Chapter 7
Protein-Ligand Interactions as the Basis for Drug Action

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Abstract Lead optimization seeks for conclusive parameters beyond affinity to profile drug-receptor binding. One option is to use thermodynamic signatures since different targets require different mode-of-action mechanisms. Since thermodynamic properties are influenced by multiple factors such as interactions, desolvation, residual mobility, dynamics, or local water structure, careful analysis is essential to define the reference point why a particular signature is given and how it can subsequently be optimized. Relative comparisons of congeneric ligand pairs along with access to structural information allow factorizing a thermodynamic signature into individual contributions.

7.1 Introduction

In a drug development program a lead scaffold, possibly discovered by high-throughput screening [1], virtual computer screening [2] or by a fragment-based lead approach, is optimized from milli via micro to nanomolar binding [3–5]. This optimization is performed by either "growing" the initially discovered scaffold into a binding site, or by exchanging functional groups at its basic skeleton by other, purposefully selected bioisosteric groups. These modifications are intended to increase the binding affinity of the small-molecule ligand toward the target protein and they usually result in an increase of the molecular mass of the candidate molecules to be improved.

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7.2 How to Measure and Rank "Affinity"

To quantify this optimization process, the binding of a ligand to its target protein is measured [6]. Usually the so-called binding constant is determined under the conditions of a chemical equilibrium, which is literally taken, either the dissociation constant K_d or its inverse, the association constant K_a. They indicate what portion of a ligand is bound to the protein according to the underlying law-of-mass. With enzymes usually the so-called inhibition constant K_i is determined in a kinetic enzyme assay. The turn-over of an appropriate substrate is followed concentration dependent. At low substrate concentration, it determines the dependence of the inhibitory concentration on the change in the reaction rate of the enzymatic turnover. Although K_i is not exactly defined as a dissociation constant, K_i, K_d, and K_a are usually referred to interchangeably and represent a kind of strength of the interaction between protein and ligand.

Frequently, instead of the binding constant a so-called IC_{50} value is recorded. This value is characterized by the ligand concentration at which the protein activity has decreased to half of the initial amount. In contrast to the K_i value, the IC_{50} value depends on the concentrations of the enzyme and the substrate used in the enzyme reaction. The obtained value is affected by the affinity of the substrate for the enzyme, as substrate and inhibitor compete for the same binding site. Using the Cheng-Prusoff equation IC_{50} values can be transformed into binding constants [7].

7.3 Affinity: A Thermodynamic Equilibrium Entity Composed by Enthalpy and Entropy

The binding constant can be logarithmically related under constant pressure and standard conditions to thermodynamic properties such as the Gibbs binding free energy ΔG, which itself partitions into an enthalpic and entropic binding contribution, whereby the latter is weighted by the absolute temperature at which the recorded process is determined [8, 9]. The enthalpy reflects the energetic changes during complex formation and can be linked to the interactions associated with the various steps important for the generation of the protein-ligand complex [8]. However, the changes in enthalpy are not the entire answer as to why such a complex is actually formed. In addition, it is important to consider changes in the ordering parameters. This involves how a particular amount of energy is distributed over the multiple degrees of freedom of a given molecular system. This comprises the ligand and the protein prior to complex formation, the formed protein-ligand complex and, important enough, all changes that occur with water and the various components solvated in the water environment (such as buffer compounds or ions to balance the charge inventory in the local environment). Only if this entire system transforms on the whole into a less-ordered state, which corresponds to a situation of increased entropy, a particular process such as the formation of a protein-ligand complex will

spontaneously occur. Important enough the entropic component is weighted with temperature. It matters a great deal whether the entropy of a system is changed at low temperature, where all particles are largely in an ordered state, or whether it occurs at high temperature where the disorder is already significantly enhanced. Spontaneously occurring processes are characterized by a negative value for ΔG. Energetically favorable, exothermic processes are defined by a negative enthalpy contribution. If entropy increases, a positive contribution is recorded; however, because the entropic term $T\Delta S$ is considered with a negative sign, an increase in the entropy will cause a decrease in the Gibbs free energy and therefore an increase in binding affinity. A detailed discussion of the various interactions possible to be formed between a protein and a ligand can be found in Ref. [8].

