 10, represents the temperature dependence of the conformational transition enthalpy. Extrapolating linearly this dependence to the intersection with the temperature axis, one gets the temperature T_H. As seen, it is highest for the acetate buffer solutions. This circumstance makes the acetate buffer most promising in studying the cold denaturation of proteins. In particular, for metmyoglobin, T_H in acetate buffer reaches 31°C, while in glycine it is about 15°C. As a consequence, the Gibbs energy functions are shifted considerably in these two buffers (Figure 11). In a glycine buffer, the low temperature slope of this function reaches zero below 0°C at all pHs, while in an acetate buffer at some pHs it reaches zero at above 0°C.

C. Influence of Urea and GuHCl

The principal feature of denaturants, such as urea or GuHCl, is that they interact extensively with proteins, solvating them. The enthalpy of their solvation is negative and large,[121] and is not strictly proportional to the denaturant concentration (Figure 12). It is only at concentrations below 3 *M* that one can assume

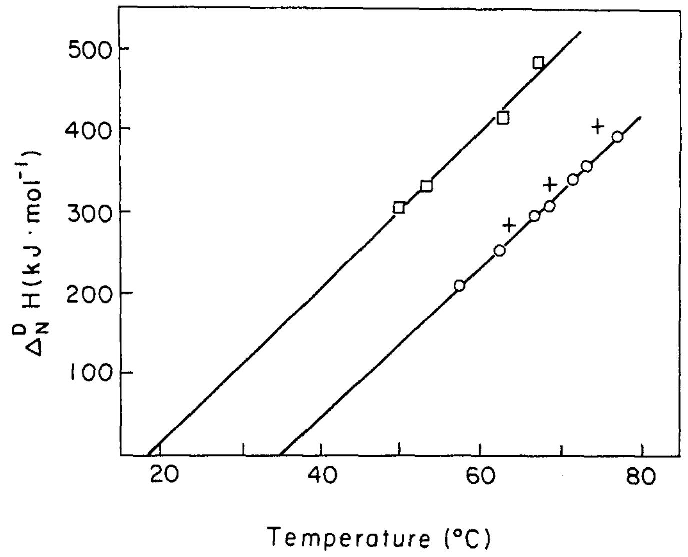

FIGURE 10. Enthalpy of metmyoglobin denaturation plotted against the temperature of denaturation for the solution, containing different buffers and various concentrations of GuHCl and urea. (According to Privalov et al.[105] and unpublished results of the laboratory.) □, Glycine buffer; ○, acetate buffer; +, 2 *M* urea with glycine buffer.

a linear dependence between the protein solvation enthalpy and the concentration, w, of denaturants

$$\Delta H^{sol} = Bw \qquad (18)$$

where B < 0. As is clear from the slopes of the corresponding functions in Figure 12, the denaturants interact much more extensively with the denatured proteins than with the native ones, i.e., $|B^D| > |B^N|$. This preferential binding to the denatured protein is what imparts to the denaturants their denaturing action.

Due to the preferential binding and the negative enthalpy of binding, the enthalpy of protein heat denaturation in the presence of urea or GuHCl is always lower than that in their absence (Figure 10), and this difference is proportional to the concentration of the denaturant if the concentration is not too high

$$\delta\Delta_N^D H(T) = \Delta_N^D H^*(T) - \Delta_N^D H(T) = (B^D - B^N)w$$
$$= \Delta_N^D B \cdot w < 0 \qquad (19)$$

Here the asterisk denotes the solution containing denaturant.

The solvation of the denaturant by protein decreases with a temperature increase because of the negative enthalpy of binding, but the desolvation process is greatly extended in temperature as the molar enthalpy of denaturant binding is not very large. Therefore, this process does not influence significantly the partial heat capacity of the protein in solution, especially the denaturation heat capacity increment, which in the first approximation can be assumed to be the same in urea or GuHCl solutions as in solutions not containing denaturants.[121-123] However, an almost parallel shift of this function in the presence of a denaturant leads to an increase of T_H by an increment proportional to the concentration of the denaturant

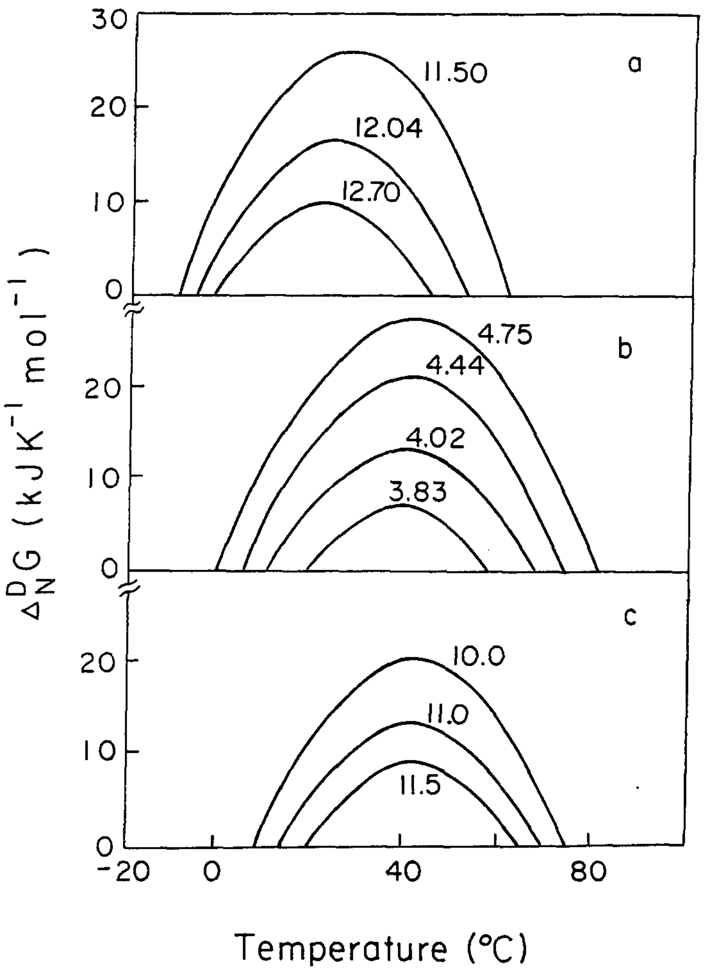

FIGURE 11. The Gibbs energy function of metmyoglobin in the glycine (a) and acetate (b) buffers and in the glycine buffer in the presence of 2 *M* urea (c) at various pHs (indicated on the curves).

$$\delta T_H = T_H^* - T_H = \frac{\delta\Delta_N^D H}{-\Delta_N^D C_p} = \frac{\Delta_N^D B}{-\Delta_N^D C_p} \cdot w \qquad (20)$$

from which

$$\delta\Delta_N^D H(T) = -(T_H^* - T_H)\,\Delta_N^D C_p \qquad (21)$$

Correspondingly, for the change of the Gibbs energy in the presence of a denaturant, we have from Equation 10

$$\delta\Delta_N^D G(T) = \Delta_N^D G^* - \Delta_N^D G$$
$$= T\left[\left(\frac{T_H^*}{T_G^*} - \frac{T_H}{T_G}\right) + \frac{T_H - T_H^*}{T} + \ln(T_G^*/T_G)\right]\Delta_N^D C_p \qquad (22)$$

