3

Experimental Approaches to Determine the Thermodynamics of Protein-Ligand Interactions

R. B. Raffa

3.1

Introduction

Used appropriately and judiciously, thermodynamic parameters can offer insight into the energetics of protein-ligand interactions that is not readily attainable by other means. The utility or application of thermodynamic analysis has traditionally been considered more the domain of (bio)chemistry than biology. However, the modern recognition of an interface in the case of protein-ligand interactions, particularly when the protein is an enzyme or a drug receptor, has kindled an integration with pragmatic benefit to basic understanding and to drug-discovery efforts [1].

Because the nature of most protein-ligand interactions involves relatively weak forces resulting from electrostatic attractions such as ion–ion, ion–dipole, dipole–dipole (hydrogen bonds), induced transient fluctuating dipoles (van der Waals), or hydrophobic effects, they are typically readily reversible and thus amenable to standard equilibrium thermodynamic analysis. Also convenient is that most protein-ligand interactions occur as closed systems, namely, they contain a fixed amount of matter, and the exchange of work is confined to expansion (\(\int P\,dV\)). Because other types of energy exchange, such as radiation, or other types of exchange of work, such as electrical, surface, or photophysical, are negligible (or are approximated to be), the thermodynamic analysis of protein-ligand interactions is simplified.

This chapter provides a broad overview of the purpose and experimental approaches for determining thermodynamic parameters of protein-ligand interactions.

3.2

Basic Thermodynamics of Protein-Ligand Interactions

Thermodynamics, originally the study of the more limited phenomena of heat and heat transfer, evolved to become the more broad study of energy and energy transfer with the recognition – through the cumulative work of Count Rumford
(Benjamin Thompson), Robert Mayer, Sadi Carnot, James Joule, and others (see [2–4] for historical accounts) – that heat is a form of energy. A vast amount of experience and experimentation can be generalized in the following way (e.g., [5]): in any defined “system,” although the work done on the system ($W$) or the heat absorbed by the system ($Q$) in going from one “state” of the system to another varies with the path taken, the sum of $W$ and $Q$ is a constant and depends only on the initial and final “states” of the “system” under consideration. This generalization is formalized as follows:

$$\Delta U = Q + W ,$$

(Eq. 3.1)

where $\Delta U$ represents the change in the energy$^{1}$ of the circumscribed “system.” This equation defines energy in terms of the measurable entities of heat and work and $\Delta U$ as dependent only on the state of the system (i.e., independent of the path by which the system moves from one state to another). $\Delta U$ around a closed path is zero, and only changes in energy can be measured (in terms of heat and work), not absolute values.

The First Law of thermodynamics (colloquially, the law of “conservation of energy”; Mayer, Helmholtz) does not explain why or guarantee that a defined system change will occur spontaneously or, if it does, in which direction the change will occur. This shortcoming is addressed by the Second Law of thermodynamics. Again, a vast amount of experience and experimentation can be generalized by (Carnot, Kelvin, Clausius),

$$\sum (Q/T) \geq 0$$

(Eq. 3.2)

or

$$\int d(Q_{\text{reversible}}/T) \geq 0$$

(Eq. 3.3)

where $T$ is temperature in Kelvin. By defining change in “entropy” as $\Delta S \equiv Q/T$,

$$\sum \Delta S_{\text{system}} + \sum \Delta S_{\text{surroundings}} \geq 0 ,$$

(Eq. 3.4)

or

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1) $U$ (or $E$) was previously termed the “internal” energy (no longer used). For a “closed” system (defined as one in which there is no exchange of mass with the ‘surroundings’) at rest, $\Delta U = Q + W$ if there is no other mechanism of exchange of energy. By convention, $Q$ is the heat absorbed by the system (hence, positive if heat flows into the system and negative if heat flows out of the system) and $W$ is the work done on the system (hence, positive if the surroundings do work on the system and negative if the system does work on the surroundings).
Spontaneous change or equilibrium is described when the RHS of Eq. 3.4 or 3.5 is $> 0$, respectively. To restrict the evaluation to measurable properties of the system rather than of the surroundings, free energy functions have been derived (Gibbs, Helmholtz). Most protein-ligand interactions occur at constant temperature and pressure, so that the only work is $-P \Delta V$. The second law then is represented by

$$
\Delta S_{\text{system}} - \frac{(\Delta U + P \Delta V)_{\text{system}}}{T} \geq 0.
$$

(Eq. 3.6)

Since $\Delta U + P \Delta V$ is the change in “enthalpy” for these conditions,

$$
\Delta S - \frac{(\Delta H)}{T} \geq 0,
$$

(Eq. 3.7)

which upon rearrangement becomes

$$
T \Delta S - \Delta H \geq 0.
$$

(Eq. 3.8)

