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A Calorimetric Approach with Structure-Based Thermodynamics for Molecular Interactions

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Calorimetry is one of the most powerful and convenient approaches for researching reactions accompanied by heat. Hence, researchers such as protein science, pharmaceutical science, and polymer science have been effectively applying calorimetric methods. We described the practical applications of calorimetric techniques to molecular interactions between proteins and small molecules such as drug candidates in terms of energetics. Given that no unified comprehensive summary of the structural thermodynamics important in understanding and designing molecular interactions and drug candidates is available, we first review structural thermodynamics based on calorimetric results in a concise way. The basis of advanced applications of empirical relations to binding reactions is described following a general and simple means of using isothermal titration calorimetry (ITC) data. We further describe our recent applications of ITC results and structural thermodynamics to protein-protein interactions. Second, the general energetic nature of molecular binding events is explained by using databases containing a number of case studies obtained by ITC and statistical analysis. Based on these trends and experimental efforts manipulating entropy and enthalpy, thermodynamic optimization for selecting and designing drug candidates has been improved. Well-rounded exploitation of calorimetry with structural thermodynamics is key to increasing the current understanding of molecular interactions.

Keywords: binding energetics, calorimetry, drug discovery, intermolecular interactions, structural thermodynamics

1. Introduction

Organic molecules represented by biopolymers such as polypeptides or nucleic acids keep and alter their physical and chemical integrity under the control of energetics and kinetics. Upon changes in environmental conditions, molecules are reorganized toward more energetically favorable states of the system. Outer stress for a molecule of interest includes a change in temperatures or pH and the appearance of binding partners. It is inevitable that these intramolecular and intermolecular interactions should be followed by the trading of heat, a cardinal extensive property, in a closed system.[1-4] Therefore, understanding heat flow at constant pressure or volume is essential in many scientific fields. Indeed, the use of calorimetric methods such as isothermal titration calorimetry (ITC) and differential scanning calorimetry (DSC) is widespread.

It is of great interest to investigate biological processes obtained by evolutionary pressure in vitro using heat as a probe and thermodynamics as an analytic tool. However, as the observed absolute amounts of heat from biological molecular interactions are generally not great, large amounts of samples were needed for calorimetric experiments. Fortunately, it has become possible to detect very small heat changes following recent developments in hardware. A good example is microcalorimetry such as VP-ITC (MicroCal), ITC_{200} (MicroCal), and VP-DSC (MicroCal). The microcalorimetry-based researches on intra- and inter-molecular interactions such as protein (un) folding, protein misfolding, and protein-ligand associations have been rapidly increasing and providing insightful thermodynamic outcomes for learning the properties of molecular interactions.[1-4] Such data and experiences have in turn produced a number of empirical relationships between

thermodynamic parameters and structural sources, so-called structural thermodynamics.[5-7] Furthermore, these cumulative results were also summarized by constructing databases.[4,8-11] The proper use of structural thermodynamics with molecular interaction databases and microcalorimetry provides an unique opportunity to predict and design molecular interactions in protein science and pharmaceutical science.

Here, we focus on ITC-based calorimetric studies for molecular interactions and survey ways of analysis and applications of experimental results with a thermodynamic parameter, mainly the changes in free energy based on the affinity constant, enthalpy, entropy, and heat capacity as well as a structural parameter, accessible surface area. Thermodynamic optimization of molecular interactions for drug candidates using modulating of enthalpy and entropy with databases is also addressed.

2. Typical analysis and structural thermodynamics -based advanced analysis of ITC data

2.1 Widely-used forms of ITC

ITC is convenient and beneficial for obtaining much information on molecular interactions, and observes directly all heat related to binding events. Modern calorimeters are sensitive enough to probe even weak biological interactions making ITC a popular choice.

A typical experiment involves the addition of one binding partner (titrant), usually a small molecule, in a syringe to another binding partner (titrate), usually a macromolecule, in the cell over time using one or more injections. Heat is measured either as a change in temperature or as a change in power required to maintain temperature between the sample and reference cells depending on the type of instrument being used. This energy is then converted into a molar binding enthalpy based on the known cell volume and the concentrations of the reactants. The heat generated (exothermic) or absorbed (endothermic) during each injection is proportional to the amount of complex formed. Thus, one expects maximal enthalpies at the earliest points in a titration with a decrease in intensity as the free titrate is consumed.