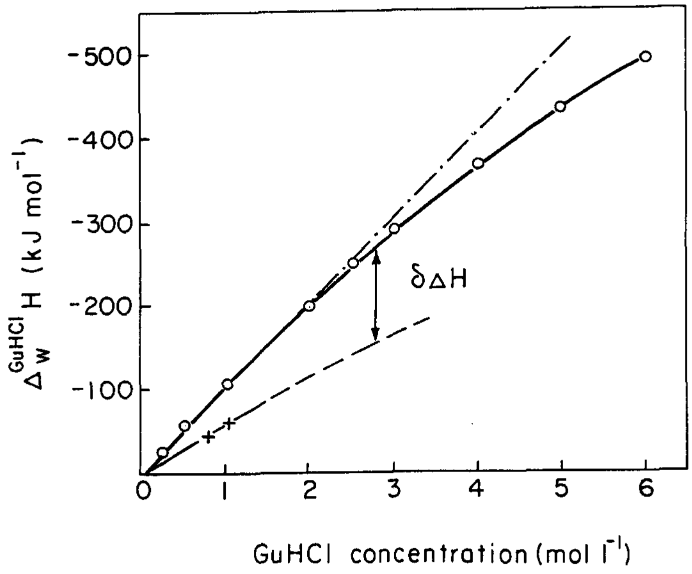

FIGURE 12. Enthalpy of titration of the native and denatured lysozyme by GuHCl at 59°C according to Pfeil and Privalov.[121] +, Native lysozyme, pH 4.5; o, denatured lysozyme, pH 1.5.

which is negative and increases in absolute value at a temperature decrease. From Equations 21 and 22, we get for the change of the denaturation entropy in the presence of a denaturant

$$\delta\Delta_N^D S(T) = \Delta_N^D S^* - \Delta_N^D S$$

$$= -\left[\left(\frac{T_H^*}{T_G^*} - \frac{T_H}{T_G}\right) + \ln (T_G^*/T_G)\right] \Delta_N^D C_p \qquad (23)$$

Since $T_H^*/T_G^* > T_H/T_G$, it follows from this equation that the entropy of the preferential binding is negative and increases in absolute value with a decrease in temperature (Figure 13). Thus, the destabilizing action of a denaturant on a protein is determined by the negative enthalpy of preferential binding.

Figure 11 presents the Gibbs energy functions, calculated for metmyoglobin in aqueous solutions in the presence and absence of 2 *M* urea. As seen, urea shifts considerably the Gibbs energy function to higher temperatures and at some pHs makes quite probable the observation of cold denaturation of metmyoglobin above 0°C. A similar conclusion on the role of GuHCl in revealing cold denaturation has been made recently by Chen and Schellman.[9]

IV. EXPERIMENTAL STUDIES OF COLD DENATURATION

A. Thermodynamic Studies on Solutions Which Do Not Contain Denaturants

As follows from the previous section, the most promising object for studying cold denaturation is myoglobin in acetate buffer solutions, which probably should denature upon cooling before reaching the freezing point of water. This was observed first by Cho et al.,[124] who studied the thermal stabilities of various liganded forms of horse heart myoglobin by following the changes in light absorption in the Soret band. A detailed study of this phenomena by various methods, including light absorption, CD and NMR spectroscopies, viscometry, and calorimetry, has been carried out by Privalov et al.[105]

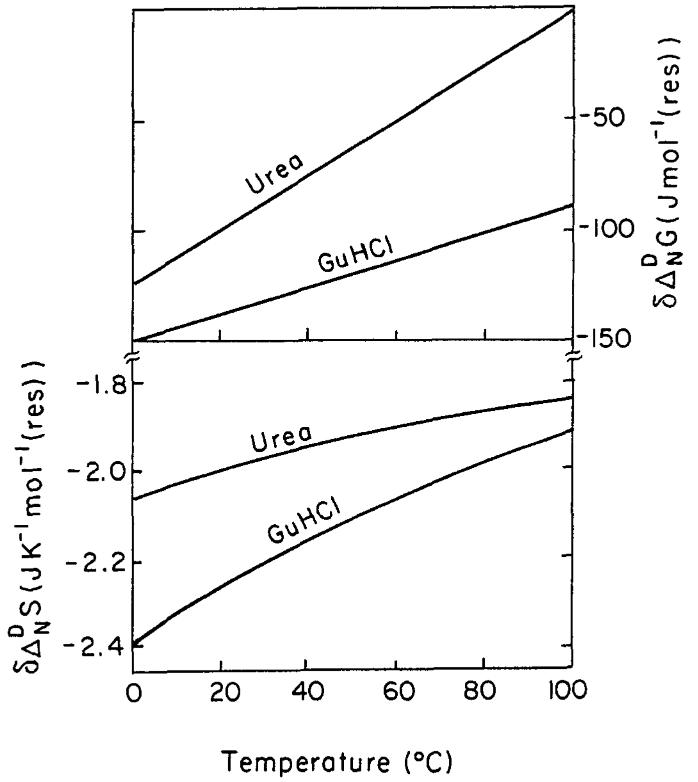

FIGURE 13. Temperature dependence of the Gibbs energy and entropy of preferential binding of urea and GuHCl of protein upon denaturation calculated per amino acid residue for 1 *M* of the denaturant in solution.

It was shown that at pHs of acetate buffering, metmyoglobin unfolds almost completely upon cooling; its intrinsic viscosity is raised to the value characteristic for a random coil (Figure 14); in its NMR and CD spectra, all signs of tertiary structure disappear and only some residual ellipticity in the far ultraviolet suggests slight helicity in the conformation of an unfolded polypeptide chain (Figure 15).

The calorimetric experiment shows that the cold denaturation of metmyoglobin is an exothermic process (Figure 16a). On subsequent heating of the precooled solution, the heat effect observed in the low temperature region reverses its sign. This endothermic process corresponds to the renaturation of the cold-denatured protein, which denatures again with heat absorption on further heating. Thus, heat and cold denaturations are processes which proceed with the enthalpies of opposite signs. The shift and deformation of the observed heat effect toward lower temperatures upon cooling and toward higher temperatures upon heating show that the kinetics of the process

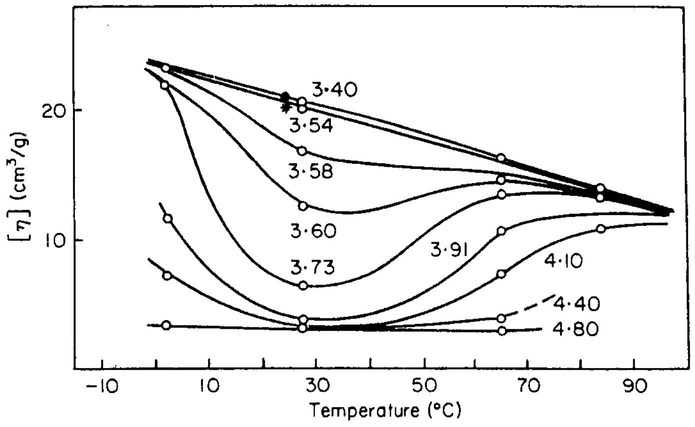

FIGURE 14. Temperature dependence of the intrinsic viscosity of metmyoglobin in Na-acetate solutions with various pHs.[105]

