Determination of Binding Thermodynamics

This unit serves as a starting point for exploring the thermodynamic properties of small molecule–DNA interactions, in theory and practice. The treatment of thermodynamics here will be limited to determination of simple, apparent association/dissociation constants, a necessary limitation due to the complexity of DNA as a "receptor" and the variety of different mechanisms by which small molecules recognize DNA sequence, structure, and dynamics. The spectrum of drug-DNA interactions ranges from sequence specific to sequence nonselective. For example, the intercalator ethidium bromide is relatively sequence nonselective in its binding to DNA compared to the enediyne calicheamicin, which binds to the 3′ ends of purine tracts. For both molecules, the DNA-binding affinity can be determined on a global scale for a mixture of DNA sequences with a spectrum of different binding sites. This type of thermodynamic study is useful for defining general modes of binding such as intercalation, as described in *UNIT 8.1*, or as a first step in structure-function studies with ligand congeners. However, the model used to calculate binding constants must take into account the fact that there is a spectrum of binding sites with differing affinities for the ligand, and that binding of a ligand molecule to one site may influence subsequent binding of other ligand molecules (i.e., cooperative interactions).

The basic approach described in this unit consists of data gathering and curve fitting. The first step in all cases is to determine, under a defined set of conditions, both the concentration of DNA-bound ligand and the concentration of ligand "free" in solution. This information can be obtained by any technique that measures a change in some property of the ligand or DNA that occurs upon binding—including UV/visible (UV/vis) and fluorescence spectroscopy, circular dichroism, and DNA cleavage analysis—or by a technique that allows separation of bound and unbound ligand, such as equilibrium dialysis, centrifugation, or solvent partitioning. Of necessity, this unit will focus on a single method, spectroscopic titration, to quantify bound and unbound ligand. A binding constant is then extracted from the data using any of several mathematical models for DNA-ligand thermodynamics, including the neighbor-exclusion models of McGhee and von Hippel.

SPECTROSCOPIC TITRATION OF FIXED DNA CONCENTRATIONS WITH VARYING LIGAND CONCENTRATIONS

BASIC PROTOCOL

There are several applications in which ligand binding thermodynamics must be determined in solutions containing many copies of a long (>50 bp) "random" DNA sequences, such as in the intercalation studies of *UNIT 8.1* with plasmid DNA or sonicated calf thymus DNA. In these cases, the calculation of apparent binding constants is performed with mathematical models that take into account the possibility of cooperative interactions and the presence of many sites with differing affinities.

The binding of a ligand to DNA can be assessed by UV/vis or fluorescence spectroscopy if the ligand possesses optical properties that change upon binding to DNA. Furthermore, the optical properties must differ from those of DNA, with significant absorbance at wavelengths >300 nm. Table 8.2.1 contains examples of the optical properties of two intercalating ligands, ethidium bromide and daunomycin, that can be exploited to determine DNA binding thermodynamics.

In this protocol, varying ligand concentrations are added to a fixed concentration of DNA and the absorbance changes are recorded to determine the degree of ligand binding.

Contributed by Peter C. Dedon

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Table 8.2.1 Optical Properties of Ethidium Bromide and Daunomycin

Property	Ethidium bromide[a]	Daunomycin[b]
λ_{max} free	479 nm	480 nm
λ_{max} bound	517 nm	505 nm
ε_{480} free	5600-5800 $M^{-1}cm^{-1}$	11500 $M^{-1}cm^{-1}$
ε_{480} bound	~2500 $M^{-1}cm^{-1}$	7000 $M^{-1}cm^{-1}$
Isosbestic points[c]	390 and 510 nm	540 nm (ε = 5100 $M^{-1}cm^{-1}$)
Relative fluorescence[d]	free = 1; bound ~15	free = 1; bound = 0.05

[a]Data from Waring, 1965; Hinton and Bode, 1975; Chaires et al., 1982.

