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Microcalorimetry of Macromolecules: The Physical Basis of Biological Structures

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Abstract This manuscript focuses on a specialized field within solution thermodynamics that encompasses some of the major macromolecular components comprising all biological systems. Nucleic acids and proteins are large biomolecules that represent quazi-macroscopic systems with highly ordered structures in which each atom occupies a defined space. In view of their unique quaternary structures that resemble aperiodic crystals, one might reasonably surmise that the resultant solution thermodynamics are highly specific. Systematic investigations of these biological macromolecules have necessitated the development of ultrasensitive measurement techniques including differential scanning and isothermal titration calorimetry. These experimental approaches represent the gold standard in terms of microcalorimetric instrumentation designed to characterize complex macromolecular systems. This paper presents an overview of thermodynamic data gleaned from studies employing these calorimetric techniques as part of our comprehensive strategy to characterize the energetic basis of nucleic acid and protein stability.

Keywords Macromolecules · Nucleic acids · Proteins · Differential scanning calorimetry · Isothermal titration calorimetry

This paper represents a condensed version of the Plenary Lecture presented at The 68th Calorimetry Conference (CALCON 2013) in recognition of Professor Robert Wood's lifetime contributions to the field of Solution Thermodynamics.

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The Problem

Talking and contention of Arguments would soon be turned into labours; all the fine dreams of Opinions and universal metaphysical natures, which the luxury of subtle Brains have devised, would quickly vanish and give place to solid Histories, Experiments, and Works.

Robert Hooke (1665) [1].

1 Introduction

The intrinsic feature of all living systems that distinguishes these creatures from nonliving entities is the incredible wealth of genetic information encoded within each cell which is responsible for maintaining life and modulating specific functions. Considering the inherent complexity, Ervin Shrödinger arrived at the conclusion that living systems are characterized by large molecular scaffolds arranged in aperiodic order [2]. This conclusion derived from the notion that macromolecular structures in the form of aperiodic crystals are required to harbor the wealth of genetic information. Shrödinger's proposal has prompted a number of relevant inquiries regarding the thermodynamic properties of such aperiodic systems. Perhaps the most intriguing question relates to the role of entropy and its accurate determination. A practical approach towards characterizing the thermodynamics of such macromolecules might involve calorimetric measurement of their heat capacities at absolute zero via a similar method that is routinely employed for determining the entropy of periodic crystals. The challenges associated with elucidating the thermodynamic basis of macromolecular structure elicited my lifelong interest in calorimetry.

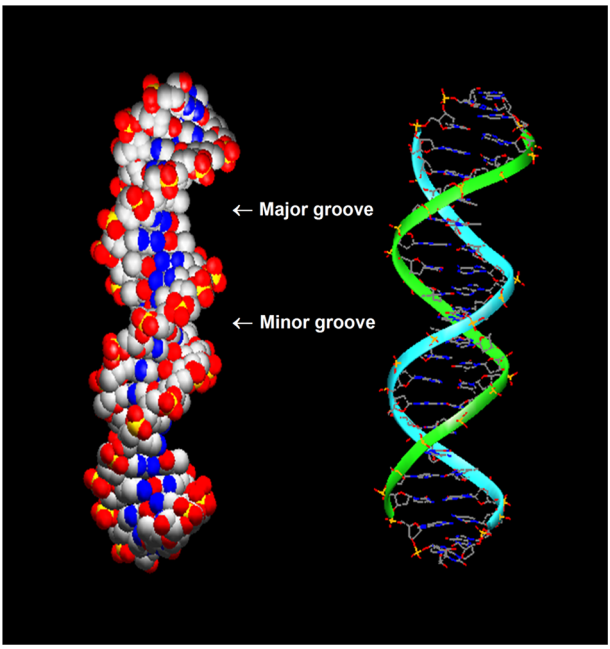

Fig. 1 Three dimensional structure of the DNA double helix illustrating both major and minor grooves

In a historical context, one must recognize the pioneering contributions of Miescher [3] who initially identified the macromolecule bearing all genetic information in a living organism. Preliminary studies conducted by Avery et al. [4] corroborated its similarity to the highly acidic polymer isolated previously from the cell nuclei and designated as a nucleic acid. Subsequently, Chargaff [5] discovered that this polynucleotide is comprised of four bases [i.e., adenine (A), guanine (G), cytosine (C). and thymine (T)] arranged in a unique ratio such that the adenine/thymine and guanine/cytosine concentrations are equivalent: [A] = [T] and [G] = [C]. This unique correspondence of base ratios suggested that deoxyribonucleic acid (DNA) might be formed by two complementary strands. Based on the crystallographic data of Rosalind Franklin, Watson and Crick envisioned two complementary single strands forming the DNA double helix [6] as illustrated in Fig. 1.

Elucidation of the DNA double helical structure gained immediate popularity given its significance in explaining the mechanism of coding genetic information based on the sequence of A–T and G–C complementary base pairs similar to the digital "Morse code". Moreover, the three-dimensional structure furthered our understanding of the mechanism governing replication, as the two complementary strands encoded with genetic information dissociate and new complementary strands are synthesized using the originals as templates. This marked a seminal period of discovery in biochemical and biophysical research as scientists with diverse backgrounds and expertise mobilized in their quest to participate in a global DNA "gold rush".

As a graduate student in physics at that time, I became particularly interested in determining the work required to dissociate the DNA complementary strands and thereby evaluate the forces involved in stabilizing the double helix. According to the Watson and Crick model, these stabilizing forces should arise from hydrogen bond (HB) formation between the bases of each complementary strand. Specifically, there are two HBs between adenine and thymine and three HBs between cytosine and guanine as illustrated in Fig. 2. Based on these considerations, one might reasonably anticipate that the enthalpic

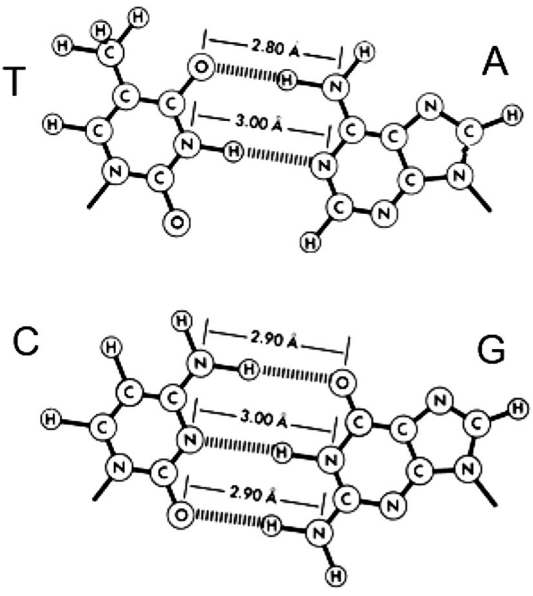

Fig. 2 Complementarity of T·A and C·G base pairing scheme according to Watson and Crick

contribution of a GC base pair to duplex stabilization is larger than that of an AT base pair. Accordingly, the overall stability of a DNA double helix might be directly correlated with its GC base pair content. Experimental studies have demonstrated enhanced duplex stabilization as a function of increased CG base pair content [7] and thereby represent a strong argument favoring the Watson–Crick DNA model.