With the definition of (J. Willard Gibbs) “free energy” as

$$
\Delta G = \Delta H - T \Delta S,
$$

(Eq. 3.9),

where $\Delta G < 0$ describes spontaneous change and $\Delta G = 0$ describes equilibrium. These and other fundamentals of thermodynamics are reviewed in several excellent texts [6–25]. In terms of protein-ligand interactions, energy changes occur in the dissociation of the ligand molecules from the molecules of the solvent and the association of ligand molecules with the protein molecules. Ligand with protein is associated with changes in $\Delta H$ and $\Delta S$. In addition, because the solvent environment is structured due to hydrogen bonds, London forces, or van der Waals interactions, particularly near membrane surfaces, the leaving of ligand molecules is associated with a reversal of the solvation process, which generally involves a decrease in entropy and an increase in energy level. Thus, the change in free energy upon protein-ligand interaction is the net result of dual rearrangement processes: first of the protein molecule (usually involving a change in degrees of freedom or

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2) Change in enthalpy is defined as $\Delta H = \Delta U + \Delta(PV)$, where $P$ and $V$ are the pressure and volume, respectively, of the system. $\Delta(PV)$ is negligibly small in most protein-ligand interactions, so $\Delta H \approx \Delta U$, and the change in the enthalpy is used as an indication of the molecular forces involved in the interaction.

3) This is the fundamental criterion for a spontaneous transformation in a system, typical of most protein-ligand interactions, of constant temperature and pressure. The interaction proceeds spontaneously in the direction in which $\Delta G < 0$. It is important to note that the rate of the interaction is not determined by the sign or magnitude $\Delta G$. 

exposure to water molecules) and then of the solvent molecules (usually involving a decrease in structural constraint and hence an increase in entropy).

### 3.3 Measurement of Thermodynamic Parameters

For an interaction between a protein (P) and a ligand (L) that forms a protein-ligand complex (PL) according to a simple, reversible, bimolecular step represented as

\[
P + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} PL
\]

(Eq. 3.10)

the reaction can be characterized, with appropriate caveats, by the equilibrium constant \(K_{eq} = [PL]/[P][L]\). In practice, the reciprocal of the equilibrium constant is commonly used and is termed the Michaelis constant \(K_M\) when the protein is an enzyme and the ligand is a substrate and is termed the dissociation constant \(K_d\) or \(K_i\) when the protein is a receptor and the ligand is a neurotransmitter, hormone, or drug.

The interaction can be visualized as a reaction-energy diagram as shown in Fig. 3.1. Changes in the energy coordinate (the ordinate) are plotted as a function of the position of the interaction as it proceeds in either direction along the reaction coordinate (the abscissa). This highly schematized representation indicates the overall change in energy \(\Delta E\) for the protein-ligand interaction and the activation energies for the association \(\Delta E_a\) and dissociation \(\Delta E_d\) steps. The diagram applies to the elementary step of the interaction. Associated processes, such as migration to the interaction site, catalytic activity (enzymes), activation of second-messenger transduction processes (receptors), etc., are not included.

For the interaction represented by Eq. 3.10, the relationship between the change in free energy \(\Delta G\), change in enthalpy \(\Delta H\), and change in entropy \(\Delta S\) is given by Eq. 3.9. There are two major ways of obtaining the thermodynamic parameters. One way is by direct measurement of the heat of reaction, which for no \(\Delta PV\) work is the same as \(\Delta H\). The recent development of highly sensitive calorimeters allows such measurement for a relatively wide variety of protein-ligand interactions and is described in more detail below. An alternative procedure employs a more indirect measure, which utilizes a simplified relationship (the van’t Hoff equation) between the thermodynamic parameters and the temperature dependence of the equilibrium constant of Eq. 3.10.

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4) The relationship between these constants and the forward and reverse rate constants of the interaction is not automatically known except for an elementary reaction step.
3.3.1 Calorimetric Determination of Thermodynamic Parameters

The use of calorimetry to measure the heat of a reaction is a time-honored technique. Presently, two modernized high-accuracy automated types of equipment are available with accompanying convenient software. One is known as “differential scanning calorimetry” (DSC), and the other is known as “isothermal titration calorimetry” (ITC). DSC measures the heat capacity (which at constant pressure is the temperature derivative of enthalpy) of the protein-ligand interaction under investigation by incrementally varying the temperature of the system over a specified range (the “scan”). Ultrasensitive isothermal titration microcalorimetry (the use of instruments for which the sensitivity is better than $1/10^8 \text{W}$) [26] measures the heat change that is associated with reactions in solution at a constant temperature and, by the sequential addition of ligand to the solution, also yields thermodynamic parameters. It is a well-characterized and widely accepted technique because the interaction is carried out at a constant pressure, $\Delta P = 0$. Therefore, the energy change associated with the interaction is $\Delta H$ the change in enthalpy ($\Delta U = \Delta H + \Delta PV$). An advantage of ITC over other methods is that it measures the enthalpy change directly. Other techniques, also described below, determine the enthalpy change indirectly. For this reason, DSC or ITC is the preferred method of obtaining interaction parameters, provided that the experimental conditions allow the use of these techniques. Because of the greater use of ITC for protein-ligand interactions to date, the details of this technique are provided below.