[b]Data from Chaires et al., 1982; conditions: 20°C, 6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM Na_2EDTA, 185 mM NaCl, pH 7; calf thymus DNA.

[c]Isosbestic point: free and bound ligand share the same absorbance at this wavelength.

[d]For daunomycin, λ_{em} = 555 nm and λ_{ex} = 480 nm; for ethidium bromide, λ_{em} = 605 nm and λ_{ex} = 525 nm. Values apply to unbound ligand.

Materials

Assay buffer: 10 mM Tris·Cl, HEPES, or other buffer, pH 7, containing 1 mM EDTA
Calf thymus DNA, sonicated (see recipe)
Ligand of interest dissolved in assay buffer

UV/vis spectrophotometer with temperature-controlled cuvette holder
1-mL cuvette
Software capable of linear regression analysis (e.g., Microsoft Excel)

Additional reagents and equipment for determination of extinction coefficients of free and DNA-bound ligands (*UNIT 8.1*, Basic Protocol 3)

Determine extinction coefficients of bound and unbound ligand

1. Using a UV/vis spectrophotometer with temperature-controlled cuvette holder and a 1-mL cuvette, determine the extinction coefficient of the ligand free in solution and fully bound to DNA as described in *UNIT 8.1*, Basic Protocol 3.

2. Determine if an isosbestic point exists by examining the absorbance spectra obtained in step 1 for a wavelength at which the absorbance is the same in the presence and absence of DNA. Determine the extinction coefficient at the isosbestic wavelength for a least three different DNA and ligand concentrations (e.g., vary each by a factor of three).

   This value allows calculation of total ligand concentration in the presence of any concentration of DNA.

Perform spectral titrations

3. Blank the spectrophotometer against 1 mL of a 1 mM DNA solution (molarity calculated as base pairs).

4. Add 1 µL of a 10 mM solution of the ligand and mix thoroughly.

   For ligands with extinction coefficients >20,000 $M^{-1}cm^{-1}$, a 10 µM ligand concentration should provide an absorbance of >0.2, which is adequate for the titration. For ligands with extinction coefficients <20,000 $M^{-1}cm^{-1}$, start with higher ligand concentrations (e.g., 50 µM). This will ensure an adequate absorbance value for the ligand. Mixing is accomplished by repeated pipetting or, ideally, on a continuous basis with a stirring bar in spectrophotometers equipped with a magnetic stirrer.

5. Record the absorbance at the λ_{max} of the ligand or perform a wavelength scan that encompasses the λ_{max}.

   If there is an isosbestic point associated with ligand binding to DNA, then record the absorbance at this wavelength as well.

6. Repeat steps 4 and 5 for each of three additional 1-µL aliquots of the 10 mM ligand solution.

7. Repeat steps 3 to 6 for DNA concentrations decreasing in half-log intervals (i.e., 300, 100, 30, . . . µM DNA base pairs) to a final DNA concentration of 1 nM.

   The broad range of DNA concentrations ensures a rigorous and complete binding isotherm.

Analyze data

8. Adjust all absorbance values to account for dilution of the sample that occurred upon addition of each aliquot of ligand.

9. For each aliquot of ligand added to the DNA solution, calculate the total ligand concentration in solution (C_t), either directly from knowledge of the concentration of the ligand stock solution or, to avoid pipetting errors, from the absorbance at the isosbestic wavelength using Beer's law (i.e., $A = \varepsilon_{isos} l C_t$, where A is the absorbance, ε_{isos} is the extinction coefficient at the isosbestic wavelength, l is the cuvette path-length in centimeters, and C_t is the total concentration of the ligand).

10. For each ligand concentration, calculate the concentration of bound ligand (C_b) according to the following equation:

$$C_b = \frac{\varepsilon_f l C_t - A}{\varepsilon_f l - \varepsilon_b l}$$

Equation 8.2.1

where A is the absorbance of the solution, ε_f is the extinction coefficient of the free (unbound) ligand, ε_b is the extinction coefficient of the fully bound ligand, C_t is the total ligand concentration, and l is the pathlength of the cuvette in centimeters.