In a parallel effort to that expended for the characterization of nucleic acids, John Kendrew applied X-ray crystallographic measurements to resolve the first protein structure [8] of myoglobin as illustrated in Fig. 3. In contrast with the four basic components of nucleic acids, proteins are comprised of a linear heterogeneous polymer arranged from twenty different amino acid residues. Paraphrasing the author's initial impression of the myoglobin structure: "Perhaps the most remarkable features of the molecule are its complexity and its lack of symmetry. The arrangement seems to be almost totally lacking in the kind of regularities which one instinctively anticipates, and it is more complicated than has been predicted by any theory of protein structure."

An intriguing question that arose immediately following determination of the first protein structure is how does a linear heteropolymer or polypeptide fold into such a unique conformation? Physical studies on macromolecular structure formation suggested that protein folding/unfolding is a reversible process. Specifically, exposure to an aggressive environment (e.g., high temperature and/or pressure, chemical denaturants, acidic or alkaline solution) induces the protein polypeptide chain to unfold. Conversely, removal of such harsh conditions facilitates polypeptide chain refolding to the native state conformation. Thus, protein folding/unfolding appears to be a reversible, thermodynamically driven process directed by the information encoded within the arrangement of amino acid residues forming its polypeptide chain [9]. A relevant inquiry that remained unresolved is whether folding/unfolding of the protein polypeptide chain proceeds in discrete steps or via a single cooperative process comparable to the phase transition in crystals. In this respect, calorimetry is the only method that permits direct investigation of the thermodynamics governing this process.

Despite advancements in elucidating the thermodynamic basis of macromolecular structure, an impediment to pursuing calorimetric studies of protein folding/unfolding and

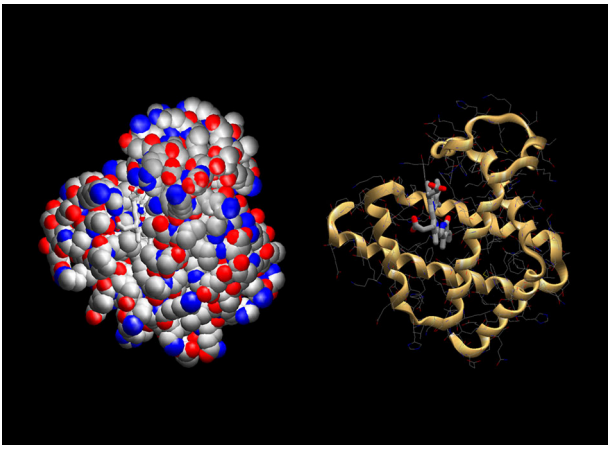

Fig. 3 Crystallographic structure of myoglobin at atomic resolution illustrating the conformation of its polypeptide chain enwrapping the heme group

nucleic acid association/dissociation resides in the need to perform such measurements on highly dilute solutions. Additional complications include expression/purification of proteins in the native state and development of facile nucleic acid synthetic approaches which generally limit the quantities of these biological macromolecules. Therefore, the rigorous calorimetric investigation of proteins and nucleic acids has required the design and development of ultrasensitive instrumentation capable of detecting and resolving microjoule reaction heats. Only recently have the advances in calorimetric instrumentation enabled us to acquire the requisite measurements in dilute aqueous solution and thereby facilitate characterization of the thermodynamic processes governing and modulating protein and nucleic acid stability.

2 Calorimetric Instrumentation

We initially addressed the problem associated with determining the heat capacity of macromolecules in dilute solution by constructing a vacuum, adiabatic, differential scanning microcalorimeter (DSC) [10]. During the ensuing five decades, we have optimized and refined this instrument by introducing several modifications to enhance its sensitivity [11–13]. The current design incorporates a pair of matched gold capillary cells for the sample and reference solutions as illustrated schematically in Fig. 4a. The profiles observed upon heating and cooling of a 12 base pair deoxyoligonucleotide duplex comprised of the non-self-complementary d(CGCAAATTTCGC) and d(GCGAAATTTGCG) strands, at a constant scan rate of 1.0 deg·min^{-1}, is depicted in Fig. 4b. The resultant heat effects appear as mirror images and thereby reflect the reversible nature of DNA duplex dissociation/association processes.

Parallel efforts to characterize the association of macromolecules at a given temperature resulted in the development of an isothermal titration microcalorimeter (ITC) designed by Stan Gill's laboratory as illustrated in Fig. 5a [14]. Within the following 5 years, John Brandts and colleagues successfully introduced the first commercial ITC capable of detecting and resolving microjoule reaction heats. Further optimization and refinement of this calorimetric technique as illustrated in the schematic of Fig. 5b has enabled isothermal measurements over a broad temperature range. A binding isotherm depicting the heat of

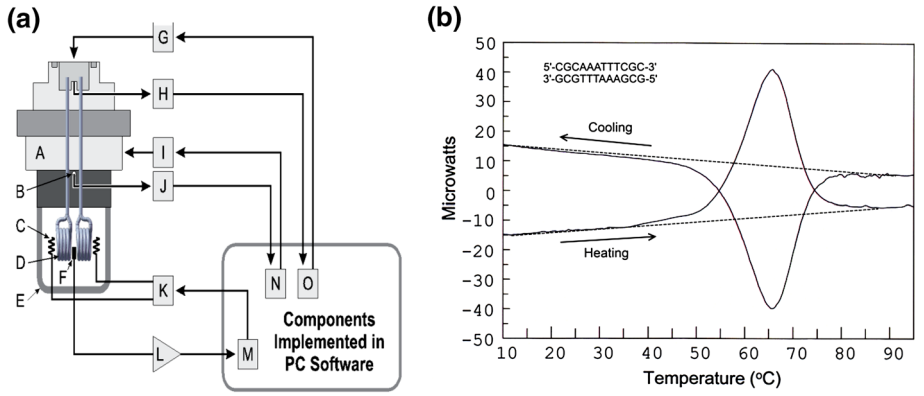

Fig. 4 **a** Capillary DSC instrument designed by the author; **b** heating and cooling scans of a 220 μmol·L^{-1} dodecamer duplex in 10 mmol·L^{-1} sodium phosphate buffer and 150 mmol·L^{-1} NaCl (pH 7.4)

