J Therm Anal Calorim (2011) 104:69–73
DOI 10.1007/s10973-011-1285-3

Microcalorimetry of heat capacity and volumetric changes in biomolecular interactions—the link to solvation?

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ISBC XVI Conference Special Chapter

Abstract Changes in solvation play a central role in the thermodynamics of non-covalent interactions in solution, especially in water, yet there are relatively few techniques available to probe this unambiguously. Experimental studies of the thermodynamics of biomolecular interactions in water have exposed two significant empirical observations. The first, well known from the very earliest applications of microcalorimetry, is that processes such as protein folding, ligand binding, and protein–protein association almost always occur with a decrease in overall heat capacity of the system (negative ΔC_p). This results in a strong temperature dependence of the enthalpy of interaction that has, historically, been usually attributed to solvation changes, though more generally it has been shown to be an inevitable consequence of processes involving the cooperative interaction of multiple weak interactions. More recently using pressure perturbation calorimetry (PPC), we have shown that such interactions in the same systems also occur with significant decreases in molar thermal expansibility (negative $\Delta E°$) that can be related to the loss of solvation during complexation. The apparently strong correlation between ΔC_p and $\Delta E°$ potentially leads to a generic picture of the thermodynamics of macromolecular interactions in water in which both solvation and conformational fluctuation play a much more prominent role than has been hitherto supposed.

Keywords Protein folding · Ligand binding · Thermodynamics · Fluctuations

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The applications of microcalorimetry techniques to biomolecular systems have expanded in recent years with the increasing availability of sensitive calorimeters suitable for the study of stability and interactions of biological macromolecules in dilute aqueous solution [1]. My remit here in this brief contribution is to give an overview of the picture now emerging of the thermodynamics of such interactions, with an attempt to understand the fundamental molecular basis for the phenomena involved. The questions we might wish to address are as follows: what makes a protein fold, and what controls its stability? Once it has folded, what does the protein bind to and what is its function?—questions central to practical applications in proteomics and drug discovery. And, at a more fundamental level, what are the thermodynamic forces responsible for ligand binding and stability, and can we use this information to design better proteins, better ligands or better drug formulations?

Calorimetry began with Joseph Black in Glasgow 250 years ago, and it is said that "*He waited with impatience for the winter*" so that he could do experiments on the freezing and melting of water and water/alcohol mixtures [2]. This led to the concept of latent heat and the earliest studies of the heat capacity of hydrogen-bonded networks. The quest continues, and current work focuses on the use of three calorimetric techniques: (1) differential scanning calorimetry (DSC) for the study of thermal unfolding and related transitions in proteins, nucleic acids and other macromolecules [3]; (2) isothermal titration calorimetry (ITC) for measurement of binding interactions in solution [4]; and (3) pressure perturbation calorimetry (PPC) for the determination of volumetric effects associated with such interactions [5]. Work from our own group and many others over recent years has established clear patterns in the thermodynamic profiles of non-covalent

interactions that can be summarized and exemplified as follows.

Protein folding, unfolding and aggregation involves significant heat capacity (ΔC_p) effects

Differential scanning calorimetry of thermal unfolding of globular proteins in solution shows consistently that the apparent excess heat capacity (C_p) of thermally unfolded protein is higher than that of the more compact folded conformation. This is illustrated in Fig. 1, showing how the unfolding enthalpy (ΔH_{unf}), obtained from the area under the DSC transition peak, increases with transition temperature, T_m, and also how the heat capacity baseline is uniformly higher after the transition. Both these effects indicate a positive ΔC_p for the unfolding transition. However, protein unfolding is more frequently accompanied by irreversible exothermic aggregation of the polypeptide chain, often resulting in aggregates resembling the amyloid plaques associated with many protein misfolding diseases. As illustrated in Fig. 2, this transition is accompanied by a decrease in heat capacity (negative ΔC_p). Both these examples show that the heat capacity of unfolded polypeptide chains in solution is higher than more compact states, and is reduced either by folding to a specific monomeric native conformation, or by more non-specific oligomeric aggregation.

