Thermodynamic analysis of biomolecular interactions

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Direct measurement of the thermodynamics of biomolecular interactions is now relatively easy. Interpretation of these thermodynamics in simple molecular terms is not. Recent work shows how the multiplicity of weak noncovalent interactions, and the inevitable enthalpy/entropy compensation that these interactions engender, lead to difficulties in teasing out the different components.

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Abbreviations

DSC	differential scanning calorimetry
ITC	isothermal titration calorimetry
MB	Mercedes Benz
PrP	prion protein

Introduction

Thermodynamics impinges on most aspects of biomolecular interactions, and recent improvements in sensitivity and usability of instrumentation have made measurements of thermodynamic parameters relatively straightforward. Calorimetric methods, in particular isothermal titration calorimetry (ITC) and differential scanning calorimetry (DSC), are now popular both as general analytical tools and as seductively direct routes to fundamental data about intermolecular and intramolecular forces. What is less secure is our understanding of what these parameters might mean at the molecular level. This 'snapshot' review illustrates some of the uses (and abuses) of thermodynamics in biomolecular systems appearing (mainly) during the past year (up to May 1999), discussing how far we might have come since earlier work [1,2].

Thermodynamics can be daunting, so it is worth starting with a brief reminder of the important parameters and where they come from. The key thermal parameter is the heat capacity difference (ΔC_p), since all other relevant parameters derive from this; Equations 1 and 2 show examples:

$$\Delta H = \int_0^T \Delta C_p \cdot dT + \Delta H(0) \quad (1)$$

$$\Delta S = \int_0^T (\Delta C_p/T) \cdot dT \quad (2)$$

where ΔH is the enhalpy change, $\Delta H(0)$ is the enthalpy change for the process at 0K and ΔS is the change in entropy. Equation 1 emphasises how enthalpy changes reflect differences in the amount of heat energy required to achieve a particular state, whereas Equation 2 shows that the entropy change is a measure of how easy it might be to distribute that energy amongst the various molecular energy levels [3]. (Note: $\Delta H(0)$ dominates covalent interactions because there are large changes in ground state energies, but it is usually less significant in noncovalent interactions. That is why heat capacity effects are so important here.)

The Gibbs free energy (G) is the parameter that really matters in determining (bio)molecular equilibria: it shows the direction in which processes will tend to go, or the amount of work that needs to be done to make them go. Equation 3 shows how the change in free energy, for any process at constant pressure, is made up of two contributions:

$$\Delta G = \Delta H - T \cdot \Delta S \quad (3)$$

At the molecular level, this reflects the opposition of two fundamental effects — the tendency to fall to lower energy (bond formation, negative ΔH), offset by the equally natural tendency for thermal (Brownian) motion to disrupt things (bond breakage, positive ΔS). Equation 4 shows the standard free energy change:

$$\Delta G^\circ = -RT \cdot \ln K \quad (4)$$

where R is the gas constant and K is the equilibrium constant. This shows the change that would take place under some arbitrary and usually very unrealistic standard conditions. It is probably better viewed as a convenient way of expressing the equilibrium constant for the process on a logarithmic scale, with units of energy. With this background (and apologies to those who are already familiar with the basics) we may now explore some recent experimental and theoretical developments in biomolecular thermodynamics.

Models, theories and databases

Much of what we know about the thermodynamics of interactions in complex biomolecular systems is based on what we hope are relevant model systems, together with theoretical analysis and correlations from experimental databases.

With characteristic flair, the Dill group has recently tackled the theory of hydrophobic interactions using a two-dimensional 'Mercedes Benz (MB)' model of water [4,5••]. Despite its relative simplicity — adopted for ease of computation and clarity of interpretation — this model reproduces many of the anomalous properties of liquid water and the transfer of hydrophobic solutes between aqueous and non-polar solvents. The two-dimensional MB model simulations support the classical picture of hydrophobic interactions [1,6•] in which, at least at lower temperatures, small hydrophobic groups are surrounded by

a shell of more ordered water molecules with stronger hydrogen bonding than that found in bulk water. This ordering difference between shell and bulk water accounts for the observed negative ΔH and ΔS of transfer of such groups to water from non-polar environments, the empirical characteristic of the hydrophobic interaction. Both the MB model and another theoretical approach based on information theory [7] give partial explanations for the convergence temperature (T_S), a common empirical temperature at which entropies of transfer become zero. Other work [8•], however, rightly questions the experimental validity of the concept because of the dependence of the absolute values of ΔS on an arbitrary choice of standard state and concentration units, as well as inherent in accuracies in ΔC_p data used for extrapolations.

