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Isothermal titration calorimetry: application to structure-based drug design

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Abstract

The road to market for drug compounds is a treacherous one, generally involving a huge temporal and financial investment. The role of structure-based drug design or lead optimisation ranges wildly in importance in different pharmaceutical companies. The adoption of these aids to provide routes to high affinity ligands has not received widespread acceptance. This is based on a number of factors, from the perceived failings of such methods, to the belief that rapid screening of compound libraries alone is the most effective way to discover drugs.

The panacea of being able to take a computer generated representation of the structure of a target site of a given biomolecule and rationally design an high affinity inhibiting compound has proved seemingly unreachable for three major reasons: (1) current capabilities in computing; (2) the requirement for atomic resolution structural detail; and (3) determination of how structural features can be related to the thermodynamics of interactions. It is the last of these points that this review seeks to address. In particular the use of isothermal titration calorimetry is discussed in the light of the accumulation of accurate thermodynamic data and examples are given where this has been applied to understand the structural aspects of formation of drug–biomolecular complexes.

Keywords: Thermodynamics; Drug–receptor interactions; Drug–protein; Drug–DNA

1. Introduction

The target site of an enzyme can be characterised at very high resolution from X-ray crystal structural analysis. Using this structural detail, computer representations of compounds of suitable size, and complementarity of charge, polarity and hydrophobicity can then be inserted into the target site. Using a range of docking programs or molecular dynamics simulations, usually based on theoretical thermodynamic values, a term representing the affinity of the modelled interaction can be calculated. If this compound has a calculated affinity higher than other inhibitors or the substrate itself, this virtual molecule can be synthesized and the drug can begin the voyage through clinical trials to market. If, on the other hand, this affinity is not optimal, changes in functional groups on the virtual molecule template can be effected *in silico* and the process re-iterated until the required affinity is achieved. As simple and effortless as this process appears, many compounds so derived, when synthesized and tested have very different affinities to those predicted from the computer. The failing of this process can, at least partially, be blamed on our lack of understanding of how structural detail is related to the thermodynamics of an interaction. More specifically, this relates to our ignorance of what are the respective contributions from the changes in enthalpy (ΔH) and entropy (ΔS) to the change in free energy

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(ΔG) of an interaction. Typically this form of drug design is based only on the prediction of the ΔG of an interaction. This is generally assimilated by the addition of the non-covalent bond terms derived from theoretical enthalpy. The entropic terms, particularly those associated with solvent contribution, are less well characterised, and as such are frequently ignored.

To rectify this situation, being able to understand the correlation between structural detail and thermodynamic measurement of an interaction is of central importance. This requires the accurate measurement of thermodynamic changes on interaction. Isothermal titration calormetry (ITC) provides a method for the measurement of the ΔH of an interaction at constant temperature and pressure (for reviews of the method see, for example [1–6]). Since calorimetric methods directly measure the ΔH of an interaction at a specified temperature, they avoid the necessary indirect calculation of this term used in other methods (e.g. spectroscopic techniques) which is inherently less accurate. In ITC, the enthalpy of the interaction is measured as one component of an interaction is titrated into the other. The concentration regime of the interacting components is usually set up to ensure that the binding sites of the moiety in the calorimeter cell are saturated. The heat change of interaction (exothermic or endothermic), therefore, is a probe of the amount of ligand bound at each injection. Therefore, from the ratio of bound and free interactants, the binding constant ($K_{B,obs}$) can be determined [7]. Having established the $K_{B,obs}$ at a given experimental temperature (T), the free energy can be simply determined as

$$\Delta G_{obs} = -RT \ln K_{B,obs} \quad (1)$$

Furthermore, knowing the ΔG_{obs} and the ΔH_{obs}, the change in entropy can be calculated as

$$\Delta S_{obs} = \frac{(\Delta H_{obs} - \Delta G_{obs})}{T} \quad (2)$$

Thus, in one experiment, a full thermodynamic characterisation of the interaction at one temperature can be obtained.