7.4 If a Complex Forms: Two Particles Merge into One

Prior to complex formation, protein and ligand are separately solvated and move freely in the bulk solvent phase. Upon complex formation the two independent particles merge into one species. By this they sacrifice their independent rotational and translational degrees of freedom as two independent particles reduce to one [10]. This loss of about 15–20 kJ/mol is associated with a price in Gibbs free energy to be afforded. This value has been nicely confirmed by a study of Nazare et al. who studied binding of two non-overlapping fragments to FXa [11] and by Borsi et al. [12] who investigated the assembly of an acethydroxamate and a benzenesulfonamide fragment as a potent MMP-12 inhibitor. Comparing the binding affinity of the two individual fragments with that of the merged supermolecule reveals a value of approximately 14–15 kJ/mol. These values match very well with the price to be paid for the loss of degrees of freedom for merging two into one particle.

7.5 How Gibbs Free Energy Factorizes into Enthalpy and Entropy

This fact also sets a lower affinity limit to be expected for complex formation. Only if the newly assembled complex experiences interactions, which will overcome this intrinsic lower barrier of about 15 kJ/mol, a complex can be observed. This finding is nicely reflected by a compilation published by Olsson et al. [13]. The authors have collected the available thermodynamic data in literature and mapped the information in a ΔH versus $-T\Delta S$ diagram (Fig. 7.1). The main diagonal in the $\Delta H/-T\Delta S$ plot corresponds to the observed data scatter in the Gibbs free energy, which covers a range from approx. -15 to -60 kJ/mol. This distribution reflects the range accessible for ligand optimization from milli- to subnano-molar affinity. The diagonal perpendicular to the ΔG distribution reflects the mutual scatter of enthalpy

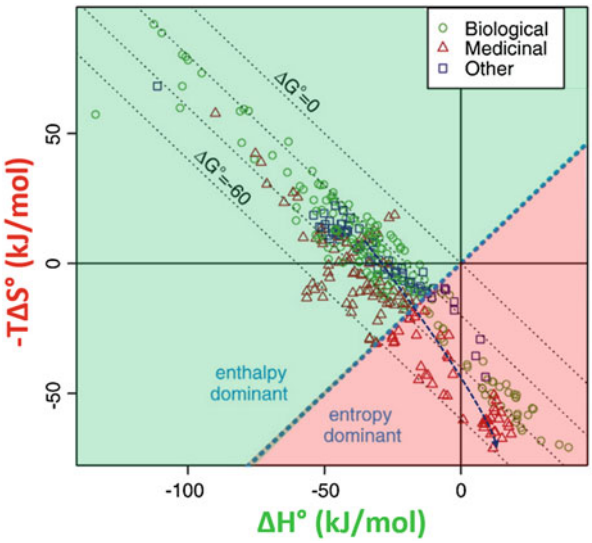

Fig. 7.1 Thermodynamic data of protein-ligand complexes measured by ITC and plotted in a ΔH versus –TΔS diagram. The change in Gibbs free energy is shown along the main diagonal (*dotted line*), perpendicular the scatter in enthalpy and entropy is indicated. In the *dark grey* area enthalpic binding, in the *light gray* area entropic binding preveals. Ligands from medicinal chemistry programs (Δ) tend towards entropically driven binding with increasing affinity (*lower right*) (The figure was adapted from Ref. [13])

and entropy with opposing contributions to ΔG. As this distribution spreads over a very large range, it discloses an intrinsic enthalpy/entropy compensation that must be in operation, leading to the rather small scatter in ΔG.