Much of what we know about hydrophobic interactions is based on model studies involving transfer or partitioning between solvents that are supposed to mimic the various biomolecular environments, but these models neglect the possibly more structured environment, both polar and nonpolar, that might be found within macromolecular structures. Careful experiments comparing partitioning in amorphous or partially ordered environments show that the structure of the environment can completely change the thermodynamic signature of the hydrophobic interaction [9••].

Compared with the effort expended on the hydrophobic effect, relatively little effort has gone into understanding hydrophilic effects, such as hydrogen bonding and electrostatic interactions, in aqueous environments. Despite indications from the behaviour of small model compounds, which suggest that H-bonds should be at best thermodynamically neutral in aqueous systems, some experiments (but by no means all; see below) show that, in protein folding, hydrogen bonding might be as significant as hydrophobic interactions (e.g. [10••,11•]). Compilations of the very extensive literature on the thermodynamics of protein folding are now available [12•–14•], including a web version [13•].

Isotope effects

One way in which the various components of interactions might be disentangled is to compare effects in H_2O and ^2H_2O. Studies of protein folding stability in H_2O versus ^2H_2O show that the small increase in stability in ^2H_2O cannot be explained entirely by isotopic responses to changes in accessible nonpolar surface areas [15••], implying that other factors such as hydrogen bonding must play a significant role. The relative strengths of H-bonds can be probed using measurements of the partitioning of H and ^2H into exchangeable protein sites in H_2O/D_2O mixtures. Recent work [16••] supports previous indications that the strength differences between intramolecular amide–amide and amide–water H-bonds are small. This work also emphasises how the apparent strengths of H-bonds can be very context-dependent and hard to predict. Model studies [17•] suggest that the sign of ΔC_p for transfer from ^2H_2O to H_2O is different for polar and nonpolar groups, and this could be a useful diagnostic.

Aqueous solvation effects and the direct involvement of solvent water are likely to be particularly important in protein–carbohydrate interactions because of the very hydrophilic nature of sugars. The importance of solvation can be demonstrated by comparing the interactions in ^2H_2O and H_2O, as illustrated by a continuing series of ITC studies [18,19•]. Although it is not possible to predict reliably the magnitude (or even sign) of the effects of $H_2O/^2H_2O$ exchange on thermodynamic parameters, the observation of large effects on, for example, ΔH has been correlated with structural evidence for ordered water in lectin binding sites and the changes in this ordered water upon binding of saccharides. Studies with a range of saccharide ligands show that binding free energies and enthalpies are generally non-additive, with no simple correlation, for example, with the number of hydrogen bonds involved in the complex. Effects of other solvent additives on the thermodynamics of protein–carbohydrate interactions have been alternatively interpreted in terms of the effect of osmotic stress on the differential uptake of water molecules [20•]. It is likely that the overall thermodynamics will involve both direct structural participation of water molecules and more indirect, non-local effects on bulk solvent structure (as reflected, for example, in osmotic stress).

Pressure effects

After a period of absence, high pressure experiments on protein interactions are now returning to some popularity [21••,22•,23,24•], though their execution and proper interpretation remains difficult [21••]. There is a long-standing paradox here [1,24•] because the effects of high pressure on protein stability do not correlate with pressure effects on transfer of small molecules into solution. Such model compound experiments cannot easily take account of specific macromolecular structure effects, and some experiments [22•] have confirmed that it is changes to internal void volume upon unfolding that contribute most to pressure unfolding, rather than more general hydration effects. Some theoretical calculations, however, reach an opposite conclusion [24•]. Inevitably, both effects will be present in most systems, and which (if any) dominates in any particular circumstance will most likely depend on specific structural details of the folded and unfolded macromolecules.