In the ITC experiment, the heat of interaction measured includes contributions from all the equilibria that occur on the interacting components going from the free to bound state. Thus, the thermodynamic terms are considered as 'observed' to signify that they are derived from experiment and the subscript 'obs' is inserted. If the enthalpy is measured at a range of temperatures, then the change in constant pressure heat capacity ($\Delta C_{p,obs}$) can be determined based on the following equation:

$$\Delta C_{p,obs} = \frac{\Delta H_{T_1,obs} - \Delta H_{T_2,obs}}{T_2 - T_1} \quad (3)$$

where T_1 and T_2 are two different experimental temperatures. The change in heat capacity is affected by several important contributions which characterise binding (see Section 3).

2. Enthalpy and entropy data and drug design

The determination of the thermodynamic parameters for the formation of a complex provides an invaluable characterisation of the interaction. The enthalpic term gives a direct readout of the total contribution from the formation, or removal, of non-covalent bonds in the system. Determination of the entropic term from the ITC data (see Eq. (2)) gives quantitative value for the change in order of the system. This incorporates effect of changes in conformation (i.e. a tightening, or loosening, including folding or unfolding of the structure) as well as effects resulting from the binding or release of solvent atoms/molecules to or from the interacting macromolecules. Thus, determination of these terms for compounds displaying systematic, subtle changes can give a clearer understanding of the important attributes of binding.

The interaction of potential inhibitors to the binding of the SH2 domain from the protein Src has been examined using ITC and high resolution crystal structures [8]. SH2 domains bind to ligands containing phosphorylated tyrosine residues. As they play a role in intracellular signal transduction pathways, SH2 domains feature in many disease states where the pathways become aberrant. Studies using short tyrosyl phosphopeptide sequences suggested that these could mimic physiological ligands, and furthermore be used to inhibit interactions. Specificity for individual SH2 interactions generally appeared to be derived from the recognition of residues immediately proximal and C-terminal to the phosphotyrosine residue by the domain [9]. Several pharmaceutical companies embarked on attempting to discover drugs based on the interactions

observed in peptide studies. The thermodynamic data revealed several key features of the binding of potential novel inhibitors. For example, the modification of the phosphotyrosine residue to a non-hydrolysable, phosphotase resistant analogue was important for efficacy of any drug compound. The binding of a peptidomimetic drug compound with the phosphotyrosine substituted by a benzylmalonate moiety showed a significantly weaker affinity ($K_B = 8.5 \times 10^4\ M^{-1}$) compared to a tyrosyl phosphopeptide standard ($K_B = 2.5 \times 10^6\ M^{-1}$) [8]. This reduction in affinity was reflected in a significant reduction in entropy, which could be partially attributed to the restriction of the degrees of freedom of the additional bonds and comparative change of the hydration state of the benzylmalonate group. Structural determination also revealed that a further entropic cost was paid by the malonate group which was responsible for restricting the motion of a loop of the SH2 domain. The additional bonds resulting between the potential drug and the domain could, at least in part, account for the additional observed favourable change in enthalpy for the interaction. This enthalpic contribution was of insufficient size to compensate for the additional entropic cost. This route to novel inhibitors could, therefore, be disregarded.

Triplex-forming oligonucleotides (TFOs) are of major interest as therapeutic agents particularly as part of a DNA duplex-targeted antigene strategy. TFOs are designed to control the expression of regulatory genes by inhibition of transcription or binding of regulatory proteins. The TFO consists of a short sequence of DNA capable of sequence-selective recognition and hybridisation to a target double-stranded DNA site forming a triplex structure. Triplex instability under physiological conditions is a major limiting factor in the therapeutic use of the TFO strategy. One approach to enhance stability of these structures, and hence, improve efficacy, is to use adjuncts that show preferential binding to triplex DNA. ITC binding studies of two disubstituted anthracene-9,10-diones revealed that one had a defined preference to bind triplex DNA [10]. The binding of 2,6-bis[3-9dimethylamino)propionamido]-anthracene-9,10-dione was largely due to the enthalpic contribution from the apolar interactions derived from intercalation into the triplex DNA. The affinity was weak ($K_B = 2 \times 10^5\ M^{-1}$) leaving scope for structure-based design approach which could incorporate additional interactions outside the aromatic interface of the intercalating groups to give a route to an improved therapeutic.

