Thermochimica Acta, 193 (1991) 363–390
Elsevier Science Publishers B.V., Amsterdam

Proteins in aqueous solutions. Calorimetric studies and thermodynamic characterization

Giuseppina Castronuovo

Department of Chemistry, University Federico II of Naples, via Mezzocannone, 4–80134 Naples (Italy)

(Received 4 January 1991)

INTRODUCTION

Calorimetric techniques applied to the study of the thermal denaturation of proteins, and of their interaction with various ligands, have permitted, over the last two decades, a better insight to be gained into our understanding of complex biological phenomena. Improvements in the design of calorimetric equipment have enabled the detection of very small thermal effects associated with the formation or disruption of non-covalent bonds in a macromolecule. Only through differential scanning calorimetry has it become possible to assess the two-state nature of the thermal unfolding of many globular proteins in aqueous solution. It is mainly by means of this technique that we are now able to deal satisfactorily with a phenomenon known for a long time, but experimentally difficult to study: the "cold" denaturation of proteins. Why does a protein unfold at a temperature so much different from that of the more usual "heat" denaturation? What are the forces controlling this phenomenon? The thermodynamic stability of a protein conformation is the result of several non-covalent interactions which may occur intramolecularly or with the solvent. The importance of the role of water in biochemical processes has long been recognized, starting with the analysis of the solvent effect made by Kauzmann [1]. This is the reason why it is impossible to discuss the thermodynamic stability of proteins without considering studies of the thermodynamics of model systems in aqueous solution. Besides, the ability of proteins to interact with small and large ligands (for instance, with other proteins, as in self-association) is the basis of many biological phenomena, such as specific binding, cooperative binding, catalysis, and ligand-induced structural changes. Isothermal calorimetry acquires special significance, because it provides all the thermodynamic parameters characterizing the association process.

DIFFERENTIAL SCANNING CALORIMETRY

Differential scanning calorimetry (DSC) has proved to be a powerful technique for studying the thermally induced conformational transitions of

biopolymers, such as proteins and nucleic acids [2–4]. The high reliability of the thermodynamic data obtained allows one to test rigorously the assumptions related to the reaction mechanism, and provides additional details of the molecular aspects of the reactions involved. In the present contribution, attention will be focused mainly on differential scanning calorimetry and on the results obtained in the last few years by this technique. However, because of the wealth of information appearing in the literature during this period, the discussion will be restricted to those studies which, in the author's opinion, have provided useful thermodynamic information. Other calorimetric methods, such as isoperibol calorimetry, and its many applications to proteins and other biomolecules will not be considered here. However, the extensive reviews existing on this subject will show the reader the advantages and the unique *features of every calorimeter.*

Recently, Privalov has reviewed the three historical and technical stages in the development of scanning calorimeters and the practical and technical reasons which stimulated the development of each stage [5]. Readers are therefore referred to that article for the technical features of these instruments, and for an insight into the future progress of their design.

Heat capacity data on the unfolding of RNase A [6] and lysozyme [7] have been reported for use in calibration of scanning calorimeters. The conformational transitions exhibited by the two proteins are suitable sources of accurate, reproducible thermal data for evaluating calorimeter performance.

A typical scanning calorimetric curve enables the evaluation of the thermodynamic parameters necessary for characterizing the denaturation process. From the curve, it is possible to obtain the temperature dependence of the heat capacities of the native and denatured protein below and above the transition region, the change of the heat capacity at the transition, the transition temperature, the calorimetric enthalpy change directly from the area under the curve, and the van't Hoff, effective, enthalpy by the sharpness of the curve. An important point to underline is that the determination of the calorimetric enthalpy does not require any a priori assumption relating to the thermodynamic mechanism of the reaction. One of the greatest advantages of scanning calorimetric traces is the possibility of obtaining simultaneously the real, calorimetric enthalpy of the transition process and also the effective (van't Hoff) enthalpy. Comparison of these values permits hypotheses to be formulated regarding the cooperativity and nature of the denaturation process.

HEAT DENATURATION OF PROTEINS

According to Kauzmann [1], the denaturation of a protein is a process during which the spatial disposition of the polypeptide chain changes

TABLE 1

Calorimetric and van't Hoff enthalpies for the denaturation of Staphylococcal nuclease at various pH values [a]

pH	T_d (°C)	$\Delta_d H_{cal}$ (kJ mol^{-1})	$\Delta_d H_{v.H.}$ (kJ mol^{-1})	R
7.0	51.7	353	323	1.09
6.8	50.0	332	312	1.06
5.0	48.3	290	372	1.07
4.5	43.3	268	238	1.13
3.98	35.0	186	159	1.17

[a] Data from ref. 11. R is the ratio between the calorimetric and the van't Hoff enthalpies.

toward a more disordered one. An approach which is useful for studying the stability of the native conformation is to perturb it through the change of a physico-chemical parameter, and to evaluate the work required for complete denaturation. A very effective agent for such thermodynamic studies is the temperature. Simply by using temperature as a variable, it is possible to gain information about a protein conformation on an energetic basis. In fact, the dependence of enthalpy on the temperature gives the partition function, which permits the statistical description of any macroscopic system. Calorimetric studies have shown that the denaturation process for many small, compact, globular proteins can be described according to the two-state theory [8–10]. This theory implies a discontinuity between two thermodynamic states, one being stable below and the other above the transition temperature. Every thermodynamic state is the macroscopic resultant of many microscopic states allowed to the system under the conditions of temperature and pressure employed. The transition from the native, folded state to the denatured, unfolded form is a highly cooperative process involving disruption of intramolecular hydrogen bonds, hydrophobic interactions, and other types of non-covalent interactions. If the two-state model actually describes the process being considered, the ratio of the enthalpy experimentally determined to that evaluated by the van't Hoff approach should be near to unity. In Table 1, the calorimetric and van't Hoff enthalpies are reported for the denaturation of Staphylococcal nuclease at various pH values [11]. Their ratios slightly exceed unity, as for many other small, globular proteins. In fact, an average ratio of 1.05 ± 0.05 for the thermal unfolding of several proteins is reported by Privalov [9]. A larger deviation from unity can be considered as evidence of the inadequacy of the two-state model, or of the existence of other macroscopic states between the native and the denatured one.

Small, compact, globular proteins denature according to the two-state model: the protein, in this case, behaves as a single cooperative system. However, most of the proteins we know are not small and globular, but rather, they are formed by various cooperative units, or domains [12]. Their

TABLE 2

Calorimetric and van't Hoff enthalpies for the thermal denaturation of pepsinogen under various conditions [a]

pH	Solvent	T_d (°C)	$\Delta_d H_{cal}$ (kJ mol^{-1})	$\Delta_d H_{v.H.}$ (kJ mol^{-1})	R
6.0	buffer	66.0	1133	728.0	1.6
6.5	buffer	64.5	1069	548.1	2.0
7.1	buffer	57.5	975.2	544.3	1.8
8.0	buffer	51.1	761.1	511.7	1.5
6.5	buffer + 1 M urea	61.0	1004.2	523.8	1.9
7.1	buffer + 1 M urea	54.5	819.2	500.4	1.6

[a] Data from ref. 14. R is the ratio between the calorimetric and the van't Hoff enthalpies.

properties do not change abruptly, and thus their denaturation cannot be considered as a simple two-state transition. As an example, the ratio $\Delta H_{cal}/\Delta H_{v.H.}$ for papain (mol. wt. = 23000) is 1.80 ± 0.01 [13]. For pepsinogen (mol. wt. 40000), the data reported in Table 2 show that, whatever the conditions, the denaturation is not a two-step process [14]. The explanation for this is that these proteins consist of two independent and equally cooperative regions. In general, large proteins are made up of more or fewer cooperative domains. The native structure is disrupted in several discrete steps, each one assumed to be a two-state transition. In many cases, each domain behaves as a single cooperative unit almost independently of the others.

DSC permits the study of a protein's thermal stability directly, over a broad concentration range, and in the absence of light, thus overcoming the problem of photosensitivity which, in the case of some proteins, tends to complicate spectroscopic studies. These features have made DSC particularly attractive for studying the thermal stability of bovine lens crystallins. Structural stability is an essential prerequisite for the behaviour of these proteins: in fact, there is practically no turnover during the life of a mammalian lens. The inherent transparency of these crystallins, a consequence of short-range order between them, is greatest at the high protein concentrations found *in vivo* (300–600 mg ml^{-1}). The thermal stability of the major crystallin fractions has been studied [15], together with the effects of protein concentration on each of their thermally induced conformational transitions. All the major lens crystallin fractions were found to be thermally stable; indeed, α and β crystallins increase their thermal stability at increasing protein concentrations, an effect having physiological relevance. One of the main components of the core region of the eye lens, γII-crystallin, has been studied in detail very recently [16]. It is known to consist of two homologous domains, each composed of two similar "Greek key" motifs. Equilibrium and kinetic studies on the domain folding of this

crystallin show biphasic transition profiles. In particular, calorimetry has shown that the enthalpy of unfolding of the complete molecule is twice the van't Hoff enthalpy. Bimodal scans give two temperatures corresponding to the independent "melting" of the –COOH and $-NH_2$ terminal domains.

Complex thermal behaviour is, obviously, more easily explained when the structure of the protein in the solid state is known. For instance, DSC experiments have shown that the tryptophan repressor of *E. coli* offers an unusual stability against thermal unfolding and an unusual resistance against the degradation of its separate polypeptide chains [17]. The protein is a dimer, the monomeric subunit of which consists of helical regions that are intertwined with the corresponding helices of the other subunit, thus forming significant inter-subunit contacts. Such a type of interaction gives to the dimer the high stability detected by calorimetry.