In the standard ITC apparatus, the protein-ligand interaction proceeds in a sample cell of relatively small volume (usually 1–3 mL). One component (e.g., protein) of the reaction is placed in the reaction cell, and the other component (e.g., ligand) is added in stepwise fashion by an automated injection system in preset measured amounts for preset measured times. A built-in stirrer ensures that the reaction is continuously and well mixed. The reaction cell is composed of material
that has high thermal conductivity such that energy changes (heat of reaction) that occur within the reaction cell are transmitted with minimal loss as changes in temperature. In modern ITC equipment, the change in temperature is measured as the amount of differential current (power) that is required to maintain the reaction cell at the same preset temperature as that of a reference cell filled with distilled water or the same buffer solution as the reaction cell. As a consequence of this design, the measurements are extremely precise because the dependent variable is power and essentially the only limitation is the electronic thermal motion.

If the protein-ligand interaction is endothermic, more power (\(\mu\text{cal s}^{-1}\)) is required relative to the reference cell. The power that is required, over baseline, comprises the raw data output of the ITC equipment. If the reaction is exothermic, less power is required, which is recorded as a downward deflection in output (Fig. 3.2). The overall interaction between a protein (enzyme or receptor) and a ligand (substrate, inhibitor, neurotransmitter, hormone, or drug) is carried out in a sequence of automated titrations. At each injection step, the power is recorded as a function of time. Each subsequent injection in the series is made after the power function returns to baseline. The output, therefore, forms an S-shaped curve, mirroring the progression of binding of the interacting species from initial

![Diagrammatic representation of typical results obtained in an ITC study of a protein-ligand interaction. The raw data output (peak) accompanying each injection of ligand is the power (\(\mu\text{cal s}^{-1}\)) that is required to maintain the sample cell at the same temperature as a reference cell. A downward deflection indicates an exothermic reaction; an upward deflection indicates an endothermic reaction. Multiple ligand injections are made at preset intervals. The progressively smaller heat outputs correspond to progressively greater protein-ligand binding until saturation is achieved. The residual deflections at the end of the run yield the heat of dilution, which is subtracted from the other deflections prior to further analysis.](image-url)
injection to full saturation (Fig. 3.3). At the end of each run, all of the binding sites are occupied and no further heat of reaction is detected. Any residual power differential is a measure of the heat of solvation of the injected species. In modern ITC equipment, this heat is usually automatically subtracted from the heat of reaction. The raw data obtained for each injection (peak) are then integrated with respect to time, and the integrated heats that are derived from the raw data are plotted against the molar ratio of the interacting species. A best fit of the data is obtained using a non-linear algorithm. From this fit, the stoichiometry, $K_d$, and $/C_68/H$ of the interaction are obtained. From the $K_d$ and $/C_68/H$, the other thermodynamic parameters, $/C_68/G$ and $/C_68/S$, are easily calculated from standard relationships. Additional details of the design and application of ITC are available in several excellent reviews [27–29].

3.3.2
van’t Hoff Determination of Thermodynamic Parameters

3.3.2.1 Relationship to Equilibrium Constant
In the simplest case, the protein-ligand interaction can be represented as, or modeled as, a reversible bimolecular reaction such as depicted by $P + L \leftrightarrow PL$. The change in Gibbs free energy ($\Delta G$) for the interaction in the direction indicated is related to the standard free energy change ($\Delta G^\circ$) by the following equation:

$$\Delta G = \Delta G^\circ + RT \ln \left( \frac{[PL]}{[P][L]} \right),$$  
(Eq. 3.11)

where the brackets indicate concentration, $R = 1.99$ cal/mol·K ($=8.31$ J/mol·K), and $T$ is the absolute temperature in Kelvin (°C+273.15). Most protein-ligand in-
teractions are examined at steady state, at which ΔG = 0 (the process is not capable of producing work), so that Eq. 3.11 becomes

\[ ΔG^0 = -RT \ln \left( \frac{[PL]}{[P][L]} \right), \]  
(Eq. 3.12)

The ratio of complex concentration to the reactant concentrations can be represented by the equilibrium constant \( K_{eq} \), the reciprocal of the equilibrium constant (e.g., \( K_m, K_d \), or \( K_i \)), or by some alternative designation in other types of studies. For the example of \( K_d \), substitution into Eq. 3.12 yields

\[ ΔG^0 = -RT \ln (K_{eq}) = -RT \ln (1/K_d) = RT \ln (K_d). \]  
(Eq. 3.13)

Hence, for the conditions under which most protein-ligand interactions are studied, Eq. 3.13 describes the relationship between the thermodynamic parameter \( ΔG^0 \) and a reaction characteristic (the equilibrium constant) that can be measured experimentally. Because the change in Gibbs free energy is related to the change in enthalpy and entropy by \( ΔG^0 = ΔH^0 - TΔS^0 \), Eq. 3.13 can be rearranged to

\[ \ln (K_d) = \left( \frac{ΔH^0}{R} \right) \left( \frac{1}{T} \right) - \frac{ΔS^0}{R}. \]  
(Eq. 3.14)