This equation is derived from Beer's law in which the total absorbance is the sum of contributions from the free and bound ligand: $A = \varepsilon_f l C_f + \varepsilon_b l C_b$. The equation is solved for C_b by substitution from the equation $C_t = C_f + C_b$.

11. Calculate C_f according to the following equation:

$$C_f = C_t - C_b$$

Equation 8.2.2

12. Calculate r, the number of moles of ligand bound per DNA base pair, according to the following equation:

$$r = \frac{C_b}{C_{DNA}}$$

Equation 8.2.3

where C_{DNA} is the concentration of DNA as base pairs.

13. Plot values of r/C_f versus r for each ligand concentration at each DNA concentration (i.e., a Scatchard plot).

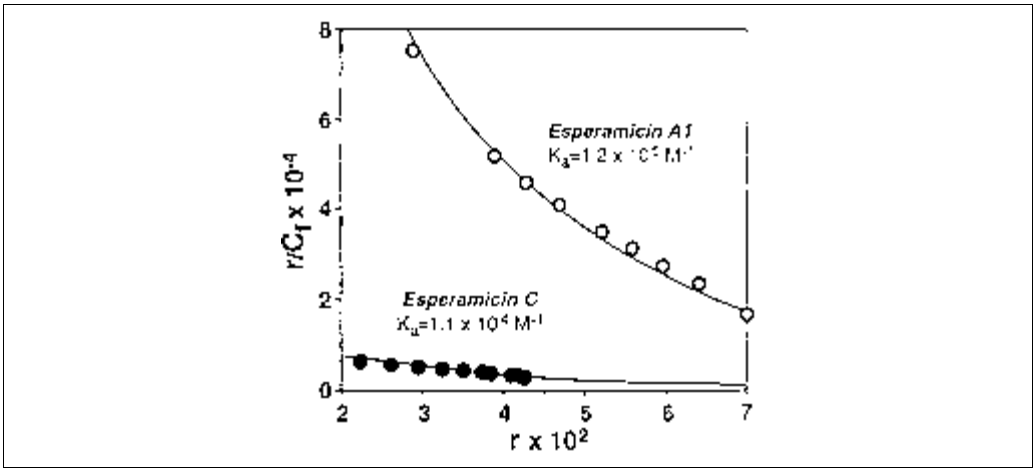

Figure 8.2.1 Example of a DNA binding isotherm with data fit to the McGhee and von Hippel equation. Esperamicin A1 is an enediyne antibiotic that intercalates in DNA; esperamicin C is an analog of A1 missing the intercalating moiety. Adapted with permission from Yu et al. (1994). Copyright 1994, American Chemical Society.

If there is no positive cooperativity in the binding of ligand to DNA, the plot should appear as a downward-sloping curve as demonstrated in Figure 8.2.1. Positive cooperativity will be apparent as an initial upward slope in the plot followed by a downward-sloping curve.

14. Using any of several software programs that allow nonlinear regression analysis (e.g., Microsoft Excel), calculate the apparent binding (association) constant from the r/C_f and r values according to the neighbor exclusion model of McGhee and von Hippel:

$$\frac{r}{C_f} = K_{obs}(1 - nr)\left[\frac{1 - nr}{1 - (n-1)r}\right]$$

Equation 8.2.4

where K_{obs} is the intrinsic (apparent) binding constant (units of M^{-1}) for the ligand and the DNA species studied, and n is the neighbor exclusion parameter that represents the size of the ligand binding site in base pairs.

The software should calculate both K_{obs} and n for the fitted data. One can vary the values of either n or K_{obs} and observe the effect of the changes on the standard deviation of the fit. For further information, the reader is referred to the work by Johnson and Faunt (1992), which provides a general review of least-squares fitting methods.