Bovine seminal ribonuclease, RNase BS, isolated from bull seminal plasma is a natural dimer of pancreatic RNase A. The two subunits are covalently linked by a pair of interchain consecutive disulphide bridges: structural studies have shown that the subunits exchange the $-NH_2$ terminal peptides. DSC studies of the thermal denaturation of the monomeric and dimeric proteins show that the thermal denaturation is a two-state transition characterized by the same transition temperature [18]. The enthalpic change for the dimer is much less than twice that relating to RNase A. This fact indicates denaturation processes which are only partially comparable, probably because the denatured state of the dimeric covalent protein has fewer degrees of freedom than two separate monomeric units.

An important step in the study of complex proteins was the introduction of the statistical deconvolution of thermally induced melting profiles, a procedure which makes it possible to dissect the melting profile of a multistate transition [19,20]. In physics, statistical deconvolution is often known as function analysis, and deconvolution 'desmearing' yields the true kinetic heat flow rate from calorimetric curves for instruments with high time constants. A classical example of this type of study is that of plasminogen [21]; deconvolution of the experimental calorimetric curve has shown that the thermal denaturation involves seven transitions. The results obtained by analysing the fragments obtained by 'cutting' the molecule by means of various proteolytic enzymes showed that the various fragments behave as they do in the intact molecule, thus indicating that the parts are independent, each one resembling a small globular protein. Another interesting case is fibrinogen, which according to calorimetric studies has twelve domains [22]. Sometimes the deconvoluted DSC curves unravel more complex behaviour with respect to that shown by the melting curves obtained by optical methods. Very complex melting profiles were detected for the thermal unfolding of myosin and two of its proteolytic subfragments [23]. The deconvolution of the DSC curves showed that myosin rod con-

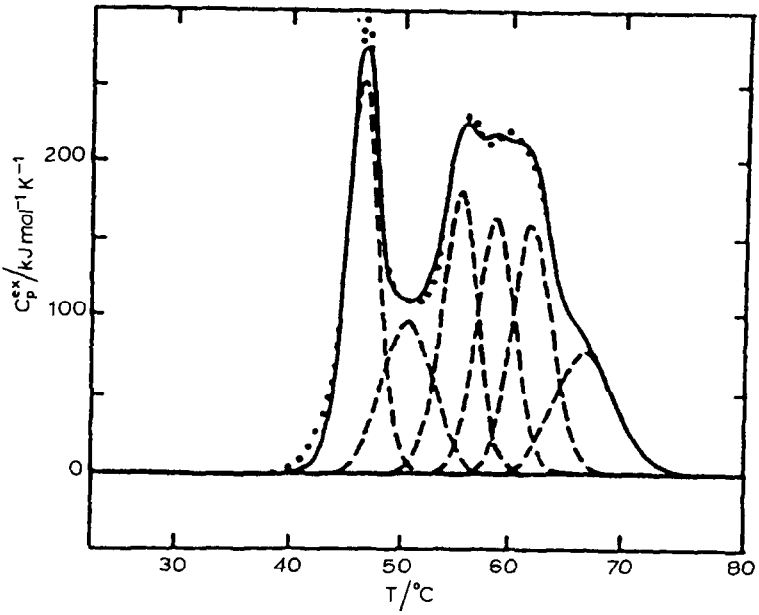

Fig. 1. Experimental curves (·····), resolved by computer deconvolution components (– – – –), and sum of the resolved transitions (———) of the excess heat capacity for myosin rod in 0.5 M KCl, 0.2 M phosphate (pH 7.0). From ref. 23.

tains six independent cooperative domains (Fig. 1), each of which can undergo a two-state thermal transition. The calorimetric results led to the possibility of tentatively locating the two-state domains along the myosin rod.

TREATMENT OF DSC DATA

The denaturational behaviour of complex systems has commonly been treated by an independent-domains approach. This approach assumes the independence of the structural domains and their melting as two-state processes. However, when the domains are not independent, there is a need to take into account all interactions between cooperative domains: that becomes increasingly difficult with increasing numbers of domain interfaces. In these cases, the most important interactions are provided by a least-squares analysis of the data.

In contrast to the assumption of the independence of structural domains of complex macromolecules, an allosteric approach [24] has been utilized to study the effects of aggregation, buffers and ligation upon the thermal denaturation of proteins as measured by DSC [25]. The various thermal processes are treated in terms of overall macromolecular states, thus overcoming the problem of domain composition of the protein involved. Least-squares fitting methods permit one to check whether an assigned

equilibrium scheme is correct with respect to the experimental observation. Kidokoro et al. have developed the mathematics of the deconvolution procedures [26] as they apply to self-dissociation/association processes, but without discussing the ligand binding effects [27].

Interactions between domains, for multi-domain proteins, provide the intraprotein communication necessary to coordinate function. However, the thermodynamic models available to interpret the DSC results for these proteins are insufficient for giving quantitative information on this interaction or for analysing its dependence on the variation of the experimental conditions. In fact, either the interaction is simply neglected, or it is assumed to be strong enough to render the sequence of unfolding unique. Recently, a simple thermodynamic model has been formulated which quantifies this interaction by the inclusion of an interface free energy term [28]. This term is assumed to vanish when either domain involved in pairwise interaction unfolds: hence, domain–domain interaction seems to affect only the domain unfolding at lower temperature. The model has been applied successfully to the DSC data for several proteins, in the absence or presence of substrates, and also to ligand binding of proteins having communicating domains.

HEAT CAPACITY OF AQUEOUS SOLUTIONS OF PROTEINS

The transition of a protein from the native to the denatured state is a process which occurs with increase in the heat capacity. The heat capacity is the derivative of the enthalpy with respect to the temperature, and this function thus describes in greater detail the changes in the state of the macromolecule with temperature. A consequence of the difference in the heat capacities of the two states is the dependence of the thermodynamic parameters on temperature. In fact, under fixed pH and environmental conditions

$$\Delta_d H(T) = \Delta_d H(T_d) + \int_{T_d}^{T} \Delta_d C_p \, dT \tag{1}$$

$$\Delta_d S(T) = \Delta_d S(T_d) + \int_{T_d}^{T} (\Delta_d C_p / T) \, dT \tag{2}$$

Here, T_d is the transition temperature, where the populations of the native and denatured states are equal and the Gibbs free energy difference is zero.

Many effects have been supposed to contribute to the increase in the heat capacity of a protein, the main one being a more extended hydration of non-polar groups exposed to water upon unfolding. The increase of the configurational freedom of the polypeptide chain upon the disruption of the compact native structure and the gradual melting of the residual

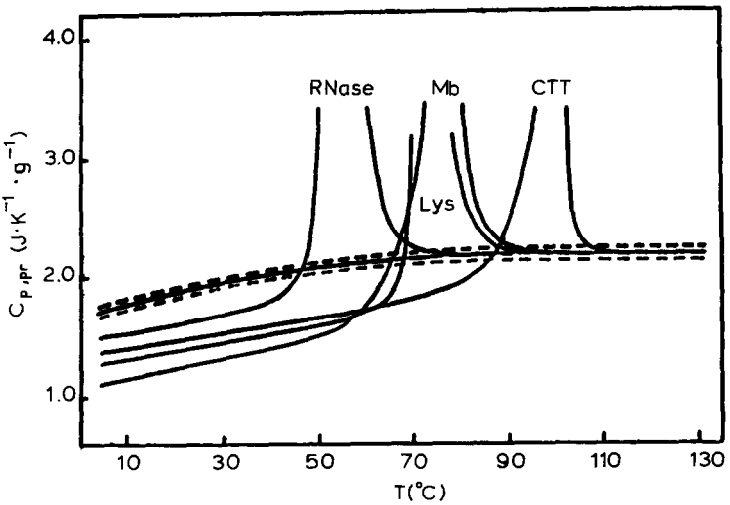

Fig. 2. Heat capacity as a function of temperature for ribonuclease A, lysozyme, myoglobin and catalase from *Thermus thermophilus*. The flattened curves refer to ribonuclease, lysozyme and apomyoglobin having the polypeptide chains in a random coil conformation. From ref. 30.

structure of the denatured protein at increasing temperature must also be considered. However, the extent of these last two contributions has been a controversial matter, and recent studies on the heat capacity of proteins have shed new light on this subject.

One of the problems that has attracted the attention of researchers concerns the denatured states of proteins. Is the denatured state of a protein in guanidine hydrochloride (GuHCl) or urea the same as that obtained by acid or heat denaturation? The last named was thought of as maintaining a residual structure, and then it was not seen as a completely unfolded state. However, for lysozyme, direct calorimetric experiments showed that, when the solvation of the denaturant was considered, the enthalpy of denaturation by heat agreed with that obtained in GuHCl [29]. Moreover, the partial heat capacity of heat- and GuHCl-denatured lysozyme are indistinguishable. Recently, the heat capacity of several proteins has been studied calorimetrically in a wide temperature range, −5–130 °C, using a third generation capillary scanning microcalorimeter, which allows exploration of temperatures below 0 ° and above 100 °C [30]. To reach 130 °C, measurements were performed under an excess pressure of 5.066 $\times 10^5$ Pa. Five globular proteins have been examined: pancreatic ribonuclease A, staphylococcal nuclease, hen egg-white lysozyme, sperm whale myoglobin and horse heart cytochrome *c* (Fig. 2). To study better the heat capacity of the native state over a broad temperature range, another globular protein was also studied, catalase from thermophilic microorganisms, on account of its extreme thermostability. Among others, two impor-

tant findings of that study are: (a) the heat capacity of the native protein is much lower than that of the denatured one, which in turn closely resembles that of an unfolded polypeptide chain calculated from the heat capacities of the constituent amino acid residues; (b) the heat capacity of the native protein increases linearly with temperature, while that of the denatured form is not a linear function of the temperature. The slope of the latter function decreases asymptotically and vanishes at about 100 °C. The similarity of the heat capacity of the denatured protein and of the completely unfolded polypeptide chain is evidence that the increment of the denaturational heat capacity is due, not to a gradual melting of the residual structure, but rather is caused prevailingly by the hydration of the non-polar groups exposed to water following the unfolding process. The heat capacity increase is different for different proteins, and it is correlated with the number of contacts between non-polar groups in native proteins. The different dependences on temperature of the heat capacities of the two forms of the protein make the heat capacity increment a decreasing function of the temperature. Thus, $\Delta_d H$ and $\Delta_d S$ should asymptotically approach a constant value at the temperature where $\Delta_d C_p$ becomes zero. That actually happens at a temperature of ≈ 140 °C, as has been shown for two proteins, ribonuclease A and myoglobin [31,32]. This temperature should be the same for globular proteins. In fact, the heat capacity

