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Protein-binding pockets are usually thought to be solvated. However, recent studies indicate that this may not necessarily be the case, leading to unexpected gains in ligand binding affinity.

Water, water everywhere — except where it matters?

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Biological processes depend on specific recognition between molecules with carefully tuned affinities. Because of the complexity of the problem, binding affinities cannot reliably be computed from molecular structures. Modern biophysical techniques can decompose the problem to determine the thermodynamic contributions from protein, cognate ligand and solvent. Such studies applied to a model protein with a hydrophobic binding pocket have resulted in some surprising findings. For example, binding is not driven by the favourable entropic loss of solvent water from the binding pocket, but rather by favourable dispersion interactions arising from suboptimal hydration of the protein-binding pocket. Under these circumstances, one can anticipate particularly dramatic gains in binding affinity using shape complementarity to optimise solute–solute dispersion interactions, since these will not be offset by opposing solute–solvent dispersion interactions.

All biological processes depend critically on highly specific recognition between molecules with carefully tuned affinities. Despite the universal nature of these interactions, our understanding of their molecular basis is severely limited. For example, despite a number of successes, it is still extraordinarily difficult to exploit routinely high-resolution structural data for a given complex in order to design molecules that inhibit binding. In other words, it is not trivial to predict binding affinity from structure. Thus, with notable exceptions, 'structure-based' ligand design has enjoyed limited success. In the face of the emergence or re-emergence of diseases such as age-related neurodegenerative disorders or the relentless progress of antibiotic resistant bacterial strains that may soon reach epidemic proportions, the ability to design novel ligands at will that inhibit biomolecular interactions remains one of the major challenges in contemporary science.

Our limited ability to predict affinity from structure is due in large part to the complexity of the problem, whereby competing thermodynamic processes all contribute to binding affinity. The standard free energy of binding, which determines the strength of the interaction, not only is governed by structural terms (loosely, enthalpy) but also involves the dynamics (loosely, entropy) of the interacting partners (Box 1). Thus, the affinity of a protein for a given ligand depends not only on the spatial positions of the interacting atoms but also on their dynamics, that is, how their positions change with time. To further complicate the problem, a full understanding of the binding process requires knowledge of not only the structure and dynamics of the protein and ligand but also solvent water, which can have a dramatic influence on binding (Box 2).

The hydrophobic effect

A typical case in point is the classical hydrophobic effect, whose driving force is widely accepted as arising from solvent reorganisation [1]. Water molecules cannot form hydrogen bonds with nonpolar solutes, and this results in a disruption of the favourable hydrogen-bonding network of bulk water. Water molecules that are in contact with the solute are bonded more strongly to their neighbours, which results in an ordering of water molecules around the solute. The nature of the resulting ordering has been described in various ways over the years, such as 'clathrates', 'icebergs' and 'flickering clusters' [1–3]. More recent theoretical analyses [4] suggest that water molecules move away from the solute surface to form an interface that bears a similarity to that between a vapour and a liquid, such that the hydrophobic surface is 'dewetted'. This solvent-ordering phenomenon is consistent with the data on the solvation thermodynamics of small hydrophobic molecules [5]. The standard entropy of hydration, that is, the change in the standard entropy

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BOX 1

The essentials of binding thermodynamics for a ligand–protein association.

The interaction of a ligand with a protein can be written in the form of a standard chemical equilibrium:

$$P + \mathrm{L} \Leftrightarrow \mathrm{PL} \quad (1)$$

The association constant K_a (or equivalently, the reciprocal of the dissociation constant $1/K_d$) for this reaction is related to the standard free energy of binding as follows:

$$\Delta G_b^\circ = -RT \ln K_a \quad (2)$$

Note that the *standard* free energy of binding ΔG_b° must not be confused with the free energy of binding ΔG_b, which by definition is zero at equilibrium. The standard free energy of binding is in turn comprised of the standard enthalpy of binding ΔH_b° and the standard entropy of binding ΔS_b° (multiplied by the absolute temperature T):

$$\Delta G_b^\circ = \Delta H_b^\circ - T\Delta S_b^\circ \quad (3)$$

The enthalpy can be loosely defined as the static, structural component of the association, whereas the entropy is related to the dynamics of the interacting partners.