Eq. 3.14 is an integrated form of the van't Hoff equation

\[ \frac{d(\ln K_{eq})}{dT} = \frac{ΔH^0}{RT^2}, \]  
(Eq. 3.15)

and is an approximation valid when \( ΔH^0 \) and \( ΔS^0 \) are not temperature dependent. Noting that Eq. 3.14 represents a linear relationship between \( \ln (K_d) \) and 1/T with the y-intercept = -\( ΔS^0/R \) and the slope = \( ΔH^0/R \), it is a common practice in thermodynamic analysis of protein-ligand interactions to determine \( K_d \) at several different temperatures and then construct a “van’t Hoff plot” from which \( ΔH^0 \) and \( ΔS^0 \) are determined from the slope and y-intercept of the resultant data plotted as \( \ln (K_d) \) against 1/T (which is a straight line if the heat capacity is independent of temperature). A smaller error in \( ΔH^0 \) can be obtained if \( ΔS^0 \) is determined first from the van’t Hoff plot and then \( ΔH^0 \) from \( ΔH^0 = ΔG^0 + TΔS^0 \).

Not all such plots turn out to be linear, indicating that in those cases the heat capacity change (\( ΔC_p \)) is not independent of temperature for the interaction under study. It has also been suggested that \( ΔH^0 \) values determined using the van’t Hoff plot method can differ from the same values determined using direct calorimetric measurement [30]. However, it has subsequently been reported that discrepancies are relatively minor [31].
3.3.2.2 Obtaining the Equilibrium Constant

In order to apply the van’t Hoff method of obtaining thermodynamic parameters, some means of measuring the association or dissociation constant of the protein-ligand interaction must be used. The basic principles and many of the experimental methodologies available for obtaining these constants have recently been summarized [32, 33] and are the subject of more extensive coverage in recent reviews (e.g., [34]) and monographs (e.g., [12, 35]). The methods include (extracted from [32] and [33]):

- **Equilibrium dialysis** – Two compartments of a dialysis cell are divided by a semi-permeable membrane. The protein-ligand complex is allowed to associate or dissociate across the membrane until equilibrium is attained. By measuring the constituents of the interaction, the binding constant can be obtained from standard formulas.
- **Steady-state dialysis** – The equilibrium dialysis technique is accelerated by having buffer flow at a constant rate on one side of the semi-permeable membrane and by stirring both sides in order to minimize the concentration gradients [36].
- **Diafiltration** – A type of dialysis equilibrium in which pressure is used to force the ligand-containing solution from one chamber into the protein-containing chamber [37].
- **Ultrafiltration** – Pressure or centrifugation is used to force a mixture of known total concentrations of protein and ligand through a semi-permeable membrane [38].
- **Partition equilibrium** – Separation occurs between two phases rather than across a semi-permeable membrane. Examples include partition between aqueous and lipid phases or partition between a liquid and a solid phase (e.g., where the binding sites are embedded on a solid matrix).
- **Gel (exclusion) chromatography** – Counterpart to equilibrium dialysis when there is sufficient difference in size between protein and ligand and when the protein and protein-ligand complexes co-migrate.
- **Spectroscopy** – Binding-induced changes in either a chromophore or fluorophore absorbance or emission are used to measure the ratio of free to bound ligand concentration. Examples include circular dichroism (differential absorption of left- and right-handed circularly polarized light), fluorescence emission (energy loss as radiation as a fluorophore returns to ground state from photon-excited state) methods, including fluorescence anisotropy (binding of ligand changes the relative depolarization of the emission spectrum compared with that of a polarized exciting light).
- **Electrophoresis** – The components are separated on the basis of differential rates of migration toward an anode or cathode.
- **Sedimentation equilibrium** – An analytical ultracentrifuge is operated at a relatively slow speed that leads to a measurable equilibrium distribution of the constituents of a protein-ligand interaction.
- **Radioligand binding** – The most commonly used technique for the determination of binding to receptors is commonly called radioligand binding because of...
the use of a radioactive-labeled ligand for the quantification of the amount of bound material. As typically used, a radiolabeled ligand is incubated with the receptor preparation for a time sufficient for equilibrium to be attained. Bound and unbound ligands are then separated using any of a variety of techniques such as dialysis, centrifugation, or vacuum filtration (the most widely used method) (see [33] and [39] for details).

- Others – Affinity chromatography, biosensor techniques, and radioimmunoassay are among some of the other available techniques. In addition, perhaps a special mention should be made of the technique of estimating dissociation constants in pharmacological studies using irreversible antagonists (for the $K_d$ of an agonist) or a reversible antagonist (for the $K_d$ of the antagonist). These estimates, although not as intimate to the receptor-ligand interaction as some of the others, nevertheless have been used to some distinct advantage.

3.4 Applications

3.4.1 Calorimetric Determination of Thermodynamic Parameters

There are now well over 200 publications in which microcalorimetry has specifically been used to study protein-ligand interactions of a variety of types. A list of these studies is readily available by a MEDLINE search or from ITC equipment suppliers. Since the studies are too numerous to review here, perhaps a recent one might serve as a representative example of the technique and of its application. In this example [40] we determined the thermodynamic parameters associated with the binding of the reversible inhibitor 2'-CMP (2'-cytidine monophosphate) to RNAse-A (ribonuclease A). We were specifically interested in the binding under conditions that were relatively “physiological,” i.e., at body temperature and in a buffer that contained multiple ions at roughly cellular concentrations.