TABLE 3

Partial molar heat capacities (kJ K^{-1} mol^{-1}) of proteins [a] calculated for the unfolded polypeptide chain (C_p^U) and experimental values for the denatured and native states (C_p^D) and (C_p^N), and contribution of hydration ($\Delta_d C_p^{hydr}$) to the total heat capacity change ($\Delta_d C_p^{tot}$) on protein unfolding

Heat capacity	Temperature (°C)					
	5	25	50	75	100	125
Lysozyme						
C_p^U	26.7	29.1	31.1	31.8	32.4	32.5
C_p^D	25.2	27.5	29.5	31.1	31.4	31.5
C_p^N	18.2	20.0	22.2	24.4	26.7	28.9
$\Delta_d C_p^{hydr}$	6.6	7.8	8.4	8.1	7.7	6.8
$\Delta_d C_p^{tot}$ [b]	8.5	9.1	8.9	7.4	5.7	3.6
Ribonuclease						
C_p^U	24.1	26.0	27.8	28.5	29.3	29.5
C_p^D	23.1	26.0	27.7	29.2	29.8	30.1
C_p^N	19.5	20.8	22.5	24.2	25.8	27.5
$\Delta_d C_p^{hydr}$	3.7	4.9	5.7	5.5	5.5	4.9
$\Delta_d C_p^{tot}$ [b]	4.6	5.2	5.3	4.3	3.5	2.0

[a] Selected data taken from ref. 34. [b] $\Delta_d C_p^{tot} = C_p^U - C_p^N$. The hydration heat capacity change from the native to the completely unfolded state was calculated as described in refs. 33 and 34.

increment for the transfer to water of non-polar substances decreases asymptotically at increasing temperature. Consequently, also for a protein, the effect of exposing its non-polar groups to the solvent should become less and less pronounced with increasing temperature, and finally disappear. The asymptotical value of $\Delta_d H$ and $\Delta_d S$ should thus indicate the absence of hydration effects.

As for the increase of configurational freedom gained by the polypeptide chain upon unfolding, recent studies have quantified the extent of this effect. Partial molar heat capacities of all amino acid residues and their constituent groups in aqueous solution have been determined over a broad temperature range (5–125 ° C) [33,34]. The calculated heat capacities of the completely unfolded polypeptide chains have been compared with the experimental values for the denatured and native states of lysozyme, ribonuclease, myoglobin and cytochrome. This operation has permitted the calculation of the contributions of the hydration and configurational effects to the total heat capacity change computed with respect to the ideal random coil, and obtained by the simple summation over all amino acid residues. As can be seen from Table 3, the main contribution to $\Delta_d C_p^{tot}$ originates from the hydration effect. The difference between the total and the hydration heat capacity changes gives the small contribution of the configurational freedom gained by the polypeptide chain.

THERMAL DENATURATION OF PROTEINS IN THE PRESENCE OF STABILIZING OR DENATURING AGENTS

The structural stability of proteins is extensively controlled by the interactions between the proteins and the surrounding solvent molecules. Various substances stabilize or destabilize the native structure as a reflection of their effect on the water structure around the protein. Polyhydric alcohols, such as glycerol and sorbitol, and sugars prevent the loss of enzymatic activities and raise the denaturation temperature of proteins, probably through the strengthening of hydrophobic interactions [35]. In contrast, monohydric alcohols are found to weaken the native conformation. Studies of the thermal denaturation of RNase A and chymotrypsinogen in aqueous solutions of ethylene glycol at several acidic pH values [36], and of RNase A in aqueous solutions of 2-methyl-2,4-pentanediol [37], reveal evidence of the role of solvent water in such processes. The plot of $\Delta_d H$ versus $\Delta_d S$ at T_d gave a linear correlation between these parameters, thus indicating an enthalpy–entropy compensation effect, evidence that the thermal stability of these proteins is closely related to the water structure around the protein molecule. RNase A increases its stability against thermal denaturation in the presence of maltitol [38]. Even in the presence of guanidine hydrochloride, polyols induce stabilization of the protein, competing with the effect of the denaturant, as detected by DSC and by

other methods [39]. The thermodynamics of unfolding of intact lysozyme and of lysozyme linked between Glu35 and Trp108 was studied in aqueous solutions in the presence of various concentrations of propan-1-ol by DSC [40]. The dependence of the thermodynamic parameters on the concentration of the alcohol gave useful information on the stabilization mechanism in the cross-linked protein.

Concentrated aqueous solutions of urea and guanidine hydrochloride are often used as denaturing media for proteins. The high concentrations necessary for these conformational perturbants to be effective are an indication of their unspecific action. Competitive solvation for the polar groups on the surface of the protein, interactions between water and denaturant molecules, and competitive intermolecular interactions of the denaturant with peptide groups are among the effects which can lead to the disruption of the intramolecular peptide–peptide hydrogen bonds. These effects are not easily taken into account, and there are not, as yet, reliable procedures to evaluate the thermodynamic parameters associated with the conformational transitions caused by these denaturants. In fact, in those cases where a denaturant is used, there is the need to extrapolate the free energy of stabilization to zero concentration of denaturant. The problem is how this extrapolation should be done. The DSC results, reported for lysozyme [29] and α-lactalbumin [41] in the presence of various concentrations of GuHCl, have been used to obtain the $\Delta_d G^{H_2O}$ at zero denaturant concentration by the linear extrapolation method. The values were found to be comparable with those obtained from the average change in accessibility to solvent of the component groups of the protein [42].

EFFECTS OF LIGANDS ON THE THERMAL UNFOLDING OF PROTEINS

Differential scanning calorimetry is commonly used to study the effect of ligands on the thermal denaturation of proteins. As an example, in an extensive study concerning cytoplasmic aspartate transaminase [43], various problems were addressed: the stability of the various forms of apo- and holoenzyme in the absence or presence of ligands, sensitivity of subunits to the degree of occupancy of specific sites by substrates or coenzyme, and thermal unfolding in the presence of covalently bound ligands as compared with non-covalently bound analogues. In general, when a ligand is bound by the native form of a protein but not by the denatured one, the denaturational temperature will increase at increasing ligand concentration according to the van't Hoff equation [4], as shown by the binding of *Streptomyces* subtilisin inhibitor to α-chymotrypsin [44], or of L-arabinose or D-galactose to the L-arabinose binding protein of *E. coli* [45]. In the case of Taka-amylase A [46], the release of the tightly bound Ca^{2+} ion occurs during the unfolding of the last of the three domains identified by the

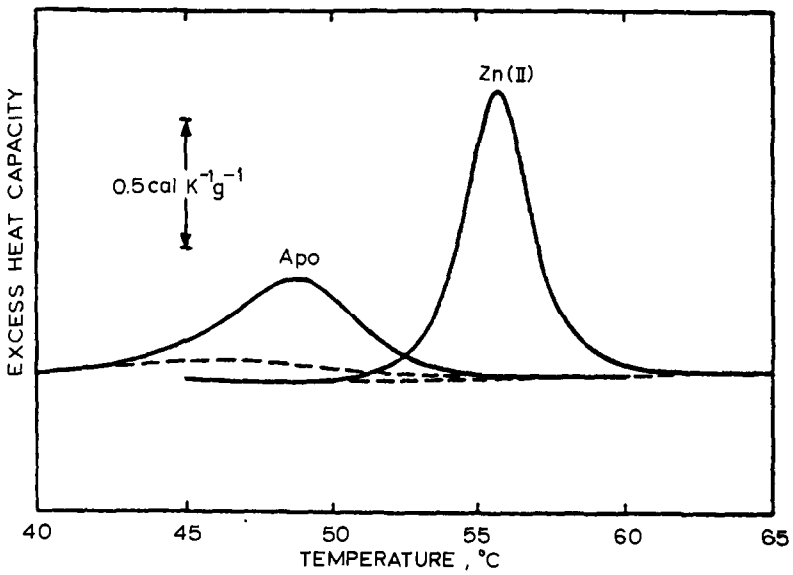

Fig. 3. Differential scanning calorimetric curves showing the effects of Zn^{2+} removal on the thermal denaturation of T4 gene 32 protein. From ref. 47.

deconvolution of the experimental curve. Gene 32 protein from T4 bacteriophage is a single-stranded DNA binding metallo-protein required for DNA replication, recombination and repair. DSC studies of the thermal unfolding of native Zn^{2+} gene 32 protein and its Co^{2+} and Cd^{2+} substituted forms in the presence and absence of poly(dT) have shown [47] that the metal ions increase the thermal stability of the protein with respect to that of the apo form (Fig. 3), especially when bound to single-stranded DNA. Studies of thermal denaturation in the presence of ATP, CTP and *N*-(phosphonoacetyl)-L-aspartate gave important information about the interactions between subunits in the case of aspartate transcarbamoylase from *E. coli* [48]. Catabolite activator protein from *E. coli* has been studied in the absence and in the presence of adenosine cyclic 3′,5′-phosphate and guanosine cyclic 3′,5′-phosphate [49]. In contrast to a single peak obtained for the thermal denaturation of the protein alone, multipeaked denaturation curves were obtained in the presence of the ligands. The data suggested the presence of independent domains for the nucleotide-bound protein, and an increased extent of oligomerization of the protein in the presence of either ligand.