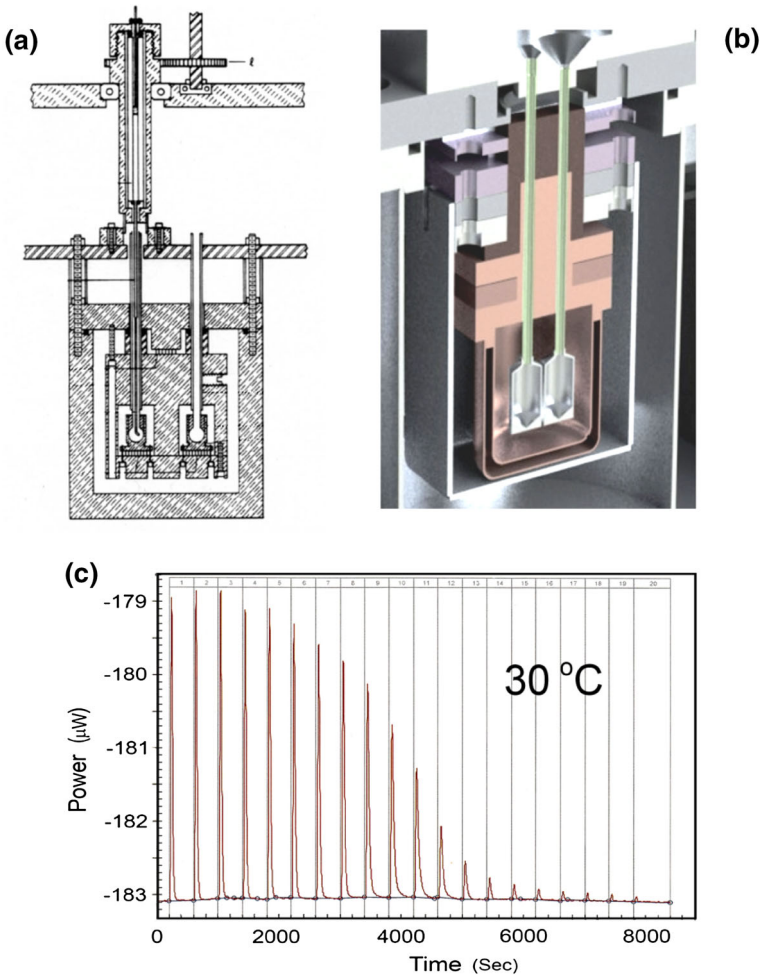

Fig. 5 **a** Isothermal titration calorimeter designed by Gill [14]; **b** ITC instrument designed by the author; **c** ITC profile of complementary deoxyoligonucleotide association for the titration of 200 μmol·L^{-1} d(GCGGCGGCGGCG) into a 1.0 mL sample cell containing 5 μmol·L^{-1} d(CGCCGCCGCCGC) at 5.0 °C

association measured for two deoxyoligonucleotide strands is presented in Fig. 5c. Advances in the design, development, and introduction of ultrasensitive microcalorimeters have effectively revolutionized the field of biothermodynamics by affording biophysicists an enhanced ability to characterize the energetics of macromolecular interactions in dilute solutions.

3 Energetic Basis of the DNA Double Helix

Our interest in elucidating the forces stabilizing nucleic acids dates from the early 1960s [15–17], immediately following the structural determination of the double helix by Watson and Crick [18]. In subsequent years, several laboratories dedicated significant efforts

towards elucidating the forces stabilizing nucleic acid duplexes and DNA–ligand interactions [19–24]. A combination of calorimetric and spectroscopic approaches led to the resolution of nearest neighbor contributions [22], which culminated in the development of predictive capabilities [25] that find numerous applications in the fields of biophysics, biochemistry, and molecular biology. Recent progress in the development of ultra-sensitive differential scanning calorimeters (DSC) (for reviews refer to [26, 27]), coupled with advances in oligonucleotide synthetic capabilities, have facilitated measurements of DNA duplex energetics with improved accuracy and reliability. Considering the abundance of empirical data and vast array of applications emerging from the analysis of nucleic acid energetics, there are a number of fundamental questions that remain unresolved.

Initial calorimetric measurements of DNA duplexes presumed that unfolding/dissociation does not proceed with an appreciable heat capacity increment. Subsequent attempts to characterize nucleic acid energetics have challenged this widely invoked assumption [28–31] as studies employing synthetic deoxypolynucleotides [29] and deoxyoligonucleotides [28, 30] have conclusively demonstrated that the helix-to-coil transition is accompanied by a measureable ΔC_p value. Recent investigations have adopted empirical approaches that specifically incorporate heat capacity contributions when evaluating DNA duplex energetics [31–34]. Several of these methods have embraced standard DSC protocols developed for the analysis of proteins [35, 36], in which heat capacity changes are determined from the difference in pre- and post-transition baselines extrapolated to the midpoint. The calorimetric enthalpy (ΔH_{cal}) is determined via integration of the resultant profile, while the transition temperature (T_M) corresponds to the maximal heat capacity [35–37].

These attempts to define the pre- and post-transition baselines generate a significant degree of uncertainty, particularly in terms of adequately describing the pre-denaturational duplex state that is often characterized by gradual thermal-induced fluctuations. As a consequence, the measured ΔC_p values of 130–423 $J{\cdot}K^{-1}{\cdot}(mol\text{-}bp)^{-1}$ {i.e., $\sim$30–100 $cal{\cdot}K^{-1}{\cdot}(mol\text{-}bp)^{-1}$} reported for DNA/RNA base pairing [29, 31, 34] reflect the overall uncertainty and variability in defining the thermodynamic parameters of duplex formation over a broad temperature range. Despite concerted efforts to reconcile nucleic acid association/dissociation energetics, the lack of a defined methodological protocol coupled with the dearth of experimental data assessing the impact of sequence- and length-dependence on DNA thermodynamics warrants further systematic investigations. A combination of DSC and ITC studies have revealed that DNA dissociation enthalpies measured at elevated temperatures are significantly higher than the corresponding association enthalpies of the complementary strands determined at lower temperatures. These findings suggest the need to explicitly incorporate the energetic contributions of competing equilibria such as the temperature-dependent intra/inter single strand residual structure formation [38]. Subsequent studies have advanced an experimental approach to characterize the energetics governing duplex to single strand equilibria [28, 30].