RNAses are exo- and endonucleases (EC 3.1.27.5), present in vertebrates and also in several bacteria [41–43], mold [44], and plant species [45, 46], that participate in a variety of RNA-processing pathways. Several members of the RNAse superfamily, commonly referred to as the “non-secretory” type, function in predominantly intracellular roles, whereas others, termed the “secretory” type, have evolved [47] roles that are predominantly extracellular, presumably contributing to digestive and cytoprotective functions. (There are actually several systems of nomenclature for RNAses. This came about through historical factors, such as different names for the same RNAse being studied in different species and subsequently recognized as the same RNAse, identification of RNAse activity after naming the enzyme for other reasons, etc.). For the cytoprotective function of RNAses, cytotoxicity against external threats is a desirable and self-protective characteristic that is manifested under normal physiologic conditions. Usually, an intracellular ribonuclease inhibitor (RI) with exceptionally high affinity for RNAse protects the
cell from any secretory RNAs that does not leave the cell. However, under two circumstances the secretory RNAses can be cytotoxic: failure of RI activity or unchecked RNAse activity. The first circumstance is a consequence of genetic defects that result in deficiencies in RNAse production or function. The second is a consequence of excess activity or inappropriate activity in pathological states. Perhaps the best-known example of the latter is the enhanced tumor growth that is attributed to angiogenesis stimulated by the blood-borne RNAse angiogenin. However, there are other RNAses, specifically those designated as the ribonuclease 2 type, that are implicated in pathophysiological conditions where eosinophils appear in increased numbers, as in asthma and other inflammatory disorders in which tissue damage occurs as part of an allergic response [48–50].

Members of the human RNase-A superfamily include

- (“secretory”) pancreatic type (ribonucleases 1);
- (“non-secretory” or “neurotoxin” type) liver, spleen, and urine (Us) RNAses (ribonucleases 2), also known as eosinophil-derived neurotoxin (EDN);
- plasma RNAse (HT-29) (ribonucleases 4);
- and angiogenins [47].

They constitute a group of homologous enzymes that display a preference for pyrimidine bases of RNA. Although some of the details are yet to be delineated, the catalytic mechanism of RNA cleavage by RNAses is hypothesized to occur as depicted in Fig. 3.4. The overall reaction is thought to occur in two steps [51]. In the first step, a 2',3'-cyclic phosphodiester is formed by a “transphosphorylation” reaction from the 5' carbon (starting from the base) to the 2' carbon of the next nucleotide in the RNA chain (Fig. 3.5). The catalytic reaction domain is formed by specific amino acid residues of the RNAse (Fig. 3.6), the details of which have been investigated by several strategies such as chemical modification and site-di-
rected mutagenesis studies (e.g., [52–55]). The reaction products of the first step are not enzyme bound and therefore migrate into the solvent [56]. In the second step, which is believed to occur within the solvent, the product of the first step (2',3'-cyclic phosphodiester) is hydrolyzed to a 3' nucleotide [57, 58]. These reactions can then be represented as follows [51]:

Step 1: \[ \text{RNA} \leftrightarrow 2',3'-\text{cyclic phosphodiester} + \text{R-OH} \]
Step 2: \[ 2',3'-\text{cyclic phosphodiester} \rightarrow 3'-\text{phosphomonoesters} \]

Step 1 is the primary one that is catalyzed by RNAses. It is a fairly straightforward reaction and therefore is amenable to analysis by standard procedures [59]. RNase is also susceptible to inhibition by substances such as 2'-CMP. In our study [40], we used ITC to determine the binding affinity and thermodynamic parameters associated with the reversible inhibition of RNase-A by 2'-CMP at body temperature (37°C) and in a more “physiologically relevant” (i.e., multi-ion)
buffer. These data ultimately might be helpful in drug-design efforts. Consistent isotherms with stable baselines were obtained. Maximal output to the injections of 2'-CMP was about –1.5 to –2.5 μcal/s, the negative deflection indicative of an exothermic reaction. As conventional for studies of this sort, the transposed data were plotted as the integrated heats (kcal/mol of 2'-CMP) for each injection against the 2'-CMP/RNase-A molar ratio, and fitting parameters for the single-site nonlinear regression computer-fit of the raw data points yielded values for $S$ (stoichiometry of the interaction), $K_{eq}$, and $\Delta H^\circ$ for each run. The calculated stoichiometry was very close to 1:1, consistent with previous measures by others of a 1 to 1 interaction between 2' CMP and RNase-A (e.g., [59]). The other estimated parameters, means (± S.D.) of triplicate runs, were $K_d=13.9$ (± 3.9) μM; $\Delta G^\circ=–6.90$ (± 0.16) kcal/mol; $\Delta H^\circ$ (kcal/mol)=–15.7 (± 2.0) kcal/mol; and $\Delta S^\circ=–0.028$ (± 0.006) kcal/mol · K. The observed negative entropy change is consistent with the location of the ribonucleolytic reaction active site within a cleft that binds and cleaves RNA [60]. The interaction proceeds because of a larger decrease in enthalpy. These results, which were determined in multi-ion buffer, were notably different from those determined in single-ion buffer [61] (Tab. 3.1). This single example, hopefully, serves as an example of the methodology of ITC and also a sense of its versatility.