BOX 2

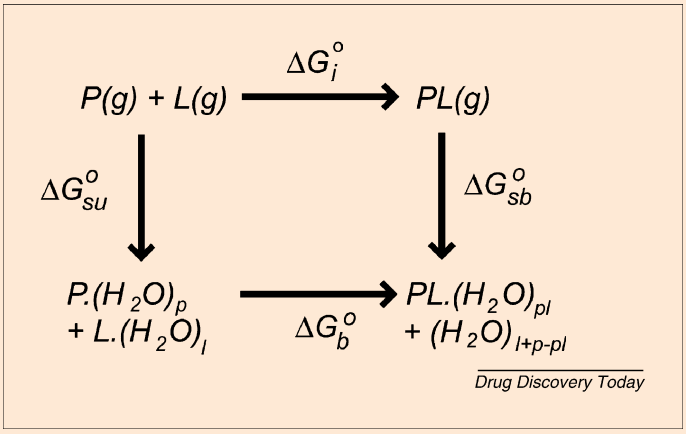
The formulation of the interaction in terms of Equation 1 (Box 1) is in fact a gross oversimplification of the binding process, since it ignores solvent water. A complete formalism involves a thermodynamic cycle as shown, where we account for the fact that the ligand and protein are associated with H_2O (Scheme 1). Here, ΔG_b° is the observed standard free energy of binding, ΔG_i° is the 'intrinsic' solute–solute interaction in the absence of solvent, and the terms ΔG_{sb}° and ΔG_{su}° are related to the solvation of the complex and the free species, respectively. Since G is a state function, the sum over the cycle is zero, and we can thus write:

$$\Delta G_b^\circ = \Delta G_i^\circ + \left\{\Delta G_{sb}^\circ - \Delta G_{su}^\circ\right\} \quad (4)$$

We can equate ΔG_{sb}° with the solvation free energy of the complex ΔG_{solvPL}°, whereas ΔG_{su}° comprises the sum of the solvation free energies of the free protein and free ligand, $\Delta G_{solvP}^\circ + \Delta G_{solvL}^\circ$. Thus, finally we can write:

$$\Delta G_b^\circ = \Delta G_i^\circ + \left\{\Delta G_{solvPL}^\circ - \Delta G_{solvP}^\circ - \Delta G_{solvL}^\circ\right\} \quad (5)$$

A similar equation can be written for the enthalpy and entropy of binding since these are also state functions. The thermodynamic decomposition approach involves the determination of each component on the right-hand side of Equation 5.



SCHEME 1

Thermodynamic cycle for a ligand–protein interaction in solvent water.

when the molecule is transferred from the gas phase to water solution, is invariably negative. It therefore follows that the association of two hydrophobic molecules in aqueous solution with the burial of hydrophobic surface area will be characterised by a favourable entropy of binding because of the expulsion (and increase in entropy) of solvent water molecules at the solvent interface. Entropy-driven binding has thus long been taken as a characteristic thermodynamic binding signature of hydrophobic associations.

A second parameter that has been ascribed to hydrophobic association is a negative change in the heat capacity at constant pressure (ΔC_p). Again, this is related to solvent organisation. Experimental data for the transfer of small hydrophobic molecules from non-aqueous to aqueous solution are usually accompanied by a positive ΔC_p [6]. This can readily be understood in terms of the above model of the hydrophobic effect — the ordered solvent molecules surrounding the solute are able to 'soak up' more thermal energy without a concomitant rise in temperature because they have a lower kinetic energy than bulk solvent. Conversely, the loss of ordered solvent water molecules from a binding interface will result in a negative ΔC_p for the binding process.