By fitting an ITC thermogram to a theoretical equation, the affinity constants (the association and dissociation constants), the binding stoichiometry (n), and the binding mode are obtained. The enthalpy change (ΔH) is calculated from ITC peaks. Using ΔH and the affinity constant (K), the Gibbs free energy change (ΔG) and the entropy change (ΔS) are obtained without relying on van't Hoff eqution as follows:[1,3]

$$\Delta G = -RT\ln K \quad (1)$$

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

where R and T are the gas constant and the absolute temperature, respectively.

The most simple usage of ITC is to judge whether molecular interactions occur or not by simply observing thermograms. Often, interactions not detected by optical methods can be discovered by ITC. ITC directly measures binding constants at, 10^2 to 10^9 M^{-1}, and using competitive binding techniques, at 10^9 to 10^{12} M^{-1}. Additionally, ΔH and ΔS values can be directly used for simple discussions of driving force.[1,3]

However, mechanical stirring in the reactor can cause protein destabilization and aggregation. These in turn hamper the incorporation of an exact concentration into the fitting procedure and make noise. Furthermore, to extract heat from only intermolecular binding reactions, other factors such as (de) protonation and conformational changes of molecules should be investigated (see **2.2.2** for further information).[3]

2.2 Advanced application of ITC results with structural thermodynamics

2.2.1 Enthalpy changes and heat capacity changes with changes in the accessible surface area

Based on protein (un) folding and binding data, the groups of Makhatadze and Privalov, Spolar and Record, Freire, and Robertson and Murphy suggested useful empirical relationships for structural thermodynamics.[3,5-7,9,12,13] They established the insightful structural thermodynamic parameters, ΔH, (partial molar) heat capacity changes (ΔC_p), and ΔS, in relation to the accessible surface area (ASA).

$$\Delta H\ (60)_{\mathrm{Freire}} = 31.4\Delta ASA_{\mathrm{pol}} - 8.44\Delta ASA_{\mathrm{apol}} \quad (3)$$

$$\Delta H\ (60)_{\mathrm{RoMur}} = 20.54\Delta ASA_{\mathrm{pol}} - 1.91\Delta ASA_{\mathrm{apol}} \quad (4)$$

ΔH (60) represents the enthalpy change at 60 ℃ in kJ K^{-1} mol^{-1}. $\Delta ASA_{\mathrm{pol}}$ and $\Delta ASA_{\mathrm{apol}}$ indicate the changes

in the solvent accessible polar and apolar surface area in Å^2, respectively. The equations for ΔH $(60)_{\text{Freire}}$ and ΔH $(60)_{\text{RoMur}}$ were suggested by the group of Freire[6] as well as Robertson and Murphy[7], respectively. The different scaling factors may be ascribed to the distinct model systems used.

ΔH_{bind} values at each temperature were plotted against temperature to deduce ΔC_{p} with $\Delta C_{\text{p}} = \partial \Delta H / \partial T$ (5). ΔC_{p} is closely related to surface burial and dehydration upon folding and binding. Five empirical equations (6)-(10) were proposed by Makhatadze and Privalov,[1] Murphy and Freire,[6] Spolar and coworkers,[9] and Myers and coworkers,[11] and Robertson and Murphy,[7] respectively.

$$\Delta C_{\text{p MakPri}} = 0.88\Delta ASA_{\text{pol}} - 2.14\Delta ASA_{\text{apol}} \quad (6)$$

$$\Delta C_{\text{p MurFre}} = 1.09\Delta ASA_{\text{pol}} - 1.88\Delta ASA_{\text{apol}} \quad (7)$$

$$\Delta C_{\text{p SpoRec}} = 0.59\Delta ASA_{\text{pol}}\text{l} - 1.34\Delta ASA_{\text{apol}} \quad (8)$$

$$\Delta C_{\text{p Myers}} = 0.38\Delta ASA_{\text{pol}} - 1.17\Delta ASA_{\text{apol}} \quad (9)$$

$$\Delta C_{\text{p RobMur}} = 0.66\Delta ASA_{\text{pol}}\text{l} + 0.12\Delta ASA_{\text{apol}} \quad (10)$$

ΔC_{p} is expressed as $\text{J K}^{-1}\text{mol}^{-1}$. Opposing signs of scaling factors between ΔASA_{pol} and ΔASA_{apol} represent well the nature of (de) solvation of water molecules (from) to hydrophilic and hydrophobic surfaces except for the equation.[10].

2.2.2 Additional enthalpic contributions

The observed enthalpy changes include not only heat of binding between molecules, but also additional sources of heat such as solvent effects, molecular reorganization and conformational changes, heats of dilution, and purely mechanical artifacts of sample stirring.