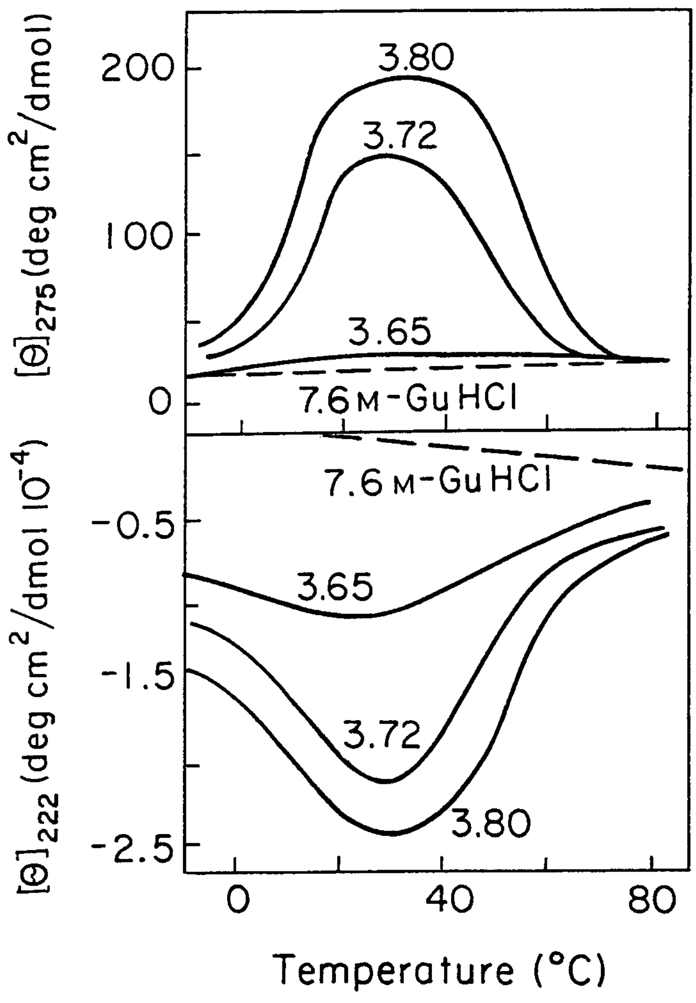

FIGURE 15. Temperature dependence of the ellipticity in the near and far ultraviolet for metmyoglobin in Na-acetate solutions with various pHs.

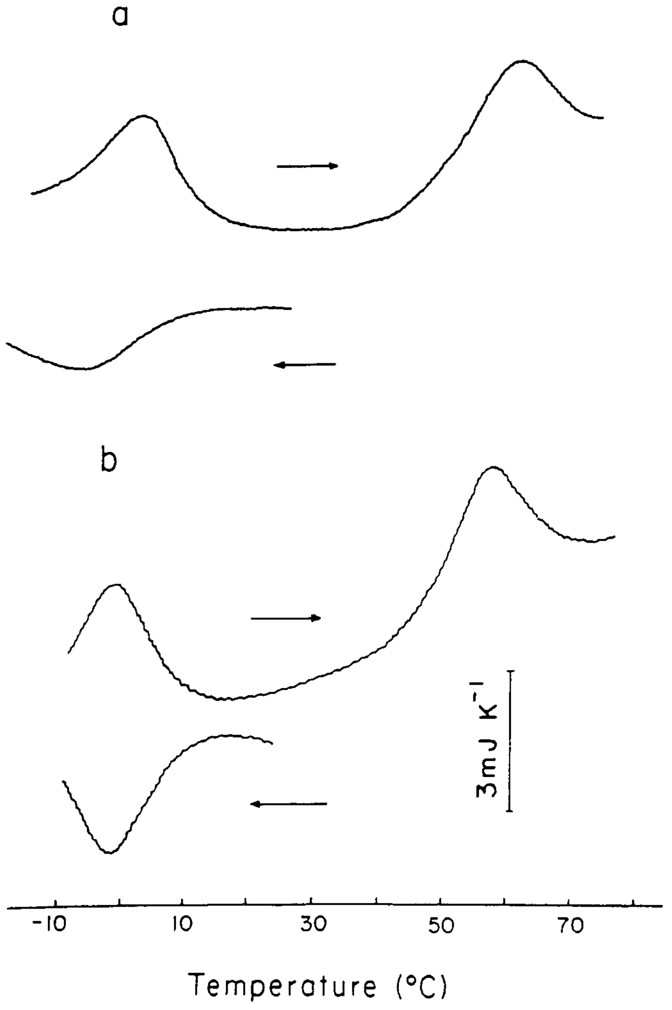

FIGURE 16. Original microcalorimetric recordings of the heat effects upon cooling and consequent heating with the scan rate 0.5 kal·min^{-1} of metmyoglobin (a) and apomyoglobin (b) in Na-acetate solution.[105,125]

which takes place in the low temperature region are rather slow. When the pH is lowered, the two heat absorption peaks observed on heating decrease and approach each other. At pH 3.5, the gap between them vanishes and they disappear (Figure 17), as was predicted thermodynamically (see Figure 8). Thus, it follows that the cold-denatured protein has the same heat capacity as the pH-denatured protein at a low temperature, and the pH-denatured protein at a high temperature has the same heat capacity as the heat-denatured protein, i.e., the heat capacity of the protein, denatured by cooling, heating, or a pH change, is universal and has a significantly higher value than the heat capacity of the native protein. This difference is almost independent of environmental conditions in the range of temperatures from −7 to about 80°C.

Similar effects are observed on apomyoglobin (Figure 17). At 30°C and neutral pH, apomyoglobin has a compact native-like structure, which disrupts cooperatively upon heating and cooling.[125] In contrast to myoglobin, the cold denaturation and renaturation of apomyoglobin are very fast processes. Therefore,

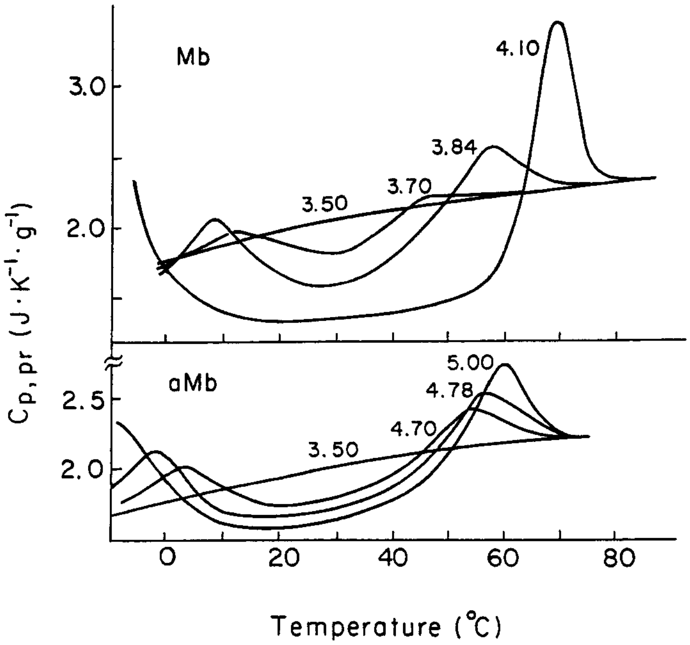

FIGURE 17. Partial molar heat capacity of metmyoglobin (Mb) and apomyoglobin (aMb) in Na-acetate solutions with various pHs.[105,125]

the scanning microcalorimetry recordings of the respective heat effects appear as mirror images (Figure 16b).