The space covered in the enthalpy/entropy diagram can be split into an area where enthalpic binding contributions prevail (dark gray) and an opposing one where entropic contributions (light gray) dominate. It is remarkable to note that ligands originating from medicinal chemistry optimization tend toward enhanced entropic binding profile with growing potency. This immediately calls for the question whether a more enthalpically or entropically driven binding is desired [14–19] and whether such a binding profile of a ligand to be developed can be designed at will [20]? The immanent enthalpy/entropy compensation already suggests that both properties are interdependent, but can they be optimized independently? Most efficient ΔG optimization could be achieved if ΔH and –TΔS could be enhanced simultaneously; however, is such a strategy achievable without getting stuck in an enthalpy/entropy compensation trap? Even though there is no physical law, which argues for mutual enthalpy/entropy compensation many considerations on the molecular level suggest that the two opponents will at least partially cancel out [21]. However, strong enthalpic interactions will fix a ligand at the binding site, which is entropically unfavorable. In contrast, pronounced residual mobility in the bound state is entropically beneficial, as a smaller amount of degrees of freedom is lost upon complex formation. Nonetheless, the quality of the formed interactions will be less efficient leading to a minor enthalpic contribution.

7.6 What Profile Is Required: Enthalpy Versus Entropy Driven Binding

This suggests that it is obviously difficult to optimize both properties independently and the tailored design of a predominantly enthalpic or entropic binder represents a major challenge. Notwithstanding, different targets require ligands with different thermodynamic profiles. A CNS drug needs different properties compared to a drug addressing an extracellular target, e.g. in the blood stream. High target selectivity can be of utmost importance, to avoid undesirable side effects, in contrast, promiscuous binding to several members of a protein family can be essential to completely down-regulate a particular biochemical pathway, e.g. in case of kinases, or to achieve a well-balanced binding profile at a given GPCR. In case of viral or bacterial targets, rapid mutational changes can create resistance against a potent ligand. The strategies followed by the pathogens span from steric mismatch in the active site to changes in the protein dynamics to diminish affinity of a bound active agent [22, 23]. As the molecular foundations of these mechanisms are quite distinct well-tailored thermodynamic signatures are required to escape resistance. Freire et al. have suggested improved susceptibility to resistance mutations for ligands optimized enthalpically as they still exhibits sufficient flexibility to evade geometrical modifications of the target protein upon mutational variations [19, 24]. However, equally well ligands binding with entropic advantage due to high residual mobility allowing for multiple binding modes might provide some benefit to escape resistance development. This has remarkably been demonstrated by the superior resistance susceptibility of dapivirine or etravirine over other compounds inhibiting HIV reverse transcriptase [25]. The two inhibitors are characterized by the ability to reorient into alternative binding modes. A firm mapping of the optimal thermodynamic profile to the requirement of a given target is yet not evident and subject to current research.

Drug development based on rational concepts requires detailed understanding of the interactions of a small molecule drug with its target protein. Therefore, increasingly structural and thermodynamic properties of ligand-protein binding in terms of enthalpy/entropy profiles are correlated [26]. It has been proposed to use such profiles to support the decision making process which ligands to take as lead candidates to the next level of development [14–20]. From a theoretical point of view it appears promising and advisable to focus on the most enthalpic binders, as optimization steps governed by entropic factors will be followed unavoidably during late stage optimization. However, at this stage the reasons for a resulting thermodynamic binding signature must be fully characterized to correctly assign ‘largest enthalpic efficiency’ to a prospective lead.

7.7 Isothermal Titration Calorimetry: Access to Thermodynamic Data

The method of choice to record thermodynamic data is isothermal titration calorimetry (ITC). It provides direct access to ΔG and ΔH in one single experiment, $T\Delta S$ is calculated from their numerical difference. Any error or deficiency in the measurement of these two properties will cause an inevitable $\Delta H/T\Delta S$ compensation, apart from the heavily discussed intrinsic enthalpy/entropy compensation in biological systems (s. above). Not to get trapped in an error-prone compensation, thorough analysis and correction of superimposed effects of ITC data has to be performed and it is highly advisable to only correlate matching ligand pair series relative to each other.

7.8 Contributions to the Thermodynamic Profile: H-bonds and Lipophilic Contacts

Hydrogen bonding usually relates to an enthalpic signal which increases once growing charges of the interacting functional groups are involved [27–29]. However, with larger charges also a detrimental entropic contribution is experienced which reduces, due to enthalpy/entropy compensation, the overall free energy contribution of an H-bond. Lipophilic contacts buried upon complex formation result in an increasing entropic signal, but only, if ordered water molecules are displaced from the binding pocket [29–31]. Mobile water molecules displaced upon ligand binding can also give rise to a more enthalpy-driven binding [32, 33]. If no permanent and strong charges of the interacting species are involved, the release or pick-up of water molecules upon ligand binding seems to be virtually balanced out in the Gibbs free energy inventory, but huge effects are experienced with respect to the enthalpy/entropy partitioning [31, 34]. This observation demonstrates that the sole determination of free energy will hardly unravel involvement of water molecules in binding. This also explains why surprisingly many computer modeling approaches can still generate reasonable ΔG predictions neglecting water, but geometries will be predicted incorrectly.