The effect of (de) protonation is often overlooked in spite of the accessibility of measurements. Therefore, ITC measurements should be repeated in buffers of distinct ionization enthalpies. In terms of the following relation (11), (de) protonation can be confirmed.[3]

$$^{\text{obs}}\Delta H_{\text{bind}} = {}^{\text{total}}\Delta H_{\text{bind}} + n_{\text{proton}}\ \Delta H_{\text{buffer}} \quad (11)$$

where $^{\text{obs}}\Delta H_{\text{bind}}$ is the observed apparent enthalpy changes obtained from ITC, and $^{\text{total}}\Delta H_{\text{bind}}$ is the enthalpy changes except (de) protonation. ΔH_{buffer} represents heat of ionization of the buffer, and n_{proton} is the number of protons released or taken up from the buffer. The positive and negative slopes, a sign of ΔH_{buffer}, mean protonation and deprotonation, respectively.

As we described, conformational changes may be further involved. Consequently, the equation (11) is divided as follows:

$$^{\text{total}}\Delta H_{\text{bind}} = {}^{*}\Delta H_{\text{bind}} + \Delta H_{\text{conf}} \quad (12)$$

where $^{*}\Delta H_{\text{bind}}$ is intrinsic binding heat which comes purely from interactions between the molecules, and ΔH_{conf} indicates the enthalpic contribution arising from conformational changes. It is difficult to parameterize ΔH_{conf} in terms of ΔASA. Freire and coworkers obtained ΔH_{conf} by using a nonlinear least squares analysis.[14] On the other hand, Privalov and coworkers corrected $^{*}\Delta H_{\text{bind}}$ by considering ΔH_{conf} rising from binding induced folding with DSC measurements.[15] Much more precise and accurate ways of correction are required.

2.2.3 Case by case application

2.2.3.1 Estimation of ΔH and ΔC_{p} using ΔASA

We often encounter the necessity for predicting the energy terms ΔH and ΔC_{p} based on the 3D structure of protein-protein or protein-small complexes determined by X-ray crystallography or NMR spectroscopy. By using the relationships of (3)-(10) as well as ΔASA_{pol} and ΔASA_{apol}, one can calculate the values of ΔH and ΔC_{p} on complexation. For instance, incorporating the values of ΔASA_{pol} and ΔASA_{apol} to the equation (3) or (4) produces ΔH at 60 ℃. Next, ΔH at a temperature of interest is easily obtained using the slope of ΔH, *i.e.*, ΔC_{p} which is now the known value (Eq.5).

2.2.3.2 Estimation of ΔASA using ΔH and ΔC_{p}

If the values of ΔH and ΔC_{p} from ITC are available, a simultaneous equation of one from (3) or (4) and one from (6)-(10) provides ΔASA_{pol} and ΔASA_{apol} on the molecular interactions. As in the case of **2.2.1**, ΔH at a temperature of interest is obtained from ΔC_{p}.

In addition, although Spolar and Record also suggested the following relation, $\Delta C_{\text{p}} = (0.25 \pm 0.02)$ ΔA_{apol} (13),[10] there are questions about using this relation due to a lack of consideration of the contribution from polar interactions.

2.2.3.3 Comparison of experimentally obtained and calculated values

As we can see with equations (11) and (12), if two proteins interact without conformational changes in the absence of (de) protonation, one can expect the similar ΔH values obtained by ITC and by structural thermodynamic parameters, i.e., rigid body association. Accordingly, differences in the two ΔH values even in

the absence of (de) protonation imply the presence of enthalpic contributions from structural changes.

2.2.4 Deconvolution of entropy terms

The thermodynamic quantity deduced from ITC includes all contributions of the binding system. To gain more detailed information on entropy changes upon molecular interactions, the net entropy changes (ΔS_{bind}) obtained from ITC are divided into the three terms as follows:[2,12)]

$$\Delta S_{bind} = {}^{trans}\Delta S_{bind} + {}^{hydr}\Delta S_{bind} + {}^{conf}\Delta S_{bind} \quad (14)$$

where ${}^{trans}\Delta S_{bind}$ indicates the entropy change in the overall rotational and translational degrees of freedom, *i.e.*, the mixing entropy (ΔS_{mix}) calculated as the cratic entropy as follows:[12)]

$${}^{trans}\Delta S_{bind} = \Delta S_{mix} = R\ln(1/55.5) \quad (15)$$

For 1:1 stoichiometric binding, ${}^{trans}\Delta S_{bind}$ is calculated as -33.5 J K^{-1} mol^{-1}. ${}^{hydr}\Delta S_{bind}$ is the entropy change on the dehydration of polar and apolar surfaces, and can be estimated according to the following relation,[13)]

$${}^{hydr}\Delta S_{bind} = -\Delta C_p \ln(T/385) \quad (16)$$

Spolar and Record[10)] as well as Jelesarov and Bosshard[16)] introduced and applied the following equation. However, this relation does not consider dehydration from polar surfaces.