Recent studies concern the thermal denaturation of bovine [50] and human albumins [51,52]. The mechanism of denaturation of the latter, the consequence of removing endogenous long-chain fatty acid (LCFA) on the observed biphasic unfolding of undefatted monomer, and the effects of protein concentration on the unfolding of defatted and undefatted monomer were explored. Denaturation of defatted human albumin

monomer is monophasic. In the presence of subsaturating concentrations of various ligands, biphasic or monophasic unfolding processes take place, according to the affinity of the native protein for the ligands. The greater the affinity, the greater the tendency for biphasic denaturation. The biphasic unfolding process is related to the uneven fatty acid distribution, which leads to the denaturation of different protein molecules, LCFA-poor and LCFA-rich. Any relationship of such a process to the unfolding of different domains within the molecule is excluded. Thermodynamic calculations of the excess heat capacity, based on experimental observations which account for the effects of the ligand binding on the two-state thermal denaturation of a protein, have recently been reported [53]. These results confirm that the ligand-induced biphasic protein denaturation is caused by a perturbation of the ligand binding equilibrium occurring during the thermal event.

EFFECTS OF MUTATIONS ON THE THERMAL TRANSITION

The study of mutant proteins offers the possibility of determining the role of individual amino acids in both the stability and the biological function of a protein. In fact, the marginal stability of the secondary and tertiary structure of proteins makes them susceptible to stability perturbations even by a single amino acid replacement [4,54].

Very recently, a DSC study has been carried out on *E. coli* aspartate transcarbamylase [55]. In particular, the denaturational thermodynamics of the holoenzyme with single-site mutations in the catalytic chain have been explored. The effects on the thermal stability of mutations at the interface between catalytic chains, either within a catalytic subunit or between catalytic subunits, have been considered. The advantage in using DSC relies on the fact that the effects of mutations on catalytic and regulatory subunits can be resolved, since each subunit melts at a characteristic temperature.

IRREVERSIBLE THERMAL DENATURATION OF PROTEINS

Thermodynamic analysis of DSC curves relative to protein unfolding relies upon the assumption that chemical equilibrium exists throughout the temperature-induced unfolding process. The procedure which is usually followed is an examination of the reproducibility of the calorimetric curve upon a second heating. In many cases the second curve shows no thermal effect and, therefore, thermodynamic functions cannot be calculated. However, it has been shown that DSC curves for the thermal denaturation of several proteins can be interpreted in terms of equilibrium thermodynamics, notwithstanding the calorimetric irreversibility [56–58].

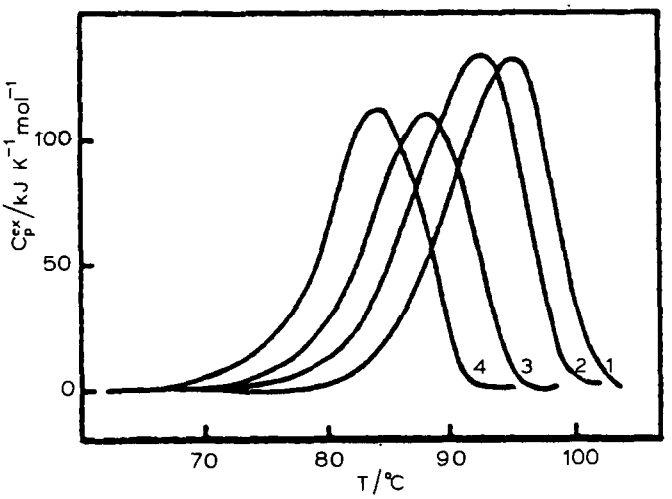

Fig. 4. Excess heat capacity vs. temperature for thermolysin at pH 7.5 at four different scan rates: (1) 1.9, (2) 1.0, (3) 0.5, and (4) 0.2 K min^{-1}. From ref. 61.

The irreversible thermal denaturation of a protein can be represented according to the scheme [59]

$$N \underset{k_2}{\overset{k_1}{\rightleftharpoons}} D \xrightarrow{k_3} I$$

where I is a final state which is attained from D through an irreversible process. Assuming that the three processes are first-order ones and that $k_3 \gg k_2$, most of the D molecules will convert to I, instead of returning to N. Hence there will be no equilibrium between N and D, and the overall denaturation process must be considered as an irreversible one, N $\rightarrow$ I, kinetically controlled by the slow conversion from N to D. In the case of *Bacillus thermoproteolyticus rokko* thermolysin [60] and of porcine procarboxypeptidase A [61], the thermal denaturation was found to be irreversible and dependent on scan rate (Fig. 4). The results were interpreted according to a model which is a particular case of that outlined above. A kinetic scheme $N \xrightarrow{k} D$ holds, where k is a first-order kinetic constant, the change of which with temperature is given by the Arrhenius equation, and D is the unfolded state or a state given by the irreversible alteration of the unfolded form.

In contrast, equilibrium thermodynamics have been used where the equality holds between the van't Hoff enthalpy calculated from the shape of the calorimetric curve and that calculated from the effects of ligands and protein concentration on the transition temperature. In this case, it is assumed (referring to the model N $\rightleftharpoons$ D $\rightarrow$ I) that $k_3 \ll k_2$ within the temperature range of the transition. However, the irreversible alteration of the unfolded state, which occurs with a small thermal effect, may be faster

at a temperature higher than that of the N → D thermal transition, giving rise to calorimetric irreversibility. In addition, the scan rate parameter can be used as an equilibrium criterion [62]. The dependence of the calorimetric traces on this parameter is a clear indication of an kinetically controlled process and, hence, of the impossibility of using equilibrium thermodynamics.

The mechanisms of the thermal inactivation of some proteins have been extensively explored [63–68]. The irreversibility of these processes has been found to be caused by a variety of processes occurring at increasing temperature. For hen egg white lysozyme, the processes which lead to thermoinactivation were found to be deamidation of Asn/Gln residues, hydrolysis of peptide bonds at Asp residues, destruction of S–S bonds and formation of incorrectly folded and kinetically trapped structures. In the case of pancreatic RNase A at pH 4 and 90 °C, the enzyme inactivation is due partly to the same kinds of process, namely deamidation and hydrolysis. At pH 6–8 and 90 °C, the enzyme inactivation is caused by a combination of disulphide interchange and β-elimination of cystine residues, coupled with the deamidation of Asn/Gln residues. In some cases, the process of proline isomerization must be considered: it is slow, occurring with almost no thermal effect, but able to hinder sterically the refolding of the polypeptide chain into the folded native structure. In addition, the aggregation of the unfolded chains, which depends on the concentration and on the experimental conditions, interferes with the folding process.

COLD DENATURATION OF PROTEINS

The process transforming a native structure of a protein into a disordered one can be assimilated to an order–disorder transition. As will be stated below, the process called "cold" denaturation should occur with a decrease of entropy, notwithstanding the breakdown of the ordered native structure. This phenomenon was first predicted more than 20 years ago, but it was based on an extensive extrapolation of indirect data [69,70]. Experimental difficulties caused cold denaturation to be little studied, because for all proteins studied the predicted values of the cold denaturation temperature (T_L) are below the freezing point of water. This factor made T_L inaccessible to experimental determination in a homogeneous system [71,72]. In fact, freezing leads to many phenomena which inactivate the protein, and different techniques [73] were tried to overcome this problem. The stability of chymotrypsinogen at low temperatures [74] was studied, and T_L was identified as 240 K employing the water-in-oil droplet emulsion technique to inhibit the nucleation of ice in the supercooled solution. Lactate dehydrogenase in aqueous methanol at low temperature was successfully investigated [75,76]. However, other authors [77] did not detect the cold denaturation of RNase A in 70% aqueous methanol. On

the basis of the parabolic relationship of $G(T)$, they expected T_L under these conditions to be around 228 K. However, enzyme activity was found even at 203 K, although the activity at this temperature was probably due to the need to use high protein concentrations to enhance the stability against denaturation.

The direct experimental evidence of this phenomenon obtained by differential scanning calorimetry has offered new possibilities for studying the forces involved in the formation of the native structure. Assuming, as a first approximation, that the difference in the heat capacity between the native and the denatured states does not depend on the temperature, we have

$$\Delta_d H(T) = \Delta_d H(T_d) - \Delta_d C_p(T_d - T) \tag{3}$$

$$\Delta_d S(T) = \Delta_d H(T_d)/T_d - \Delta_d C_p \ln T_d/T \tag{4}$$

where T_d is the transition temperature at which the Gibbs free energy difference is equal to zero

$$\Delta_d G(T_d) = \Delta_d H(T_d) - T_d\, \Delta_d S(T_d) = 0;\ \Delta_d S(T_d) = \Delta_d H(T_d)/T_d \tag{5}$$

From the linear relationship (3) it can be seen that, with decreasing temperature, $\Delta_d H$ decreases, at a certain temperature T_i its value becomes zero, and finally it changes its sign

$$\Delta_d H(T_d) = \Delta_d C_p(T_d - T_i);\ T_i = T_d - \Delta_d H(T_d)/\Delta_d C_p \tag{6}$$

The entropy also decreases with decreasing temperature, but with a non-linear trend: it reaches zero at a slightly higher temperature than the enthalpy does. The Gibbs energy is given by

$$\begin{aligned}\Delta_d G(T) &= \Delta_d H(T) - T\Delta_d S(T)\\ &= \Delta_d H(T_d) - (T_d - T)\, \Delta_d C_p - T\, \Delta_d H(T_d)/T_d + T\, \Delta_d C_p \ln T_d/T\\ &= \Delta_d H(T_d)(T_d - T)/T_d - (T_d - T)\, \Delta_d C_p + T\, \Delta_d C_p \ln T_d/T\end{aligned} \tag{7}$$

The maximum is obtained from the condition

$$(\delta\, \Delta G/\delta T)_{T_{max}} = 0 = -\Delta_d S(T_{max}) \tag{8}$$

At the temperature T_{max}, at which ΔS between the native and denatured state is zero, the stability of a small, globular protein is maximal, and it is governed only by the difference in the enthalpies of the two states. On both sides of this maximum the free energy values must decrease, and then reach zero at two different temperatures. The higher temperature is the temperature of heat denaturation, where the denaturation process occurs with the absorption of heat, namely with an increase of enthalpy and entropy. The lower temperature is the temperature of cold denaturation, a process happening with a decrease of enthalpy and entropy. The relationships reported above assume a constant value for the heat capacity differ-

ence. However, there have been some attempts to consider the variation of this parameter with the temperature [72], also on the basis of DSC data on synthetic polymers [78]. This treatment provides that, as well as enthalpy and entropy, ΔC_p could also change its sign. Conceptually, this is more satisfactory, in that the forces and mechanism ruling cold denaturation are probably not the same as those ruling heat denaturation. It is well known that hydrophilic interactions (dipole–dipole, hydrogen bonds) weaken and hydrophobic interactions strengthen with increasing temperature. The latter interactions play a major role in stabilizing the protein native structure at physiological temperature, while hydrophilic interactions give the major contribution at low temperature. It should consequently be found that, at the temperature where cold denaturation occurs, the low-temperature induced denatured state differs from that obtained at high temperature in respect of the intactness of the hydrophilic interactions.

