# Probing the Binding Entropy of Ligand–Protein Interactions by NMR

Steve W. Homans*[a]

## Biomolecular Interactions

The draft human genome sequence has revealed about 30 000–40 000 protein-coding genes in the human genome[1]—roughly twice as many as in a fly. Thus, it follows that the complexity of higher organisms lies in part in the interactions between gene products. Despite their fundamental importance, it is perhaps therefore surprising that our knowledge of the driving forces that govern biomolecular interactions is very rudimentary. For example, our ability to predict protein folds from sequence (an intramolecular-recognition problem) is currently a distant dream, as is our ability to design novel lead-drug candidates from high-resolution structures of target biomolecules.

## Thermodynamic Principles

The affinity of one biomolecule for another can be defined by application of the basic principles of chemical thermodynamics. In particular, the association constant ($K_a$) for complexation is defined by :

$$\Delta G^\circ = -RT \ln K_a \quad (1)$$

$\Delta G°$ is the *standard* free energy change for the association (not to be confused with $\Delta G$ which is zero at equilibrium), $R$ is the gas constant and $T$ is the absolute temperature. The standard free energy change is in turn comprised of the standard enthalpy change $\Delta H°$ and standard entropy change $\Delta S°$ for the association.

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ \quad (2)$$

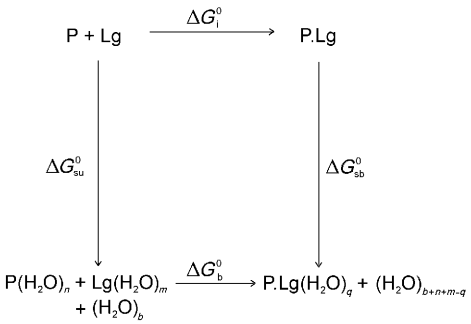
For the purposes of this discussion, the enthalpy can loosely be thought of as the "structural" component of the association and the entropy can be thought of as the "dynamic" component. Equation (2) reflects a balance between the tendency of the system to minimise its energy during association and to maximise its entropy. It follows that a full understanding of molecular interactions requires a complete knowledge of both the enthalpies and entropies of the species before and after association.

This minireview concerns the contribution of NMR-relaxation measurements to the derivation of the entropic component of the free energy of binding. Important recent developments (vide infra) permit the measurement of binding entropies on a per-residue basis—data that are unavailable through any other experimental approach. In what follows, we focus on binding thermodynamics rather than NMR relaxation measurements per se. The theory of the latter is adequately covered in several excellent recent reviews.[2–11]

## Thermodynamics of Ligand–Protein Association in Solution

The derivation of enthalpies and entropies for interacting species is a formidable task. This is especially true for the entropy, which depends on all degrees of freedom of the system. However, our understanding is further frustrated by the presence of solvent water in all biomolecular interactions. Since the thermodynamics of binding depends upon all interacting partners, the solvent contribution cannot simply be ignored.[12] Indeed, there is evidence that solvation (or desolvation) might be the dominant driving force in certain systems.

Since $\Delta G$ is a state function, a binding event can conceptually be represented in terms of a conventional Born–Haber cycle (Scheme 1).[12] In this cycle, $\Delta G_i^\circ$ represents the "intrinsic" standard free energy of binding of the solutes, $\Delta G_b^\circ$ represents the "observed" standard free energy of binding in solution, and $\Delta G_{su}^\circ$ and $\Delta G_{sb}^\circ$ represent the standard free energies of solvation of uncomplexed and complexed solutes, respectively. Equivalent cycles can be drawn for the enthalpy and entropy




**Scheme 1.** Born–Haber cycle for the association between protein (P) and ligand (Lg) showing the relationship between the observed free energy of binding $\Delta G_b$, the solute–solute free energy of binding $\Delta G_i$ and the solvation free energies of the unbound ($\Delta G_{su}$) and bound ($\Delta G_{sb}$) species.