One of the differences in the thermodynamic properties of apomyoglobin and metmyoglobin is in the heat capacity increment, which is much smaller for apomyoglobin (4.7 kJ $K^{-1}mol^{-1}$) than that for metmyoglobin (11.0 kJ $K^{-1}mol^{-1}$). This difference results from a much higher partial specific heat capacity of native apomyoglobin relative to metmyoglobin (Figure 17). Since the main contributors to the heat capacity of proteins are the hydrated nonpolar groups, one can conclude that apomyoglobin has a smaller hydrophobic core than metmyoglobin because there are more nonpolar groups exposed to water. This conclusion is confirmed by the lower solubility of apomyoglobin in water and its enhanced tendency to form aggregates.

Notwithstanding the lower heat capacity increment, the cold denaturation of apomyoglobin takes place at rather high temperatures due to its much lower denaturation enthalpy and, correspondingly, the lower stability of the native state; according to the calorimetric data, the maximal stability of apomyoglobin at 31°C and pH 5.0 is only 11 kJ mol^{-1}, while that of metmyoglobin amounts to 34 kJ mol^{-1} at the corresponding conditions.[125]

Another well-studied example of cold denaturation is staphylococcal nuclease. It has rather large $\Delta_N^D C_p$, almost of the same magnitude as metmyoglobin. However, since it does not have so many buried histidines, its total denaturation enthalpy in acetate buffer is much higher than that of metmyoglobin. Correspondingly, it has a much lower T_H value, about 15°C, and, consequently, one can expect that its cold denaturation should take place at considerably lower temperatures than that of metmyoglobin and apomyoglobin. This is just what has been found in the calorimetric studies of staphylococcal nuclease.[126] Figure 18 presents the partial specific heat capacity of staphylococcal nuclease at different pHs, which shows that when protein stability decreases the second heat absorption peak appears from the low temperature side. This corresponds to the heat effect of renaturation of the cold-denatured protein. The minimum between the two peaks is just at 15°C in solutions with pH ≤ 4.5. Since the denaturation heat effect is zero at this temperature, it is clear that the somewhat higher heat capacity of protein observed at these pH values is caused by the presence of partially denatured protein in the solution. The amount of denatured protein increases on heating or cooling of the solution from this temperature. Unfortunately, the low-temperature part of the heat capacity function, which is related to cold denaturation, takes place at a temperature too low to be traced even in a supercooled solution.

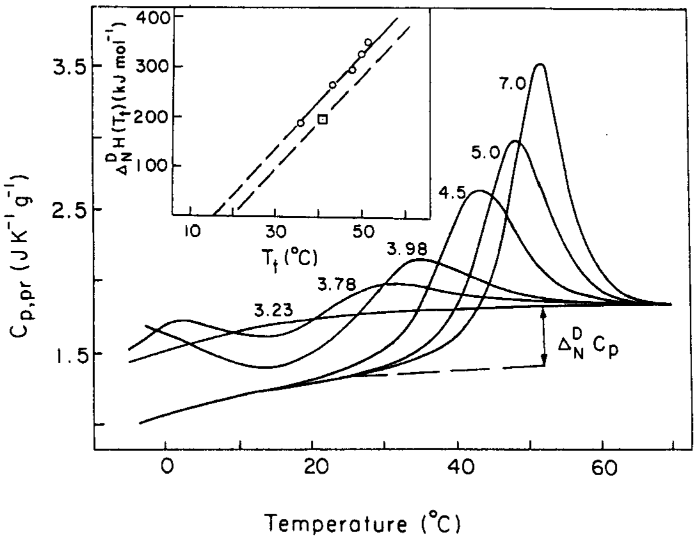

FIGURE 18. Partial molar heat capacity of staphylococcal nuclease in Na-acetate solution with various pHs. Insert: the enthalpy of denaturation plotted against the denaturation temperature (○, without urea; □, in the presence of 2 *M* urea).[126]

B. Thermodynamic Studies on Solutions Containing Denaturants

As follows from Figure 11, in alkaline solutions one can hardly observe the cold denaturation of metmyoglobin, but in the presence of 2 *M* urea, it seems to be apparent. The results, obtained by Griko and Privalov[127] on metmyoglobin in an alkaline glycine buffer solution containing 2 *M* urea, are presented in Figure 19.

Addition of 2 *M* urea helped also to shift the Gibbs energy function of staphylococcal nuclease to a higher temperature and made observable its cold denaturation to completeness (Figure 20).[126] The perfect correspondence of the expected and

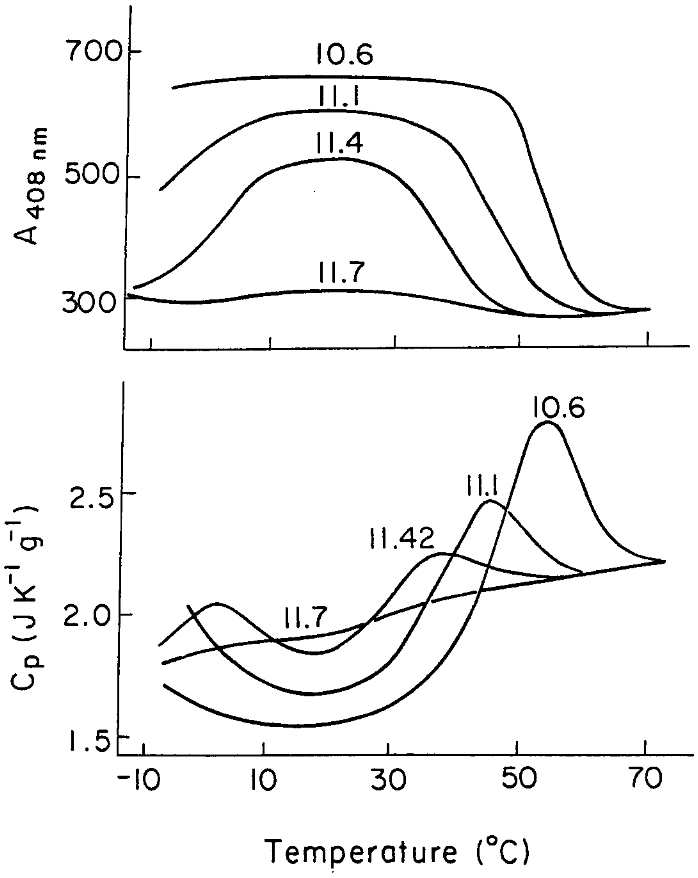

FIGURE 19. Ellipticity and heat capacity of metmyoglobin in the glycine solution in the presence of 2 *M* urea.[127]

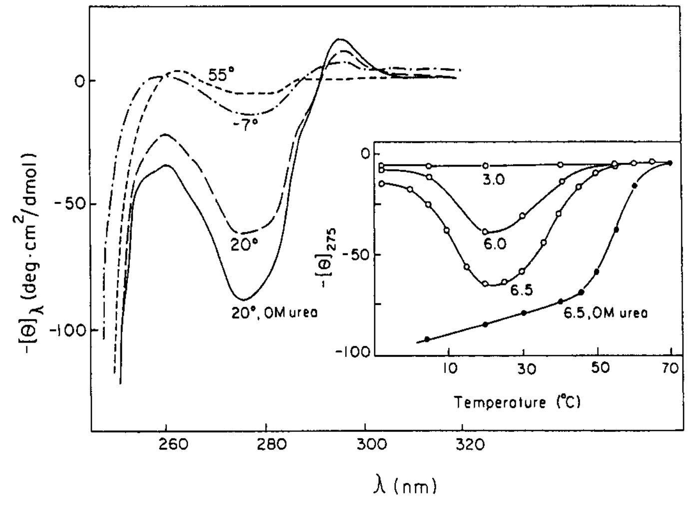

FIGURE 20. The ellipticity of staphylococcal nuclease in acetate solutions containing 2 *M* urea.[126]

experimental results shows that the role of urea in the protein solution has been taken into account correctly.