A related matter of inquiry that necessitates further exploration is the impact of heat capacity effects on nearest neighbor thermodynamics. The acquisition of such databases preceded discussions on the relevance of incorporating ΔC_p contributions and the resultant algorithms are derived exclusively in the absence of ΔC_p corrections. Although nearest neighbor algorithms are remarkably accurate in predicting global duplex dissociation enthalpies at the transition temperature (T_m), the thermodynamic parameters are often compromised when data are extrapolated to a desired reference temperature without explicit incorporation of ΔC_p contributions. In fact, there is ample evidence to suggest that the sequence-dependence of enthalpies/entropies determined at higher temperatures are

somewhat compressed when ΔC_p corrections are introduced and the parameters are extrapolated to lower reference temperatures [29, 34]. In recognition of the fundamental role that heat capacity changes contribute to duplex energetics, our ultimate goal is to assemble a database of nearest neighbor thermodynamic parameters that explicitly incorporates heat capacity changes and thereby facilitates elucidation of the molecular forces driving base-pair formation within the nucleic acid duplex at a given reference temperature.

Over the past decade, we have developed systematic methodologies employing isothermal and scanning microcalorimetric approaches to characterize the thermodynamic forces driving protein–DNA recognition [39–48]. We are now focusing attention on unraveling intrinsic DNA sequence-specific thermodynamic properties that govern protein binding (as reviewed in [46]). A natural extension of these studies is to evaluate the impact of endogenous/exogenous damaging agents on DNA structure, energetics, and recognition by specialized enzyme repair machineries [49–56]. In conjunction with our concerted effort to elucidate the energetic basis of helix to single strand transitions [28], the realization that DNA dissociation processes are accompanied by a defined heat capacity increment [29, 30] has prompted us to develop a comprehensive experimental strategy that characterizes duplex energetics as a function of sequence context and nearest-neighbor contributions. This quantitative approach incorporates the impact of single strand residual structures, gradual pre-melting thermal fluctuations, and heat capacity changes (ΔC_p) accompanying cooperative duplex-to-coil transitions. Our systematic analysis yields heat capacity corrected values for duplex dissociation enthalpies, entropies, and Gibbs energies. Such studies are required for a complete understanding of the thermodynamic forces governing nucleic acid stability and protein–DNA interactions [46].

4 Temperature-Induced Protein Unfolding

The application of differential scanning calorimetry to characterize protein energetics and stability is best illustrated by exploring the temperature-induced unfolding of myoglobin. Inspection of the DSC profile in Fig. 6 reveals the partial heat capacity function of myoglobin over a broad temperature range. The heat capacity of myoglobin increases linearly upon heating up to a critical temperature at which extensive heat absorption occurs and the protein unfolds. While the heat capacity decreases at the conclusion of this process,

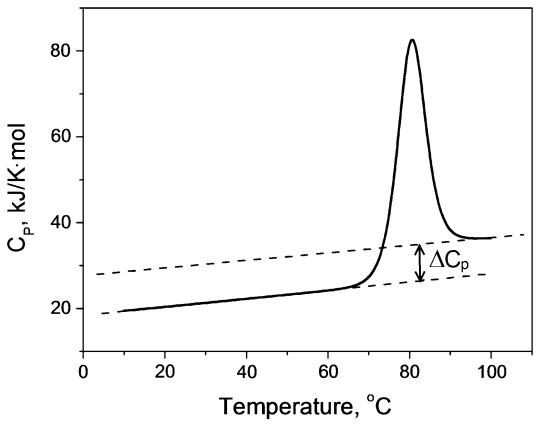

Fig. 6 Partial molar heat capacity of myoglobin in acetate buffer (pH 4.1)

its magnitude is sufficiently higher than expected for a linear extrapolation of the initial heat capacity. Thus, the unfolding of myoglobin results in a significant heat capacity increment. One may therefore surmise that the heat capacity of an unfolded protein is significantly higher than its compact native state.

A thermodynamic description of myoglobin unfolding may be viewed by invoking the Kirchhoff equation, which defines the heat capacity increment as follows:

$$\partial \Delta H / \partial T = \Delta C_p \tag{1}$$

The thermodynamic properties of protein unfolding may be evaluated in accordance with the following standard relations:

$$\Delta H(T) = \Delta H(T_t) - \Delta C_p(T_t - T) \tag{2}$$

$$\Delta S(T) = \Delta H(T_t)/T_t - \Delta C_p \ln(T_t/T) \tag{3}$$

$$\Delta G(T) = \Delta H(T) - T\Delta S(T) \tag{4}$$

Based on the thermodynamics prediction, one might reasonably anticipate that the enthalpy of protein unfolding increases linearly with temperature. Conversely, the enthalpy would decrease and might even change sign at sufficiently low temperatures as illustrated in Fig. 7.

Correspondingly, the entropy factor ($T\Delta S^{unf}$) is a nearly linear function of temperature. The difference between $\Delta H(T)$ and $T\Delta S(T)$ is essentially a parabolic function that represents the Gibbs energy of protein unfolding (ΔG), which is characterized by a maximum at approximately 35 °C. The Gibbs energy corresponds with the work required to unfold the protein at a given temperature and is usually considered a measure of protein stability. Inspection of the thermodynamic functions presented in Fig. 7 suggests that myoglobin in sodium acetate buffer (pH 4.0) exhibits a greater stability within the physiological temperature range. Upon increasing the temperature above 35 °C, myoglobin stability decreases and the protein undergoes unfolding. In an analogous manner, myoglobin stability should decrease at temperatures sufficiently below the physiological range.

Assuming that the thermodynamic formalisms are appropriate in describing the protein folding/unfolding process, myoglobin unfolding upon cooling should proceed with both the release of heat and a decrease in entropy. Corroboration of the expected behavior is

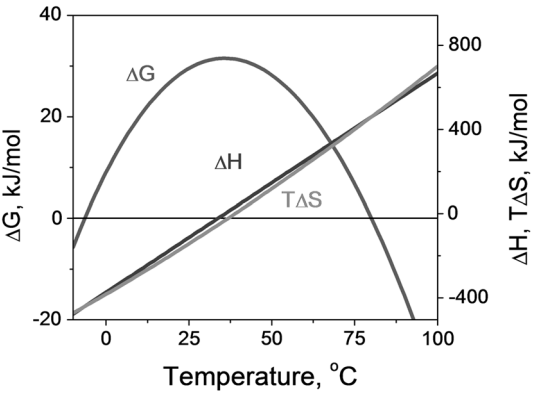

Fig. 7 Thermodynamic functions specifying myoglobin stability: unfolding enthalpy (ΔH), entropy factor ($T\Delta S$) and Gibbs energy (ΔG)

