Pressure perturbation calorimetry, heat capacity and the role of water in protein stability and interactions

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Abstract

It is widely acknowledged, and usually self-evident, that solvent water plays a crucial role in the overall thermodynamics of protein stabilization and biomolecular interactions. Yet we lack experimental techniques that can probe unambiguously the nature of protein–water or ligand–water interactions and how they might change during protein folding or ligand binding. PPC (pressure perturbation calorimetry) is a relatively new technique based on detection of the heat effects arising from application of relatively small pressure perturbations (±5 atm; 1 atm = 101.325 kPa) to dilute aqueous solutions of proteins or other biomolecules. We show here how this can be related to changes in solvation/hydration during protein–protein and protein–ligand interactions. Measurements of ‘anomalous’ heat capacity effects in a wide variety of biomolecular interactions can also be related to solvation effects as part of a quite fundamental principle that is emerging, showing how the apparently unusual thermodynamics of interactions in water can be rationalized as an inevitable consequence of processes involving the co-operative interaction of multiple weak interactions. This leads to a generic picture of the thermodynamics of protein folding stabilization in which hydrogen-bonding plays a much more prominent role than has been hitherto supposed.

Introduction

When proteins fold, or when molecules come together in solution, there will be changes in solvation that inevitably contribute to the thermodynamic stability of the folded protein or the associated complex. Changes in the molecular surfaces exposed to solvent, predominantly water in this context, will result in displacement of solvent molecules from the solute/water interface. Alternatively, water molecules may become trapped in cavities formed in the folded protein or at interfaces in protein–ligand or protein–protein interfaces. In some cases, these changes can be visualized, at least in part, by high-resolution crystallographic or other structural studies, especially where the waters are well ordered. Unfortunately, such studies will almost always tell only half the story, since we will almost always lack information about the less ordered waters or, even more important, the state of solvation of the unfolded protein or of the free ligand. Since thermodynamic stability depends on changes in properties during folding or binding, looking at just the final state is only partly helpful. This is a major technical challenge. If solvation changes contribute to the thermodynamics of protein folding or protein binding, then we need to be able to see the solvation states of not only the final folded protein or the protein–ligand complex, but also where they came from, i.e. the unfolded polypeptide or the free ligand and protein in solution. As far as we are aware, there are no structural studies, as yet, that have achieved this for all partners in the process. Consequently, we must resort to less direct methods to establish what solvation changes might occur, and how they contribute to the overall thermodynamic signatures of protein stability and interactions. Water is an unusual liquid. By virtue of an extended hydrogen-bonded structure that perpetuates, at least dynamically, in the liquid state, it has unusual volumetric and heat capacity properties that should be manifest in solvation effects. Here, we will summarize some of our work illustrating how we can use sensitive calorimetric techniques to examine these properties for a range of biomolecular and model interactions in water.

PPC (pressure perturbation calorimetry)

PPC is an experimental technique based on measurement of differential heat effects (ΔQ) in response to small pressure pulses (±5 atm; 1 atm = 101.325 kPa) [1]. These small heat effects arise from the pressure × volume (PV) work done when the sample is gently squeezed. For molecules in solution, this heat is directly related to the difference in thermal expansivity of the solute molecules compared with the volume of solvent that they have displaced, and recent advances in microcalorimetry technology mean that this can now be applied to the study of relatively dilute solutions [1–4]. This is of particular interest for biomolecular interactions in solution, where such volumetric properties and associated thermodynamic quantities are related directly to solvation...
effects that can be difficult to determine by other techniques. Using a combination of calorimetric (PPC) and volumetric measurements (see the Experimental section for details), we can measure directly the changes in thermal expansion coefficient ($\alpha$) and molar expansivity ($E^c$) associated with binding or other processes. Thermal expansion in any substance arises from the increase in average intermolecular distances with increase in temperature. This arises predominantly from non-covalent contacts, since covalent bond vibrations are not excited at normal temperatures and the intrinsic volume of any molecule generally remains constant. Consequently, any changes in apparent molecular volume for a (rigid) molecule in solution will reflect the changes in average intermolecular separation between the molecule and its solvation shell.

Previous work has shown how PPC can be used to probe the volumetric changes associated with conformational changes and co-solvent effects in relation to protein folding stability [1,2,5]. These studies have shown, for example, that protein unfolding in aqueous solution and co-solvent mixtures can be accompanied by either an increase or a decrease in partial molar volumes, depending on the protein and on experimental conditions. This reflects the balance of (often opposing) effects arising from changes in solvation and changes in macromolecular conformational packing during the protein folding/unfolding process that can be difficult to resolve unambiguously.