15. Finally, fit the data to an extended version of the above equation that takes into account cooperative interactions between ligands:

$$\frac{r}{C_f} = K_{obs}(1 - nr)\left[\frac{(2\omega - 1)(1 - nr) + r - R}{s(\omega - 1)(1 - nr)}\right]^{n-1}\left[\frac{1 - (n+1)r + R}{2(1 - nr)}\right]^2$$

Equation 8.2.5

where ω is the cooperativity parameter (ω >1 indicates positive cooperativity and ω <1 indicates negative cooperativity).

The use of Equation 8.2.5 is justified only if the standard deviation of the fitted line is smaller than that derived from Equation 8.2.4 or if there is prior knowledge of cooperativity of the ligand/DNA interaction. If there is little difference between binding constants obtained by either equation, use the values obtained from Equation 8.2.4.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX.***

Calf thymus DNA, sonicated

Prepare sonicated calf thymus DNA in assay buffer (10 mM Tris·Cl, HEPES, or other buffer, pH 7, containing 1 mM EDTA) as described in *UNIT 8.1*, Support Protocol; use the preparation recommended for Basic Protocol 3 in that unit—i.e., 10 to 15 min sonication and no size fractionation.

COMMENTARY

Background Information

The experiments in this unit represent just one of many approaches to quantifying the DNA binding affinity of a ligand. While the unit focuses on the use of UV/vis spectroscopy, virtually any technique can be used as long as some property of either the DNA or the ligand changes upon interaction of the two, or the unbound ligand can be physically separated from bound ligand. Examples of applicable techniques include: equilibrium dialysis (Chaires et al., 1982); fluorescence titration (Chaires et al., 1982); centrifugation (Minton, 1990; Yu et al., 1994); solvent partitioning (Waring et al., 1975); and DNA cleavage (Krishnamurthy et al., 1995).

The calculation of C_f, C_b, and r from data obtained with these methods is straightforward. For example, for fluorescent ligands whose fluorescence decreases upon binding to DNA (e.g., daunomycin), C_f can be calculated as follows:

$$C_f = C_t\left[\frac{F - F_{min}}{F_0 - F_{min}}\right]$$

Equation 8.2.6

where F is the fluorescence of the ligand in the presence of some quantity of DNA, F_{min} is the fluorescence of the ligand in the presence of a large excess of DNA (i.e., fully bound ligand), and F_0 is the fluorescence of the ligand in the absence of DNA. For a fluorescent ligand whose fluorescence increases upon binding to DNA (e.g., ethidium bromide), C_b can be calculated as follows:

$$C_b = C_t\left[\frac{F - F_0}{F_{max} - F_0}\right]$$

Equation 8.2.7

where F_{max} is the fluorescence of the ligand in the presence of a large excess of DNA (i.e., fully bound ligand). In either case, C_b or C_f is calculated by difference.

Ideally, the binding data should be acquired by more than one method, since the physicochemical properties of the ligand may affect the data obtained with different techniques. This is illustrated in studies performed by Chaires et al. (1982), in which the binding of daunomycin to DNA was assessed by solvent partitioning and spectral titration techniques.

Once the concentrations of bound and free ligand have been determined, the binding affinity can be quantified by analysis of the ligand-DNA isotherm. In most cases, a Scatchard plot of the data (i.e., a plot of r/C_f versus r) will not produce a straight line as one would expect for ligand binding to a simple receptor with one or a few discrete binding sites. Instead, there is usually a pronounced downward curvature to the plot, as shown in Figure 8.2.1. The curvature arises from the binding of one drug molecule that excludes the binding of other ligand molecules nearby along the DNA polymer, a phenomenon referred to as neighbor exclusion.

Extraction of a binding constant in the face of the neighbor-exclusion phenomenon can be achieved by several different numerical analysis methods, the most convenient of which are the equations derived by McGhee and von Hippel. Equation 8.2.4 is applied to the binding of a ligand to a DNA lattice of identical, noninteracting binding sites. The value n, the exclusion parameter, describes the size of the binding site in base pairs and it is best determined with data approaching saturation of the DNA binding sites with ligand (Fig. 8.2.2).