[a] *Prof. S. W. Homans*
*School of Biochemistry and Microbiology, University of Leeds*
*Leeds LS2 9JT (UK)*
*Fax: (+44) 113-343-3167*
*E-mail: s.w.homans@leeds.ac.uk*

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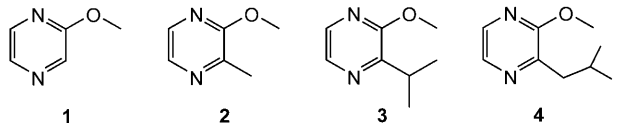
of association since these are also state functions. From Scheme 1 it is clear that :

$$\Delta G^{\circ}_{b} = \Delta G^{\circ}_{i} + (\Delta G^{\circ}_{sb} - \Delta G^{\circ}_{su}) \quad (3)$$

In other words, the observed standard free energy of binding comprises the "intrinsic" standard free energy of binding between solutes plus the difference in solvation free energies of the bound and free species.

## Importance of "Per-Residue" Thermodynamics

The advent of sensitive isothermal titration calorimetry (ITC)[13] has enabled the accurate determination of $\Delta G^{\circ}_{b}$, $\Delta H^{\circ}_{b}$ and $\Delta S^{\circ}_{b}$ for biomolecular complexes in aqueous solution. However, this technique measures the global thermodynamics of binding including solvation effects, as defined in Equation (3). In many cases it is therefore practically impossible to delineate the factors responsible for the association process. As a simple example, we refer to data obtained in my own laboratory concerning the binding of a panel of pyrazine-derived ligands (Scheme 2) to the mouse major urinary protein (MUP), a promiscuous binder of small hydrophobic molecules (Table 1). Apart from the fact that the affinities of these four ligands increase in the order that one might anticipate based on hydrophobicity, the global thermodynamic data do not shed light on the magnitudes of the affinities. In particular, the nature of the forces governing affinity are particularly obscure from $\Delta H^{\circ}_{b}$ and $T\Delta S^{\circ}_{b}$ values for each ligand. Intuitively, one would expect the interaction of these pyrazine ligands with MUP, which has an extremely hydrophobic binding site, to be entropy driven through the classical hydrophobic effect—yet it is clear from Table 1 that association is enthalpy driven in each case. Clearly, it is necessary to probe the interaction in atomic detail in order to appreciate the dominant factors governing affinity.




**Scheme 2.** Four pyrazine derivatives whose thermodynamics of binding to MUP are given in Table 1.

**Table 1.** ITC-derived thermodynamic parameters for binding of the four pyrazine derivatives illustrated in Scheme 2 to mouse MUP.

| Ligand | $\Delta G°$ [kJ mol$^{-1}$] | $\Delta H°$ [kJ mol$^{-1}$] | $\Delta S°$ [kJ mol$^{-1}$ K$^{-1}$] | $K_d$ [μM] | Stoichiometry |
|---|---|---|---|---|---|
| **1** | −23.9 | −42.0 | −18.1 | 67 | 0.98 |
| **2** | −26.3 | −35.3 | −9.0 | 25 | 0.95 |
| **3** | −33.9 | −44.5 | −10.7 | 1.8 | 0.97 |
| **4** | −38.5 | −47.9 | −9.4 | 0.3 | 1.01 |

## Per-residue Thermodynamics from NMR Relaxation Measurements

In the last decade, a number of important papers appeared in which an attempt was made to correlate protein dynamics with binding thermodynamics by using a variety of NMR relaxation techniques.[14–23] These studies are potentially of great value since in principle it is possible to obtain *per-residue* thermodynamic parameters by measurement of relaxation data at individual sites.

Pioneering work of Akke et al.[14] showed how it is possible to derive free energies of binding from differences in the square of the NMR-derived generalised order parameter $S^2$[24] determined from backbone-$^{15}$N relaxation data for calbindin in the "apo", half-saturated [$(Cd^{2+})_1$] and fully saturated [$(Ca^{2+})_2$] states. The dominant contribution to the free energy of binding was found to originate from the first binding event, with $\Delta G_b$ ranging from $-13.2 \pm 3.5$ to $-11.6 \pm 3.2$ kJ mol$^{-1}$ depending on the residue for which $S^2$ was measured. The authors interpreted this result as the free-energy cost of stiffening the backbone of the protein. As mentioned above, it would be of enormous benefit to decompose the per-residue free energies of binding thus obtained into the corresponding entropic and enthalpic components. In an important step towards this goal, Li et al.[25] used a simple model (a one-dimensional vibrator) to illustrate the relationship between dynamics measured by NMR relaxation methods and the local residual entropy of proteins. They concluded that dynamics of methyl-containing side chains correspond to a significant entropic contribution to the free energy of ubiquitin of approximately 40 kcal mol$^{-1}$ at 300 K. Subsequently, Yang, Kay et al.[15,26] examined the relation between the order parameter and conformational entropy from ns–ps bond-vector dynamics considering a number of simple models describing bond-vector motion. Although it was not possible to derive equations relating the order parameter to conformational entropy for the majority of models considered, an approximate relation was found to describe order parameters versus entropy profiles extremely well:

$$S_p/k = A + \ln \pi[3-(1+8S)^{1/2}] \quad (4)$$

in which $A$ is a model-dependent constant.