Protein folding and misfolding

There is much discussion, relevant to the choice of appropriate small molecule model systems, as to whether the native fold of a protein is best viewed as analogous to a macroscopic solid or liquid. Experimental data can be ambiguous, though both views are not incompatible with the mesoscopic nature of proteins [25,26]. An analysis of molecular dynamics simulations and X-ray diffraction data [27] concludes, perhaps not surprisingly, that the interiors of globular proteins are more akin to solids in some respects than the more fluid protein surfaces. A similar analysis of unfolded and other intermediate states might be useful, especially in view of the ongoing controversy [28•,29•] as to whether the 'molten globule' is a truly discrete thermodynamic state, or

whether it is just a convenient phrase to encompass the myriad of interconverting conformational states of a polypeptide that are neither 'folded' nor 'unfolded' [3].

Controlling the folding stability of proteins by solvent additives or mutagenesis is of considerable practical importance. Large increases in stability in the presence of carboxylic acid salts (which can cause a change in melting temperature [ΔT_m] of up to 22°C at 1 M concentrations) have been correlated with changes in solvent surface tension [30•]. The inherent difficulties in interpreting, far more in predicting, the effects of mutagenesis on the thermodynamics of protein folding are highlighted by the significant consequences of introduction of a simple amino-terminal methionine [31•].

The thermodynamic basis for prion protein (PrP) activity has been explored using recombinant variants of human PrP representing known familial variations with enhanced pathogenicity [32•]. PrP is thought to be involved in the development of spongiform encephalopathies such as scrapie and, in humans, Creutzfeld–Jacob disease. It is thought that conformational changes in PrP are responsible for the brain pathology. The 'protein only' hypothesis suggests that the key event is conformational transition from the mainly α-helical, 'benign', native conformer (PrP^C) to the 'pathological', predominantly β-sheet conformer (PrP^{Sc}). The latter is unlikely to be thermodynamically stable by itself, and its accumulation probably results from aggregation or other non-equilibrium intermolecular interactions — a feature that might be common to many proteins that aggregate when (partially) unfolded. It is possible, however, that thermodynamics might play a role in determining the ease with which PrP might undergo the conformational switch. Studies of the folding stability of PrP mutants using the usual thermal and chemical denaturant guanidine hydrochloride (GdnHCl, a protein-denaturing agent that disrupts ion–ion associations and H-bonding patterns) methods show that this is unlikely to be the case. Mutations associated with enhanced PrP activity do not show any significant thermodynamic destabilization of the native conformer [32•].

Protein–protein and protein–ligand interactions

One contribution to the thermodynamics of association of two molecules is the loss of translational and rotational entropy that this entails, and one might have expected the effects of losses of degrees of freedom to be well understood. Unfortunately this is not the case [33•,34•], since there seems still to be considerable distance between theory [33•] (which may be too simplistic) and experiment [34•] (which may be too complex to extract the desired effect).

An example of how difficult it can be to rationalise even simple mutation effects is given by work from the Fersht group [35•,36•]. Using a chymotrypsin inhibitor–subtilisin BPN′ system (EC 3.4.21.62; a member of the serine endopeptidase family) [35•], they show that independent mutation of residues at separate sites in the inhibitor — one directly involved in the inhibitor–subtilisin interface and one in a loop distant from this interface — have similar, destabilising effects on the free energy of binding of the two proteins. In the case of mutations in the protein–protein interface such changes are easily rationalised in terms of direct effects on packing or other interactions. For mutations remote from the site of structural contact, however, one has to invoke arguments based upon indirect effects such as changes in chain conformation or flexibility. This begs the question: to what extent might such effects also be present, even in cases of mutations in the interface contact region? Just because you can 'see' the interaction in the protein structure does not rule out the possibility of contributions from more subtle, indirect, nonlocal effects, which can have similar magnitudes. Careful experimental examination of the barnase–barstar system [36•] emphasises the difficulties, especially when entropy/enthalpy compensation is involved (as it almost always is — see below) and when water molecules tend to fill the cavities created by mutations or other packing defects [36•–38•].

Nucleic acid–ligand interactions

Large ΔC_p changes are usually associated with changes in hydrophobic or polar group hydration, but theoretical calculations have shown that more general, long-range electrostatic interactions can also make a significant contribution [39•]. This may be of real significance in interactions with nucleic acids because of the highly charged polynucleotide backbone. Direct measurements of the thermodynamics of drug–DNA interactions are relatively sparse (in comparison with those for protein studies), but have been reviewed recently by Chaires [40]. Such interactions can be difficult to interpret because of the heterogeneity of binding sites and the large conformational changes that can be induced in the DNA, especially by intercalating molecules, but they show the same range of ΔC_p and entropy/enthalpy compensation effects to be expected for interactions made up of multiple, weak, noncovalent components.