Proteins with the largest $\Delta_d C_p$ and the smallest $\Delta_d H$ values are the most suitable for such studies, since their adequately high T_L values permits observation before freezing. Myoglobin [79] and apomyoglobin [80] are among the proteins most extensively studied, by DSC and other techniques. On heating, two peaks are observed, the first corresponding to the formation of the native structure and the second to the disruption of this structure. On cooling, again two peaks are obtained, but for the

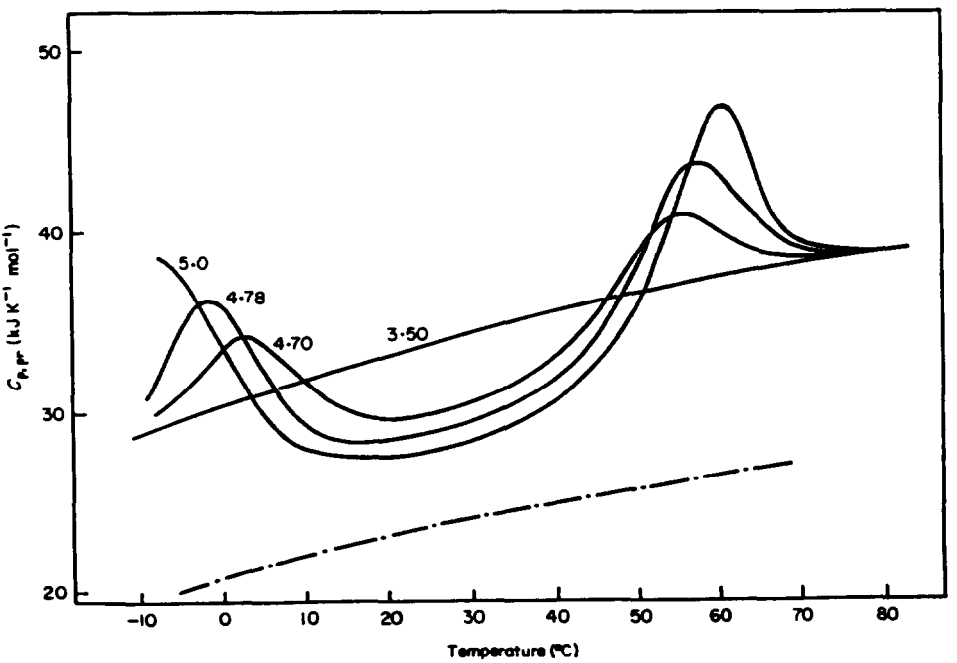

Fig. 5. Heat capacity vs. temperature for apomyoglobin at various pH values. The broken line refers to the heat capacity of native metmyoglobin at pH 5.0. From ref. 80.

opposite processes (Fig. 5). There is evidence strongly supporting the assumption of a two-state transition for the denaturation processes of both proteins. Another suitable protein is staphylococcal nuclease [11], heat denaturation of which had previously been shown [81] to occur through a rather large increase of heat capacity. For all proteins, it was clearly found that the heat capacity of the protein denatured either by heating or cooling is significantly higher than that of the native state.

STUDIES ON MODEL SYSTEMS

Weak, non-bonding interactions are important in macromolecular assembly processes, and their change or disruption may lead to the disruption of the native form of a protein. The native structure of a globular protein is determined by a balance of the interactions between the amino acid residues with each other and with the aqueous environment.

Among the weak, non-bonding, interactions occurring in aqueous solutions, the so-called hydrophobic interaction [82] has been the most widely studied, especially in relation to protein stability [32,83,84]. It is commonly asserted that these interactions are the most important driving forces for many biochemical processes. Correlation of the thermodynamic properties of protein folding with data on hydrophobic model compounds has allowed better analysis of the role of non-polar interactions. The enthalpy of dissolution of various apolar substances in water is negative, and the absolute value is proportional to the accessible surface area. The heat capacity change for the dissolution in water of apolar substances is positive and proportional to the surface area of the substance. Thus the heat capacity increment observed upon the unfolding of a protein is due to the structuring of water molecules around non-polar residues. Calorimetric data for the thermodynamics of transfer of liquid hydrocarbons in water, combined with solubility data, led to the proposal of a model for the temperature dependence of the hydrophobic interactions in protein folding [85].

The removal of non-polar surfaces from water, the transfer of non-polar solutes to a non-aqueous phase and the folding of proteins are processes characterized by negative heat capacity changes. This last quantity has been proposed for use in quantifying how the stability of a globular protein depends on the contribution of the burial of non-polar surfaces [85], or how it correlates with the change in water accessible non-polar surface area [86]. Both the correlations lead to consideration of the contribution of the hydrophobic effect as the main driving force for folding, while the contribution of polar groups is considered to be insignificant. In a complex system, such as a protein, it is difficult to separate the effect of the hydrophobic interaction from that of the other types of interaction. In the case of the sequential polypentapeptide of elastin and its more hydrophobic analogues,

the absence of polar chains has allowed the thermodynamic analysis of the DSC traces, focusing on the hydrophobic features of the polypentapeptide [87]. The properties of hydration water are examined in order to understand the inverse temperature transition of the aqueous solutions of these substances, namely the reasons for the increased order of intra- and intermolecular interactions at increasing temperature. In some particular cases, as for phage Cro protein, a reverse hydrophobic effect is observed [88]. The wild type and variant proteins, obtained by the substitution of Tyr-26 with other hydrophobic side chains, are destabilized, since the side chain is more exposed to the solvent in the native than in the denatured state.

However, very recently, some authors began casting doubt upon the major importance of hydrophobic interactions. The latest articles by Ben-Naim and coworkers [89–92] show indeed that intramolecular hydrophilic interactions are strong, and are highly dependent on orientation and on the properties of the solvent. They are thus probably as important as hydrophobic interactions in highly specific processes such as protein folding and molecular recognition. Moreover, other papers have appeared which, on the basis of experimental data of heats of dilution, show the importance of the hydrophilic–hydrophilic interaction. In fact, studies on the excess enthalpies of aqueous solutions of alkane-*n*,*m*-diols [93,94] have led to the conclusion that two hydrated molecules interact through a preferential orientation, stabilized by the double contemporaneous CHOH–CHOH interaction, that makes more effective the interaction between hydrophobic groups. This finding seems to be a general rule for substances bearing groups through which favourable interactions can be established. In fact, in the case of α-amino acids [95], the 'forced' interaction between the zwitterions enhances steric differences, making it possible to detect chiral recognition, which disappears in the absence of the zwitterions, as in H_2O–HCl mixed solvent [96]. It can thus be safely affirmed that the interaction between hydrophilic groups is one of the leading factors in the interaction of these substances in water.

The hydrophobic core of a protein has long been thought to behave as an organized liquid, rather than as an organized solid: thus, attention has been focused on the solution properties of model compounds. However, much evidence supports the view that the hydrophobic core resembles an organic solid. Thermodynamic studies of the dissolution of solid model compounds should thus give useful information on the interaction ruling protein folding. A good model molecule for the intramolecular peptide–peptide hydrogen bonds in a protein is diketopiperazine, a cyclic dimer of glycine: there are no end charge effects, and hydrogen bonding occurs between peptide linkages. The enthalpies of dissolution of this substance, scaled either to its surface area or to two glycyl residues, are compared with the enthalpies of denaturation of lysozyme, ribonuclease and myo-

TABLE 4

Comparison, with reference to 110 °C, of the enthalpy of solution of diketopiperazine (DKP) with the enthalpies of denaturation for the three-proteins reported, scaled either to 'buried' area (kJ mol^{-1} $Å^{-2}$) or 'buried' residues (kJ mol^{-1}) [a]

	kJ mol^{-1} $Å^{-2}$	kJ mol^{-1}
DKP	93.8	11.6
Ribonuclease	57.7	9.3
Lysozyme	56.5	9.2
Myoglobin	51.9	9.1

[a] Data from ref. 98.

globin, scaled to either the 'buried' area or 'buried' residues [97]. The comparison is made with reference to 110 °C, the temperature at which hydrophobic solvation is essentially enthalpic. As can be seen from Table 4, the values for the proteins and for the model molecule are comparable: the higher values for diketopiperazine are probably due to the higher density of interactions within the regular crystal lattice of the small molecule. The quantitative agreement of the energetics of crystal dissolution and protein denaturation characterizes the cyclic compound as a good model of the hydrogen bonding and van der Waals interactions in a folded polypeptide chain.

INTERACTION OF PROTEINS WITH SMALL LIGANDS

An important feature of biological macromolecules is their ability to interact with small molecules with a high degree of specificity: such an interaction is a determining factor in molecular recognition. Isoperibolic and isothermal calorimeters, the most used among the calorimetric techniques for such kinds of problem, allow not only the detection of the thermal effects associated with the binding reaction, but also the evaluation of the binding constants and of the stoichiometry of the reaction. These values can, in turn, be used for obtaining free energy and entropy. The extensive review by Eftink and Biltonen [98] on the thermodynamics of interacting biological systems is focused especially on the use of microcalorimetric techniques. The many effects contributing to the values of the thermodynamic parameters are examined, as well as the relationship between thermodynamic quantities and macromolecule conformational changes and cooperative binding phenomena.

