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Quantitative Thermodynamic Analyses of Spectroscopic Titration Curves[§]

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Abstract

Elucidation of ligand - macromolecule interactions requires detailed knowledge of energetics of the formed complexes. Spectroscopic methods are most commonly used in characterizing molecular interactions in solution. The methods do not require large quantities of material and most importantly, do not perturb the studied reactions. However, spectroscopic methods absolutely require the determination of the relationship between the observed signal and the degree of binding in order to obtain meaningful interaction parameters. In other words, the meaningful, thermodynamic interaction parameters can be only determined if the relationship between the observed signal and the degree of binding is determined and not assumed, based on an *ad hoc* model of the relationship. The approaches discussed here allow an experimenter to quantitatively determine the degree of binding and the free ligand concentration, *i.e.*, they enable to construct thermodynamic binding isotherms in a model-independent fashion.

Keywords

Spectroscopic Titrations; Thermodynamics; Fluorescence; Binding Isotherms

INTRODUCTION

A comprehensive understanding of the ligand - macromolecular interactions ultimately requires information about the energetics and kinetics of the interacting molecules.

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Thermodynamic studies give the information necessary to understand the molecular forces that drive the complex formation. Moreover, they can provide information about functionally significant structural changes occurring in participating reacting species, as a result of the interactions [1–21]. Major goals of quantitative binding studies are to obtain the chief characteristics of the binding reaction that include: (1) The maximum stoichiometry of the formed complexes; (2) The presence, character, and extent of cooperative interactions; (3) Macroscopic and intrinsic binding constants; (4) Nature of interactions involved in formation of the studied complexes, *i.e.*, the dependence of equilibrium binding parameters on solution variables (temperature, pressure, pH, salt concentration, type of salt, *etc.*).

Equilibrium binding isotherms for the ligand association with a macromolecule represent the relationship between the total average degree of binding (number of ligand molecules bound per macromolecule) and the free ligand concentration. True thermodynamic isotherms reflect only this correlation and cannot be dependent upon any assumptions, or models concerning the relationships between the observable physico-chemical parameter used to monitor the binding, and the total average degree of binding. Thermodynamic isotherms alone already provide information about the ligand macroscopic affinity and the maximum stoichiometry of binding process. On the other hand, in cases where multiple ligand molecules bind to a macromolecule, the experimental binding isotherms can be analyzed using statistical thermodynamic models that incorporate known molecular aspects of the system, such as intrinsic binding constants, cooperativity parameters, allosteric equilibrium constants, discrete character of the binding sites, or overlap of potential binding sites, *etc.* Only then can one make rational molecular interpretations of the nature of the observed interaction processes. Equilibrium ligand - macromolecule interactions, including protein - nucleic acid interactions, have been directly examined by the methods in which binding is directly monitored [16,22–30]. These direct approaches comprise analytical ultracentrifugation, column chromatography, affinity chromatography, filter binding assays, and gel electrophoresis. Although the direct methods are straightforward, some, like filter binding, or gel shift assays are non-equilibrium techniques, which require several controls before true equilibrium data can be obtained [16,22–30].

The indirect methods, which include spectroscopic titration techniques, are based on observing a physico-chemical signal (*e.g.*, fluorescence intensity), proportional to the macromolecule or ligand concentrations and accompanying the formation of the complex, not the total average degree of binding [1–6,8–10,12–15,17–21]. These changes must then be correlated with the degree of binding and the concentration of the free ligand. Nevertheless, the functional relationship between the observed spectroscopic signal and the total average degree of binding is never *a priori* known. It is commonly assumed that the observed spectroscopic signal change is directly proportional to the degree of saturation of the macromolecule and/or the ligand, *i.e.*, it is a linear function of the degree of binding. However, if a “binding isotherm” is obtained by indirect methods that involve the assumption, then the “binding isotherm” and obtained interaction parameters will be no more accurate than the assumption. This may be particular serious problem if the isotherm is being used to differentiate between alternative models for the interactions, since if a model

does or does not "fit" the isotherm, this may be due either to the failure of the model or the failure of the assumption on which the "isotherm" is based.

There are several reasons why the observed spectroscopic signal may not depend in linear fashion as a function of the degree of binding. For instance, structurally or functionally different sites on the macromolecule, each possessing different spectroscopic properties, may be differently affected by the ligand, or the spectroscopic properties of the ligand may be different in different sites. Possible cooperative interactions may affect the physical state of the bound ligand, or binding sites. Moreover, the number of cooperative contacts among bound ligand molecules may not be a linear function of the degree of binding. A ligand may bind to the macromolecule in different binding modes characterized by different responses of the spectroscopic signal accompanying the formation of the individual mode [1–3,5–7,17]. Other situations may include combinations of all mentioned above situations.