3. Change in heat capacity reveals the nature of the complex interface

Establishing a direct link between structural detail and thermodynamic data has been a goal of a significant body of work over the last decade. With respect to the rational design of pharmaceuticals this would enable the prediction of binding data based purely on information from high resolution structural determination, thus, circumventing the temporally and financially expensive process of synthesising molecules, and performing binding studies. Based initially on calorimetrically determined data from heats of transfer of organic compounds to aqueous solvents and studies on protein folding/unfolding equilibria, a clear correlation was observed between the burial of biomolecular surface area and the ΔC_p [11,12]. Water molecules on a hydrophobic surface become ordered and their non-covalent bonds have energetically different vibrational modes resulting in a different heat capacity to water in the bulk solvent. On forming a macromolecular complex, the resulting burial of hydrophobic surface means that this order is removed from the system and there is a net change in heat capacity. The largest contribution to ΔC_p results from this burial of hydrophobic surface area, however, the correlation has been fine-tuned to include the effects of polar surfaces ([13,14]). The accurate determination of the ΔC_p using ITC is obviously of fundamental importance in exploring these correlations.

ITC studies on the interaction of the immunosuppressive agents FK506 and rapamycin with the peptidylproline *cis–trans* isomerase known as FK506 binding protein (FKBP-12) provided the one of the earliest attempts to use the correlation of thermodynamic data with structure to enhance a drug design programme [14]. These interactions involve the burial of significant hydrophobic surface area on forming the complexes. ΔC_p determinations for the individual interactions enabled some understanding of the potential contributions from aromatic and non-aromatic non-polar groups.

In a study involving ITC measurement, the correlation with ΔC_p was adopted to determine the likely surface area buried on the formation of a complex between the fibroblast growth factor and its cognate receptor. From this, the structure of the complex could be inferred and hence, the potential to develop inhibitors for this interaction was evoked [15].

DNA as a potential target for therapies has become increasingly important in recent years. Indeed, several anti-cancer and antibiotic compounds are aimed at interfering with the template function of DNA and either blocking gene transcription or inhibiting DNA replication. It is now clear that there is a huge potential for the development of sequence-specific drugs with high affinity [16–18]. The rational design of new structure- or sequence-selective and specific DNA binding agents is now receiving a great deal of attention. The application of calorimetric measurement to attempt to understand the thermodynamic basis of interaction has been successful and the information gleaned is being used to improve the efficacy of drug compounds.

Most DNA-binding drug compounds can be divided into two distinct modes of interaction: intercalators and minor groove binding. Complexes involving intercalating compounds are stabilised by van der Waals interactions between the bases of the oligonucleotide and the usually aromatic, hydrophobic region of the drug. The minor groove binders are almost exclusively found to favour A–T sequences of the DNA and interact via specific hydrogen bonds to the C2 carbonyl oxygen of the thymine and the N3 nitrogen of the adenine. These minor groove binders are usually based on compounds with aromatic groups linked in such a way as to allow significant torsional freedom (see below).

The ΔH and ΔC_p for the binding of a series of DNA intercalators including daunorubicin and adriamycin to oligonucleotides have been determined using ITC [19]. The measured ΔC_p values were found to correlate well with changes in buried surface area calculated from high resolution structural detail. A refinement of the correlation between ΔC_p and surface area burial was proposed based on the data for the DNA binding compounds. The ITC experiment was modified from the typical binding experiment where the concentration regime is set up so as to result in saturation of the interacting compounds. In this case, since only the ΔH at a given temperature was required, the experiment was set up with a large excess of DNA in the calorimeter cell such that on each injection the drug was completely bound. Thus, after integration of the raw data and subtraction of heats of dilution, the experiment provided a large number of independent determinations of the molar enthalpy from which a statistically relevant mean value could be obtained.

In a clever use of ITC, the negative cooperativity in the binding of inosine monophosphate (IMP) to IMP-dehydrogenase was established [20]. Furthermore, it was shown that the binding of the inhibitor, mycophenolic acid was critically dependent on the presence of the IMP. The large ΔC_p value measured exhibited an unusual temperature dependence. This provided the basis for the existence of two forms of the apoenzyme. Furthermore, the requirement of the presence of the IMP for inhibitor action led to the proposal of a model incorporating subunit–subunit interactions via allosteric change. This provided a starting point for the development of a nested allosteric model and ultimate routes to ''allosteric-effector'' inhibitors

The correlation of ΔC_p and surface area burial on complex formation is subject to some caveats. Since the original correlation was based on the data from heats of transfer of organic compounds and protein folding/unfolding equilibria, discrepancies arise in the correlation when effects, which are not accounted for in these empirical data sets, appear. For example, the incorporation of chemical moieties that are not present in original data set into predictions (e.g. phosphate groups, furan derivatives) could be expected to give aberrancies. This makes any application in drug design somewhat limited without further optimisation. In addition, the incorporation of water molecules in a complex interface appears to result in significant discrepancies [21,22]. Thus, the correlation between ΔC_p and the burial of either hydrophobic, or hydrophilic surface area has to be considered as simplistic at best.