We are interested in the potentially simpler problem of solvation/hydration changes during protein–ligand or protein–protein interactions, although even here the interpretation is not necessarily straightforward. Preliminary experiments using PPC to explore volumetric changes upon binding of simple inhibitors at enzyme active sites, using both lysozyme–trisaccharide and RNase–2′-CMP, have shown significant changes in thermal expansivity upon complexation [3]. More recently, we have performed similar experiments for an azurin–cytochrome peroxidase–protein interaction, the thermodynamics of which we have previously studied by a variety of biophysical techniques [6]. In all cases, the thermal expansion coefficient ($\alpha$) for the enzyme–inhibitor or protein–protein complex is less than the sum of the thermal expansion coefficients for the separate components alone. This suggests that most of the $\alpha$ comes from the solvated water in the vicinity of the binding site and the ligand that is lost upon complex formation. The difficulty with this interpretation is that the components involved, particularly the protein molecules, are inherently flexible structures, and we cannot rule out volumetric changes related to ligand-induced changes in structural rigidity and packing densities within the protein–inhibitor complex. Consequently, we have also investigated cyclodextrin–adamantane host–guest complexes as a simpler model for non-covalent ligand binding in aqueous solution. Cyclodextrins are doughnut-shaped cyclic oligosaccharides that can readily form non-covalent complexes with small non-polar molecules in aqueous solution [7], including adamantanes. These complexes have been well characterized, both in terms of their structures and their binding thermodynamics [4,8–12], and both host (cyclodextrin) and guest (adamantane) are sufficiently small and rigid that we can reasonably ascribe any volumetric changes solely to solvation effects without complications from macromolecular conformational changes.

Preliminary results from PPC examination of expansivity changes in a variety of non-covalent interactions are summarized in Table 1, covering a range of protein–ligand, protein–protein and model compound examples. These results show consistently that in all cases the $E^c$ of the complex is less than that for the free molecules. This is in line with expectation, assuming that the main source of expansivity is the solvated molecular surface. Although the absolute magnitude of the effect is smaller with the cyclodextrin–adamantane model system, presumably reflecting the smaller molecular dimensions, the trend is similar and shows that decreases in expansivity are unlikely to arise solely from macromolecular conformational change. (We should emphasize that, in any case, there is no structural evidence for significant ligand-induced conformational change in any of the protein systems examined here.) Using empirical data for the approximate difference in $E^c$ of water in the bulk compared with solvation shell [9,13], we can relate these observations to the apparent numbers of water molecules displaced in the binding process ($\Delta n_h$, Table 1). Despite numerous approximations and simplifying assumptions, the numbers obtained for displaced waters are not unreasonable. For the cyclodextrin–adamantane system, the results ($\Delta n_h \approx −12$) are consistent with the approx. 11 cavity water molecules observed in the crystal structure of $\beta$-cyclodextrin and the approx. 15–25 water molecules released upon adamantane binding observed by less direct techniques [8].

### Heat capacity changes

The most significant consequences of solvation changes on interaction thermodynamics probably relate to changes in heat capacity ($\Delta C_p$). This has long been recognized as a signature of hydrophobic interactions [14] since, qualitatively at least, the relatively more ordered waters in the solvation layers around non-polar molecules will probably have a higher heat capacity than bulk water, and the loss of some of this solvation layer during interactions will result in a lower overall heat capacity (negative $\Delta C_p$). This shows up as a significant temperature dependence of the enthalpy (heat) of interaction ($\Delta C_p = d\Delta H/dT$) that is measured most directly by microcalorimetry, and this effect is now commonly observed. Interestingly, although originally thought to be mainly a

<table>
<thead>
<tr>
<th>Compound</th>
<th>$\Delta E^c$ (cm$^3$·mol$^{-1}$·K$^{-1}$)</th>
<th>$\Delta n_h$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme–trisaccharide</td>
<td>−0.46</td>
<td>−37</td>
</tr>
<tr>
<td>RNase–2′-CMP</td>
<td>−0.35</td>
<td>−28</td>
</tr>
<tr>
<td>Azurin–cytochrome peroxidase</td>
<td>−0.63</td>
<td>−50</td>
</tr>
<tr>
<td>$\beta$-Cyclodextrin–adamantane</td>
<td>−0.153</td>
<td>−12</td>
</tr>
</tbody>
</table>
characteristic of hydrophobic interactions, more recently it has become clear that this is a feature of any process involving a multiplicity of weak interactions [3,15–17]. For example, using combinations of DSC (differential scanning calorimetry) and ITC (isothermal titration calorimetry), we have determined significant ΔC_p effects not only in protein folding thermodynamics [15], but also in amyloid fibril formation [17], and in high-affinity protein–protein interactions dominated by electrostatic interactions [18]. We have also shown similar effects in the binding of trisaccharides to a lectin, ConA (concanavilin A) [17,19], and others have reported similarly large ΔC_p effects for the binding of di- and tri-saccharide inhibitors to lysozyme [20]. The significance of these latter observations is that these protein–sugar complexes are dominated by hydrogen-bonding interactions, with scarcely a hint of hydrophobic interactions in the crystal structures.