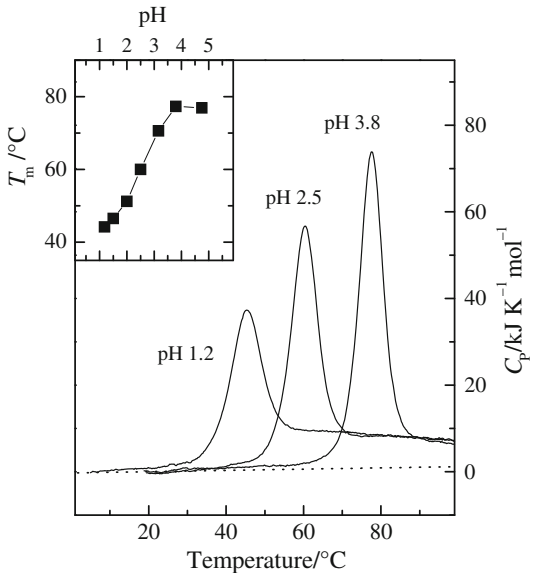

Fig. 1 Typical DSC data for the unfolding of a small globular protein (lysozyme) in solution at various pH values. The insert shows the variation in mid-point unfolding temperature (T_m) as a function of pH. The increase in area under each endotherm with higher T_m, and the higher heat capacity baselines after the unfolding transitions, are both indications of the significant positive ΔC_p commonly associated with such processes. (Adapted from [10], with permission)

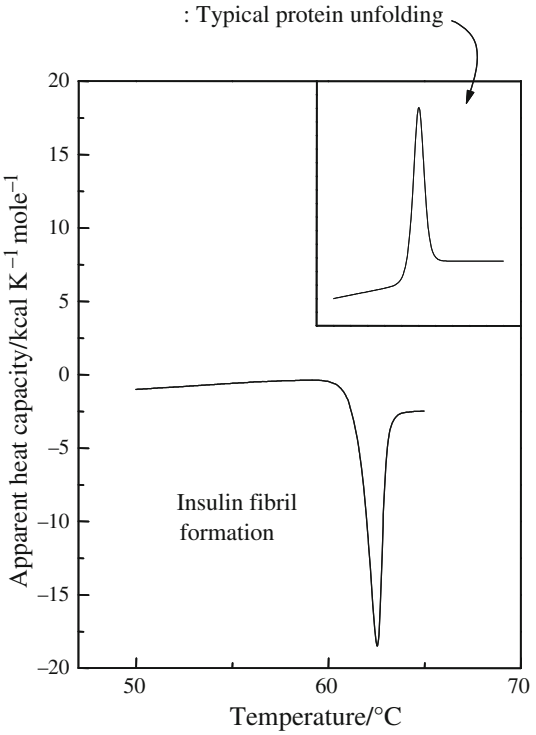

Fig. 2 DSC of thermal aggregation of insulin in solution under conditions where the unfolded protein undergoes a kinetically limited exothermic transition to a condensed protein aggregate similar to amyloid fibrils. The *inset* shows the more typical thermogram seen for protein unfolding (without aggregation). (Adapted from [9] with permission)

Protein–ligand binding and protein–protein interactions in solution are also accompanied by significant heat capacity (ΔC_p) changes

Isothermal titration calorimetry (ITC) gives direct and (usually) unambiguous data on the enthalpy (ΔH), stoichiometry, and equilibrium constant (K) for non-covalent binding interactions. This is now most often the experimental method of choice for studying such interactions, and it has been widely applied to a range of biomolecular interactions including protein–ligand (drug) binding and protein–protein association. Interestingly, in almost all cases, the measured enthalpies show a strong temperature dependence, regardless of the nature of the interactions, that implies a finite and significant ΔC_p. A particularly striking example is shown in Fig. 3, where the ΔH for association of two protein subunits varies with temperature to such an extent that it changes sign over a quite small temperature range, being exothermic at high T and endothermic at low T. By chance, the ΔH for this interaction becomes zero at 25 °C, though the binding affinity remains high throughout. There are now many examples of such behaviour, and further details can be found in [6–8]. Surprisingly, similar ΔC_p effects are seen for binding of relatively small molecules to proteins and other systems [9–11].