However, Equation 8.2.4 does not address cooperative interactions between ligands. To account for positive and negative cooperativity in ligand binding, McGhee and von Hippel (1974) introduced an additional parameter, ω, into Equation 8.2.4 to yield Equation 8.2.5. If ω >1, binding of one ligand molecule will promote the binding of another ligand nearby (positive cooperativity), while ω <1 implies that binding of one ligand molecule will inhibit

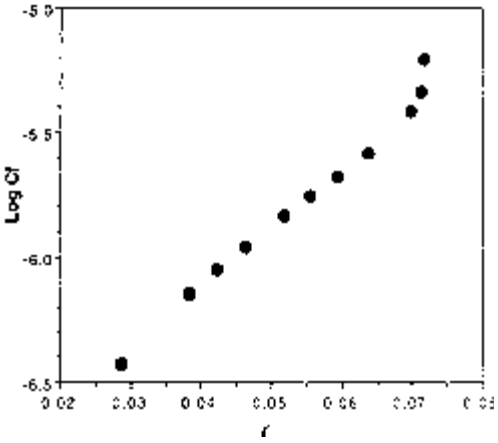

Figure 8.2.2 Plot of log(C_f) versus r for the binding of esperamicin A1 to DNA to demonstrate completeness of the binding isotherm. The curve adopts a roughly sigmoidal shape and becomes nearly asymptotic at low and high r values, which reflects complete binding and saturation, respectively. Data adapted from Yu et al. (1994).

the binding of another molecule nearby (negative cooperativity). The case of negative cooperativity is extremely difficult to distinguish from the neighbor-exclusion phenomenon, and the reader is referred to the work of Correia and Chaires (1994) for approaches to resolving the contributions of these two features of ligand binding. Furthermore, the use of the more complicated Equation 8.2.5 can sometimes produce a poorer fit of the data than Equation 8.2.4. The best approach is to fit the data to both equations and compare the standard deviation of the plots. One can also apply the F test to the resulting standard deviations to determine the goodness of fit (Chaires, 1992).

At this point, it is important to discuss the allosteric binding model of Dattagupta et al. (1980). This model is more rigorous for small molecules than the McGhee and von Hippel model, but it requires greater sophistication in statistical-mechanical approaches to fitting data. The McGhee and von Hippel model assumes that the conformation of DNA is not affected by ligand binding and that positive cooperativity arises instead from ligand-ligand interactions, such as that occurring between protein ligands. The allosteric model of Dattagupta et al. (1980), however, takes into account changes in DNA conformation caused by ligand binding, which is usually what is occurring. Positive cooperativity in this model arises when ligand binding changes DNA conformation in such a way as to promote binding of other ligands. The only drawback to the model is that the data cannot be fit using simple nonlinear regression programs and instead requires a statistical-mechanical model to extract the binding constant. Interested experimentalists are encouraged to explore the work of Dattagupta et al. (1980).

Further extraction of thermodynamic information from the binding isotherms, such as enthalpy and entropy, is beyond the scope of this unit. However, one can begin to parse the contributions made by electrostatic and nonelectrostatic interactions by measuring the binding constants in the presence of varying concentrations of NaCl. For each salt concentration, the apparent binding constant, K_{obs}, can be used to calculate the observed Gibbs free energy (ΔG_{obs}°) from the relation:

$$\Delta G^{\circ}_{obs} = -RT\ln K_{obs}$$

Equation 8.2.8

where R is the gas constant and T is the temperature in degrees Kelvin. The ΔG_{obs}° can be viewed as the sum of two contributions:

$$\Delta G^{\circ}_{obs} = \Delta G_{nonel} + \Delta G_{el}$$

Equation 8.2.9

where ΔG_{nonel} and ΔG_{el} represent the nonelectrostatic and electrostatic contributions to free energy. For several different NaCl concentrations, it has been shown by Record et al. (1978) that a plot of K_{obs} versus salt concentration yields a slope of $\mathbf{Z}\varphi$ according to the equation:

$$\frac{\delta \ln K_{obs}}{\delta \ln(Na^+)} = -\mathbf{Z}\varphi$$

Equation 8.2.10

where $\mathbf{Z}$ is the charge of the ligand, and thus represents the number of counterions released during drug binding, and φ is the fraction of Na^+ associated with each DNA phosphate. The electrostatic contribution to the free energy of ligand binding can then be calculated from:

$$\Delta G_{el} = \mathbf{Z}\varphi RT\ln(Na^+)$$

Equation 8.2.11

In this way, one can begin to determine the individual contributions of electrostatic and nonelectrostatic (e.g., hydrophobic) contributions to the DNA binding energetics of a ligand.

Critical Parameters

There are several factors critical to the determination of binding constants of small molecules by the technique of spectroscopic titration

described here, and more generally for all methods used to assess binding thermodynamics. The most obvious is that the UV/vis absorbance properties of the ligand must change when the ligand binds to DNA. A 10% to 15% difference between the absorbance of bound and unbound ligand can be considered a minimum for the rigorous determination of a binding constant. The ligand should have a $\lambda_{max} > 300$ nm to avoid interference from DNA.

More generally, the titration of ligand and DNA must be performed over a broad range of DNA concentrations to ensure a complete isotherm. The completeness of the binding isotherm can be assessed by plotting $\log(C_f)$ versus r for the entire set of data. Ideally, the data will fall on a sigmoidal curve as illustrated in Figure 8.2.2 for the enediyne antibiotic esperamicin A1. At high r values, the curve becomes asymptotic due to the saturation of DNA binding sites. Though not illustrated well in Figure 8.2.2, the plot becomes asymptotic at low r values where most of the ligand is bound to DNA and the C_f becomes vanishingly small.

Another critical factor is the purity of the DNA. The presence of contaminating salts, metals, buffers, proteins, and other molecules can affect the DNA binding energetics of the ligand or block binding sites. Ideally, the DNA should be thoroughly extracted with phenol/chloroform and desalted by dialysis into the appropriate buffer; alternatively, the extracted DNA can be purified by gel-filtration chromatography as described in the Support Protocol in *UNIT 8.1*.

Troubleshooting

Several problems may be encountered during the collection of binding data. First, there may be significant scatter in the data at the extremes of the binding isotherm due to systematic errors in determination of the extinction coefficients and the sensitivity of the spectrophotometer. It is best to use data in which the concentration of bound ligand as a fraction of total ligand concentration is between 0.2 and 0.8.

Equipment errors may also pose problems. Sequential additions of ligand should be limited to only a few aliquots to avoid changes in absorbance due to drift in the spectrophotometer electronics. Pipetting errors will also introduce significant scatter in the binding data. For ligands soluble only in organic solvents, the vapor pressure created during pipetting of aliquots of the ligand in a volatile solvent can affect the actual volume of ligand transferred into the spectrophotometer cuvette. The use of nonvolatile solvents (e.g., DMSO) or positive-displacement pipets helps to prevent this problem. It is also important to use pipets that are both accurate and precise in the volume range used in the studies (1 to 10 μL).

Anticipated Results

An example of the anticipated results of these experiments is shown in the Scatchard plot in Figure 8.2.1 for the enediyne antibiotics esperamicins A1 and C. The range of r values obtained will vary as a function of the binding affinity of the ligand.

Time Considerations

A complete binding isotherm will require at least 2 weeks to obtain. The bulk of the time will be spent preparing reagents such as the DNA substrate, which must be quite pure to prevent artifacts from contaminating salts and proteins. Collection of the binding data by spectroscopic titration will require ~1 week, with the largest fraction of the time spent determining the optimal range of concentrations of ligand and DNA.

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