The above studies suggest that measurement of both $\Delta G_b$ and $T\Delta S_b$ for a biomolecular association is possible through measurement of order parameters from relaxation measurements on species before and after association, from which $\Delta H_b$ can be determined from Equation (2). Unfortunately, however, as discussed by Yang and Kay,[15] while the conformational entropy change between states derived from this approach does not depend upon differences in ground-state energies, this is not the case in the calculation of free-energy changes. Since ground-state energies of the two states are in general unavailable, NMR relaxation measurements are only able to offer reliable insight into the entropy of binding. Nonetheless, this is a major step forward since entropy is notoriously difficult to quantify by other means.

## Measurement of Per-Residue Entropies

It is important to be aware that the derivation of per-residue entropies as described in the previous section is subject to certain assumptions and limitations.[3,15] First, from Equation (4) it is clear that the per-residue entropy is model-dependent, and in general the nature of the motional model is unknown. In the case of the entropy of binding, $\Delta S_b$, this is not a severe limitation if the assumption is made that the motional models before and after association are similar, in which case the constant $A$ cancels. A second limitation is that the order parameter measured from conventional heteronuclear relaxation parameters ($T_1$, $T_2$, NOE) is sensitive only to motions on a time-scale shorter than overall rotational diffusion (picoseconds to nanoseconds), and is sensitive only to reorientational motions of the relevant bond vector. Finally, no account is taken of correlated motions between different bond vectors. Despite these limitations, work to date suggests that NMR relaxation measurements can provide reasonably accurate per-residue entropies for a variety of biomolecular associations (vide infra).

The nucleus of choice for probing backbone entropies is for practical and experimental reasons invariably $^{15}N$, whereby the conformational entropies of NH groups for each amino-acid residue can be measured (assignments and resonance overlap permitting) from $^{15}N$ relaxation data assuming a diffusion-in-a-cone model for NH vector motions.[15] In the case of side-chain entropy measurements, a natural choice at first sight might be $^{13}C$.[27–29] The use of fractionally deuterated $^{13}C$-labelled samples can eliminate $^{1}H$,$^{13}C$-dipolar cross-correlation effects that plague the effective analysis of $^{13}C$-relaxation data for, for example, methyl groups.[30] Moreover, fractional[28] or selective[31] $^{13}C$ enrichment can overcome $^{13}C$,$^{13}C$-scalar coupling interactions that complicate measurement of $^{13}C$ $T_2$ relaxation times. However, as discussed at length by Muhandiram et al.,[32] for a number of reasons $^{2}H$ relaxation-time measurements are more straightforward to interpret since relaxation of the deuteron is dominated by the quadrupolar interaction. Recently, Millet et al.[33] have described an approach whereby five relaxation rates per deuteron can be obtained in $^{13}C$-labelled and fractionally $^{2}H$-enriched proteins, enabling self-consistency of the relaxation data to be established. The merits of multifield $^{13}C$ data versus $^{2}H$ data for deriving order parameters have been the subject of two independent studies.[34,35] In the case of moderately sized proteins ($<30$ kDa) it is suggested that more accurate methyl order parameters can be estimated from $^{2}H$-relaxation data. However, for larger proteins, the sophistication of $^{2}H$-relaxation measurements comes at a cost compared with $^{13}C$-relaxation measurements due to very rapid $R_2$ relaxation.[35]

## Entropic Contributions in Biomolecular Associations

One of the first applications of the above methodologies considered the conformational entropy change associated with the folding–unfolding transition in the N-terminal SH3 domain of the *Drosophila* signal-transduction protein Drk.[15] The observed entropy change for the folding–unfolding transition averaged 12 $J\,mol^{-1}\,K^{-1}$, compared with the average entropy change per residue estimated from alternative techniques of $\sim 14\ J\,mol^{-1}\,K^{-1}$.[36] In a subsequent study, Wrabl et al.[37] used simulated order parameters for N–H bond vectors from nanosecond molecular-dynamics simulations of staphylococcal nuclease and compared per-residue entropies calculated by using Equation (4) with those estimated by using quasiharmonic analysis.[38] A positive correlation between these parameters suggested that NMR-derived order parameters provide a reasonable estimate of the total conformational entropy change on protein folding.