Recent calorimetric studies of the cold denaturation of lactoglobulin in urea solution (Figure 21) have shown that the heat effect observed in this case is quite similar to those found for myoglobin and staphylococcal nuclease[128] and is exactly what one can expect from the previous studies on denaturation of this protein in the presence of urea.[7]

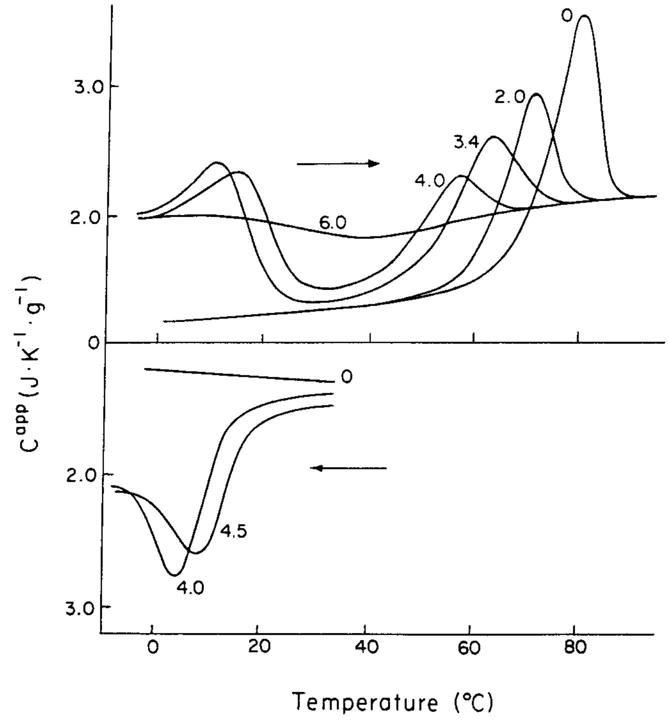

FIGURE 21. Temperature dependence of the apparent partial heat capacity of lactoglobulin in urea solutions with phosphate buffer, pH 2.0, measured by Griko and Privalov[128] in cooling and heating experiments. Concentration of urea in moles is indicated on the curves.

In all calorimetrically studied cases, the heat effect of cold denaturation is opposite that of heat denaturation, and its magnitude increases as the temperature at which the transition takes place decreases. The same has been found by Chen and Schellman,[9] who analyzed the low-temperature unfolding of a mutant of phage T_4 lysozyme observed by CD spectra. According to these authors, the cold denaturation of this protein proceeds with enthalpy and entropy decreases and a considerable heat capacity increment.

C. Kinetic Studies

Among the studies mentioned in Section I of this paper, there are only two quantitative studies on the kinetics of the cold denaturation of proteins.

Cho and Chen[129] studied the kinetics of the cold denaturation and renaturation of metmyoglobin in an acetate buffer solution without a denaturant and found that it is slow and complex and consistent with the transitions which involve all three states: native, transient intermediate, and denatured, which are converted one to another in the given order. These authors, however, did not specify an intermediate state and did not exclude the possibility that the second stage in this reaction can be related to the loss of the heme by the unfolded protein.

Chen et al.[130] have been more efficient in their study of the low-temperature unfolding of a mutant of phage T_4 lysozyme in GuHCl solution. The reversible unfolding and refolding of this protein with a single relaxation time has permitted them to accumulate a large amount of data on the effects of temperature, denaturant concentration, and pH on the rates of the forward and backward reactions and to specify thermodynamically the transient state. It has been shown that the increase of the heat capacity and the decrease of the enthalpy and entropy, which are specific for cold denaturation, take place especially during transition of the protein from the transient state to the unfolded one, while in the transition from the native to the transient state, the enthalpy increases (Figure 22). The decrease of the temperature at which the cold denaturation is induced leads to an enhancement of the entropy and a decrease of the enthalpy in the second stage of this process.

D. Multidomain Proteins

The only example of a multidomain protein on which the cold denaturation has been studied in detail is phosphoglycerate kinase (PGK). The molecular mass of this enzyme is about 48,000. According to the crystallographic studies of Blake and Rice,[131] its single polypeptide chain forms two well-defined domains. However, the calorimetric studies of the heat denaturation of this enzyme showed that this process is a two-state transition,[132] i.e., in this process the two-domain molecule figures as a single cooperative unit.

Griko et al.[133] have studied PGK in a broad temperature range and showed that at low temperatures it undergoes cold denaturation. Most interesting was the observation that while the heat denaturation of this enzyme is a two-state process (judging by the simultaneous change of various optical properties and the sharp heat absorption peak with equal van't Hoff and calorimetric enthalpies) the cold denaturation of this protein consists of two distinct and rather independent stages (Figure 23). Since a yeast PGK molecule has two tryptophans which are located in the C-terminal domain, one can easily identify the observed stages, analyzing the change of fluorescence spectra of tryptophan in the studied temperature range. This shows that the two stages in the cold denaturation of PGK correspond to independent disruption of two domains. Thus, it follows that the domain interaction in PGK is temperature dependent: it decreases as the temperature decreases. If at a high temperature the two domains are merged into a single cooperative unit, at a low temperature they figure as independent cooperative units.

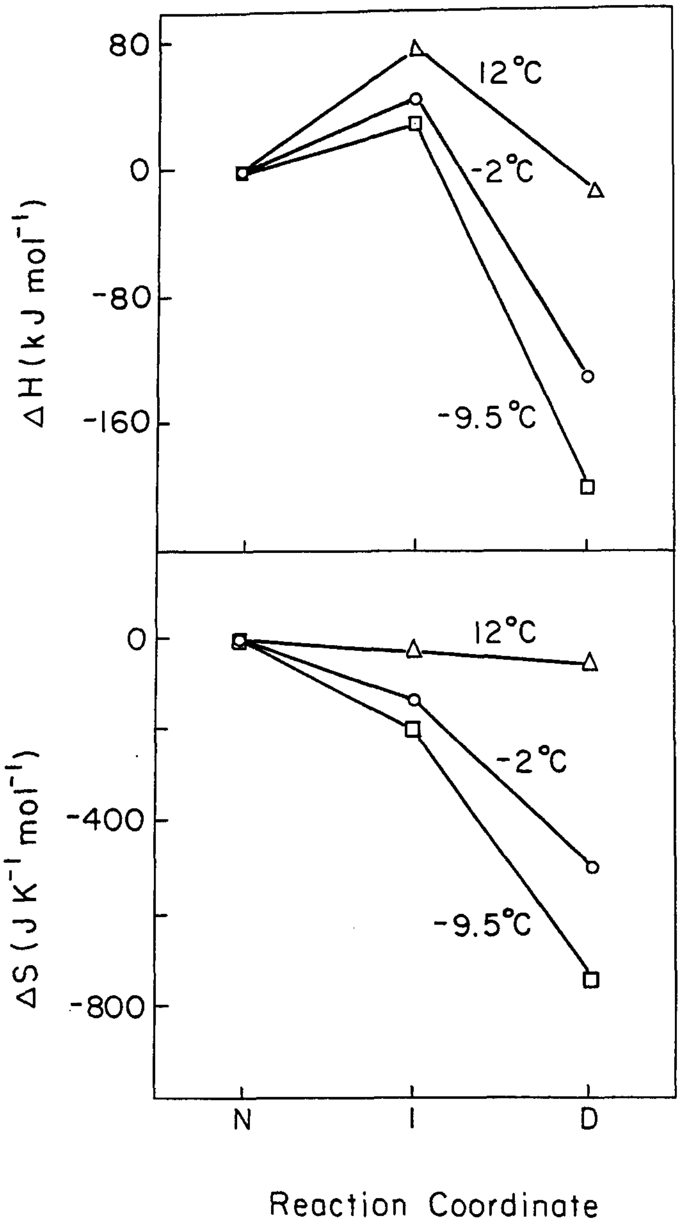

FIGURE 22. Reaction profile of the unfolding for T_4 phage lysozyme in 3 *M* GuHCl at pH 5. The abscissa indicates that the reaction proceeds from the native state "N" to the transient state "T" and then to the denatured state "D". The ordinate indicates a change in the enthalpy (a) and entropy (b) at the temperatures: −9.5, −2, and 12°C.[130]