3.4.2 van’t Hoff Determination of Thermodynamic Parameters

The van’t Hoff method has been the most commonly applied technique to determine thermodynamic parameters. A MEDLINE search of “van’t Hoff” reveals over 500 publications between 1966 and 2002. The application to enzyme reaction is well known. More recently, this method has been applied to ligand-receptor inter-

5) Bovine pancreatic RNAse-A, 2'-CMP free acid (98% purity), Na', K', Ca²⁺, Mg²⁺ acetate, and glacial acetic acid (ACS or molecular biology grade) were purchased from commercial sources. The RNAse was dissolved in deionized water and was dialyzed twice for 4 h (in 20 mL solution) in a stirred 1-L beaker maintained at 1.5 °C in a plate by immersion in an ice-bath. RNAse and salt stock solutions (in deionized water) were mixed such that the final concentrations were KCl (3 mM), CaCl₂ (0.1 mM), NaAcetate (10 mM), K₂HPO₄ (3 mM), MgSO₄ (0.4 mM), and KAcetate (50 mM) adjusted to pH 5.5 by dropwise addition of 50 mM HAcetate. The concentration of RNAse (0.04–0.05 mM), selected to be not much higher than the $K_d$ of interaction with 2'-CMP, was determined by quantitative UV spectrophotometry (277.5 nm; extinction coefficient $\varepsilon=9800 \text{ M}^{-1} \text{cm}^{-1}$). The concentration of 2' CMP (1.2 mM), selected so that the $\varepsilon$ value (equal to the product of the binding constant and the total molar concentration of RNAse) would be between 1 and 500, was prepared in the same buffer as the RNAse-A and verified spectrophotometrically (260 nm, $\varepsilon=7400 \text{ M}^{-1} \text{cm}^{-1}$). Solutions were degassed at 36.5 °C under vacuum (about 686 mmHg). The reference cell of the calorimeter contained degassed deionized water. The reaction cell contents were stirred at 400 rpm at 37 °C throughout the experiment (the frictional heat of stirring is incorporated into the baseline). 2'-CMP was introduced into the reaction cell in a series of 35 4-μL injections, each delivered over 16 s at 3-min intervals. The equipment automatically adjusts for the change in volume. The data were evaluated (sampling rate 2 s⁻¹) with computer software.
The basic principles of thermodynamics of course apply to any chemical system, and in this sense the extension of the application of thermodynamic analysis to ligand-receptor interactions is straightforward. Ligand-receptor interactions involve a ligand molecule that has "affinity" for a receptor molecule in biological tissue. There is a requisite complementary 3-D shape for the ligand to be able to "fit" the receptor and form chemical bonds – usually weak, reversible ones – with the receptor molecule. A subset of ligands, termed "agonists," is also capable of inducing a biological effect by binding to receptors. Such molecules are said to have "intrinsic activity," "efficacy," or some similar term. Agonists can be "full" or "partial," depending on their efficacy. Ligands that possess affinity but lack efficacy are "antagonists." Such ligands do not activate measurable biological effects but block the agonist's access to the receptor sites. Because it is not always possible to control all variables precisely, the application of thermodynamic analysis to drug-receptor (pharmacological) interactions involves some care in both methodology and interpretation. Nevertheless, such an endeavor is often worthwhile if there is the opportunity to learn more about such systems than can be learned using other measures. The receptor concept was originated during the latter part of the 1800s and early 1900s, but it was the development of methodological techniques during the 1970s, in particular, radioligand binding techniques (e.g., [33]), that allowed the accurate determination of the number of drug-receptor binding complexes. With the wide commercial availability of relatively stable, radioactively labeled ligands, the technique is now almost routine (e.g., [35, 39]).

The study published by Weiland et al. in Nature in 1979 [62] was perhaps the first to truly catch the attention of many biologists and remains probably the best-known thermodynamic study of drug-receptor interactions to many pharmacologists. In this study the authors measured the temperature dependency of the binding of 20 agonists and antagonists to the β-adrenoceptor located on turkey erythrocyte membranes. They reported that agonist binding affinity was greater at the lower of the two temperatures they examined. The calculated thermodynamic parameter values