A number of independent studies have suggested that changes in configurational entropy make significant contributions in ligand–protein binding processes. Bracken et al.[17] examined the dynamics of the basic leucine zipper domain of yeast transcription factor GCN4 on binding to DNA. This domain binds to DNA as a dimer in which the C-terminal residues form a parallel $\alpha$-helical coiled coil leucine zipper, and the N-terminal residues form the basic region that consists of symmetrically positioned $\alpha$-helices that contact the major groove of the cognate DNA sequence. In the absence of DNA, the basic region adopts an ensemble of transient structures, but undergoes a transition to yield a stable $\alpha$-helical structure on binding DNA. Thus, an unfavourable contribution to binding is anticipated from the change in conformational entropy of the protein backbone. Indeed, this was estimated as $\Delta S_b \sim -0.6\ kJ\,mol^{-1}\,K^{-1}$, which agrees remarkably well with theoretical predictions based on calorimetric measurements for the same system ($\Delta S_b \sim -0.5\ kJ\,mol^{-1}\,K^{-1}$). At 300 K the contribution to the free energy of binding is thus between $-150$ and $-180\ kJ\,mol^{-1}$. Although this contribution is likely offset by other factors (vide infra), it illustrates that in principle the entropic contribution from protein degrees of freedom can easily become a dominant unfavourable component of the binding free energy.

Zidek et al. have provided an independent investigation of the role of backbone dynamics to the entropy of ligand binding,[18] by examining the interaction of the pheromone 2-*sec*-butyl-4,5-dihydrothiazole to the mouse major urinary protein (MUP). In contrast to the study of Bracken et al., these workers found that the backbone conformational entropy of the protein was found to increase on ligand binding, and they estimated a resulting favourable entropic contribution to binding of $\sim 50\ kJ\,mol^{-1}$.

Lee et al. examined the entropic contribution to binding from both backbone and side-chain degrees of freedom for calcium-saturated calmodulin binding with a peptide model of the calmodulin-binding domain of myosin light-chain kinase.[21] This work is particularly notable since it illustrates a number of important features. The most remarkable of these is perhaps the fact that the protein effectively redistributes the side-chain entropy upon binding of the peptide. Thus, the side chains of binding-site residues become more rigid upon association of the peptide as anticipated, whereas certain residues remote from the binding site become more flexible, thus offsetting in part the unfavourable entropic contribution from binding-site residues. A second important result is that backbone and side-

chain dynamics do not correlate and can be clearly separated, with little change in the motional characteristics of the backbone. Once again, the overall entropic contribution to binding free energy derived from NMR relaxation measurements is in qualitative agreement with calorimetric measurements.

Very recently Bingham et al.[23] undertook a study of the binding of 2-methoxy-3-isobutylpyrazine (IBMP, **4** in Scheme 2) and 2-methoxy-3-isopropylpyrazine (IPMP, **3** in Scheme 2) to MUP. Backbone dynamics of certain regions of the protein exhibited increased flexibility on binding IBMP, whereas others, notably the loop centred on Asn99, displayed an overall reduction in flexibility (Figure 1). The overall entropic contribution from backbone dynamics was unfavourable with $T\Delta S_b = -7.4 \pm 6.5\ kJ\,mol^{-1}$. This contrasts with the above-mentioned study of Zidek et al.[18]. The reasons for this discrepancy are unclear, but might derive from substantially different ligands in each study. The overall contribution from side-chain methyl dynamics on binding IBMP (Figure 2) was also unfavourable ($T\Delta S_b = -3.4 \pm 2.8\ kJ\,mol^{-1}$) and, in common with the calmodulin–peptide complex studied by Lee et al.,[21] "Entropy–entropy compensation" is observed, that is, loss of dynamics for binding-site residues is offset by increased dynamics of side chains distal to the binding site (Figure 3).