V. MECHANISM OF COLD DENATURATION

A. Protein as a Macroscopic System

All the experimental facts reported above concerning the cold denaturation of various proteins in various solutions with and without denaturants are in perfect correspondence with the expectations on the basis of heat denaturation studies. The agreement between the predicted thermodynamically and experimentally observed effects is a strong argument for the correctness of the basic assumptions, namely, that the denaturation of a small protein or of an isolated single domain can be approximated by a two-state transition, that it is accompanied by a significant heat capacity increase, not too dependent on the environmental conditions, particularly

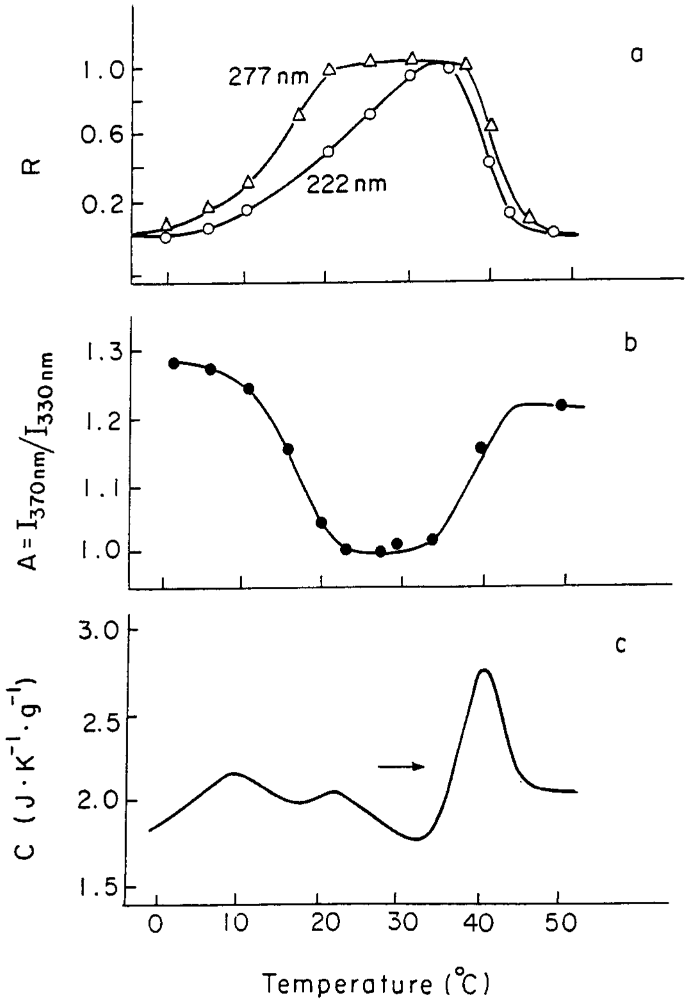

FIGURE 23. Temperature dependence of (a) relative changes (R) of phosphoglycerate kinase ellipticity at 222 nm (○) and 277 nm (△), (b) tryptophan emission spectrum maximum, and (c) partial specific heat capacity in solution containing 0.7 *M* GuHCl.[133]

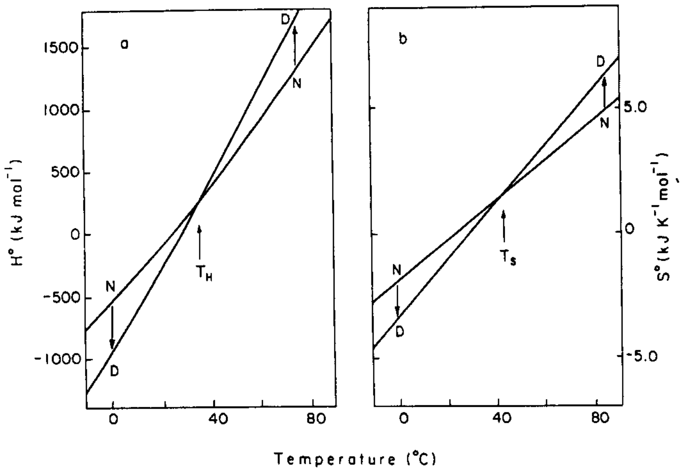

FIGURE 24. Standard enthalpy (a) and entropy (b) of the native and denatured metmyoglobin in acetate solution, pH 4.2, as temperature functions. The native protein at 25°C is taken as a standard state.

temperature, and that the influence of denaturants on proteins is provided by the negative enthalpy of preferential solvation of the denatured state which is proportional to the denaturant concentration.

The correspondence found between the thermodynamic properties (heat capacity, enthalpy, and entropy) of cold-denatured proteins and those denatured by other means (such as heating or an increase of acid or denaturant concentration)[105,121,122,134] shows that these properties depend only on the environmental conditions, i.e., that the denatured state is universal. This reinforces the idea that small compact globular proteins, or a single isolated domain of large proteins, represent an extremely cooperative macroscopic system with only two macroscopic states, the native and denatured, which could be regarded as separate phases and specified by distinctly different enthalpy and entropy functions (Figure 24).[111,112,122,123,134] Therefore, the transition between these states, which proceeds with a discontinuity of these functions, presenting the first derivatives of the thermodynamic potential, should be regarded according to the conventional classification as a first-order phase transition.[135]

However, the main thermodynamic difference between these macroscopic states is not in the enthalpy and entropy values, which could even be identical in some conditions, but in the heat capacity, which is the source of the different steepness of these parameters as a function of temperature. As a result, the functions which specify the native and denatured states always intersect at some temperatures, T_H and T_S, respectively, at which the enthalpies or entropies of these states are identical. Usually T_H and T_S do not coincide, except of the case in which the states do not differ in the Gibbs energy either. This occurs, e.g., for metmyoglobin in acetate buffer at 31°C and pH 3.73, where $\Delta_N^D H = 0$, $\Delta_N^D S = 0$, and $\Delta_N^D G = 0$.[105] However, such a singular point is not critical for proteins since the native and denatured phases of proteins differ in symmetry and, therefore, even at this point, the transition between these states represents a phase transition.[135]

The much steeper temperature dependence of the enthalpy and entropy of the noncompact and flexible denatured protein than that of the rigid compact native protein, i.e., the large heat capacity of a denatured protein, cannot be explained by gradual melting of the residual structure in the denatured protein on heating since this leads to the absurd conclusion that at a temperature below T_H, when the enthalpy of the denatured state is lower than that of the native one, the residual structure in the protein is more extensive than the native structure. Other possible causes of the observed protein denaturation heat capacity increment, such as an increase in the extent of freedom upon denaturation, were also considered,[136,137] but they cannot contribute significantly to the observed effect and cannot explain the negative denaturation enthalpy values at temperatures below

T_H. The only plausible explanation of the observed temperature dependence of the enthalpy and entropy is that it is caused by different solvation of water by the native and denatured protein and, particularly, by different solvation of nonpolar groups, which in the unfolded denatured protein are much more extensively exposed to water.