If n identical and non-interacting sites are available to the ligands on the protein molecule, the heat of binding, H_b, can be expressed by the following equation

$$1/\Delta H_B = 1/\Delta H(\max) + 1/K'_B L_F \, \Delta H(\max) \qquad (9)$$

For each value of ΔH_B, the concentration of free ligand is given by

$$L_F = L_0 - n\ \Delta H_B P_0 / \Delta H(\text{max}) \quad (10)$$

where K'_B is the apparent binding constant, L_F and L_0 are the concentration of free and total ligand, $\Delta H(\text{max})$ is the saturation enthalpy, and n is the number of binding sites. By imposing a value on n, $\Delta H(\text{max})$ and K'_B can be determined through an iterative least-squares method. The linearity of the double reciprocal plot proves the reliability of the value assigned to n. A better knowledge of the stoichiometry of the reaction would come from two kinds of experiment, one at constant concentration of protein and the other at constant concentration of ligand. It is the equality of the saturation enthalpies that ensures that the aggregate formed is of the 1:1 type, otherwise their ratio gives the number of equivalent and independent sites. However, this second experiment is usually not performed, since increasing concentrations of the protein could lead to aggregation phenomena.

The determination of the stoichiometry is particularly important when dealing with multi-subunit proteins, since the binding at the site of the first subunit can influence the binding on the other subunits, as in the case of concanavalin A.

One of the best characterized carbohydrate-mediated recognitions of the cell surface receptor is the association between lectins and the carbohydrate moiety of the glycolipids and glycoproteins present on a cell membrane. Some simple, specific sugars can inhibit this interaction, because they can compete for the combination sites on the lectin. One of the most studied lectins is concanavalin A (Con A), which is a dimer at pH < 5.8 and a tetramer at physiological pH. The subunits are essentially identical, and each of them has an active site for the binding of oligosaccharides and glycoconjugates. Extensive calorimetric studies have been reported about the interaction of this protein with simple monosaccharides [99,100], $\alpha(1 \rightarrow 4)$ linked glucosides [101], and more complex glycoconjugates [99]. The stoichiometry for the binding of Con A to mono- and disaccharides is 2 mol of ligand per protein dimer. Passing to a trisaccharide, melezitose, and to the glycoconjugates reported in Fig. 6, the stoichiometry of association becomes 1 mol of ligand per protein dimer: there is thus an apparent inactivation of the site on the second subunit. Analysis of the thermodynamic parameters led to the hypothesis that the surface of Con A can adapt to a 'polyfunctional' branched saccharide or glycoconjugate having at least three sugar rings with two terminal α-linked manno- or glucopyranoside rings. For the interaction of Con A with glycosyl-free liposomes [102], the stoichiometry has been shown to be approximately 1000 moles of lipid per mole of protein. The affinity constants are smaller than those for the binding of Con A to biological membranes. The protein–lipid association is strongly entropy-controlled: hydrophobic interactions are the pre-

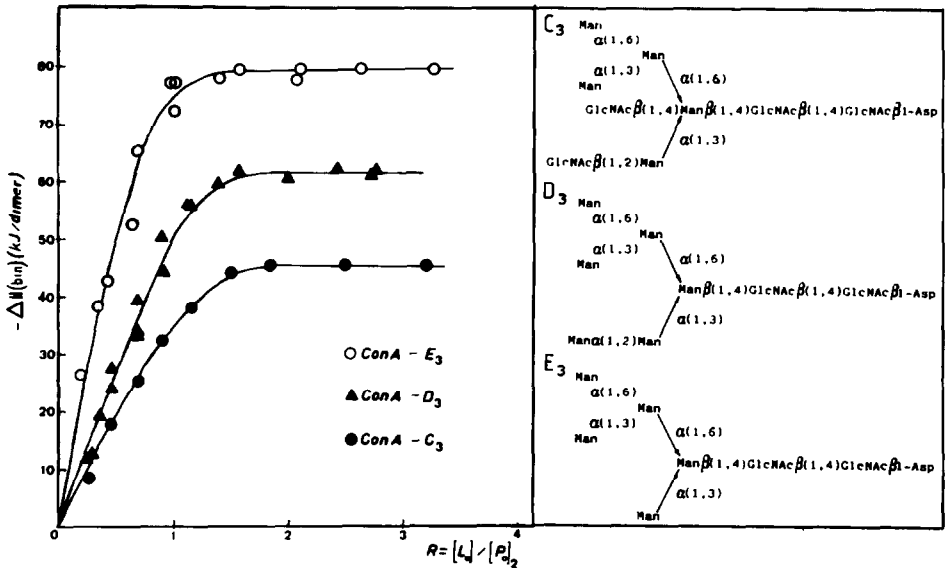

Fig. 6. Calorimetric titration at 25 °C of concanavalin A with the glycopeptides shown on the right. The apparent binding constants (l mol^{-1}) were: 3.1×10^6 for E_3, 1.7×10^5 for D_3, and 1.7×10^4 for C_3. From ref. 99.

vailing ones, while electrostatic forces appear to play a minor role. Non-specific lectin–lipid interactions seem to represent the main driving forces for the binding of the lectin to membrane surfaces, while the specificity of lectin–membrane interaction is controlled by the glycosidic receptor groups.

Con A is a metalloprotein, and in the native form it has Mn^{2+} in the S1 site and Ca^{2+} in the S2 site. An S3 site exists for lanthanide ions, and recently the stoichiometry and energetics of this last interaction have been investigated [103] using the native and the demetallized protein. The stoichiometry is different for the native protein as opposed to the apo form. The native protein metallized with Gd^{3+} was shown to be a fully functional protein: the presence of an S3 site on the Con A monomer thus provides a valuable reference point in high resolution NMR experiments relying on the paramagnetic properties of Gd^{3+}.

In the presence of two distinct, non-identical, classes of binding site, calorimetric titration curves cannot be analysed for a unique fit, since three adjustable parameters (n, ΔH_B°, K_B') exist for each class of sites. Through a 'trial and error' approach, for instance, it was shown that the dimeric bovine seminal ribonuclease (RNase BS) binds two molecules of cytidine 3′-phosphate with different affinities [104]. The higher affinity site resembles that of the monomeric RNase A, and the higher binding constant is due mainly to a less negative entropic contribution with respect to RNase A (Table 5).

TABLE 5

Thermodynamic parameters (kJ mol^{-1}) per site, at 25 °C, for the interaction of various proteins with the ligands reported

Ligand	$-\Delta H_B^\circ$	$-\Delta G_B^{\circ\prime}$	$-T\Delta S_B^{\circ\prime}$
RNase A [a]			
3'CMP	64 ±2	20.6±0.3	143±3
RNase BS [a]			
3'CMP ($n_1 = 1$)	60	28	32
3'CMP ($n_2 = 1$)	17	27	−10
Tubulin [b]			
MTC	19 ±1	32.4±0.6	−13±2
Allocolchicine	11 ±2	34.7±0.5	−24±3
Colchicine	21 ±2	43	−22
Concanavalin A [c]			
αMManP	23.8±0.5	25.4±0.6	−2±1
Melezitose	31 ±2	22.8±0.8	8±3
Glyc C3	46 ±2	28 ±1	18±3
Glyc D3	61 ±4	30 ±1	31±5
Glyc E3	79.0±0.5	37 ±2	42±3

[a] From ref. 104. [b] From ref. 105. The data for colchicine are at 35 °C. [c] From ref. 99. Abbreviations used: αMManP, methyl-α-mannopyranoside; 3'CMP, cytidine-3'-phosphate; MTC, 2-methoxy-5-(2',3',4'-trimethoxyphenyl)-2,4,6-cycloheptantrien-1-one. $\Delta H_B^\circ = \Delta H(\max)/n$.

Often, the difficulties encountered in understanding the mechanism of protein–ligand interaction make it necessary to use simpler ligands. In such a way it is easier to understand which part of the complete ligand binds specifically to the binding site, and also what are the forces ruling the association process. In the case of the interaction of tubulin with colchicine, allocolchicine and 2-methoxy-5-(2',3',4'-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one (MTC), which lacks the central ring of colchicine, the binding thermodynamics are similar, suggesting related molecular interactions of the three ligands with the protein binding site [105]. The results have been interpreted in terms of a bifunctional binding of colchicine and MTC, through their trimethoxybenzene and tropolone moieties, to a twin-focal protein binding site.

In Table 5 the thermodynamic parameters are shown for selected systems. The knowledge of these parameters has proved essential for understanding the origin of association energy, namely, of the forces controlling the protein–ligand interaction.