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<tr>
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<th>Multi-ion</th>
<th>Single-ion</th>
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<tbody>
<tr>
<td>ΔG° (kcal/mole)</td>
<td>–6.90 ± 0.16*</td>
<td>–7.46 ± 0.10</td>
</tr>
<tr>
<td>ΔH° (kcal/mole)</td>
<td>–15.7 ± 2.0*</td>
<td>–21.9 ± 0.9</td>
</tr>
<tr>
<td>ΔS° (kcal/mole · K)</td>
<td>–0.028 ± 0.006*</td>
<td>–0.047 ± 0.003</td>
</tr>
<tr>
<td>Kd (µM)</td>
<td>13.9 ± 3.9 *</td>
<td>5.6 ± 1.0</td>
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</table>

* Significant difference (P<0.05).
Table 3.2 Examples of thermodynamic studies of ligand interaction with opioid receptors (from [1])

<table>
<thead>
<tr>
<th>Preparation</th>
<th>$\Delta G^{\circ}$</th>
<th>$\Delta H^{\circ}$</th>
<th>$\Delta S^{\circ}$</th>
<th>Reference</th>
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</thead>
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<tr>
<td><strong>Agonists</strong> a. Radioligand binding</td>
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<tr>
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<td>b. Isolated Tissue</td>
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<td><strong>Antagonists</strong> a. Radioligand Binding</td>
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<td>Naloxone</td>
<td>Rat brain</td>
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<td>$&lt;0$</td>
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<td>Naloxone</td>
<td>h-DOR/(CHO)</td>
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<td>Naltrexone</td>
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<td>$&lt;0$</td>
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were reasonable for chemical reactions ($\Delta G = -6.19$ to $-12.51$ kcal mol$^{-1}$, $\Delta H = -12.75$ to $-18.86$ kcal mol$^{-1}$). In contrast, it was found that antagonist binding to the $\beta$-adrenoceptor was largely "entropy driven" (the major contribution to the negative $\Delta G$ was due to a positive $\Delta S$ of $0.013$ to $0.042$ kcal mol$^{-1}$ K$^{-1}$). Thus, there was a clean distinction between agonist and antagonist binding that coincided quite nicely with prevailing views of the actions of agonists and antagonists at receptor sites — that agonists, but not antagonists, induce conformational changes in receptors and that this could account for the induction of the biological response by agonists but not antagonists. We now know, of course, that this does not hold for all receptor binding, but the early publications by Weiland et al. [62, 63] stand out as seminal in the field. Subsequent work has provided insight into a number of drug-receptor interactions. An example of the results of thermodynamic studies on one particular receptor type, the opioid receptor, is given in Tab. 3.2 [64–76]. Similar summaries of thermodynamic studies of other receptors can be found in Raffa [1].

Another application of the method, one that was brought to bear on a puzzling ligand-receptor question, was that reported by Wild et al. [75]. Although previous pharmacological studies had suggested the existence of more than one subtype of opioid $\delta$ receptor, only one had been cloned. Wild et al. [75] reasoned that a distinction could be demonstrated if two preparations, each containing a population of opioid $\delta$ receptors, had different temperature dependency of the dissociation constant. They measured the temperature dependence of the dissociation constant (using radioligand binding techniques) of the selective opioid $\delta$ receptor ligand

### Tab. 3.2 (cont.)

<table>
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<tr>
<th>Preparation</th>
<th>$\Delta G$</th>
<th>$\Delta H$</th>
<th>$\Delta S$</th>
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<td>$&gt;0$</td>
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</tbody>
</table>

b. Isolated Tissue

| Naloxone | MVD | $<0$ | $<0$ | $<0$ | 76 |

Mixed Radioligand binding

| Bremazocine | h-DOR/(CHO) | $<0$ | $>0$ | $>0$ | 73 |

CHO = Chinese-hamster ovary cells; CTAP = D-Phe-Cys-Tyr-D-Trp-Arg-Thr-penicillamine-Thr-NH$_2$; DAMGO = [D-Ala$^2$,NMePhe$^4$,Gly-$\op$-ol$^5$]enkephalin; DADLE = [D-Ala$^2$,D-Leu$^5$]enkephalin; DPDPE = [D-Pen$^2$,5$^5$]enkephalin; EKC = ethylketocyclazocine; $h_a$s = high-affinity binding site; $l_a$s = low-affinity binding site; m-DOR-1 = cloned $\delta$ receptor from mouse brain; MVD = mouse vas deferens; NG 108-15 = mouse neuroblastoma-rat glioma hybrid; PL017 = Tyr-Pro-NmePhe-D-Pro-NH$_2$; SNC80 = (+)-4-((αR)-α-(25,5R,4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl)-N,N-diethylbenzamide; T-dep = temperature-dependent; TIPP($\psi$) = Tyr-Tic($\psi$)(CH$_2$NH)Phe-Phe-OH.
[3H]naltrindole in mouse brain tissue and mouse spinal cord tissue. Comparison of the two revealed that the van’t Hoff plots for mouse brain and mouse spinal cord had different slopes: one was positive and the other was negative. It was concluded that there are multiple subtypes of opioid δ receptors (at least functionally).