$${}^{hydr}\Delta S_{bind} = -.135\Delta C_p \ln(T/386) \quad (17)$$

Finally, the term ${}^{conf}\Delta S_{bind}$ reflects the conformational entropy change, and is simply obtained by using the values of ΔS_{bind}, ${}^{trans}\Delta S_{bind}$, and ${}^{hydr}\Delta S_{bind}$ with the equation (14).

Regarding conformational entropy involved in structural thermodynamics, intriguing points can be described. First, intrinsic conformational entropy (${}^{conf}S$) of a globular protein was analyzed by Makhatadze and Privalov.5) ${}^{conf}S$ indicates the difference in conformational entropy between the folded and unfolded states of a protein in a vacuum. At 25 ℃, ${}^{conf}S$ (in J K^{-1} mol^{-1}) of a protein correlates with its molecular mass (M, in kDa) as follows:

$${}^{conf}S = -0.54 + 0.17M - 0.0014M^2 \quad (18)$$

Second, Spolar and Record suggested an interesting correlation between ${}^{conf}\Delta S_{bind}$ and the number of ordering residues on binding from disordered states (N_{fold}) as follows:[9)]

$$N_{fold} = {}^{conf}\Delta S_{bind} / -5.6 \quad (19)$$

This relation would be useful for one of the current issues, coupled folding and binding of intrinsically unfolded proteins.

2.2.5 Estimation of the number of dehydrated water

It is worth noting that how many water molecules escape with the formation of a complex by generating a favorable entropy gain. The number can be approximated in terms of the semi-empirical estimates as suggested by Lee and coworkers.[2)]

$${}^{hydr}\Delta S_{bind} = {}^{dehydr}\Delta n_{water} \times ({}^{dehydr}S_{water} - {}^{hydr}S_{water}) \quad (20)$$

The change in entropy of hydration (${}^{hydr}\Delta S_{bind)}$ can be estimated by multiplying the number of water molecules released to the bulk (${}^{dehydr}\Delta n_{water}$) by the average difference in the partial molar entropy between water of protein hydration (${}^{hydr}S_{water}$) and bulk water (${}^{dehydr}S_{water}$). Makhatadze and Privalov examined the entropy of hydration for different amino acids.[5)] They suggested that the value $-({}^{hydr}S_{water} - {}^{dehydr}S_{water})$ is essentially independent of the chemical nature of a solvent-exposed atomic group and, on average, equals 1.3 ± 0.3 cal K^{-1} mol^{-1} at 25 ℃. Chalikian and coworkers reported a similar value.[17)] Using this value together with the value of ${}^{hydr}\Delta S_{bind}$ obtained, ${}^{dehydr}\Delta n_{water}$ can be calculated.

2.2.6 Predictions of maximal affinity using ASA

A simple probability-based estimator for the maximal affinity ($\Delta G_{max)}$ of a binding site in terms of its apolar surface area ($ASA_{apolar,bindingsite}$) was proposed by Ladbury and coworkers based on databases as follows:[4)]

$$\Delta G_{max} = -30 - ASA_{apolar,bindingsite}/10 \text{ kJ mol}^{-1} \quad (21)$$

They described that the contribution of apolar desolvation to affinity is conventionally attributed to a gain of entropy due to solvent release. Polar surface area burial also contributes substantially to affinity but is difficult to express in terms of unit area due to the small variation in the amount of polar surface buried and a tendency for cancellation of its enthalpic and entropic contributions. Further, thermodynamic changes arising from small differences between ligands binding to individual proteins are relatively large and, in general, uncorrelated with changes in solvation, suggesting that