B. Hydration of Nonpolar Groups

As has been said in Section I, studies on the dissolution of low molecular, nonpolar substances in water show that the hydration of these substances is associated with a significant entropy decrease of the system, which reflects the increase of order in the water. It appears that it is the gradual decrease of this order with temperature which provides the excess heat capacity of such a solution. This heat capacity increment is proportional to the surface area of the solute molecule and decreases asymptotically with increasing temperature, so that the ordering influence of nonpolar groups on water vanishes at high temperatures.[138,139] Correspondingly, an increase in temperature also results in a decrease of the absolute value of the negative entropy of transfer of the nonpolar solute to water, which at some temperature, T_S^{tr}, becomes zero (Figure 25).

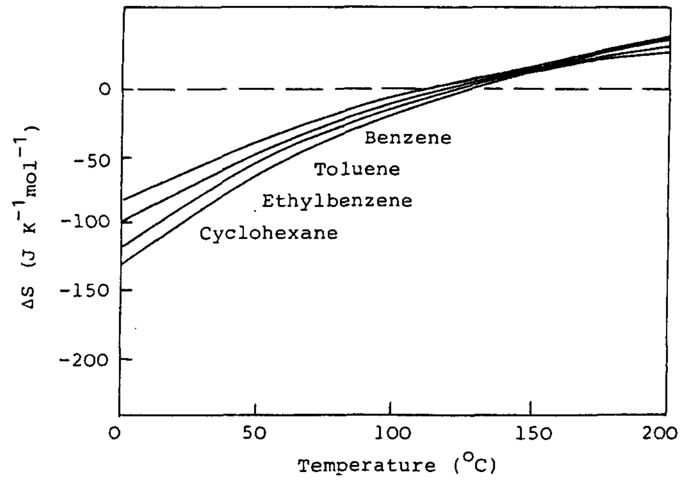

FIGURE 25. Temperature dependence of the entropy of transition of various nonpolar substances from the pure liquid phase to water.[141]

It is interesting that the temperature, T_S^{tr}, at which the entropy of transfer of a nonpolar substance from the pure liquid phase to water becomes zero is universal for all the substances studied and is about 140°C.[140-142] However, this in itself is not as surprising as it might appear because the entropy and heat capacity of transfer of a nonpolar solute from the pure liquid state (l) to water (w) are both proportional to the surface area of the solute, i.e., their ratio, $\Delta_l^w S/\Delta_l^w C_p$, which actually determines the temperature when the entropy becomes zero is a universal constant for all the nonpolar substances studied.[136]

On the other hand, since the entropy is the temperature derivative of the Gibbs energy, $\partial\Delta G/\partial T = -\Delta S$, the fact that the entropy of transfer is zero at some temperature means that the Gibbs energy of transfer has an extremum at this temperature. Thus, the Gibbs energy of transfer of a nonpolar substance from the pure liquid state to water is maximum at T_S^{tr}, which is the universal temperature for all known nonpolar substances (Figure 26). As is clear, this maximum value of $\Delta_l^w G$ is provided solely by the enthalpy of transfer.

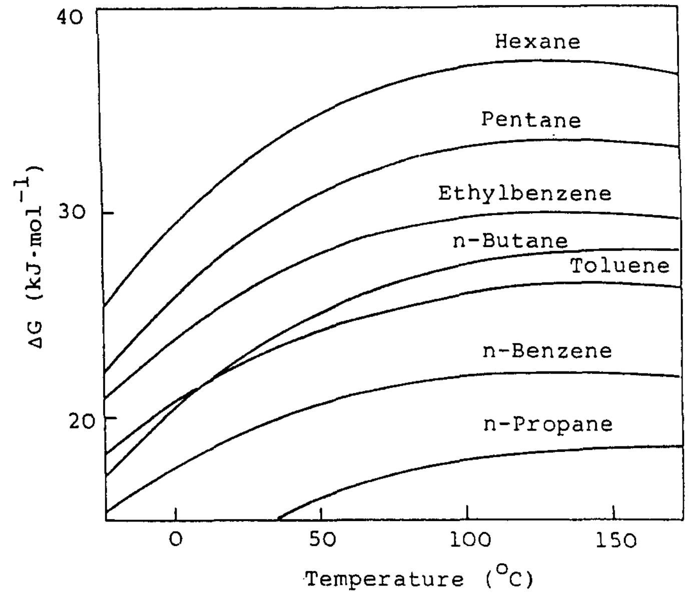

FIGURE 26. Temperature dependence of the Gibbs energy of transfer of various nonpolar substances from the pure liquid phase to water.[141]

At the temperature T_S^{tr}, the enthalpy of transfer of a nonpolar molecule from the pure liquid phase to water, $\Delta_l^w H(T_S^{tr})$, is not zero as it is at room temperature, but it is large and positive.[141-143] It is actually of the same order of magnitude as the enthalpy of vaporization of the liquid, corrected for volume expansion, i.e., it is close to the energy of the van der Waals interactions between the nonpolar molecules in the liquid phase.[142] It follows then that at this temperature, T_S^{tr}, water does not solvate the nonpolar solute, i.e., the solute molecules are not hydrated. At all temperatures below T_S^{tr}, there are clear signs of hydration of the nonpolar solute (negative entropy and lower enthalpy of transfer and a significant heat capacity increment) and all these signs become more pronounced as the temperature decreases. It becomes clear that the decrease of the Gibbs energy of transfer, which occurs at temperatures below T_S, is caused by hydration of the nonpolar solute. Without hydration and the heat capacity increment caused by hydration, the Gibbs energy of tansfer would not change as the temperature is decreased below T_S^{tr}. This means that the Gibbs energy of hydration of a nonpolar solute is negative; it is zero at T_S^{tr} and increases in magnitude as the temperature decreases.[141]

The Gibbs energy of transfer of nonpolar molecules from the pure liquid phase to water is, in fact, the only measure of the expulsion of these hydrophobic molecules from an aqueous solution, i.e., the measure of the hydrophobic interaction between nonpolar molecules. It follows from the above that the hydrophobic interactions result from the van der Waals interactions between nonpolar molecules, while hydration of these molecules has only a negative effect — it increases the solubility of these solutes in water.[141,143]

C. Stability of Protein Structure

One of the most intriguing features of the enthalpy and entropy of denaturation is that for very different small compact globular proteins these functions converge to the same value as temperature is increased (see Figure 5). The temperature of convergence T_x of these functions is about 110°C if $\Delta_N^D C_p$ is assumed to be temperature independent,[111] or about 140°C if the decrease of heat capacity is taken into account.[114] It was suggested that at this temperature, T_x, water does not solvate protein groups and the enthalpy and entropy of protein denaturation does not contain the hydration effects.[112] Later, this idea was confirmed by the finding that just at this temperature, 140°C, the entropy of transfer of a nonpolar substance from the pure liquid phase to water becomes zero[140] and the enthalpy of transfer reaches in magnitude the enthalpy of vaporization of the pure liquid substance.[142]

On the other hand, one can notice a distinct difference between the entropy functions of protein denaturation and those for the transfer of nonpolar substances to water: while the entropy of transfer of nonpolar substance from the pure liquid phase to water is zero at 140°C, the entropy of protein denaturation at this temperature is large and positive.[112]

The difference in the entropy values at a temperature at which hydration effects are absent might only mean that the protein interior is not a liquid-like, nonpolar phase, but is a crystal-like phase which is specified by a definite positive entropy of melting. The main thermodynamic consequence of this specificity of a protein molecule is that the entropy of protein transition from the native to the denatured state becomes zero at a much lower temperature than the entropy of transfer of a nonpolar substance from the liquid phase to water. Usually the temperature, T_S, for protein denaturation is only a few degrees higher than the temperature, T_H, at which the enthalpy of protein denaturation becomes zero. For most of the known proteins, this temperature is between 0 and 30°C.[112] At this temperature, the compact native protein structure is most stable because $\Delta_N^D G$ is maximal here (Figure 7).