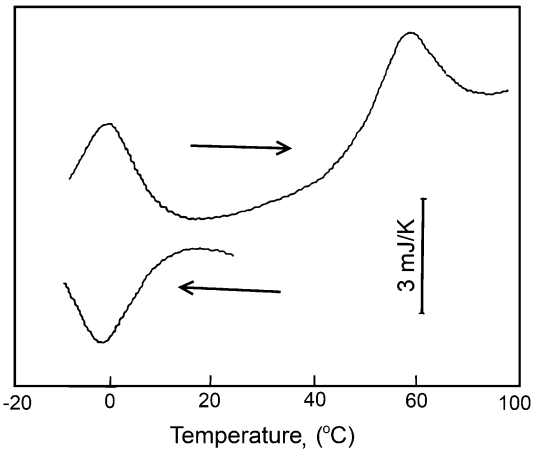

Fig. 8 The calorimetrically observed heat effect upon cooling and subsequent heating of apomyoglobin in sodium acetate buffer [57]

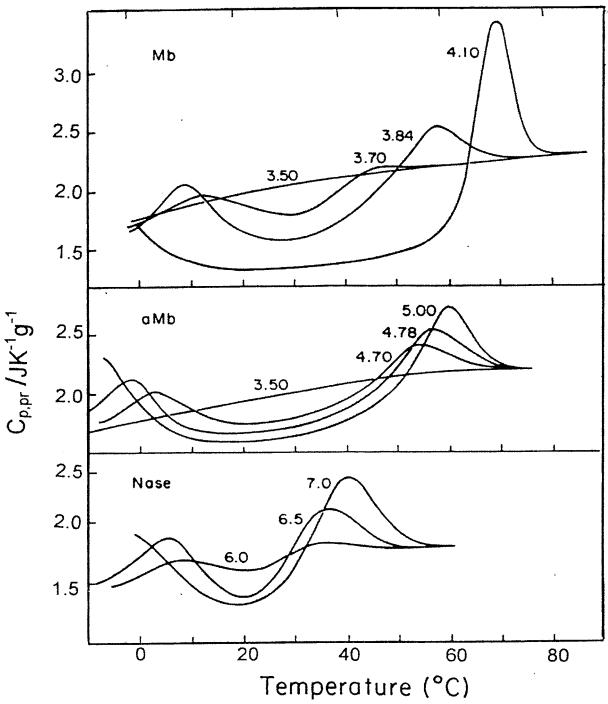

Fig. 9 Partial molar heat capacity of myoglobin (Mb), apomyoglobin (aMb), and staphylococcal nuclease (Nase) in sodium acetate buffer as a function of pH [57]

observed in DSC profiles (refer to Fig. 8), which demonstrate that myoglobin unfolds upon cooling to sufficiently low temperatures and this process is accompanied by the release of heat. In a subsequent heating cycle, the unfolded protein undergoes refolding with concomitant heat absorption followed by unfolding at higher temperatures and with corresponding heat absorption. Although cold denaturation appears to represent a general

property of globular proteins (refer to Fig. 9), experimental observation of this phenomenon is oftentimes hindered since it usually occurs at temperatures below 0 °C [57–59].

The temperature-induced unfolding of proteins is not surprising considering the fact that heat increases dissipative forces which effectively disrupt the compact, highly ordered protein structure. Conversely, dissipative entropic forces decrease with temperature, an observation that seems counterintuitive to the notion of unfolding upon cooling. A logical underlying explanation is that the enthalpy and entropy change signs at lower temperatures. Consequently, the enthalpy is a destabilizing factor whereas the entropy stabilizes the compact native state. The next question is therefore why both the unfolding enthalpy and entropy increase at higher temperatures, decrease at lower temperatures, and might even change sign? A reasonable response to these inquiries resides in a fundamental rule, namely that protein unfolding proceeds with a heat capacity increment. The heat capacity increase observed upon unfolding a compact protein might therefore actually reflect the higher degree of conformational freedom within polypeptide chains. Calculations reveal that the heat capacity change due to an increase in conformational freedom should be one order of magnitude lower than what is observed experimentally. Sources of such a disparity might include the impact of hydration effects, particularly those involving nonpolar groups.

5 Hydrophobic Forces

It is a well-established fact that nonpolar molecules are hydrophobic in nature and their transfer from the gaseous phase into aqueous solution is characterized by a large positive Gibbs energy change. An interesting observation is that the enthalpy measured for this transfer process is negligible at 25 °C [60]. Fundamental investigations on the

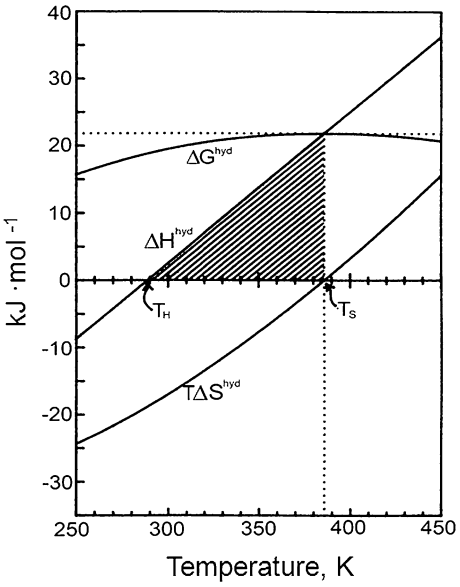

Fig. 10 Thermodynamic representation of liquid hydrocarbon (benzene) dissolution into water assuming a constant heat capacity change [65]

water solubility of hydrocarbons have provided essential information on hydrophobic effects, which are considered of pivotal importance in the formation and stability of higher order structures including proteins, nucleic acids, and cell membranes [61–63]. The transfer of nonpolar compounds into aqueous solution proceeds with a large negative entropy change, a finding that has been attributed to water ordering in the presence of nonpolar molecules. This entropy driven process has been regarded by Kauzmann [61] as the primary force responsible for protein structure compaction due to the removal of nonpolar groups from aqueous contacts. The energetically unfavorable hydrophobic force associated with water ordering of nonpolar groups has been widely invoked to describe the physics of protein folding. In subsequent years, the hydrophobic force and its implication for a number of biological processes including protein folding has been explored in greater detail and proven more complex than initially anticipated [64].

A remarkable feature characterizing the transfer of nonpolar compounds into aqueous solution is the accompanying heat capacity change. Specifically, one observes an inverse correlation as the large negative entropy decreases in magnitude upon heating and approaches zero at sufficiently high temperature (refer to Fig. 10). Conversely, the enthalpy of transferring nonpolar compounds into aqueous solution increases upon heating to the temperature at which the entropy is zero, reaching a critical value that is characteristic for the vaporization of nonpolar compounds [65]. Similarly, the Gibbs energy for transferring nonpolar groups into aqueous solution reaches its highest magnitude at the temperature of zero entropy in accordance with the conditions specifying a maximum Gibbs energy function [i.e., $(\mathrm{d}\Delta G/\mathrm{d}T)^{\max} = -\Delta S(T_s) = 0$]. In summary, a maximal hydrophobic effect is achieved at high temperatures and is characterized by an enthalpic as opposed to entropic force.