Enthalpy-driven hydrophobic effects?

Despite these apparently logical and consistent arguments, there exist discrepancies. Over two decades ago, Ross and Subramanian noted that, of the relatively limited thermodynamic data for 'hydrophobic interactions' that were available at the time, a substantial number were characterised by a thermodynamic binding signature that is enthalpy driven [7]. In order to investigate these discrepancies further, a programme of work was undertaken in our own laboratory, where we endeavoured to decompose the thermodynamics of binding of a ligand–protein interaction into contributions from the ligand, protein and solvent. We reasoned that, because of the obvious complexity of the problem, it would be appropriate to work with a structurally well-characterised model system. Moreover, it was apparent that it would be easier to try to delineate the thermodynamic *differences* in binding between a series of distinct, but structurally related, ligands for a given protein, than to attempt to dissect the interaction process for a single ligand *de novo*. With these constraints in mind, the mouse major urinary protein (MUP) was selected for further study. MUP is an abundant protein found in male mouse urine that binds pheromones, where subtle recognition of a series of related compounds underlies its biological function. The crystal structure of MUP-I isolated from urine was solved by Böcskei and co-workers [8]. The protein has a typical lipocalin fold that consists of an eight-stranded β-barrel and a single α-helix, and the interior of the barrel forms a hydrophobic cavity. A number of small hydrophobic molecules can bind within the cavity, and the protein is thus an ideal model system with which to study 'hydrophobic interactions' of a series of related ligands. The chosen ligands were 2-methoxy-3-isobutylpyrazine (a natural pheromone) and various derivatives such as 2-methoxy-3-isopropylpyrazine (Figure 1).

At the outset, we had no knowledge of the thermodynamics of binding of these ligands to MUP. Isothermal titration calorimetry

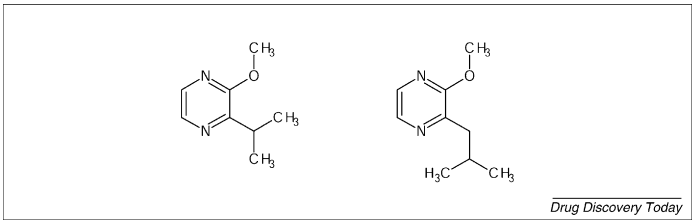


FIGURE 1

MUP ligands: 2-methoxy-3-isopropylpyrazine (IPMP, left) and 2-methoxy-3-isobutylpyrazine (IBMP, right).

(ITC) [9] was therefore used to probe global binding thermodynamics [10], which remarkably showed that binding was enthalpy driven, with an unfavourable entropy (Table 1) — a counter-intuitive result given the discussion in the previous section, but not without precedent given the earlier observations of Ross and Subramanian [7]. It was clearly important to perform a 'thermodynamic decomposition' (Box 2) in order to resolve this apparent paradox.

Protein and ligand degrees of freedom

Ignoring solvent effects for the moment, the overall entropy of binding depends not only on the dynamics (i.e. number of accessible microstates) of the complex but also on the dynamics of the cognate partners before association. In this regard, intuitively one might expect that the overall change in entropy would be negative, since both the protein and the ligand would be expected to be less dynamic following binding, as various side chains form non-bonded interactions. In order to determine whether this is the case for the protein, nuclear magnetic resonance (NMR) relaxation methods [11–15] were used to probe the dynamics of certain protein bond vectors before and after association. These methods typically enable the dynamics of backbone amide bond vectors and side-chain methyl bond vectors to be measured with relative ease, at least in proteins the size of MUP. Using straightforward data-fitting procedures, it is possible to convert the measured relaxation rates into *per residue* entropies, offering a very powerful approach to the analysis of the dynamic behaviour of a protein as a ligand binder [16–18]. A surprising observation resulted from the application of these methods to the binding of IBMP and IPMP to MUP — while side chains in the binding pocket of the protein became more rigid on ligand binding, side chains distal to the binding pocket became more dynamic [10]. Moreover, similar characteristics were apparent in backbone amide bond vectors. The net result was that the overall entropy of binding arising from protein degrees of freedom was zero within error. It seems that the protein compensates for the unfavourable loss in entropy within the binding pocket by a redistribution of dynamics over the whole protein. We have coined the term 'entropy–entropy' compensation for this phenomenon. More important, this has been observed by other researchers in very different proteins [19–23]. Indeed in some cases, it is clear that there is an over-compensation, that is, overall the protein becomes *more* dynamic, resulting in a *favourable* contribution to binding entropy [24]. The underlying molecular basis of these changes in dynamics remains obscure at present, but it is tempting to speculate that many proteins have evolved to evade the unfavourable entropic penalty arising from reduction in their degrees of freedom as the ligand binds.