In this communication, we describe thermodynamically rigorous analyses of spectroscopic approaches to study ligand - macromolecule interactions, including multiple-ligand binding phenomena, to obtain model-independent binding isotherms [1–6,8,17–21,31]. Our discussion focuses on the fundamental problem of obtaining thermodynamic and spectroscopic parameters free of assumptions about the relationship between the observed signal and the degree of ligand or macromolecule saturation. We will address the quantitative analyses as applied to the use of the fluorescence intensity as the most often encountered spectroscopic technique in biochemical studies [1–6,8–10,13–15,18–21]. However, the described thermodynamic/spectroscopic relationships are general and applicable to any physico-chemical signal used to monitor the ligand - macromolecule interactions and proportional to the concentrations of different states of macromolecule or ligand (absorbance, fluorescence emission, fluorescence resonance energy transfer (FRET) efficiency, circular dichroism, NMR line width, or chemical shift, *etc.*).

It should be noted that the discussed approaches are only valid in the absence of ligand or macromolecule aggregation/self-assembly processes, within experimental concentration ranges used. In other words, the standard reference states of the ligand and the macromolecule are their corresponding monomeric states. Hence, it is imperative that the self-assembly/aggregation properties of the examined ligand and the macromolecule are known, before any rigorous analysis of any binding reaction is performed. Moreover, we are concerned only with situations where the observed spectroscopic signal originates only from the macromolecule or the ligand, a condition, which is easily experimentally realized. The discussed specific cases of binding systems are selected from the works performed in our laboratory centered on various protein - nucleotide, protein - nucleic acid interactions and using the fluorescence intensity as the observed spectroscopic signal [1,2,5,6,17–21,32–48]. The hands-on insights how these data were obtained and analyzed allow us to achieve the best illustration of the applicability and feasibility of the discussed methods.

1. Some Possible Pitfalls in Using A Single Titration Curve

As discussed above, fluorescence titration, or any titration method based on observing a physico-chemical signal, is an indirect method in examining ligand - macromolecule

interactions. In the case of spectroscopic titrations, *e.g.*, fluorescence titrations, the observed spectroscopic signal (emission intensity) accompanying the formation of the complex can originate from the macromolecule or from the ligand [1–6,8,31,33,48] (see below). In any case, the observed spectroscopic signal of the system must be correlated with the degree of binding and the concentration of the free ligand, which is never *a priori* known. The only exception is a system in which it is known that, at saturation, a single ligand molecule binds to the macromolecule, *i.e.*, in this case, the maximum stoichiometry of the system is already known. In much more general cases, where multiple ligand molecules participate in the binding process, the maximum stoichiometry is one of the thermodynamic/structural parameters that must be determined.

For a multiple-ligand binding system, a single, equilibrium spectroscopic titration curve does not provide any information as to the maximum stoichiometry of the formed complex and can be misleading about the nature of the observed binding process [1–6,8]. An example of a theoretical fluorescence titration curve of the macromolecule possessing four independent and identical ligand-binding sites with the ligand and obtained at a single macromolecule concentration is shown in Figure 1a. The binding is accompanied by the increase of the macromolecule fluorescence, *i.e.*, the observed change of the fluorescence intensity is generated from the macromolecule. Moreover, the fractional increase of the macromolecule fluorescence, F_{obs} , is proportional to the total average degree of binding, $\Sigma\Theta_i$. The selected binding constant, K and the total macromolecule concentration, M_T , are $1 \times 10^6 \text{ M}^{-1}$, and $5 \times 10^{-7} \text{ M}$, respectively. The titration curve is quantitatively described by two parametric equations,

$$\Delta F_{\text{obs}} = \Delta F_{\text{max}} \frac{4K[L]_F}{1 + K[L]_F} = \Delta F_{\text{max}} \Sigma\Theta_i \quad (1a)$$

and

$$[L]_T = [L]_F + \Sigma\Theta_i[M]_T \quad (1b)$$

where $[L]_T$ and $[L]_F$ are the total and free ligand concentrations, respectively. The quantity,

F_{obs} , is the observed relative fluorescence increase defined as $F_{\text{obs}} = (F_i - F_o)/F_o$, where, F_i , is the value of the fluorescence intensity of the sample at “i” titration point and, F_o , is the initial value of the macromolecular fluorescence *prior* to the ligand addition. The quantity,

F_{max} is the relative maximum increase of the fluorescence intensity, defined as $F_{\text{max}} = (F_{\text{max}} - F_o)/F_o$, where F_{max} is the maximum observed fluorescence increase of the sample.

In other words, the proportionality constant between F_{obs} and $\Sigma\Theta_i$ is the maximum observed signal change, $F_{\text{max}} = 0.5$, for the ligand binding to each of the four sites. Without knowing that a multiple ligand-binding process is present, the experimenter is prone to analyze the titration curve by assuming a simple, 1:1 maximum stoichiometry of the binding process, *i.e.*, the simplest possible binding model. Also, without *a priori* knowing the affinity, the experimenter may treat the total ligand concentration as the equivalent to the free ligand concentration (see below). The analogous equations for a single ligand molecule binding to a single site on the macromolecule is then

$$\Delta F_{\text{obs}} = \Delta F_{\text{max}} \frac{K[L]_F}{1 + K[L]_F} = \Delta F_{\text{max}} \Sigma \Theta_i \quad (2a)$$

and

$$[L]_T = [L]_F + \Sigma \Theta_i [M]_T \quad (2b)$$

It should be clear from eq. 2a that assuming the 1:1 model is equivalent to assuming that

$$\Sigma \Theta_i = \frac{\Delta F_{\text{obs}}}{\Delta F_{\text{max}}} \quad (2c)$$