6 Cooperativity of Protein Unfolding/Refolding

Globular proteins comprised of a molecular mass lower than 20,000 Da generally unfold over a relatively narrow temperature range and the resultant native-to-unfolded state transition might be described as a cooperative process (refer to Fig. 11). Invoking the assumption of a two-state unfolding transition, the van't Hoff enthalpy for such a cooperative process may be determined via the following relation in which $\Delta T_{1/2}$ represents the width of a heat absorption peak at its half height [66]:

$$\Delta H^{vh} = -R\frac{\partial \ln K}{\partial 1/T} = 4RT^2/\Delta T_{1/2} \tag{5}$$

Applying a two-state analysis to the unfolding of small globular proteins reveals a general correspondence between the van't Hoff and calorimetric enthalpies. This direct correlation is often regarded as empirical evidence that unfolding/refolding approximates a two-state transition. Small globular proteins that exhibit typical two-state unfolding behavior are easily modeled as illustrated in the case of barnase (refer to Fig. 12). Visual comparison of the profiles reveals that the calorimetric endotherm and modeled transition are essentially indistinguishable, an observation which suggests that barnase unfolding/refolding proceeds without a significant population of folding intermediates.

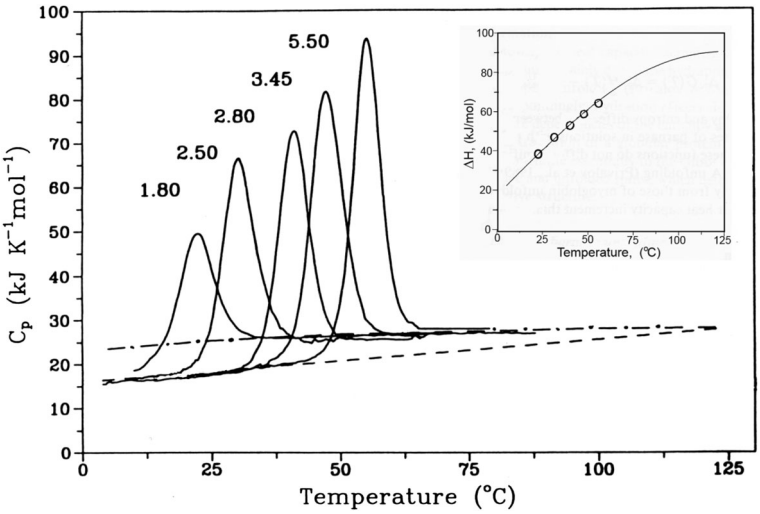

Fig. 11 The pH-dependent heat capacity profile of barnase, illustrating a progressive increase in thermostability and unfolding enthalpy at higher pH. The heat capacity difference between folded and unfolded states suggests that the enthalpy is a function of temperature and asymptotically approaches a constant value at about 120 °C (refer to *inset*)

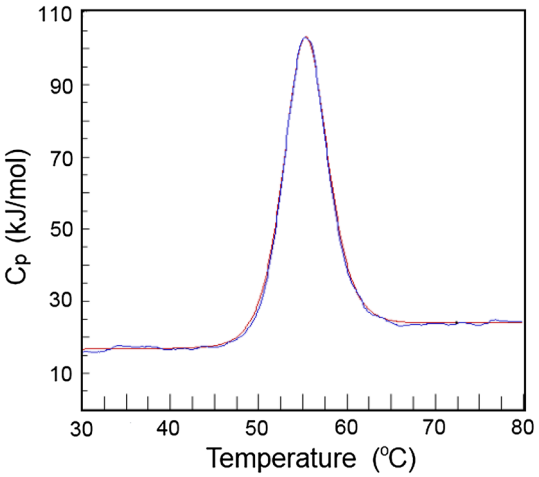

Fig. 12 Comparison of calorimetrically measured (*blue*) and computer simulated (*red*) heat capacity profiles of barnase in aqueous solution (pH 5.5). The observed overlap infers that the temperature-induced unfolding is indeed a highly cooperative process which fits a single two-state transition model (Color figure online)

7 Larger Globular Proteins

An example of a moderately large compact globular protein is papain with a molecular mass of 23,400 Da. The heat capacity profile of papain does not differ markedly from that of barnase, yet it exhibits a pH-dependent increase in peak area and sharpness (refer to Fig. 13). Qualitatively, the temperature-induced unfolding of papain appears to fit a co-operative two-state transition. Application of the van't Hoff equation (Eq. 5) for direct comparison with the integrated endotherm reveals a significant disparity in the calorimetric

and van't Hoff enthalpies (refer to Table 1). Specifically, the observed ratio of 2:1 implies that papain unfolding does not proceed via a single cooperative step. The topology of papain suggests that its polypeptide chain is folded into two domains which are separated by a deep cleft. The energetic data are therefore consistent with structural observations in that each of the papain domains unfolds/refolds more or less independently.

Another remarkable example of a large globular protein is plasminogen with a molecular mass of 90,850 Da. The heat capacity profile of plasminogen is extremely complex, suggesting that the protein unfolds via multiple steps (refer to Fig. 14). A unique advantage

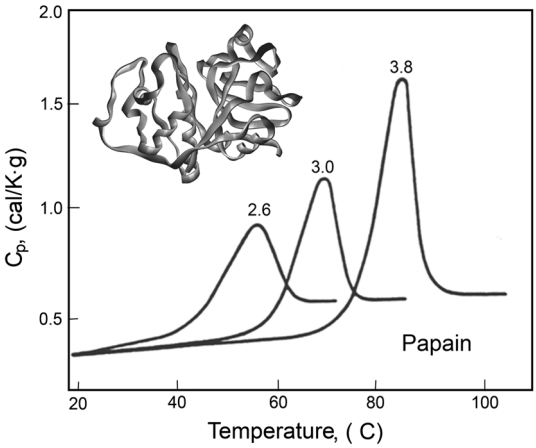

Fig. 13 The conformation of polypeptide chains comprising papain and its pH-dependent heat capacity profiles (1 cal = 4.184 J)

Table 1 Thermodynamic characteristics of papain unfolding

pH	t (°C)	ΔH_{cal} (kJ·mol^{-1})	ΔH_{vH} (kJ·mol^{-1})	$\Delta H_{cal}/\Delta H_{vH}$
2.6	56.0	122	67	1.82
3.0	69.3	168	84	2.00
3.8	83.8	216	117	1.85