The absence of any significant $H_2O/^2H_2O$ or osmotic stress (neutral solute) effects on the thermodynamics of nonspecific protein binding to DNA has been taken to mean that changes in hydration make only a minimal contribution to ΔC_p in such cases [41]. An ITC study of the effects of high concentrations of monovalent salts on interactions between protein and single-stranded DNA shows that weak-anion binding to the protein can yield large changes in the enthalpy of binding, with ΔH generally becoming less negative (less exothermic) with increasing salt concentration [42]. Normally, we would only think of ionic effects in terms of the highly charged DNA backbone, but we must not neglect the protein. Other studies of the effects of high salt concentrations in another protein–DNA system [43] have been interpreted differently in terms of large changes in hydration and ion incorporation into the protein–DNA interface, though the thermodynamic

argument is somewhat qualitative and cumbersome and there is no direct experimental evidence for either differential hydration or ion-incorporation effects in this particular system.

Entropy/enthalpy compensation

A common thread running through all these studies, especially as more comprehensive data covering a range of experimental conditions are obtained, is the way in which large variations in ΔH and ΔS appear to be correlated in such a way as to almost cancel, and give correspondingly smaller changes in ΔG. This is an old observation that (in many cases) can be attributed to experimental limitations and deficiencies in the way the data are obtained, especially when using indirect techniques [44,45•], though it now has much more substance because of direct calorimetric measurements. Entropy/enthalpy compensation due to variation in temperature, can be shown to be a simple consequence of finite ΔC_p effects [46], demonstrable from fundamental thermodynamic expressions of ΔH and ΔS (Equations 1 and 2). The effect is, however, more general than that. Frequently attributed to the peculiar properties of solvent water, it is an almost inevitable property of perturbation of any system comprising multiple, weak, intermolecular forces [47]. Intuitively, the breaking of bonds in any macromolecular system (including solvent) will be endothermic (a positive ΔH), but will be compensated by the greater entropy (a positive ΔS) that results from the increase in molecular flexibility.

The effect is very frustrating, since it means that absolute values of ΔH and ΔS cannot be viewed as diagnostic of any particular kind of interaction. In evolutionary terms, however, it might have homeostatic significance in that — regardless of the molecular basis for the compensation — mutations or changes in environment giving large changes in ΔH and ΔS can be tolerated because of the relatively much smaller effects on ΔG, which is the only parameter that really matters for the function of a system (A Cooper, CM Johnson and JH Lakey, unpublished data).

Thermodynamic fluctuations

Since heat is a manifestation of chaotic molecular motion, it is inevitable that thermodynamic systems undergo fluctuations, and this is particularly significant in small, mesoscopic systems such as individual macromolecules [25,26]. Recent papers have explored this further. Tang and Dill [48•] have used a lattice model to examine how conformational fluctuations in a macromolecule might change with temperature. In agreement with low-temperature crystallographic and spectroscopy experiments, they found that large fluctuations are frozen out at low temperatures, typically below about 200K. The observation that proteins with more stable folds tend to show fewer large fluctuations is consistent with intuition, and they make the point — not new, but worth reiterating — that protein stability is as much about unobservable conformational states as the observable native state.

Given the inherent dynamic flexibility of mesoscopic systems it is pertinent to ask whether folded proteins, and other compact macromolecules, behave thermodynamically more like liquids or solids. Experimental data are equivocal here but, as mentioned earlier, an analysis [27] using the Lindemann criterion — which compares fluctuations in the root mean square deviation in an atomic position to most probable nonbonded near-neighbour distances — concludes that the truth lies somewhere in between. That is, at physiological temperatures, native proteins behave like surface-molten solids, with essentially solid-like interiors but more fluid, liquid-like surfaces. Specific residues involved in dynamic interchange between different low-lying conformational states can be identified by NMR methods [49•]. Recent studies [50] have confirmed that, even with simple inhibitors, ligand binding affects the internal conformational motion of a protein. Such changes in dynamics must contribute to the thermodynamics of the binding process in ways that are impossible to model using a simple bond or group additivity picture. This is true also for more complex systems, as illustrated by the large contribution to entropy of protein–DNA association coming from changes in conformational motion [51]. It has also been suggested that internal vibrations of a protein might be significant in determining substrate binding energy and specificity [52], though such effects might be compensatory in terms of ΔH and ΔS [53].