4. Structural parameterisation of the free energy for an interaction

The observed thermodynamic term, free energy, can be separated into several component terms as

described for example by the following equation:

$$\Delta G_{obs} = \Delta G_{int} + \Delta G_{conf} + \Delta GE_{hyd} + \Delta G_{pe} + \Delta G_{r+t} \quad (4)$$

where ΔG_{int} is the contribution from intermolecular non-covalent bonds/interactions; ΔG_{conf} the from conformational transitions in the DNA and the ligand; ΔG_{hyd} represents the contribution from solvent effects associated with the removal of hydrophobic surface area from being exposed to aqueous solvent to burial in the complex binding site; ΔG_{pe} is the polyelectrolyte contribution; and ΔG_{r+t} is the unfavourable contribution from the loss of rotational and translational degrees of freedom resulting from the formation of a bimolecular complex.

Thus, parsing the various cumulative contributions to binding provides a full characterisation of the individual components which give rise to the interaction. Some of these will be favourable whilst other can be unfavourable. In rational drug design, the individual thermodynamic terms can be related to the observed structure of the drug and the drug-macromolecule complex revealing, which parts of the drug molecule are important to high affinity, and which can be redesigned. Therefore, potential drug compounds can be improved by making subtle structural changes to the drug and parsing the free energy terms to determine whether these changes have produced the desired effect.

This parsing of the free energy of an interaction has been successfully applied to several compounds aimed at specific sites on DNA. The thermodynamic parameters for the interaction of the compound Hoechst 33258 binding to and extended AT-tract DNA duplex, $d(CGCAAATTTGCG)_2$ have been determined. Parsing of the free energy terms for this interaction provides a clear picture of the contributions to binding [23,24]. The change in heat capacity (ΔC_p) for the interaction was derived from the temperature dependence of the ΔH_{obs} measured using ITC, and the value obtained correlated with the value predicted from surface area burial derived from structure calculations. It was shown, using the empirical relationship, $\Delta G_{hyd} = (80 \pm 10)\Delta C_{p,obs}$ [25] that the majority of the favourable contribution to ΔG_{obs} was derived from the hydrophobic surface area transfer on the Hoechst compound binding in the minor groove of the DNA. Design of molecules based on this precursor will be profoundly affected by changing the nature, and area, of the hydrophobic surface. This is an interesting example of an exercise in potential drug design since the affinity of this interaction is not only dictated by individual non-covalent bonds between interacting molecules, but rather by the entropic gain from the burial of hydrophobic surface area and the resulting liberation of water molecules into bulk solvent.

Currently, the most successful attempts at structural parameterisation for binding incorporate statistical analysis of binding data combined with rigorous statistical mechanical evaluation of those functions that can not be measured experimentally (e.g. conformational entropy). This approach has been demonstrated to be able to produce highly accurate predictions of the thermodynamics of binding on model systems [26]. This was demonstrated on the interaction of peptide inhibitors with aspartic proteinases [27]. The interaction of aspartic proteinase endopepstatin with the high affinity inhibitor pepstatin A was investigated. The calorimetrically determined enthalpic and entropic contributions to the overall free energy of binding were dissected and structure-based thermodynamic calculations allowed the estimation of the contributions from each individual residue.