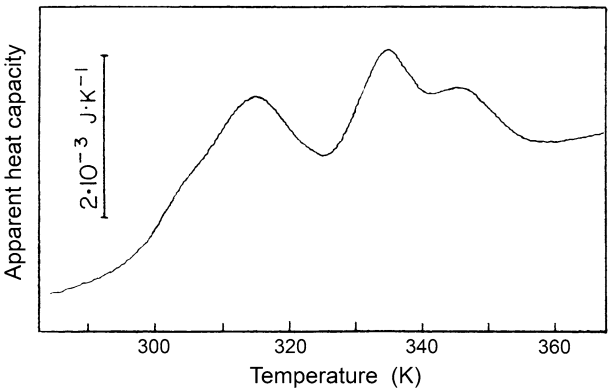

Fig. 14 Complex calorimetric profile of Lys-plasminogen in 50 mmol·L^{-1} glycine buffer (pH 3.4) [67]

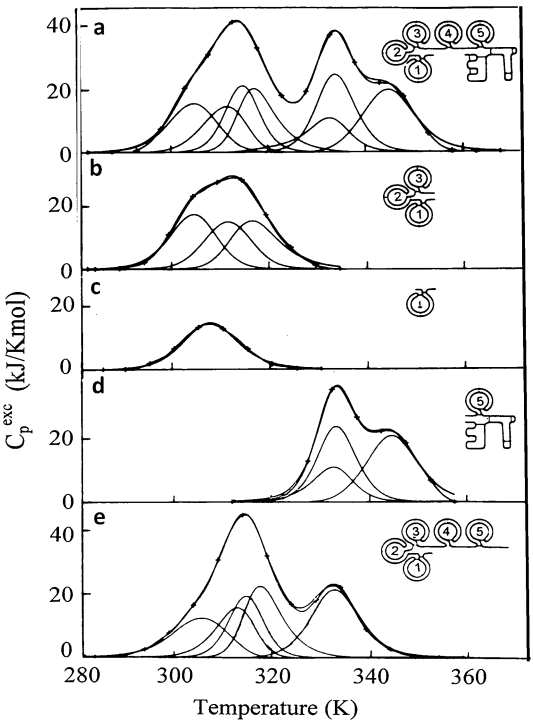

Fig. 15 Deconvolution analysis of the excess heat capacity function for **a** Lys-plasminogen; **b** fragment K 1–3 (residues 79–353); **c** fragment K4 (residues 354–439); **d** mini-plasminogen (residues 442–790); and, **e** heavy chain plasmin (residues 77–560) [67]

of differential scanning calorimetry is that the enthalpy and sharpness of each temperature-induced cooperative transition are related. One may therefore deconvolute the observed heat absorption profile into its simple components. Deconvolution analysis of the complex plasminogen endotherm yields seven independent yet largely overlapping transitions (refer to Fig. 15). Comparison of the resultant energetic data and plasminogen chemical structure suggests a direct correspondence, namely the polypeptide chain forms five loops with disulfide crosslinks (i.e., kringles) which are distinct from the domain responsible for pepsinolysis. Calorimetric analysis of the separated fragments has corroborated heat capacity deconvolution analysis and facilitated identification of the cooperative steps associated with temperature-induced unfolding of distinct domains in plasminogen [67].

8 Collagens

Collagens are of particular thermodynamic interest due to their unique thermal properties. In contrast to globular proteins, collagens are fibrillar in nature and comprised of coiled coils formed by three polypeptide chains arranged in a poly-L-proline helical conformation (refer to Fig. 16). While the third amino acid residue in every chain is glycine, the second and third are usually comprised of the imino acids proline or hydroxyproline. Although the structure resolved by Rich and Crick [68] revealed one set of HBs linking neighboring chains (refer to Fig. 17a), Ramachandran and Kartha [69] suggested the possibility of a second HB when the corresponding position is not occupied by an imino acid (refer to Fig. 17b). Consequently, Rich and Crick contended that the number of intramolecular HBs

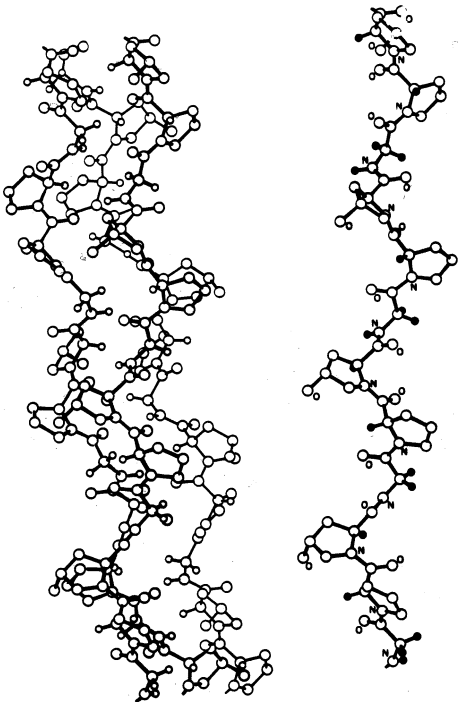

Fig. 16 Comparison of structural models for three-stranded collagen (*left*) and a single strand of the repeated sequence -Gly-Pro-Hyp- (*right*)

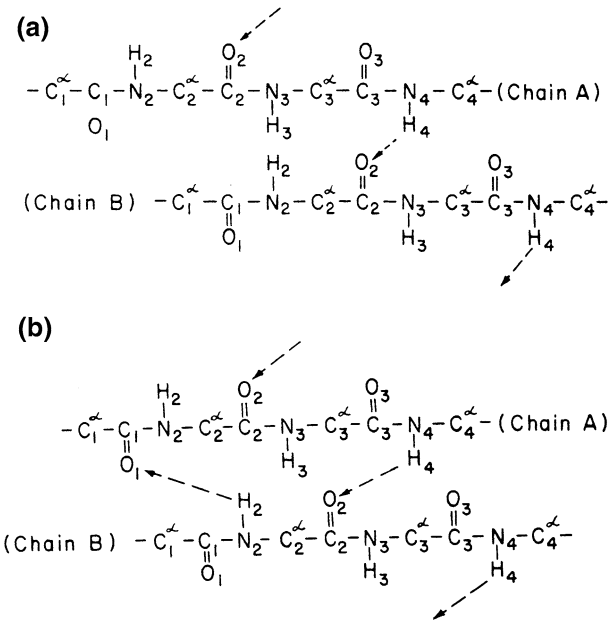

Fig. 17 Alternate schemes of hypothetical internal hydrogen bonding in collagen: **a** one-bond model proposed by Rich and Crick [68]; **b** two-bond model suggested by Ramachandran and Kartha [69]

in collagen does not depend on the amino acid content [68], in direct contrast with the findings of Ramachandran and Kartha who predicted an inverse correlation between the number of HBs and amino acid content [69].