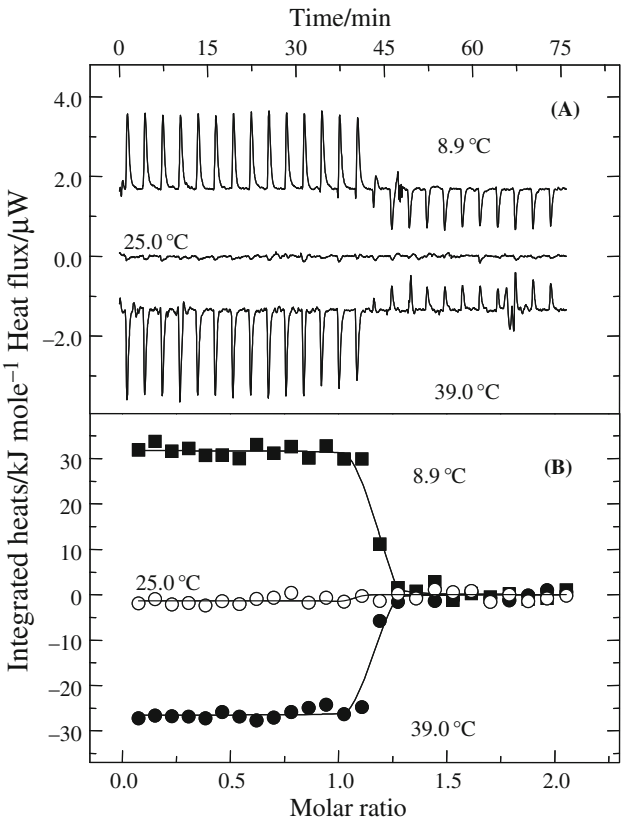

Fig. 3 Subunit interactions in the pyruvate dehydrogenase multi-enzyme complex of *B. stearothermophilus.* ITC data for the binding of di-domain and E3 subunits at different temperatures. These early data, in collaboration with the Perham group (Cambridge, UK), have since been confirmed in more detailed studies [6–8]

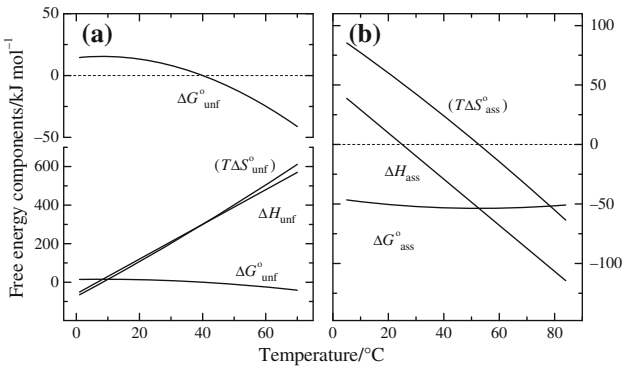

Fig. 4 Examples of the temperature dependence of thermodynamic parameters for protein unfolding and protein–protein interactions: **a** enthalpy (ΔH_{unf}), entropy ($T\Delta S^{\circ}_{unf}$) and Gibbs free energy (ΔG°_{unf}) components for thermal unfolding of a globular protein in water; **b** ΔH, $T\Delta S^{\circ}$ and ΔG° for binding of subunits of the pyruvate dehydrogenase multi-enzyme complex. Note how, despite the large temperature variation in enthalpy and entropy contributions, the free energy of binding or stability remains relatively constant. (Adapted from [9] with permission)

Almost all interactions show entropy/enthalpy compensation and thermodynamic homeostasis

A direct thermodynamic consequence of finite ΔC_p for any interaction is that both the enthalpy (ΔH) and the entropy (ΔS°) changes associated with the interaction can vary markedly with temperature, but in a compensatory fashion such that the changes in Gibbs free energy (ΔG°) are relatively much smaller. See Fig. 4 for examples. Similar compensation effects are also seen with other variants, not just temperature, and this makes it difficult to discriminate between different classes of interaction simply on the basis of their thermodynamic signatures [10]. Such compensation also means that interactions of this kind can be relatively robust to temperature fluctuations and other perturbations ("thermodynamic homeostasis"), since most functional properties rely on ΔG° rather than ΔH or ΔS°.

The more fundamental aspect of this is explored elsewhere [9, 10, 12]. What we have shown, and expanded upon more recently [12], is that changes in heat capacity of this magnitude are a common feature of any process involving molecular order–disorder transitions, and this is particularly significant in systems comprising multiple cooperative weak (usually noncovalent) interactions that are inherently flexible and dynamic. This means that most biomolecular processes will show such effects regardless of the detailed nature of the interactions involved. Consequently, and contrary to accepted wisdom, it is very hard to discriminate between such interactions solely on the basis of their thermodynamic profiles [10].