Turning now to ligand degrees of freedom, it is necessary to consider not only the reduction in internal (i.e. conformational) entropy as these are potentially 'frozen' on ligand binding but also changes in translational and rotational (i.e. configurational) entropy. Assuming the ligand is rigidly held within the binding pocket (an assumption whose validity remains to be determined for most interactions, including MUP-ligand interactions), it is straightforward to estimate the loss in conformational entropy from conventional statistical-mechanical arguments [25,26]. The loss in configurational entropy is more difficult to measure. If all of the translational and rotational motion is removed on binding, then the entropic cost of association is approximately −57 kJ/mol for a small ligand [27]. Experimental estimates based on multivalency phenomena suggest a value closer to −25 kJ/mol, suggesting that indeed there remains a residual entropic contribution in the bound state [28]. Overall, for the MUP-IPMP interaction, we estimated an unfavourable (−37 kJ/mol) contribution from ligand degrees of freedom [10,29].

Solvation contribution to entropy of binding

Returning to the contribution from solvent, rearrangements arising from the desolvation of the ligand and the protein on binding must be considered. A convenient way to do this is to consider a complete thermodynamic cycle for a protein–ligand interaction [30–32], resulting in an equation describing the standard entropy of binding ΔS_b in terms of its composite parts, in analogy with Equation 5 (Box 2). The 'intrinsic' contribution to the binding entropy comprises ligand and protein degrees of freedom described above. The term in curly brackets describes the solvation contribution. Specifically, ΔS°_{solvL} is the standard entropy of solvation of the ligand, which can be determined from the temperature dependence of the standard free energy of solvation ΔG°_{solvL}. Since the MUP ligands in question are very volatile, the latter can readily be measured experimentally using vapour–water partition experiments [29,33]. From these data, ΔS°_{solvL} for IPMP was ~ −30 kJ/mol, giving a favourable entropic contribution to binding of ~+30 kJ/mol (since binding is a *de*solvation event). The remaining contribution, $\Delta G^\circ_{solvPL} - \Delta G^\circ_{solvL}$, which represents the desolvation of the protein on binding, is very difficult to assess experimentally, except in special cases [34,35] (although theoretical estimates are available [36–38]). However, in this particular case, it is not necessary to do so, since if all the other contributions apart from this are defined, the contribution from protein desolvation can be obtained arithmetically from the overall standard entropy of binding, as shown in Table 2. Inspection of these data illustrates a second remarkable result, namely that the entropic contribution from protein desolvation is zero within error. This is again counter-intuitive in the context of the expected thermodynamic signature

TABLE 1

ITC-derived global thermodynamics of binding of IBMP and IPMP to MUP [10]

Ligand	ΔG° (kJ/mol)	H° (kJ/mol)	$T\Delta S^\circ$ (kJ/mol)	K_d (μM)
IBMP	−38.5	−47.9	−9.4	0.3
IPMP	−33.9	−44.5	−10.7	1.8

for a hydrophobic interaction but, in fact, makes perfect sense after considering the enthalpic contribution to binding.