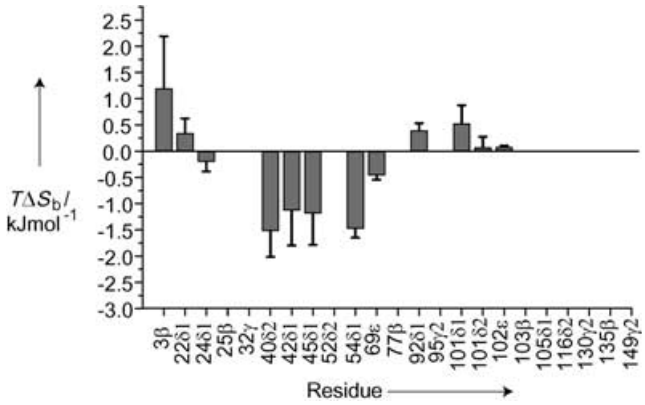



**Figure 2.** Plot of the entropic contribution to binding ($T\Delta S_b$) of IBMP to MUP derived from side-chain methyl-$^2$H-relaxation measurements. Error bars correspond with the propagated standard error, and data are plotted only for those residues for which the absolute value of $T\Delta S_b$ is greater than the standard error. Reproduced with permission from ref. [23]. Copyright 2004, American Chemical Society.

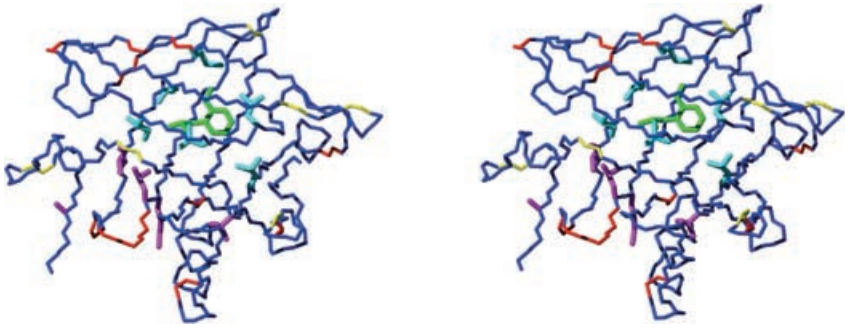


**Figure 3.** Stereo view detailing of residues that contribute to the entropy of binding of IBMP to MUP. Backbone residues that exhibit an unfavourable entropic contribution to binding are coloured red, while those that exhibit a favourable contribution are coloured yellow. Similarly, residues whose methyl-containing side chains exhibit an unfavourable contribution are coloured light blue, whereas those that exhibit a favourable contribution are coloured magenta.

## Protein Entropy in the Context of Other Contributions

Conceptually, it is clear from Scheme 1 that the overall conformational entropy of ligand–protein binding can be decomposed into four contributions: i) protein degrees of freedom; ii) ligand degrees of freedom; iii) protein solvation/desolvation; iv) ligand solvation/desolvation. We have considered (i) for a number of systems above, and it is instructive to place the entropic contribution from protein degrees of freedom in context of the other contributions.

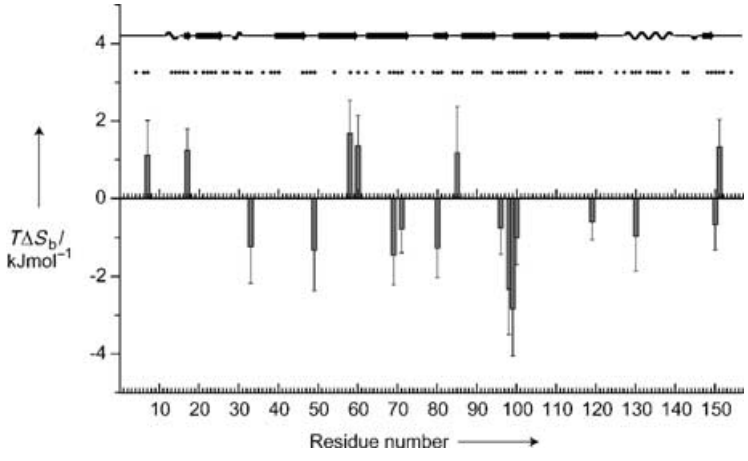



**Figure 1.** Plot of the entropic contribution to binding ($T\Delta S_b$) of IBMP to MUP derived from backbone-$^{15}$N-relaxation measurements. Error bars correspond to the propagated standard error, and data are plotted only for those residues for which the absolute value of $T\Delta S$ is greater than the standard error. Diamonds represent residues for which $^{15}$N-relaxation data were measured, and the secondary structure of the protein (derived from PROCHECK[66]) is also shown. Reproduced with permission from ref. [23]. Copyright 2004, American Chemical Society.