Discrepancies, misconceptions, and paradoxes – the 'hidden variables effect'

Confidence in experimental data is paramount, and one must be aware of some of the limitations in obtaining those data. In view of the importance placed on accessible surface area (ASA) changes in many empirical models, it is disturbing that different algorithms (or even the same algorithm used by different researchers) can lead to significantly different values (see [15••] for example).

Experimental baselines can be a problem in both direct and indirect thermodynamic measurements, where the data may span an insufficient range and results may depend on the methods used for baseline extrapolation and interpolation [21••,54•,55].

Interpretation of experiments can be equally fraught. It is sometimes implied that heat capacity changes (ΔC_p effects) are somehow decoupled from enthalpy and entropy changes, showing contributions from solvent effects not appearing in ΔH and ΔS (see [39•] for example). In view of the fundamental integral relationships (Equations 1 and 2), this is hard to defend.

Calorimetry measures the totality of heat effects in any process, and this has long been used to advantage, for example to detect otherwise unsuspected protonation changes involved in ligand binding and other processes (see [56•,57] for recent examples, though the effects has been in use for over 20 years). It is sometimes claimed that

calorimetry is unique in this respect, and that more indirect, spectroscopic or van't Hoff methods (based on the temperature-dependence of equilibrium constants [3]) do not show this. But this is incorrect, as can be shown simply from consideration of the coupled effects of temperature and pH on equilibria involving hydrogen ions, including the effects of temperature on buffer pH (refer to author for details).

Regardless of whether one measures the enthalpy directly by calorimetry or indirectly using the van't Hoff equation, the answer is the same and — importantly — includes any additional heats due to buffer protonation, whether one is aware of them or not. The same will be true for any other 'hidden variables' (i.e. additional processes that are not included explicitly in the equilibrium constant expression used in the van't Hoff analysis) as a consequence of the fundamental theories of thermodynamic linkage [57].

Thus, statements such as "...BIAcore only measures the direct binding between the two interacting partners whereas microcalorimetry also measures solvent effects..." [58•] cannot be substantiated. Any discrepancies between thermodynamic data determined by calorimetric or biosensor methods (for example [59]) are best ascribed to the inevitable perturbations introduced by macromolecular immobilisation techniques or other experimental variables, rather than to some fundamental thermodynamic distinction. That is not to deny that differences between van't Hoff and calorimetric enthalpies can be found, even when determined from the same experimental data [60,61•], but such discrepancies probably reflect the inherent inaccuracy of the van't Hoff analysis in situations where entropy/enthalpy compensation contrives to give relatively little curvature to the van't Hoff plot despite the large temperature dependence of ΔH.

New technologies

New developments in single molecule atomic force microscopy (AFM) techniques have opened up possibilities to study directly the mechanics of protein interaction and unfolding. For example, Rief *et al.* [62•] have measured the force required to mechanically unfold individual triple-helical repeats in spectrin molecules. Significantly, they were able to show that individual spectrin repeats unfold independently when stretched, confirming the relative lack of cooperative interaction between adjacent domains. Similar methods have enabled measurements of the force necessary to pull apart insulin dimers [63•], which is difficult to do by conventional calorimetric methods [64]. The forces measured in such experiments are spatial derivatives of ΔG, or work necessary to bring about the change, and are in principle independent of any model assumptions that might be needed for determination of ΔG by other methods. Provided scepticism regarding perturbations produced in single molecules by tethering or confinement methods can be overcome, such methods hold considerable promise for our understanding of the energetics of macromolecular interactions.

Conclusions

It should be easy. We have only a limited menu of noncovalent interactions, unchanged for 40 years [1]: hydrogen bonding, hydrophobic, electrostatic, dispersion and repulsive van der Waals forces, yet it is proving remarkably difficult to disentangle their separate contributions to the thermodynamics of biomolecular processes, despite a wealth of experimental data. Perhaps we need a new way of looking at things; one that, for example, treats the network of fluctuating hydrogen bonds in its entirety, rather than as an arbitrary separation into isolated components.

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