Intrinsic contributions to binding enthalpy

The enthalpic contribution to binding can also be decomposed in an analogous manner to Equation 5 (Box 2). Here, the 'intrinsic' contribution ΔH_i° comprises changes in the structure of the protein and ligand on binding, together with new non-bonded interactions that may be formed at the binding interface [39,40]. Considering first the former, it was easy to show using quantum-chemical calculations that both IPMP and IBMP are bound close to their minimum-energy configurations, resulting in a negligible contribution to binding enthalpy. Moreover, X-ray crystal structures and NMR chemical shift analyses showed no evidence of significant changes in protein structure on ligand binding, suggesting that this contribution is also very small [10]. Non-bonded interactions were therefore considered as a possible source of the favourable enthalpy of binding. It is perhaps intuitively obvious that non-bonded interactions between two species that have shape complementarity should give rise to favourable dispersion (van der Waals) contributions to the free energy of binding. However, it must be remembered that before association, both the protein-binding pocket and the ligand are surrounded by solvent water, the small size of which might be expected to ensure excellent shape complementarity (overall) with the surfaces that will become desolvated following the interaction. Thus, it has been argued that solute–solute dispersion interactions offer a minor contribution to binding because they are offset by similar solute–solvent interactions before association (reviewed by Hunter [41]). The extremely favourable enthalpy of binding of ligands to MUP was therefore very puzzling.

The key to solving this puzzle was the serendipitous discovery that MUP binds the primary aliphatic alcohols pentanol through decanol [32]. Amazingly, binding becomes increasingly favourable enthalpically and increasingly unfavourable entropically progressing upward in the series (i.e. with increasing hydrophobicity). Since the solution thermodynamics of these species have been very well documented in the literature [42], by comparing the binding thermodynamics of adjacent members of the alcohol series, together with knowledge of their solvation thermodynamics, the 'unknown' enthalpic contribution from protein desolvation cancels to first order. Thus, it is possible to derive an experimental derivation of the *difference* in intrinsic binding enthalpy across the series. Since each alcohol differs only by a CH_2 group, the difference thus corresponds to the enthalpic contribution from that group. Assuming this contribution is proportional to buried surface area, it is straightforward to estimate the contribution from dispersion interactions for the 'authentic' ligand IPMP, as shown in Table 2 [29]. At −76 kJ/mol, this contribution is very large — so what of the counterbalancing of this contribution from solute–solvent interactions? In parallel with these studies, all-atom molecular dynamics simulations on MUP were performed to study the behaviour of solvent in and around the binding pocket [43]. The pocket is 'sock' shaped and is occluded. Although the ligand is essentially completely enclosed within the protein in the complex, there is a passage for the entry and exit of water molecules in its absence. However, the results of our MD simulations indicated that the binding pocket is very poorly hydrated. Performing simulations with the binding pocket initially completely filled with solvent water molecules resulted in the rapid exit of a number of molecules with a few nanoseconds. Equivalently, simulations with the binding pocket initially empty did not result in complete solvation of the binding pocket. The density of water within the binding pocket once the simulation had equilibrated was 0.2–0.3 g/ml. These observations thus explain why the solute–solute dispersion contribution is so large and also explain the paradoxical zero contribution to the binding entropy from protein desolvation.

Of course, it is possible to criticise MD simulations on the basis that current force fields are inadequate for the 'correct' description of the degree of solvation of the binding pocket. However, to our knowledge, the desolvation of the binding pocket is the only way to explain the paradoxical thermodynamic binding signature seen with this protein. Moreover, NMR hydrogen/deuterium amide proton exchange measurements show that amide protons, which are not hydrogen bonded in secondary structural elements within the binding pocket (notably Leu 40), have exchange rates similar to those buried within the core of the protein, suggesting that these are largely solvent inaccessible (unpublished data). Finally, the modest favourable enthalpy term arising from desolvation of the binding pocket (ca. −12 kJ/mol) is consistent with 'higher energy' residual water molecules in the binding pocket that form a greater number of hydrogen bonds on returning to bulk solvent.