REFERENCES

1 W. Kauzmann, Some factors in the interpretation of protein denaturation, Adv. Protein Chem., 14 (1959) 1–63.

2 P.L. Privalov, Scanning microcalorimeters for studying macromolecules, Pure Appl. Chem., 33 (1980) 479–497.
3 P.L. Privalov and S.A. Potekhin, Scanning microcalorimetry in studying temperature-induced changes in proteins, Methods Enzymol., 131 (1986) 4–51.
4 J.M. Sturtevant, Biochemical applications of differential scanning calorimetry, Annu. Rev. Phys. Chem., 38 (1987) 463–488.
5 P.L. Privalov and V.V. Plotnikov, Three generations of scanning microcalorimeters for liquids, Thermochim. Acta, 139 (1989) 257–277.
6 F.P. Schwarz and W.H. Kirchhoff, Biological thermodynamic data for the calibration of differential scanning calorimeters: heat capacity data on the unfolding transition of ribonuclease A in solution, Thermochim. Acta, 128 (1988) 267–295.
7 F.P. Schwarz, Biological thermodynamic data for the calibration of differential scanning calorimeters: heat capacity data on the unfolding transition of lysozyme in solution, Thermochim. Acta, 147 (1989) 71–91.
8 W. Pfeil and P.L. Privalov, Conformational changes in proteins, in M.N. Jones (Ed.), Biochemical Thermodynamics, Elsevier, Amsterdam, 1979, pp. 75–115.
9 P.L. Privalov, Stability of proteins: small globular proteins, Adv. Protein Chem., 33 (1979) 167–241.
10 W. Pfeil, Protein unfolding, in M.N. Jones (Ed.), Biochemical Thermodynamics, Elsevier, Amsterdam, 1988, pp. 53–99.
11 Yu. V. Griko, P.L. Privalov, J.M. Sturtevant and S. Yu. Venyaminov, Cold denaturation of staphylococcal nuclease, Proc. Natl. Acad. Sci. USA, 85 (1988), 3343–3347.
12 P.L. Privalov, Stability of proteins. Proteins which do not present a single cooperative system, Adv. Protein Chem., 35 (1982) 1–104.
13 E.I. Tiktopulo and P.L. Privalov, Papain denaturation is not a two-state transition, FEBS Lett., 91 (1978) 57–58.
14 P.L. Mateo and P.L. Privalov, Pepsinogen denaturation is not a two-state transition, FEBS Lett., 123 (1981) 189–192.
15 B.L. Steadman, P.A. Trautman, E.Q. Lawson, M.J. Raymond, D.A. Mood, J.A. Thomson and C.R. Middaugh, A differential scanning calorimetric study of the bovine lens crystallin, Biochemistry, 28 (1989) 9653–9658.
16 R. Rudolph, R. Siebendritt, G. Nesslauer, A.K. Sharma and R. Jaenicke, Folding of an all-β protein: independent domain folding in γII-crystallin from calf eye lens, Proc. Natl. Acad. Sci. USA, 87 (1990) 4625–4629.
17 J.-J. Bae, W.-Y. Chou, K. Matthews and J.M. Sturtevant, Tryptophan repressor of *Escherichia coli* shows unusual thermal stability, Proc. Natl. Acad. Sci. USA, 85 (1988) 6731–6732.
18 G. Barone, G. Castronuovo, P. Del Vecchio, A. Di Donato and V. Elia, Thermal denaturation of ribonucleases from different sources, Proc. Int. Symp. Thermodyn. Applied to Biol. Syst., Santa Margherita Ligure, Italy, September 1988.
19 R.L. Biltonen and E. Freire, Thermodynamic characterization of conformational states of biological macromolecules using differential scanning calorimetry, CRC Crit. Rev. Biochem., 5 (1978) 85–124.
20 E. Freire and R.L. Biltonen, Statistical mechanical deconvolution of thermal transitions in macromolecules. I. Theory and application to homogeneous systems, Biopolymers, 17 (1978) 463–479.
21 V.V. Novokhatny, S.A. Kudinov and P.L. Privalov, Domains in human plasminogen, J. Mol. Biol., 179 (1984) 215–232.
22 P.L. Privalov and L.V. Medved', Domains in the fibrinogen molecule, J. Mol. Biol., 159 (1982) 665–683.
23 J.L. Lopez-Lacomba, M. Guzman, M. Cortijo, P.L. Mateo, R. Aguirre, S.C. Harvey and H.C. Cheung, Differential scanning calorimetric study of the thermal unfolding of myosin rod, light meromyosin, and subfragment 2, Biopolymers, 28 (1989) 2143–2159.

24 S.J. Gill, B. Richey, G. Bishop and J. Wyman, Generalized binding phenomena in an allosteric macromolecule, Biophys. Chem., 21 (1985) 1–14.
25 C.H. Robert, A. Colosimo and S.J. Gill, Allosteric formulation of thermal transitions in macromolecules, including effects of ligand binding and oligomerization, Biopolymers, 28 (1989) 1705–1729.
26 S.-I. Kidokoro and A. Wada, Determination of thermodynamic functions from scanning calorimetry data, Biopolymers, 26 (1987) 213–229.
27 S.-I. Kidokoro, H. Uedaira and A. Wada, Determination of thermodynamic functions from scanning calorimetry data. II. For the system that includes self-dissociation/association process, Biopolymers, 27 (1988) 271–297.
28 J.F. Brandts, C.Q. Hu, L.N. Lin and M.T. Mas, A simple model for proteins with interacting domains. Applications to scanning calorimetry data, Biochemistry, 28 (1989) 8588–8596.
29 W. Pfeil and P.L. Privalov, Thermodynamic investigations of proteins. Calorimetric study of lysozyme denaturation by guanidine hydrochloride, Biophys. Chem., 4 (1976) 33–40.
30 P.L. Privalov, E.I. Tiktopulo, S. Yu. Venyaminov, Yu. V. Griko, G.I. Makhatadze and N.N. Khechinashvili, Heat capacity and conformation of proteins in the denatured state, J. Mol. Biol., 205 (1989) 737–750.
31 P.L. Privalov, Thermodynamic problems of protein structure, Annu. Rev. Biophys. Biophys. Chem., 18 (1989) 47–69.
32 P.L. Privalov and S.J. Gill, Stability of protein structure and hydrophobic interaction, Adv. Protein Chem., 39 (1988) 191–234.
33 G.I. Makhatadze and P.L. Privalov, Heat capacity of proteins. I. Partial molar heat capacity of individual amino acid residues in aqueous solutions: hydration effect, J. Mol. Biol., 213 (1990) 375–384.
34 P.L. Privalov and G.I. Makhatadze, Heat capacity of proteins. II. Partial molar heat capacity of the unfolded polypeptide chain of proteins: protein unfolding effect, J. Mol. Biol., 213 (1990) 385–391.
35 H. Uedaira and H. Uedaira, The effect of sugars on the thermal denaturation of lysozyme, Bull. Chem. Soc. Jpn., 53 (1980) 2451–2455.
36 Y. Fujita and Y. Noda, The effect of ethylene glycol on the thermal denaturation of ribonuclease A and chymotrypsinogen as measured by differential scanning calorimetry, Bull. Chem. Soc. Jpn., 57 (1984) 2177–2183.
37 Y. Fujita and Y. Noda, Differential scanning calorimetric studies on the thermal denaturation of ribonuclease A in aqueous 2-methyl-2,4-pentanediol, Bull. Chem. Soc. Jpn., 57 (1984) 1891–1896.
38 K. Gekko and Y. Idota, Amino acid solubility and protein stability in aqueous maltitol solutions, Agric. Biol. Chem., 53 (1989) 89–95.
39 K. Gekko and H. Ito, Competing solvent effects of polyols and guanidine hydrochloride on protein stability, J. Biochem. (Tokyo), 107 (1990) 572–577.
40 S.-I. Segawa, M. Sugihara, T. Maeda, Y. Mitsuhisa, M. Kodama, S. Sek and M. Sakiyama, Calorimetric study of the effect of intrachain cross-linking on lysozyme unfolding, Biopolymers, 28 (1989) 1033–1041.
41 W. Pfeil, Thermodynamics of α-lactalbumin unfolding, Biophys. Chem., 13 (1981) 181–186.
42 F. Ahmad and C.C. Bigelow, Estimation of the stability of globular proteins, Biopolymers, 25 (1986) 1623–1633.
43 A. Relimpio, A. Iriarte, J.F. Chlebowski and M. Martinez-Carrion, Differential scanning calorimetry of cytoplasmic aspartate transaminase, J. Biol. Chem., 256 (1981) 4478–4488.
44 H. Fukada, K. Takahashi and J.M. Sturtevant, Thermodynamics of the binding of *Streptomyces* subtilisin inhibitor to α-chymotrypsin, Biochemistry, 24 (1985) 5109–5115.

45 H. Fukada, J.M. Sturtevant and F.A. Quiocho, Thermodynamics of the binding of L-arabinose and of D-galactose to the L-arabinose-binding protein of *Escherichia coli*, J. Biol. Chem., 258 (1983) 13193–13198.
46 H. Fukada, K. Takahashi and J.M. Sturtevant, Differential scanning calorimetric study of the thermal unfolding of Taka-amylase A from *Aspergillus oryzae*, Biochemistry, 26 (1987) 4063–4068.
47 K.M. Keating, L.R. Ghosaini, D.P. Giedroc, K.R. Williams, J.E. Coleman and J.M. Sturtevant, Thermal denaturation of T4 gene 32 protein: effects of zinc removal and substitution, Biochemistry, 27 (1988) 5240–5245.
48 V. Edge, N.M. Allewell and J.M. Sturtevant, Differential scanning calorimetric study of the thermal denaturation of aspartate transcarbamoylase of *Escherichia coli*, Biochemistry, 27 (1988) 8081–8087.
49 L.R. Ghosaini, A.M. Brown and J.M. Sturtevant, Scanning calorimetric study of the thermal unfolding of catabolite activator protein from *Escherichia coli* in the absence and presence of cyclic mononucleotides, Biochemistry, 27 (1988) 5257–5261.
50 M. Yamasaki, H. Yano and K. Aochi, Differential scanning calorimetric studies on bovine serum albumin: I. Effects of pH and ionic strength, Int. J. Biol. Macromol., 12 (1990) 263–268.
51 P.D. Ross and A. Shrake, Decrease in stability of human albumin with increase in protein concentration, J. Biol. Chem., 263 (1988) 11196–11202.
52 A. Shrake and P.D. Ross, Biphasic denaturation of human albumin due to ligand redistribution during unfolding, J. Biol. Chem., 263 (1988) 15392–15399.
53 A. Shrake and P.D. Ross, Ligand-induced biphasic protein denaturation, J. Biol. Chem., 265 (1990) 5055–5059.
54 M. Renner, P. Weber and H.-J. Hinz, Punktmutationen und Proteinstabilität, J. Therm. Anal., 35 (1989) 639–657.
55 D.S. Burz, N.M. Allewell, L. Ghosaini, C.Q. Hu and J.M. Sturtevant, Differential scanning calorimetric studies of *E. coli* aspartate transcarbamylase. III. The denaturational thermodynamics of the holoenzyme with single-site mutations in the catalytic chain, Biophys. Chem., 37 (1990) 31–41.
56 S.P. Manly, K.S. Matthews and J.M. Sturtevant, Thermal denaturation of the core protein of *lac* repressor, Biochemistry, 24 (1985) 3842–3846.
57 V. Edge, N.M. Allewell and J.M. Sturtevant, High resolution of differential scanning calorimetric analysis of the subunits of *Escherichia coli* aspartate transcarbamoylase, Biochemistry, 24 (1985) 5899–5906.
58 C.Q. Hu and J.M. Sturtevant, Thermodynamic study of yeast phosphoglycerate kinase, Biochemistry, 26 (1987) 178–182.
59 R. Lumry and H. Eyring, Conformation changes in proteins, J. Phys. Chem., 58 (1954) 110–120.
60 J.M. Sanchez-Ruiz, J.L. Lopez-Lacomba, M. Cortijo and P.L. Mateo, Differential scanning calorimetry of the irreversible thermal denaturation of thermolysin, Biochemistry, 27 (1988) 1648–1652.
61 J.M. Sanchez-Ruiz, J.L. Lopez-Lacomba, P.L. Mateo, M. Vilanova, M.A. Serra and F.X. Aviles, Analysis of the thermal unfolding of porcine procarboxypeptidase A and its functional pieces by differential scanning calorimetry, Eur. J. Biochem., 176 (1988) 225–230.
62 M.B. Jackson and J.M. Sturtevant, Phase transitions of the purple membranes of *Halobacterium halobium*, Biochemistry, 17 (1978) 911–915.
63 J.A. Thomson, B.A. Shirley, G.R. Grimsley and C.N. Pace, Conformational stability and mechanism of folding of ribonuclease T1. J. Biol. Chem., 264 (1989) 11614–11620.
64 T.J. Ahern and A.M. Klibanov, The mechanism of irreversible enzyme inactivation at 100 °C, Science (Washington, D.C.), 228 (1985) 1280–1284.