As a result of these direct calorimetric measurements, and many more besides from numerous groups, it is now clear that negative ΔC_p effects are a ubiquitous feature of all inter- or intra-molecular association processes in aqueous solution, regardless of the nature (hydrophobic, electrostatic, H-bonded, dispersion etc.) of the interactions involved. We have shown elsewhere that this is a natural thermodynamic consequence in weakly bonded co-operative systems [3,15,17] and have developed a simple statistical mechanical model to help rationalize these effects. One example of the application of this model is illustrated in Figure 1 and relates to the differences in thermodynamic parameters for binding to ConA of two closely related trisaccharides, in collaboration with the Boons group [19,21]. These two ligands differ only in the presence of a hydroxyethyl group in ligand 2 that displaces a water molecule that is otherwise trapped in the crystal structure of the active site complex with ligand 1. Simple model calculations based solely on the changes in solvation give quite reasonable estimates for the differences in thermodynamic binding parameters that agree well with experimental observation (Table 2). We have also used a similar model to rationalize the unusual thermodynamics of protein folding [15,17]. Thus we can be cautiously optimistic that a more complete understanding of the effects of solvent can be reasonably understood, provided we take a comprehensive approach that incorporates not just the final state of the complex, but also includes the solvation (hydration) states of the binding partners in solution before complexation occurs.

**Experimental**

See primary references [3,4,6] for details of the proteins and ligands used here. All samples were prepared in standard aqueous buffer systems at protein concentrations of less than 5 mg·ml⁻¹.

**Volumetric measurements**

Partial molar volumes in aqueous solution were obtained from density measurements (±1 × 10⁻⁵ g·cm⁻³) using a vibrating tube density meter [DA-510; KEM (Kyoto Electronics Manufacturing Co.), Kyoto, Japan] calibrated with dry air and degassed deionized water. Molal volumes of solutions (V; cm³·kg⁻¹) were calculated using V = (1000 + m·M_r)/d_s, where M_r is the relative molecular mass of the solute (g·mol⁻¹), m is the molality (mol·kg⁻¹) of the solute and d_s is the density of the solution (g·cm⁻³). For a mixture comprising n_w moles of water (or buffer) and n_s moles of solute, this is related to partial molar volumes by:

\[ V = n_w·V_w^0 + n_s·V_s^0 \]

where V_w^0 (cm³·kg⁻¹) is the partial molar volume of solvent or solute, as appropriate. V_s^0 values were determined from linear-regression slopes of experimental data over a range of solute concentrations (molalities, m).

**ITC**

Protein–ligand, protein–protein, or cyclodextrin host–guest complexation thermodynamics in solution were measured by ITC (Microcal VP-ITC) in the 15–55°C temperature range following standard instrumental procedures [22,23]. A typical experiment involved 25 sequential 10 µl injections of ligand solution into the ITC cell containing protein or other host compound. Titration data were corrected for dilution heats (measured separately) and analysed by using a single-set-of-sites equilibrium binding model (Microcal Origin™) to give the apparent binding stoichiometry (N), association/dissociation constants (K_A = 1/K_D) and enthalpy of binding (ΔH°). Other thermodynamic quantities were

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**Table 2 | Comparison of observed and calculated thermodynamic parameters for binding of trisaccharide ligands to ConA**

<table>
<thead>
<tr>
<th>Ligand 1</th>
<th>Ligand 2</th>
<th>Observed</th>
<th>Predicted</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔG° (kcal·mol⁻¹)</td>
<td>-7.6</td>
<td>-6.4</td>
<td>1.2</td>
</tr>
<tr>
<td>ΔH° (kcal·mol⁻¹)</td>
<td>-13.3</td>
<td>-11.0</td>
<td>2.3</td>
</tr>
<tr>
<td>ΔS° (cal·mol⁻¹·K⁻¹)</td>
<td>-19</td>
<td>-15</td>
<td>4</td>
</tr>
<tr>
<td>ΔC_p (cal·mol⁻¹·K⁻¹)</td>
<td>-109</td>
<td>-92</td>
<td>17</td>
</tr>
</tbody>
</table>

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**Figure 1 | Cartoon illustrating the effects of trapped (a) compared with displaced (b) water molecules in ConA-trisaccharide interactions**

Adapted from [17] with permission.