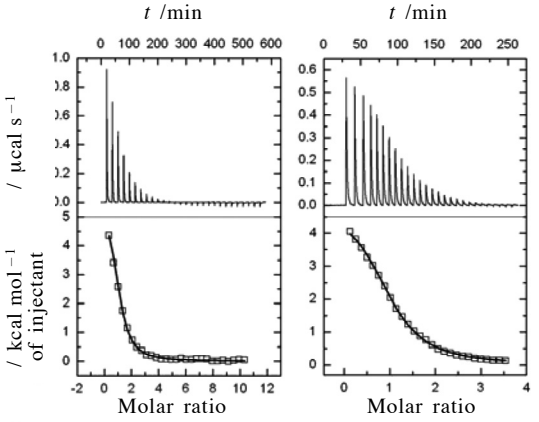

Fig.1 ITC measurements of the binding reaction between Fd and FNR in 25 mM sodium phosphate buffer at pH 6.0 and at 20℃ (left) and 27℃ (right). The raw data from calorimetric titration and the curve fitting of the observed heat values are shown in the upper and lower panels, respectively. Figures were taken with permission from ref.2.

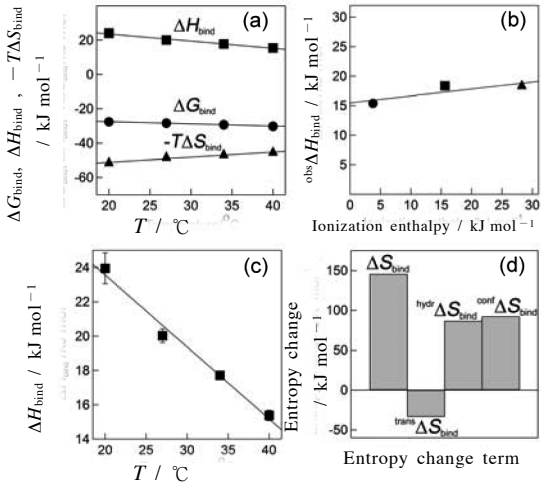

Fig.2 (a) ΔG_{bind} (●), ΔH_{bind} (■), and $-T\Delta S_{bind}$ (▲) obtained from ITC are plotted against temperature. (b) $^{ITC}\Delta H_{binds}$ is plotted against the ionization enthalpy of each buffer: 25 mM sodium phosphate (●), MES (■), and BisTris (▲) buffers. The straight line indicates the linear least-squares fitting to equation 11. (c) The temperature dependence of ΔH is shown. ΔC_p was obtained from the slope of the fitted line (equation 5). (d) The net entropy change, ΔS_{bind}, of Fd-FNR complexation obtained by ITC at 40℃ is deconvoluted into $^{trans}\Delta S_{bind}$, $^{hydr}\Delta S_{bind}$, and $^{conf}\Delta S_{bind}$ (equation 14). Figures were taken with permission from ref.2.

trends identified across widely differing proteins are of limited use in explaining or predicting the effects of ligand modifications.

Although it is difficult to understand the desolvation of water together with polar or apolar surfaces, this relationship may be useful for approximating an affinity.

2.3 Case study of an electron transfer protein complex

As we described above, binding reactions are dominated by ΔG, and ΔG is further balanced by ΔH and ΔS. Therefore, it is important to clarify the driving force and to know how binding affinity is adjusted. Decreasing ΔG makes a reaction favorable, hence decreasing ΔH and increasing ΔS make ΔG lower. Electrostatic interactions such as hydrogen bonds, van der Waals interactions, and salt bridges lower ΔH. Dehydration and elevated structural flexibility increase ΔS. By using structural parameters, the thermodynamic parameters of a binding reaction between molecules can be predicted. However, if no complex structure is available, it is difficult to predict an energetic parameter such as driving force.

In this context, an electron protein complex is a good model system because each protein in a complex interacts using opposing charges. Although complexation is usually thought to be enthalpy driven, binding reactions need to be experimentally characterized. We recently reported detailed energetic studies of the formation of an electron transfer complex between ferredoxin (Fd) and ferredoxin-$NADP^+$ reductase (FNR) using mainly calorimetry, structural thermodynamics, and NMR.[2] The negatively-charged Fd and positively-charged FNR interact electrostatically and have been thought to form a Fd-FNR complex using favorable enthalpy changes. Contrary to the conventional concept, the formation of the Fd-FNR complex was followed by positive ΔH (**Fig.1**) and positive ΔS at various temperatures and at pH 6 (**Fig.2(a)**).

The absence of (de) protonation was confirmed using three different buffers (**Fig.2(b)**). On the basis of various relations of structural thermodynamics and the molecular dynamics simulation, it was found that complexation was driven by detachments of about 20 water molecules and elevations of protein backbone dynamics (**Fig.2(d)**). The value of ΔC_p obtained by ITC was similar to the values calculated using structural thermodynamics

(**Fig.2(c)**).