7.9 Preorganization and Rigidization of Ligands, Cooperative Effects

Ligand pre-organization and rigidization of the protein-bound conformation can result in large beneficial free energy contributions, mainly due to an entropic advantage. These generalized signatures often become only transparent once a congeneric series of ligands is evaluated as the overall thermodynamic profile of

the binding process can be superimposed by multiple effects arising from changes in the dynamics of either protein and/or ligand, rearrangements of the protein and most important by changes of the solvation pattern of discrete water molecules. Furthermore, puzzling cooperativity between hydrogen bonding and hydrophobic contacts can be given resulting from changes in the dynamics of protein-ligand complexes and modulations of residual solvation pattern [35–37].

7.10 The Role of Water in Ligand Binding and Thermodynamics

Remarkable effects arise from rearrangements of surface water molecules wrapping around newly formed protein-ligand complexes [37–39]. Water networks span across the newly created complex surfaces and exhibit geometric and energetic fits of deviating quality. Ideal fit results in an affinity enhancement of the bound ligand; imperfect and fragmented water networks reduce affinity of the bound ligand. Moreover, such changes are reflected by major modulations of the enthalpy/entropy signature and easily provoke a mutual ΔH vs. $T\Delta S$ shift of ± 5–10 kJ/mol. If the residual solvation pattern takes such an enormous impact on the thermodynamic signature, classification of a given ligand as "more enthalpic" or "more entropic" binder appears rather meaningless without full information about the structural properties of the formed complex e.g. by means of high-resolution crystal structure analysis. Only then the thermodynamic profile can support the decision making process which ligand to take to the next level of development. Nonetheless, deviating thermodynamic profiles recorded across congeneric ligand series unambiguously indicate differences in the binding patterns, be it for deviations in binding poses, residual solvation patterns or intrinsic dynamics.

ITC measurements can also help to record whether a change in protonation state occurs when a ligand binds to a protein. Therefore the thermodynamic parameters have to be measured from different buffer conditions. The obtained results can be used to drive the tailored design of pK_a properties of ligands [40]. If in a congeneric ligand series thermodynamic data show an unexpected shift between enthalpy and entropy even though the Gibbs free energy of binding remains virtually unchanged among the different ligands, usually a remarkable effect or change of the system is superimposed to the binding event. Clearly such effects cannot be seen considering solely affinity data. Since the involvement of water molecules in the binding interface takes mostly minor impact of the free energy but huge effects are seen in the enthalpy/entropy inventory, thermodynamic data can uncover the importance of water on ligand binding. For the same reasons the influence of water often foils a straight forward comparison of thermodynamic signatures across ligand series without having access to structural information in parallel, as the entrapping or release of a single water molecule can easily invert the thermodynamic profile. Through thermodynamic data impressive cooperative effects resulting either from

deviating dynamic behaviour of the formed complexes [35, 36] or changes in the surface water structure became evident [37–39]. These effects became only obvious by carefully analyzing the deviating trends in the thermodynamic profiles of the formed complexes. Finally, the partitioning of the Gibbs free energy of binding in enthalpy and entropy can help to understand flat structure-activity relationships and distinguish ligand binding to deviating conformations of the target protein, an effect not to be unravelled purely considering affinity data [41].

Even though we can establish some general rules how to fight enthalpy/entropy compensation and substantiate the reasoning why to start with leads of "high enthalpic efficiency", the overall binding event shows many additional phenomena giving rise to an undesired compensation. It remains in question whether they can always be fully elucidated and avoided. But they provide an explanation why it is still not trivial and straight forward possible to factorize a thermodynamic signature into individual contributions that can be attributed to single interactions formed between a lead candidate and its target protein.

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