3.5 Caveats

The measurement of thermodynamic parameters for protein-ligand interactions can provide valuable insight into aspects of the interaction that are not easily obtainable by other techniques. However, as with all techniques, there are certain limitations in the approach – some related to the methodology and some related to the complexities of the systems under investigation. It is necessary to remember, for example, that the parameters determined apply to the overall reaction being measured. For most protein-ligand interactions, more than one process may be involved. For receptor-ligand interactions, this is almost certainly the case. For example, as a drug molecule interacts with a receptor and makes the transition from free to bound state, energy changes occur as the result of the alteration of the arrangement of receptor molecules as well as of the solvent molecular matrix from which the ligand leaves. Ion displacement, proton transfer, and other processes can be involved. The thermodynamic parameters that are measured for the interaction include these processes.

Some of the more likely encountered possible limitations in thermodynamic analysis, particularly for ligand-receptor interactions, include the following:

- Most receptors are membrane bound. Thus, the interaction of the receptor with the membrane must be considered (constraints on degrees of freedom, changes in the degrees of freedom upon ligand binding, etc.)
- The thermodynamic analyses most often used, particularly the van’t Hoff method, require that measurements be made at steady-state conditions. In the case of radioligand binding determination of equilibrium constants, the time required for the protein-ligand interaction to reach steady state depends on the incubation temperature, and, therefore, the equilibrium constant must be determined for each temperature studied. For the most accurate results, the determination needs to be made at more than two temperatures in order to detect non-linearity. The integrated form of the van’t Hoff equation takes the simple form that is commonly used only if ΔH° and ΔS° for the interaction are not temperature dependent; otherwise, non-linearity in the van’t Hoff plot can arise. Meaningful information can still be obtained in such cases, but more complex analysis is required.
- The relevant affinity state is not always obvious. If the binding reaction is complicated by other processes, such as degradation of the ligand or internalization of the ligand, receptor, or both, then the data cannot be analyzed by simple ther-
modynamics methods – unless the system is defined in a way to incorporate these additional phenomena.

- Although thermodynamic parameters can be obtained for interaction mechanisms that are complex, interpretation of the results is greatly simplified when the interaction mechanism is simple. For example, tissues in which multiple receptor types are expressed will yield results different from tissues expressing only one type, unless a type-selective ligand is used.

- In radioligand binding studies, non-linear Scatchard plots or competition curves that have abnormally steep slopes imply complex binding phenomena, possibly involving multiple receptor types or affinity states. In such cases, the thermodynamic parameters should be separately determined for each receptor type or affinity state.

- The equilibrium (dissociation) constant (binding affinity) that is measured might depend upon the receptor affinity state, G protein coupling, allosteric influences, or other factors distal to the actual binding site. According to most present models, this is more likely for agonists than antagonists.

### 3.6 Summary

The determination of thermodynamic parameters of chemical reactions is extremely useful for the characterization and understanding of chemical reaction processes. The recent extension of this strategy to protein-ligand interactions has yielded equally significant insight into the more intimate details of these complicated and intransigent systems. In addition, the pragmatic application of the information obtained from thermodynamic data of protein-ligand interactions to novel drug-discovery efforts offers exciting new opportunities for creative and valuable work.

Prior to the introduction of modern, automated, high-sensitivity calorimetry equipment, the van't Hoff technique (which is based on the temperature dependence of the equilibrium constant of the reaction) was the primary experimental approach available to determine the thermodynamics of protein-ligand interactions. It remains a mainstay of such determinations. The technique requires the measurement of the equilibrium constant (or of its reciprocal, the dissociation constant), and a large variety of methods have been developed to accomplish this. Radioligand binding is presently the most commonly used method for measuring the reaction constants of ligand-receptor interactions. In its most simplified form, the van't Hoff equation assumes a temperature independence of enthalpy, and this requirement is unfortunately not always verified by experimentalists who use it. However, this problem can be easily avoided or overcome by appropriate experimental design or data analysis.

The introduction of highly sensitive and automated calorimetric equipment has added new options for the measurement of thermodynamic parameters of protein-ligand interactions. By varying either the temperature – as in differential
scanning calorimetry (DSC) – or the ligand concentration – as in isothermal titration calorimetry (ITC) – thermodynamic parameters are obtained directly. As with the van’t Hoff technique, there are limitations of practice and of interpretation. Perhaps the major pragmatic limitation at the present time is the relatively large amount of sample required for high-affinity interactions. As new strategies are devised to overcome these drawbacks, the application of calorimetric approaches will expand even further.

The ever-increasing interest in the folding and interaction of large biomolecules with endogenous or designed ligands will provide the impetus for continued improvement and implementation of experimental approaches to determine the thermodynamics of protein-ligand interactions. The formalization of this interest in the new field of “proteonomics” will provide a framework for its development, and its application to drug-discovery efforts will demonstrate, as thermodynamics has always done, its utility.

3.7 References

11. M. Planck, Treatise on Thermodynamics, Dover, USA, 1945.
Experimental Approaches to Determine the Thermodynamics of Protein-Ligand Interactions

L. E. Limbird, W. F. Blatt, S. M. Robinson, H. J. Bix


J. J. Beintema, J. Hofsteeenge, M. Iwama, T. Morita, K. Ohgi, M. Irie, R. H.
3.7 References