65 S.E. Zale and A.M. Klibanov, On the role of reversible denaturation (unfolding) in the irreversible inactivation of enzymes, Biotechnol. Bioeng., 25 (1983) 2221–2230.
66 S.E. Zale and A.M. Klibanov, Why does ribonuclease irreversibly inactivate at high temperature?, Biochemistry, 25 (1986) 5432–5444.
67 M.P. Tombs, Stability of enzymes, J. Appl. Biochem., 7 (1985) 3–24.
68 A.M. Klibanov, Stabilization of enzymes against thermal inactivation, Adv. Appl. Microbiol., 29 (1983) 1–28.
69 J.F. Brandts, Thermodynamics of protein denaturation. The denaturation of chymotrypsinogen, J. Am. Chem. Soc., 86 (1964) 4291–4301.
70 J.F. Brandts, Conformational transitions of proteins in water and in aqueous mixtures., in S.N. Timasheff and G.D. Fasman (Eds.), Structure and Stability of Biological Macromolecules, Dekker, New York, 1969, pp. 213–290.
71 F. Franks, Biophysics and Biochemistry at Low Temperature, Cambridge University Press, Cambridge, 1985.
72 F. Franks, R.H.M. Hatley, and H.L. Friedman, The thermodynamics of protein stability. Cold destabilization as a general phenomenon, Biophys. Chem., 31 (1988) 307–315.
73 R.W. Michelmore and F. Franks, Nucleation rates of ice in undercooled water and aqueous solutions of polyethylene glycol, Cryobiology, 19 (1982) 163–171.
74 F. Franks and R.H.M. Hatley, Low temperature unfolding of chymotrypsinogen, Cryo-Letters, 6 (1985) 171–180.
75 R.H.M. Hatley and F. Franks, Denaturation of lactate dehydrogenase at subzero temperatures, Cryo-Letters, 7 (1986) 226–233.
76 R.H.M. Hatley and F. Franks, The cold-induced denaturation of lactate dehydrogenase at sub-zero temperatures in the absence of perturbants, FEBS Lett., 257 (1989) 171–173.
77 A.L. Fink and B. Painter, Characterization of the unfolding of ribonuclease A in aqueous methanol solvents, Biochemistry, 26 (1987) 1665–1671.
78 F. Franks and T. Wakabayashi, Heat capacities of undercooled aqueous solutions of polyvinylpyrrolidone, Z. Phys. Chem., N.F., 155 (1987) 171–180.
79 P.L. Privalov, Yu. V. Griko, S. Yu. Venyaminov and V.P. Kutyshenko, Cold denaturation of myoglobin, J. Mol. Biol., 190 (1986) 487–498.
80 Yu. V. Griko, P.L. Privalov, S. Yu. Venyaminov and V.P. Kutyshenko, Thermodynamic study of the apomyoglobin structure, J. Mol. Biol., 202 (1988) 127–138.
81 R.O. Calderon, N.J. Stolowich, J.A. Gerlt and J.M. Sturtevant, Thermal denaturation of staphylococcal nuclease, Biochemistry, 24 (1985) 6044–6049.
82 J.J. Kozak, W.S. Knight and W. Kauzmann, Solute–solute interactions in aqueous solutions, J. Chem. Phys., 48 (1968) 675–690.
83 P.L. Privalov, Protein stability and hydrophobic interactions, Biofizika, 32 (1987) 742–760.
84 K.P. Murphy, P.L. Privalov and S.J. Gill, Common features of protein unfolding and dissolution of hydrophobic compounds, Science, 247 (1990) 559–561.
85 R.L. Baldwin, Temperature dependence of the hydrophobic interaction in protein folding, Proc. Natl. Acad. Sci. USA, 83 (1986) 8069–8072.
86 R.S. Spolar, J.-H. Ha and M.T. Record, Jr., Hydrophobic effect in protein folding and other noncovalent processes involving proteins, Proc. Natl. Acad. Sci. USA, 86 (1989) 8382–8385.
87 C.-H. Luan, R.D. Harris, K.U. Prasad and D.W. Urry, Differential scanning calorimetric studies of the inverse temperature transition of the polypentapeptide of elastin and its analogues, Biopolymers, 29 (1990) 1699–1706.
88 A.A. Pakula and R.T. Sauer, Reverse hydrophobic effects relieved by amino acid substitutions at a protein surface, Nature (London), 344 (1990) 363–364.

89 A. Ben-Naim, Solvent-induced interactions: hydrophobic and hydrophilic phenomena, J. Chem. Phys., 90 (1989) 7412–7425.
90 A. Ben-Naim, Solvent-induced forces in protein folding, J. Phys. Chem., 94 (1990) 6893–6895.
91 M. Mezei and A. Ben-Naim, Calculation of the solvent contribution to the potential of mean force between water molecules in fixed relative orientation in liquid water, J. Chem. Phys., 92 (1990) 1359–1361.
92 A. Ben-Naim, Solvent effects on protein association and protein folding, Biopolymers, 29 (1990) 567–596.
93 C. Cascella, G. Castronuovo, V. Elia, R. Sartorio and S. Wurzburger, Hydrophobic interactions of alkanols. A calorimetric study in water at 298.15 K, J. Chem. Soc. Faraday Trans., 86 (1990) 85–88.
94 A. Andini, G. Castronuovo, V. Elia and L. Fasano, Hydrophobic interactions in the aqueous solutions of alkane-1,2-diols. Calorimetric and spectroscopic studies at 298.15 K, J. Chem. Soc. Faraday Trans., 86 (1990) 3567–3571.
95 G. Barone, G. Castronuovo, P. Del Vecchio, V. Elia and S. Puzziello, Chiral recognition between enantiomeric α-aminoacids. A calorimetric study at 25 °C, J. Solution Chem., 18 (1989) 1105–1116.
96 G. Castronuovo, V. Elia, C. Giancola and S. Puzziello, Chiral interactions in aqueous solutions of aminoacids. A calorimetric study of the protonated forms in H_2O/HCl mixed solvent at 25 °C, J. Solution Chem., 19 (1990) 855–866.
97 K.P. Murphy and S.J. Gill, Calorimetric measurement of the enthalpy of dissolution of diketopiperazine in water as a function of temperature, Thermochim. Acta, 139 (1989) 279–290.
98 M. Eftink and R.L. Biltonen, Thermodynamics of interacting biological systems, in A.E. Beezer (Ed.), Biological Microcalorimetry, Academic Press, London, 1980, pp. 343–411.
99 R. Ambrosino, G. Barone, G. Castronuovo, C. Ceccarini, O. Cultrera and V. Elia, Protein–ligand interaction. A calorimetric study of the interaction of oligosaccharides and hen ovalbumin glycopeptides with concanavalin A. Biochemistry, 26 (1987) 3971–3975.
100 M. Dani, F. Manca and G. Rialdi, Calorimetric study of concanavalin A binding to saccharides, Biochim. Biophys. Acta, 667 (1981) 108–117.
101 G.R. Munske, H. Krakauer and J.A. Magnuson, Calorimetric study of carbohydrate binding to concanavalin A, Arch. Biochem. Biophys., 233 (1984) 582–587.
102 M. Luscher-Mattli, The thermodynamic parameters of the interaction of concanavalin A with glycosyl-free liposomes: a microcalorimetric study, Biopolymers, 26 (1987) 1509–1526.
103 G. Barone, G. Castronuovo, P. Del Vecchio, V. Elia and P. Guarrata, Binding of lanthanum and gadolinium ions to cancanavalin A studied calorimetrically at 25 °C, J. Mol. Recogn., 2 (1989) 147–151.
104 R. Ambrosino, G. Barone, G. Castronuovo, O. Cultrera, A. Di Donato and V. Elia, A calorimetric approach to the study of the interactions of cytidine-3′-phosphate with bovine seminal ribonuclease, Biopolymers, 28 (1989) 1403–1411.
105 M. Menendez, J. Laynez, F. Medrano and J.M. Andreu, A thermodynamic study of the interaction of tubulin with colchicine site ligands, J. Biol. Chem., 264 (1989) 16367–16371.