Likewise, the positively charged cytochrome c and negatively charged cytochrome c oxidoreductase form the electron transfer complex, and we observed endothermic reactions at various temperatures in several buffers regardless of redox states (Y. H. Lee, K. Ishimori, and Y. Goto, in preparation). Obviously, an electrostatic interaction between oppositely charged proteins itself is favorable and energetically important. However, the energetic penalty for the detachment of water molecules around charged side chains is more unfavorable thereby producing an apparently positive ΔH.[2,19]

3. A thermodynamic approach to the development of drug candidates by affinity optimization.

Freire and his coworkers and John E. Ladbury and his group have been suggesting guidelines for identifying drug candidates based on thermodynamic data obtained by ITC.[4] Meroueh's group constructed a comprehensive dataset on intermolecular interactions and suggested the presence of enthalpy-entropy compensation in molecular recognition.[8] Thermodynamic optimization plots constructed with ΔH and ΔS and the concept of enthalpy-entropy compensation are mainly described, and these features will serve as a good reference and a tool for a deeper understanding of the relation between structures and thermodynamics as well as for the development of better models to predict and design binding affinity in drug discovery.

3.1 Enthalpy-entropy compensation and driving forces

For improving the affinity of a drug or a ligand for a target protein, changes designed solely based on structures are often do ineffective because of entropy-enthalpy compensation.

Thermodynamic data along with structural information provide an understanding of this effect and can be used to refine scoring functions employed in computational drug design. Thus, to gain insights into protein-small ligand interactions, Meroueh and his colleagues constructed a useful dataset that contains both structural and thermodynamic data, more than 400 cases, from ITC (PDBcal, http://www.pdbcal.org).[8]

The dataset reveals a lot of interesting features of binding thermodynamics. While the free energy of binding for one protein and several compounds is very similar,

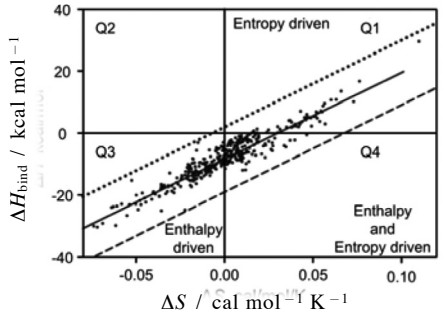

Fig.3 Enthalpy-entropy compensation in weakly interacting systems. The solid line corresponds to a linear fit of the data. Data reproduced with permission from Ref.8.

the enthalpy and entropy of binding are found to vary significantly in some cases. In addition, the free energy reveals a significant difference in the enthalpy of binding. However, this large difference in magnitude is nearly completely compensated by entropy. Compensatory effects between enthalpy and entropy are often referred to as enthalpy-entropy compensation (**Fig.3**). The upper (dotted line) and lower (dashed line) limits of binding free energy correspond to the lowest affinity with a dissociation constant in the mM region ($\Delta G = -2.1$ kcal mol^{-1}) and the highest affinity with a dissociation constant in the pM region ($\Delta G = -19.1$ kcal mol^{-1}l) (**Fig.3**).

The classification of the driving forces for binding is the most interesting. A close inspection of **Fig.3** reveals that enthalpy and entropy do not always contribute in the same manner to affinity. In the Q3 region, the negative enthalpy is favorable and the negative entropy is unfavorable. Binding would be significantly stabilized by creating order resulting in an entropy penalty. In the Q4 region, systems exhibit both favorable entropy and enthalpy. Finally, a non-negligible minority of complexes is found Q1, where binding is entirely entropically driven. The internal energy of the system actually increases as a result of the binding's unfavorable contribution to free energy, but the large degree of disorder created is significant enough to offset the enthalpy costs, resulting in a favorable free energy.

3.2 Statistical analysis of binding and entropic controls

Ladbury and coworkers also conducted a concise and straightforward analysis using the SCORPIO database (http://www.biochem.ucl.ac.uk/scorpio/scorpio.html) including information on intermolecular interactions to

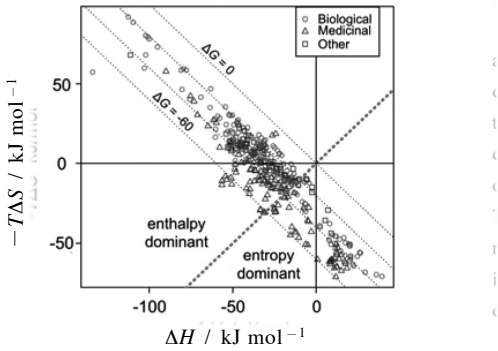

Fig.4 The interactions are categorized as natural biological ligands, synthetic ligands, and 'others'. Diagonal black dotted lines of constant ΔG are spaced at 20 kJ mol^{-1} intervals. The diagonal blue broken line represents $\Delta H = T\Delta S$. Interactions above and below the line have a favorable enthalpic and entropic contribution to ΔG. Data were reproduced from Ref.4 with permission and with slight modifications.