### Ligand degrees of freedom

Entropic contributions from the ligand include the loss of rotational and translational degrees of freedom on binding, together with internal degrees of freedom, all of which are assumed to be "frozen out" on binding. The loss of translational and rotational entropy, which is weakly dependent upon the molecular mass of the ligand, has been estimated as $\sim 50\ kJ\,mol^{-1}$.[39] An experimental measure of $\sim 25\ kJ\,mol^{-1}$ for this contribution was obtained by Turnbull et al.[40] from ITC measurements by using a ligand-fragmentation approach exploiting the concepts described by Jencks.[41] Various

estimates have been made regarding the unfavourable entropic contribution arising from the loss of internal degrees of freedom, but a value of $\sim 6\ kJ\,mol^{-1}$ per rotor appears to be a generally accepted value.[39] Thus, if we take as an example the binding of IBMP to MUP, the overall contribution to the entropy of binding from protein and ligand degrees of freedom is strongly unfavourable, in the range of $-40$ to $-90\ kJ\,mol^{-1}$. Other systems, such as the binding of GCN4 to DNA mentioned above, will exhibit an even more unfavourable contribution because of the large contribution from protein degrees of freedom.

#### Protein solvation

While the entropic contributions from ligand and protein degrees of freedom estimated above are strongly unfavourable, the overall entropy contribution $T\Delta S_b$ to the free energy of binding is generally more modest. For example, the overall entropic contribution of binding of IBMP to MUP is $\sim -10\ kJ\,mol^{-1}$.[23] The difference is in part attributable to the favourable contribution to binding from desolvation of the protein binding pocket. Prior to association, the binding site will be occupied by solvent water molecules, many of which are partially ordered because of hydrogen-bonding with binding-site residues or by virtue of the structuring of water around hydrophobic residues. On ligand binding, the majority of these water molecules are released into the solvent with a consequent increase in conformational entropy. The magnitude of this effect has been subject to intense debate. On the basis of thermodynamic data for crystal hydrates, Dunitz has shown that this cannot exceed $\sim 8\ kJ\,mol^{-1}$ per water molecule.[42] In the case of the MUP, it is estimated that eight water molecules are displaced, giving rise to a favourable entropic contribution of $\sim 24\ kJ\,mol^{-1}$.[23] Clearly there is potential for a much greater favourable contribution to binding when the ligand is considerably larger.

#### Entropy of protein solvation from NMR measurements

Current knowledge about buried water molecules in proteins is largely derived from high-resolution X-ray diffraction data.[43] It has long been known that it is also possible to detect bound water molecules by using NMR methods,[44,45] which offers the advantage that occupancies as low as 10% can be detected. More importantly, NMR dispersion (NMRD) methods can provide intramolecular order parameters that report on the orientational fluctuations of buried water molecules, thus offering a per-residue experimental measure of the entropic contribution to binding. Such measurements have been pioneered by Denisov, Halle et al.[46] In the application of this method to bound water molecules in bovine pancreatic trypsin inhibitor (BPTI), and assuming that the translational entropy of these water molecules is essentially the same as in ice, it was concluded that the buried water molecules, despite extensive hydrogen-bonding to protein, do not have significantly lower entropy than bulk water. This conclusion has very interesting implications for the estimated contributions to binding entropy mentioned in the previous section.

#### Ligand solvation

Prior to complexation, the ligand will also be associated with solvent water molecules. As can be seen in Scheme 1, the entropic contribution to binding is closely related to the solvation entropy, that is, the entropy of solvation of transfer of the ligand from the gas phase into solution. While solvation entropies have not been reported for the majority of "interesting" ligands, these have been experimentally determined for a number of simple organic molecules. For example $T\Delta S$ for solvation of aliphatic alcohols varies from $+20\ kJ\,mol^{-1}$ for methanol to $+48\ kJ\,mol^{-1}$ for hexanol.[47] This increasingly unfavourable contribution with aliphatic chain length can be interpreted as an ordering of water molecules around the hydrophobic hydrocarbon chains. To the extent that ligand–protein association represents a desolvation process, this translates to a favourable contribution to the free energy of binding, through the classical hydrophobic effect.