A remarkable feature of collagen is that the structure unfolds over a relatively narrow temperature range which correlates closely with the physiological temperature of the corresponding species. In accordance with the model proposed by Rich and Crick, collagen stability is attributed to an increase of amino acid content and associated pyrrolic ring rigidity which is reflected by a corresponding decrease in entropy upon thermal-induced unfolding. One might therefore expect that the thermostability of collagen is not accompanied by an increase in its unfolding enthalpy. Conversely, the model of Ramachandran and Kartha predicts a decrease in enthalpy upon increasing the collagen amino acid content. In view of these contrasting perspectives, calorimetric analysis of the unfolding pathway appeared crucial for the ultimate determination of which model most accurately describes collagen's structure.

Calorimetric heat capacity profiles of collagens derived from various species with differing thermostability are presented in Fig. 18. Analysis of the resultant endotherms reveals the following general observations: (1) collagen unfolding enthalpies increase with melting temperature; (2) collagen unfolding proceeds without a noticeable heat capacity increment; and, (3) collagen unfolding enthalpies calculated per amino acid (or per gram) are several time larger than those of globular proteins. The most significant finding is that the collagen unfolding enthalpies are directly correlated with their amino acid content as illustrated in Fig. 19. Collectively, the calorimetric studies furnish unequivocal evidence that neither of these proposed models adequately describes the thermodynamic properties of collagen. Specifically, the correlation of collagen unfolding enthalpies with thermostability and increasing hydroxyproline content clearly indicates that the main stabilizing effect arises from the presence of hydroxyl groups in lieu of pyrrolidine ring rigidity.

A unique feature of collagen structure is that the hydroxyl groups are unable to form HBs within the triple helix and only interact with surrounding water. Considering the well known tendency of water to cooperate with neighboring molecules, it's entirely plausible

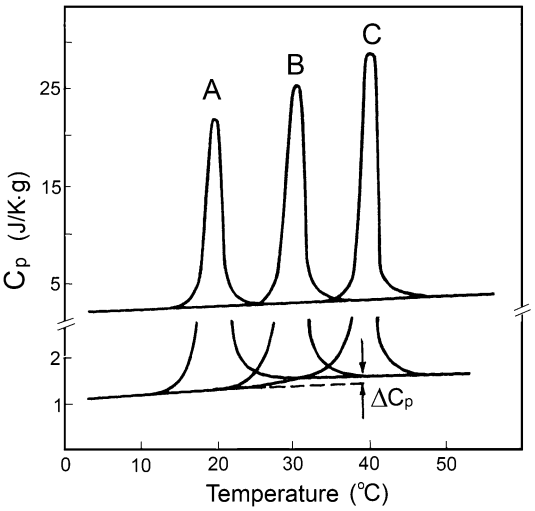

Fig. 18 Temperature-dependent partial specific heat capacity of cod (*A*), pike (*B*), and rat (*C*) skin collagens in salt-free aqueous solution (pH 3.5). A fragment of the profile depicted at magnified scale illustrates the denaturational heat capacity increment

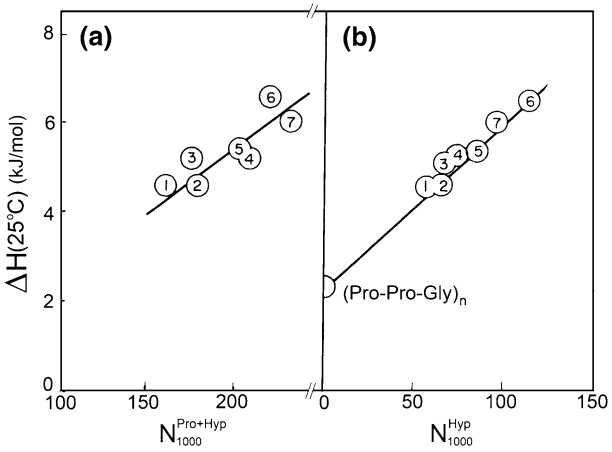

Fig. 19 Unfolding enthalpies of various collagen species expressed per mole of residue and extrapolated to 25 °C as a function of: **a** total prolyl and hydroxyprolyl content, and **b** hydroxyprolyl content

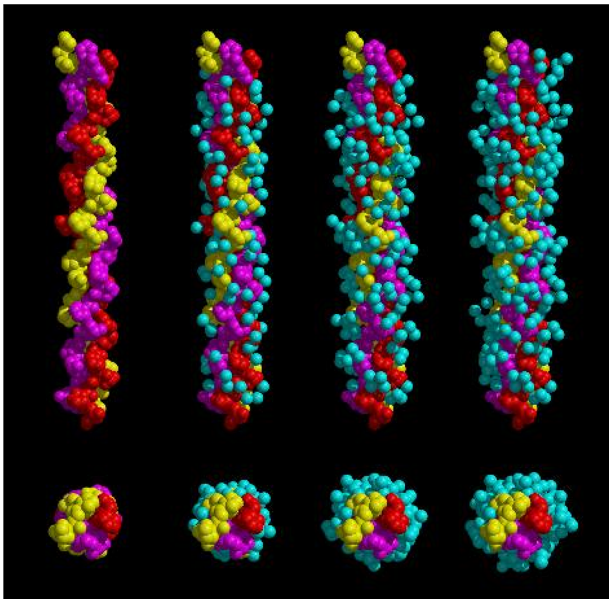

Fig. 20 Layers of fixed water molecules encompassing the collagen triple-helix [70]

that exposed hydroxyl groups can initiate formation of an extensive cooperative network of hydrogen bonding enveloping collagen. This frame of ordered water might be responsible for the exceptionally large enthalpy associated with collagen unfolding. Progress in the crystallization of synthetic models and subsequent high resolution X-ray structure analysis [70] has confirmed that collagen is surrounded by several layers of ordered water that stabilize the coiled-coil conformation (refer to Fig. 20).

9 Concluding Remarks

Nucleic acids and proteins are integral cellular components of all living organisms. The most distinguishing features of these intriguing molecular machines are the size and irregular aperiodic ordering of their spatial organization. The unique three-dimensional structure of proteins and nucleic acids are governed by the sequence of their amino acids and nucleotides, respectively. Knowledge of the structural properties at atomic resolution represents a necessary yet insufficient parameter for understanding the physical characteristics of these biological macromolecules. Complementary experimental strategies are therefore required to characterize the thermodynamic basis of these aperiodic quazi-macroscopic systems. Calorimetry represents the only model-independent approach that affords direct measurement of the energies governing the formation and stability of these macromolecular structures. The advent of ultrasensitive differential scanning and isothermal titration calorimeters has accelerated advances and progress in the field of biothermodynamics. This manuscript is intended to provide readers with a brief overview of critical insights gained from calorimetric measurements and their fundamental role in elucidating the energetics of biological macromolecules.

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