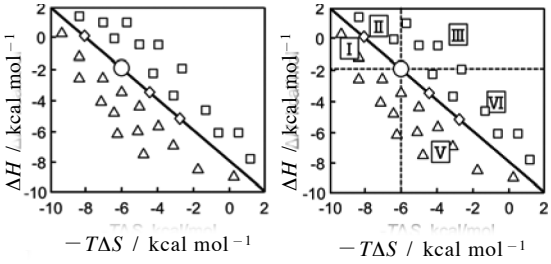

Fig.5 After a round of optimization, the points for all compounds are plotted. Compounds with better affinity (△) fall below the optimization line while compounds of lower affinity (□) occur above the optimization line. Compounds with the same affinity (◇) as the parent compound (○) fall on the optimization line. By tracing a vertical and horizontal line through the coordinates of the parent compound, six different regions can be defined. These regions define distinct strategies for optimization as described in the text. Data were reproduced with permission from Ref.1

reveal general thermodynamic properties of protein-ligand interactions.[4)]

The SCORPIO data set deals with a variety of ligands ranging from natural substrates to synthetic inhibitors and drugs. This breadth provides a firm basis for establishing general patterns and principles for the thermodynamics of protein-ligand interactions.

The most outstanding finding from SCORPIO is that the overwhelming majority of interactions are enthalpically favored. As seen in **Fig.4**, synthetic inhibitors and biological ligands form two distinct subpopulations, with the former having greater average affinity due to more favorable entropy changes on binding.

To investigate the possibility of enhancing affinity, the complexes were divided into three categories, native biological interactions, interactions involving synthetic compounds designed by medicinal chemistry programs, and 'others', a category comprising pseudo-substrates and lead-like compounds. The different distribution of ΔG for these groups is as expected: biological and lead-like interactions are typically of lower affinity. Although the mean ΔH was similar for all three groups, the distribution of $T\Delta S$ differed markedly between the biological and synthetic groups. Native protein-ligand interactions are dominantly driven by enthalpy, while products from medicinal chemistry programs have almost equal contributions to the affinity from enthalpy and entropy changes. The greater affinity of the synthetic inhibitors is thus ascribed to a proportionally greater entropic contribution to binding than found in native biological interactions. They proposed that improvements of ΔS by chemical modification may be physically easier than improvements of ΔH.

3.3 Thermodynamic optimization plot

The optimization of drug candidates would be greatly accelerated if enthalpy and entropy correlations associated with chemical modifications could be effectively manipulated in terms of enthalpy-entropy compensation. Freire and coworkers showed a thermodynamic algorithm for affinity optimization based upon experimental data obtained by ITC.[1)] This approach allows the identification of lead molecule regions and the type of chemical functionalities that will improve the binding affinity by simultaneously optimizing enthalpy and entropy for binding (**Fig.5**).

The thermodynamic optimization plot provides a platform that classifies modified compounds into six regions characterized by different enthalpy-entropy profiles (**Fig.5**). Compounds that fall into any of these regions can be further ranked by their enthalpic and entropic gains, thus giving a precise map of the localization and type of modifications that maximize the enthalpy and entropy of binding. The combination of those

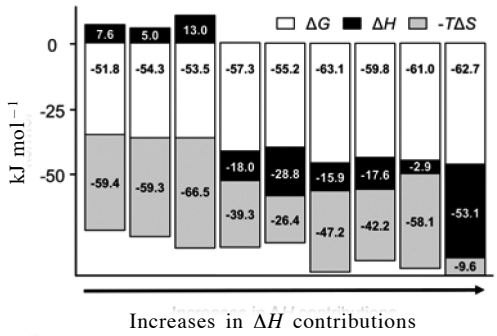

Fig.6 Examples of enthalpic optimization towards best-in-class compounds. A thermodynamic profile of the binding of a series of statins to HMG-coA reductase. The sum of Δ*H* (black) and Δ*S* multiplied by *T* (gray) gives ΔG (white). Analyzing the various compounds approved in this class over time (left to right) reveals enhancement of the contribution of Δ*H*. The HIV-1 protease inhibitors are presented in chronological order of FDA approval from left (1995) to right (2006). Data were taken with permission from ref.18 and with slight modifications.