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calculated using standard expressions: \( \Delta G^2 = -RT \cdot \ln(K_X) = \Delta H^2 - T \cdot \Delta S^2; \Delta C_P = d\Delta H^2/dT; 1 \text{ cal} \approx 4.184 \text{ J} \).

**PPC**

Thermal expansivity (expansibility) data \( (E^0 = \partial V^0/\partial T = \alpha^0 V^0) \) for proteins, ligands, cyclodextrins, adamantanes and their complexes in solution at 25°C were obtained using a Microcal VP differential scanning calorimeter equipped with a PPC accessory [1]. Differential heat effects \( (\Delta Q) \) in response to pressure pulses \( (\Delta P, \pm 5 \text{ atm}) \) were determined with the sample cell containing appropriate solutions of host, guest, or mixtures of the two, as well as for the buffer alone with respect to water or buffer in the reference cell. Each series of experiments comprised at least 50 pulses for each sample, subsequently averaged for analysis using the expression:

\[
\alpha_V^2 = \frac{\Delta Q}{\left( T \cdot \Delta P \cdot g_s \cdot V_s^0 \right)}
\]

where \( \alpha_V^2 \) and \( \alpha_s^2 \) are the thermal expansion coefficients of the solute and pure solvent respectively, \( g_s \) is the mass of solute, \( V_s^0 \) is the partial specific volume of solute, and \( T \) is the absolute temperature.

Following Chalikian [13]:

\[
V^0 = V_M + \Delta V_s = V_M + n_b(V_b - V_s)
\]

where \( V_M \) is the intrinsic (i.e. physical) volume of the solute molecule, and \( \Delta V_s \) is the total hydration-induced change in the volume of the solvent. The latter might be visualised as arising from a solvation layer of \( n_b \) solvent molecules with average molar volume \( (V_b) \) compared with the bulk solvent \( (V_s) \).

This might be further broken down, at least in principle, to contributions from different groups, \( i \):

\[
\Delta V_b = \sum n_b(V_b - V_s)
\]

where \( n_b \) and \( V_b \) are the solvation number and solvent molar volume associated with the solvated group.

Thermal expansivity:

\[ E^0 = \frac{\partial V^0/\partial T}{\partial V_M/\partial T} + \frac{\partial \Delta V_s/\partial T}{\partial V_M/\partial T} \]

Since thermal expansion arises mainly from changes in intramolecular distances, \( \partial V_M/\partial T \) is very small for rigid, covalent molecules (covalent bond vibrations are not excited at normal temperatures). Consequently, at least for rigid molecules in solution, \( E^0 \) arises predominantly from solvation layer effects, that is:

\[ E^0 \approx \partial \Delta V_b/\partial T \]

Changes in expansivity are related to solvation (hydration) changes using the empirical relationship:

\[ (E_b - E_s) \approx 0.0125 \text{ cm}^3 \cdot \text{mol}^{-1} \cdot \text{K}^{-1} \]

which expresses the difference in \( E^0 \) of water in a solvation shell \( (E_b) \) compared with bulk water \( (E_s) \).

**Conclusions**

Using a combination of direct experimental measurements using a variety of microcalorimetry techniques, we have shown the following.

1. All non-covalent interactions show very similar thermodynamic signatures, regardless of whether they arise from hydrophobic, hydrogen-bonding, electrostatic or other forces.

2. The so-called ‘anomalous’ heat capacity effects that have been described in numerous systems are nothing of the sort: they are exactly what we should have expected from the classical physical chemistry of order–disorder transitions in solid/liquid media.

3. In particular, positive heat capacity changes are to be expected for any process (protein unfolding, protein–protein and protein–ligand dissociation) involving the disruption of a co-operative lattice of multiple weak interactions.

4. Pressure perturbation calorimetry offers a way to explore hydration effects directly.

The present study was supported by grants and studentships from EPSRC (Engineering and Physical Sciences Research Council) and BBSRC (Biotechnology and Biological Sciences Research Council), including funding of the BBSRC/EPSRC Biological Microcalorimetry Facility in Glasgow, Scotland, U.K. We are grateful to Margaret Nutley for technical support.

**References**