5. The role of water molecules in drug interactions

Structural detail has revealed the importance of the incorporation of water molecules in biomolecular interfaces. The liberation of water molecules from a biomolecular interface is generally considered to provide a favourable contribution to the free energy of binding based on the increase in the overall degrees of freedom of the system on going from free to bound state. This has led to the general disregard of water molecules in drug design. In fact, most computer-assisted drug developments disregard water and begin the design process on a dehydrated target binding site. In some cases, the incorporation of water molecules into biomolecular interfaces have been hypothesised to provide a favourable contribution to binding [28–31]. This can be reconciled based on the concept that, although an entropic cost accompanies the ‘pinning down’ of a water molecule in the complex interface,

this can be overcome by the enthalpic contribution resulting from hydrogen bonds that the water molecule makes with the interacting macromolecules. This requires that the water molecule is bound in a site where it is able to make optimal hydrogen bonds. The role of water molecules can not, therefore, be ignored in rational drug design [29].

The role of water molecules in drug–protein interactions has been investigated using data derived form ITC. ITC was used in a study which included the determination of the contribution from a hydrogen bond to water molecules in the binding site on formation of the complex between FK506 (and rapamycin) and FKBP-12. This involved ITC experiments to obtain thermodynamic data in H_2O- and D_2O-based solvents [32].

The interaction of the novobiocin to an antibiotic resistant mutant of DNA gyrase was studied using ITC [33]. The loss of the guanidinium group resulting from the substitution of an arginine for a histidine residue in the mutant protein resulted in the incorporation of an ordered water molecule (as seen in the X-ray crystal structure). This is reflected, at least in part, by a more unfavourable ΔS contribution to the binding free energy. Interestingly, the mutation and the concomitant incorporation of the water molecule led to a significant increase in affinity, supporting the idea of 'optimal' water molecules previously proposed [29,31].

There is no doubt that some of the of water molecules observed in biomolecular interfaces play a role of stabilising the interaction. Often, scanning the complex interface reveals that these water molecules are in sights of low potential energy and high electrostatic complementarity. As a result, it is better to design a drug compound to incorporate, and benefit from these water molecules. In view of the importance of water molecules in binding interfaces thermodynamic measurement of the effects of their positioning would seem to be a requisite of any drug design strategy [29].

6. Conclusions

It is clear that the potential of a structure-based approach to drug development is still only in its infancy with respect to its general acceptance and widespread use by the pharmaceutical industry. It is also clear that one factor that is potentially retarding its efficacy in the process of screening for novel drug compounds is the lack useful data which can be used to optimise and perfect the programs developed to correlate thermodynamic data with structural detail. The development of ITC methodology means that these data can be obtained. Highly accurate determination of enthalpy and full charcterisation of the thermodynamic parameters of an interaction are now possible using ITC. The application of these data to the development of drug compounds provides a qualitative readout of the thermodynamic effects of altering lead compound structures, providing a language that can link the medicinal chemist with the molecular modeller. Thus, the determination of ITC data has to be a fundamental step in the drug design process (Fig. 1). As more information is accrued the process will become more honed and the success rate in structure-based drug design and/or optimisation will increase dramatically.

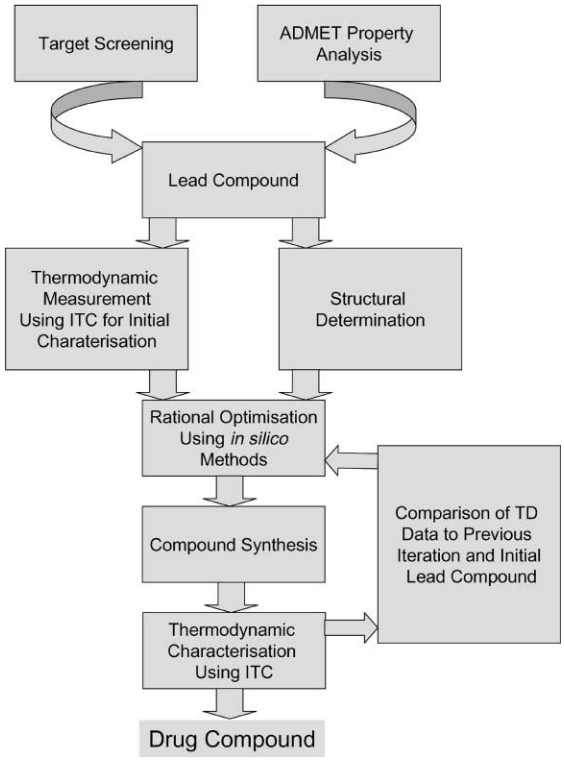

Fig. 1.

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