Conclusions

So why are these data of relevance to drug discovery? Principally, they illustrate that, contrary to popular belief, protein-binding pockets are not necessarily optimally hydrated. One often hears the adage 'Nature abhors a vacuum', which is a glib expression of the Second Law of Thermodynamics. It is, of course, true that a perfect gas will expand from one container when connected to a second evacuated container, since this results in an increase in entropy of the system. However, biomolecular interactions take place in the condensed phase — water molecules prefer to bind to

TABLE 2

Thermodynamic decomposition of binding of IPMP to MUP [10,29]

Entropic contribution		Value (kJ/mol)	Enthalpic contribution	Description	Value (kJ/mol)
$T\Delta S_i^\circ$	Protein degrees of freedom	-0.8 ± 3.8	ΔH_i°	New solute–solute interactions	~−76
	Ligand degrees of freedom	~ −37		Changes in ligand/protein structure	~0
$-T\Delta S_{solvL}^\circ$	Ligand desolvation	$+26.7 \pm 8.4$	$-\Delta H_{solvL}^\circ$	Ligand desolvation	$+43.8 \pm 8.2$
$T\Delta S_{solvPL}^\circ - T\Delta S_{solvP}^\circ$	Desolvation of protein/complex	$+0.4 \pm 9.2$	$\Delta H_{solvPL}^\circ - \Delta H_{solvP}^\circ$	Desolvation of protein/complex	-12.3 ± 8.4
$T\Delta S_{obs}^\circ$	Observed entropy	-10.7 ± 0.5	ΔH_{obs}°	Observed enthalpy	-44.5 ± 0.4

other water molecules. Although in the case of MUP, entropically the water molecules 'prefer' to occupy the binding pocket, enthalpically they prefer to form hydrogen bond with their neighbours. In the case of MUP, they are simultaneously incapable of satisfying both criteria, and enthalpy 'wins' (but only marginally). In this respect, differences in hydration dependent on the shape of hydrophobic surfaces have long been recognised [4,44,45].

Subsequent to our original discovery, it is becoming apparent that other protein receptors are suboptimally hydrated too. For example, very recently Berne and co-workers discovered that the Cox-2 receptor is suboptimally hydrated [46]. Thus, under circumstances of suboptimal hydration, one can anticipate that significant gains in binding affinity can be achieved by focusing on shape complementarity to optimise solute–solute dispersion interactions, since these will not be offset by opposing solute–solvent dispersion interactions. The key to employing this strategy will require us to recognise when a binding-pocket is optimally hydrated and when it is not. Cleft-shaped pockets such as that found in trypsin might be expected to be optimally hydrated since water molecules can simultaneously occupy the pocket and form hydrogen bond effectively with bulk solvent. Indeed, it is notable that ligands for trypsin with increasing aliphatic (i.e. hydrophobic) chain length demonstrate a binding signature that is increasingly entropy driven, in accordance with expectations based on the hydrophobic effect [47]. In contrast, occluded hydrophobic pockets are more likely to be suboptimally hydrated since water molecules cannot simultaneously occupy these pockets and form hydrogen bond efficiently with bulk water. More generally, one might anticipate that certain *regions* of otherwise well-solvated binding pockets are poorly solvated, in which case optimisation of shape complementarity exclusively in those regions might bear fruit. A forthcoming challenge will be to identify such regions quickly and easily. In this regard, thermodynamic decomposition, although a valuable approach to understanding the intricate details of protein–ligand interaction, is currently far too slow and cumbersome for use in any drug discovery programme. However, we are hopeful that these data will be of value in the development of the next generation of molecular mechanical force fields, leading to thermodynamic decomposition *in silico*.

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