Since the $\Delta_N^D G$ function is insensitive to the assumption as to whether $\Delta_N^D C_p$ is temperature dependent or temperature independent, in its analysis it is convenient to consider the latter case when the enthalpy and entropy contributions can be presented as

$$\Delta_N^D H(T) = \Delta_N^D H(T_x) - \Delta_N^D C_p L(T_x - T) \tag{24}$$

$$\begin{aligned}\Delta_N^D S(T) &= \Delta_N^D S(T_x) - \Delta_N^D C_p \ln(T_x/T) \qquad (25)\\ &= \Delta_N^D S(T_x) - \Delta_N^D C_p \frac{T_x - T}{T} + \tfrac{1}{2}\Delta_N^D C_p \left(\frac{T_x - T}{T}\right)^2\end{aligned}$$

where $\Delta_N^D H(T_x)$ and $\Delta_N^D S(T_x)$ are the temperature-independent parts of the enthalpy and entropy of protein denaturation, which do not include the effects of water ordering by the protein nonpolar groups. Then, we have for the Gibbs energy of stabilization of the native protein structure

$$\begin{aligned}\Delta_N^D G(T) &= \Delta_N^D H(T) - T\Delta_N^D S(T)\\ &\cong \Delta_N^D H(T_x) - T\Delta_N^D S(T) - \tfrac{1}{2}\Delta_N^D C_p \left(\frac{(T_x - T)^2}{T}\right)\end{aligned} \tag{26}$$

In this expression, only the first term is positive, i.e., the transition enthalpy in the absence of water solvation effects by the protein nonpolar groups. It represents the temperature-independent contribution of the van der Waals and hydrogen bonds in the stabilization of the protein compact structure. The second term, which is negative and increases with temperature, represents the disordering action of the dissipative force. The third term merely expresses the contribution of water solvation of nonpolar groups in the stabilization of the protein native structure. The most remarkable feature of this term is that it is negative and that its value decreases to zero when the temperature increases to T_x. Therefore, at all temperatures below T_x, the water solvation of protein nonpolar groups leads only to a decrease of stability of the compact native state, which is maintained only by the enthalpic interactions, i.e., by van der Waals and hydrogen bonding.[105,113,141] As seen from Figure 27, at some sufficiently low temperature, this negative term could reach a value, at which it compensates completely the positive contributions of the van der Waals and hydrogen bonds. As a consequence, the protein denatures.

D. Hydrophobic Interactions

As we see from the results obtained by studying the dissolution of nonpolar hydrophobic substances in water and the protein denaturation, the effect which is usually called "hydrophobic interaction" represents, in fact, a combined action of van der Waals interactions between nonpolar groups and hydration of these groups. This integral effect increases with increasing temperature. However, the "hydrophobic interaction" increases as a result of the decrease in the hydration contribution as temperature increases (and not to its increase, as was believed earlier) because van der Waals and this contribution differ in their sign.

An important consequence of the fact that the van der Waals and hydration effects contribute to the hydrophobic effect with opposite signs is the biphasic character of this integral effect.[142] Indeed, since the van der Waals interaction is a short range one and the hydration effect is a long range one, it is evident

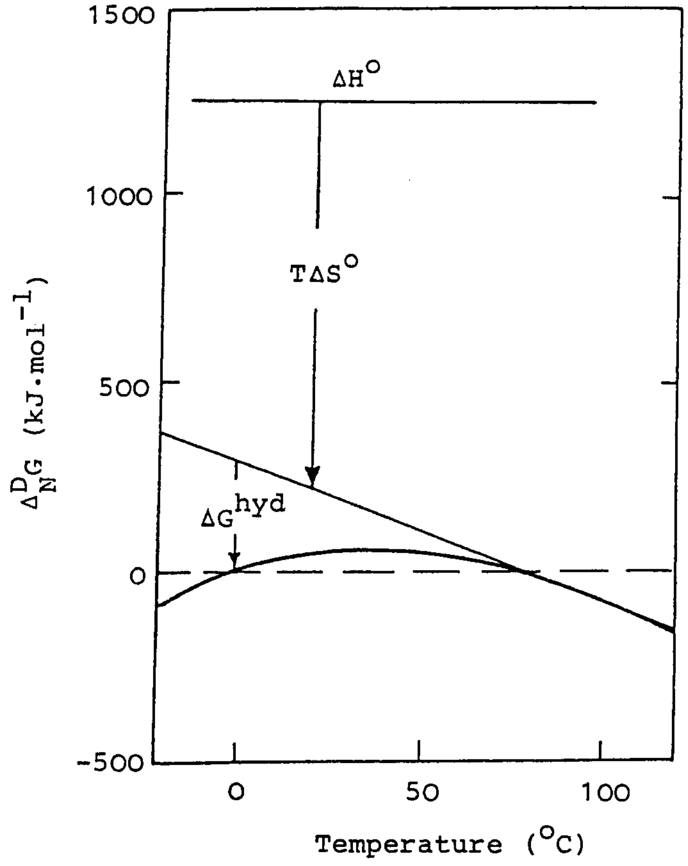

FIGURE 27. Contribution of the dissipative force ($T\Delta S$) and water solvation effect $\frac{\Delta C_p(T_x - T)^2}{2T}$ to the stabilization of an abstract globular protein, consisting of 200 amino acid residues.[141]

that the "hydrophobic interaction" should be attractive at short distances and repulsive at long distances (exceeding the size of a water molecule). This might be one of the reasons for the extreme cooperativity of the tightly packed native protein domain which is disrupted in the "all-or-none" way and always involves the penetration of water inside the protein structure. The results, discussed above, of the kinetics of the cold denaturation of phage T_4 lysozyme obtained by Chen et al.[130] bring out clearly this process by separating the two phases in the disruption of the "hydrophobic interaction" as a function of the reaction coordinates (see Figure 22). Similar, but even more pronounced results were obtained earlier by Segawa and Sugihara,[144] who studied the kinetics of lysozyme denaturation and found that the protein transition to a transient state proceeds with a positive change of enthalpy, which does not depend on temperature, i.e., the heat capacity does not increase at this stage. This means that, in the first stage, the enthalpic interactions between the protein groups are disrupted, but water does not penetrate inside the globule yet. This occurs only in the second stage of its unfolding.

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ERRATA

In Volume 25, Issue 3 (1990), the article beginning on page 155 — the first reference should read:

1. Green, P. J., Pines, O., and Inouye, M., The role of antisense RNA in gene regulation, *Annu. Rev. Biochem.*, 55, 569, 1986.