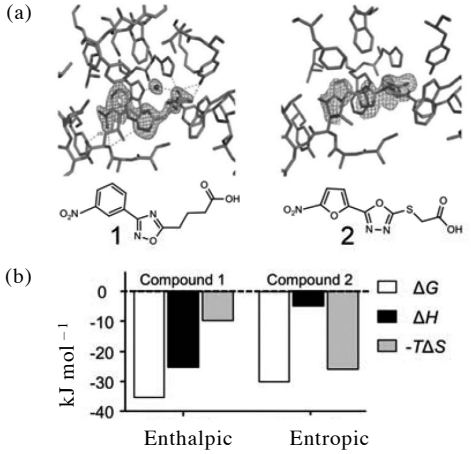

Fig.7 (a) Complexes of aldose reductase with a ligand differ by the binding of one water molecule. (b) The inclusion of water in the complex (a) results in a less favorable entropy contribution (gray) but a more favorable enthalpy contribution (black), owing to the additional hydrogen bonds. Figures were adapted from ref.20 with permission and modified slightly.

modifications into subsequent rounds is expected to accelerate binding affinity optimization.

3.4 Enthalpic adjustments for optimal binding reactions

Recently, enthalpy changes were suggested to be more efficient than entropy changes for selecting lead compounds and aiding optimization.[18-20] As described in **3.3**, the entropic term usually improves as the program proceeds because *de novo* design of non-covalent bonds between a protein and a lead compound in a binding site to enhance Δ*H* is exceptionally difficult. However, there is a limit to the improvement due to the solubility of hydrophobic compounds.

It is generally accepted that a more favorable Δ*H* signifies better non-covalent bond complementarity between proteins and compounds. Interestingly, the study of the drug classes of several approved members, the HIV protease inhibitors, showed that enthalpically optimized compounds are best-in-class rather than first-in-class compounds (**Fig.6**).[18,19]

The progression towards the best-in-class compound is related to a gradual improvement in Δ*H*. The contribution of Δ*H* to Δ*G* increases considerably from the first drug to the most recently approved drug. The example of elevated enthalpic contributions to improving drugs in a given class reveals the potential value of determining thermodynamic data.

Simultaneously, other contributions to binding enthalpy are worth considering. In fact, a number of factors can impinge on this determination. Among them, the role of water itself is highly important. For example, differences in the residual solvation structure of two similar ligands can considerably alter the characteristics of the thermodynamic profile.

The superimposed release or acquisition of a water molecule into the binding interface shifts the thermodynamic properties toward an entropic or enthalpic advantage, respectively. **Fig.7** shows the effect of inclusion of water on the thermodynamic parameters. Compound 1, which includes an interfacial water molecule, has a less favorable Δ*S* term than compound 2, but the energetic gain in Δ*H* owing to the additional hydrogen bonds with the water molecule has a substantial effect on the affinity. Structural information about the corresponding complexes is required to make an assessment of this effect.[19,20]

Besides the factors contributing to a favorable Δ*H*, more contributors such as (un)binding of protons or ions as well as static and dynamic structural changes should be considered for the design of molecular

interactions and thermodynamic optimization.

4. Perspective

Molecular interactions are fundamental to living organisms. Calorimetry-based experimental data and empirical relationships on molecular interactions improve our understanding. The application of these approaches to protein science and pharmaceutical science as well as thermodynamic optimization for the development of drug candidates and drug delivery system is very promising. It will also be helpful for understanding the mechanisms and conformational features of disease-related amyloid fibrils and designing inhibitors of amyloid fibril formation

Because binding affinity is determined by enthalpy and entropy, a more efficient optimization can be obtained by better manipulating the delicate balance between these two functions. There is the suggestion of entropy-based or enthalpy-based adjustments for optimal affinity. Surely the best way of achieving optimal affinity is to concomitantly change the enthalpy as low as possible and change the entropy as high as possible, although an energetic gain from the two terms simultaneously is not easy.

Together with the development of theoretical research, much more calorimetry-based and flexibility-incorporated thermodynamic data is required to obtain more insightful and reliable relationships for the thermodynamic optimization of molecular interactions. Other approaches such as computer-assisted computation, X-ray crystallography, NMR spectroscopy, and various optical spectroscopies provide an opportunity for creating new relationships with data from calorimetry and for increasing the reliability of empirical relationships.

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