Pressure perturbation calorimetry reveals significant thermal expansibility (ΔE°) effects for all biomolecular interactions

The relatively new technique of pressure perturbation calorimetry (PPC) is now providing an opportunity to look at the same interactions in a slightly different light [13–17]. PPC measure the small heat energy changes (ΔQ) when the sample solution is subjected to small pressure pulses, typically ±5 atm [5]. When combined with measurements of partial molar volume (V°), ΔQ can be used to estimate the solute thermal expansivity $E^{\circ} = \partial V^{\circ}/\partial T = \alpha^{\circ} V^{\circ}$, where α° is the thermal expansion coefficient of the molecule in solution. The interest here lies in the observation that, at least to a first approximation, the thermal expansion coefficient for rigid molecules in solution depends mainly on the properties of the solvation layer.

This techniques have been applied to a range of macromolecular unfolding transitions [5, 16, 18–23], but the results are difficult to interpret because of the multiplicity of

interactions involved and complications arising from conformational flexibility and the effects of cavities and voids in the macromolecular structures. More recently, we have addressed the potentially simpler problem of expansibility changes accompanying the formation of non-covalent complexes in aqueous solution [13–15]. Results show that for a wide range of cyclodextrin host–guest complexes, and for inherently more flexible protein–ligand and protein–protein interactions, there is a consistent reduction in E° upon complex formation. This negative ΔE° occurs regardless of the nature of the non-covalent forces involved: hydrophobic, electrostatic or H-bonded systems all show similar trends. This parallels similar observations in relation to heat capacity (ΔC_p) effects in the same systems, and there is a reasonable apparent correlation between ΔC_p and ΔE° when compared under the same conditions (Fig. 5). At first sight, this is consistent with the initial hypothesis that the major contribution to both ΔC_p and ΔE° comes from changes in solvation during complexation. Indeed, using simplifying assumptions, it is possible to use such ΔE° data to estimate the numbers of water molecules displaced in the binding process, with results that are reasonably consistent with expectations [14, 15]. However, subsequent more comprehensive analysis now shows that, although ΔC_p and ΔE° effects do share a common underlying molecular origin, this more likely involves not only changes in surface solvation but also more disseminated changes in molecular flexibility and conformational fluctuations induced by binding, particularly in cases involving proteins. This is discussed in more detail in [12, 14].

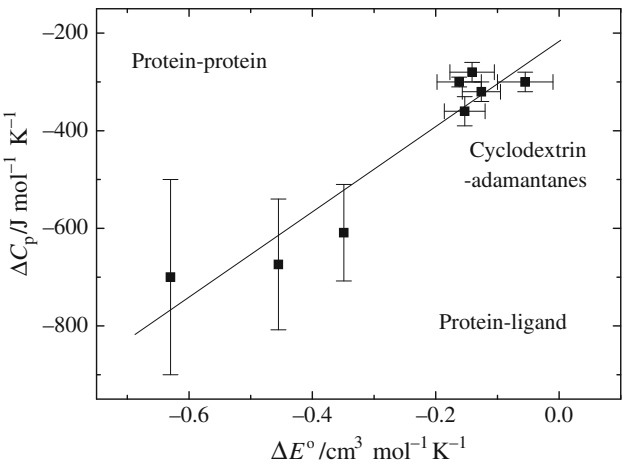

Fig. 5 Correlation of changes in heat capacity (ΔC_p) and expansibility (ΔE°) for various non-covalent interactions in water. Primary data are taken from [14]. Heat capacity data for protein–ligand (lysozyme–trisaccharide and ribonuclease-CMP) and cyclodextrin–adamantane complexes were obtained directly by ITC. ΔC_p for a representative protein–protein interaction (cytochrome *c* peroxidase–pseudoazurin) was estimated from modelling of the buried surface area in the macromolecular complex

Conclusions

- All non-covalent interactions show very similar "thermodynamic signatures".
- So-called "anomalous" heat capacity effects are nothing of the sort—they are exactly what we should have expected from the classical physical chemistry of order–disorder transitions.
- In particular, positive heat capacity changes are to be expected for any process (protein unfolding, protein–protein, protein–ligand dissociation) involving the disruption of a cooperative lattice of multiple weak interactions.
- What we can not see (solvent) is just as important as what we can (structure).
- Pressure perturbation calorimetry offers a way to explore hydration and conformational fluctuation effects directly.

Acknowledgements The Biological Microcalorimetry Facility in Glasgow was funded by the UK Biotechnology and Biological Sciences Research Council (BBSRC).

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