## The Dominant Driving Force

So which of the driving forces described above is dominant? This appears to be highly dependent upon the system under investigation. In the case of ligand binding to the major urinary protein, for example, the favourable entropic contribution from desolvation appears to offset the unfavourable contribution from "freezing out" ligand degrees of freedom almost exactly, and the measured overall binding entropy derives from protein degrees of freedom.[23] However, the free energy of binding is still dominated by enthalpy over a wide temperature range. In contrast, the binding of a number of *p*-alkylbenzamidinium chloride inhibitors to trypsin varies from strongly entropic at low temperature to strongly enthalpic at high temperature.[48] The large negative heat capacity of binding $\Delta C_p$ is a signature of the hydrophobic effect; this suggests that the solvent plays a dominant role in binding. However, the architecture of the trypsin-binding site is not substantially more hydrophobic than that of MUP. Clearly, it will be necessary to characterise fully a wide variety of systems in order to ascertain if any "rules" can be discerned.

## Future Perspectives

Despite the clear potential of NMR methods for the derivation of per-residue thermodynamic parameters for biomolecular associations, many uncertainties remain.

Turning first to the contribution of protein degrees of freedom to the entropy of binding, the major limitation is perhaps the timescale of dynamics over which the majority of studies have focussed to date, which is shorter than overall rotational diffusion (typically 10 ns). While the slowest vibrational modes of proteins fall within this range,[49] it is nonetheless important to determine the contributions of slower motions to binding entropy, which collectively could be significant. Some progress

has already been made in this direction. For example, NMR relaxation techniques are available for the quantification of microsecond-to-millisecond motions in biological macromolecules (reviewed by Palmer et al. in ref. [5]). Moreover, substantial progress has been made regarding the measurement of modulations that influence isotropic properties that are not averaged by isotropic tumbling, such as cross-correlated chemical-shift modulation[50] and heteronuclear relaxation-dispersion methods.[51,52] Methods have also been described that depend upon scalar and residual dipolar couplings for the detection of motions over the entire range from microseconds to milliseconds.[53–57] Applications include the derivation of order parameters for side-chain rotamer "jumping"[58] and domain reorientation.[59] Details of the structural and dynamic events that give rise to "entropy–entropy" compensation phenomena described above might be revealed at these longer time-scales.

A second aspect which requires considerable development concerns the solvation contribution to binding entropy. Here, scope for application of NMR methods might be rather limited since it is often impossible to perform measurements on individual water molecules which are typically in fast exchange with bulk solvent, unless a single water molecule can be "engineered out" by mutagenesis.[60] A possible avenue for development involves all-atom molecular-dynamics simulations of ligand–protein complexes with explicit inclusion of solvent water. Computation of solvation free energy, entropy and enthalpy differences between panels of related ligands is possible at modest computational cost by use of free energy perturbation or thermodynamic integration methods.[61,62] These can be extended to ligand–protein complexes albeit with a significant increase in computational cost.[48] Molecular dynamics simulations are also likely to be of increasing importance for the derivation of per-residue entropies[63] for side-chain residues that are not readily amenable to experimental measurement using NMR methods (such as the side chains of Asp and Glu). Validation of these simulations can be achieved by comparison of back-calculated per-residue entropies for side chains that can be studied experimentally.

Finally, a complete understanding of the forces that drive ligand–protein interactions will require a parallel study of binding enthalpies. For example, as mentioned above, the binding site of MUP is very hydrophobic, yet the binding of cognate hydrophobic ligands appears universally to be enthalpy-driven,[23,64] in contrast to expectations based upon our current understanding of the hydrophobic effect. As a second example, the binding of hydrophilic monosaccharides to the arabinose-binding protein is enthalpy-driven but is accompanied by a very large unfavourable binding entropy ($T\Delta S_b \sim -60$ kJ mol$^{-1}$ at 308 K),[65] which is again counterintuitive based upon the favourable entropy gain anticipated from release of binding-site water molecules. Possibly, there is a substantial entropic penalty to binding from protein degrees of freedom. A multidisciplinary approach using ITC, molecular modelling, site-directed mutagenesis, and NMR relaxation measurements is ideally suited to resolve this and other major unsolved issues that govern the affinities of ligand–protein interactions.

**Keywords:** calorimetry · dynamics · NMR spectroscopy · proteins · thermodynamics

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