The plot of the fluorescence titration curve, as defined by eqs. 2a and 2b, is included in Figure 1a, with $K = 4.8 \times 10^5 \text{ M}^{-1}$ and $F_{\text{max}} = 2.0$. It may seem that eq. 2a is equivalent to eq. 1a. However, in eq. 2a the value of $[L]_F$ is calculated by assuming that the macromolecule binds one ligand molecules instead of four ligand molecules as in eq. 1a. Within typical experimental accuracy of the titration experiments, the correct plot for four independent sites and the semi-quantitative plot for the assumed single-binding site are indistinguishable. There is an error (~a factor of 2) in determining the binding constant, which is probably not that significant in typical biochemical studies. It should be noticed that such a “small” error results from the fact that the selected concentration of the macromolecule is much lower than the total concentration of the ligand, *i.e.*, the concentration of the bound ligand, L_B , is negligible, as compared to the total ligand concentration, L_T . Nevertheless, the nature of the observed binding reaction, structural aspects of the macromolecule (four binding sites), and all conclusions as to the functionality of the macromolecule and its complexes with the ligand, is completely lost and/or simply ignored.

Performing a spectroscopic titration at a single concentration of the macromolecule can particularly be misleading when the ligand affinity is unknown. If the titration is performed at a single, high macromolecular concentration, the concentration of the bound ligand, L_B , constitutes a significant part of total ligand concentration, L_T . In general, the experimenter does not *a priori* know that. A single titration curve does not provide any clue about the ligand affinity. As an example, a theoretical titration curve for ligand binding to four independent binding sites on the macromolecule, monitored by the fluorescence emission, with $F_{\text{max}} = 0.5$, the binding constant, K , and the total macromolecule concentration, M_T , $1 \times 10^6 \text{ M}^{-1}$ and $4 \times 10^{-6} \text{ M}$, respectively, is shown in Figure 1b. The spectroscopic titration curve is described by eq. 1. Even a less quantitatively oriented experimenter would realize that the single-site binding model could not fit the curve in Figure 1b. It is because the plot does not spend ~2 orders of magnitude of the ligand concentration scale between the ~10% and ~90% of the observed total signal. In this case, the apparent behavior of the plot suggests a positive cooperativity in the binding process and the experimenter would feel justified to use the next simplest model, which would be the ligand binding to two sites with cooperative interactions and accompanied by the same fluorescence emission changes upon binding to each site. The binding model is then defined by expressions

$$\Delta F_{\text{obs}} = \Delta F_{\text{max}} \frac{2K[L]_F + 2K^2\sigma[L]_F^2}{1 + 2K[L]_F + K^2\sigma[L]_F^2} = \Delta F_{\text{max}} \Sigma\Theta_i \quad (3a)$$

and

$$[L]_T = [L]_F + \Sigma\Theta_i[M]_T \quad (3b)$$

From eq. 3a, after assuming the 2:1 model and proportionality between the observed signal and the total average degree of binding, one has that

$$\Sigma\Theta_i = \frac{\Delta F_{\text{obs}}}{\Delta F_{\text{max}}} \quad (3c)$$

The quantity, s , is the cooperative interaction parameter. The plot of eqs. 3a and 3b is shown in Figure 1b and it fits the experimental titration curve with commonly acceptable accuracy. Nevertheless, the assumed model, based on a single titration curve, completely misses the maximum stoichiometry and the nature of the examined binding process, defined by eq. 1a, not by eq. 3a. The determined binding constant, $K = 1.27 \times 10^5 \text{ M}^{-1}$, and cooperativity parameter, $s = 2.7$, respectively, are in this case fitting constants, not the thermodynamic parameters characterizing the system. The simple examples discussed above do not exhaust all possible pitfalls when analyzing a single spectroscopic titration curve. More complex situations are still possible, which would entirely obscure the observed binding reactions. The examples above are designed to simply reflect the fact that a single spectroscopic titration curve, where a spectroscopic or a physico-chemical signal is used to monitor the binding, is not by definition a thermodynamic binding isotherm (see above).

2. Thermodynamic Bases of Quantitative Equilibrium Spectroscopic Titrations Using the Spectroscopic Signal Generated From the Macromolecule

2. 1. Theory

The main task in examining the macromolecule - ligand interactions using a spectroscopic titration method is to convert the titration curve, in our discussion a fluorescence titration curve, into a model-independent, thermodynamic binding isotherm, which can then be analyzed, using an appropriate, statistical thermodynamic binding model, to extract binding parameters. First, we address the spectroscopic titrations where the observed signal (fluorescence) originates from the macromolecule [1–3,18–21,34–47]. This type of titration, where the saturation of the macromolecule increases in the course of binding experiment, is called the “normal” titration [1–6]. The thermodynamic method of the spectroscopic titration analysis relies on the fact that for the total macromolecule concentration, M_T , the equilibrium distribution of the different macromolecule states with a different number of bound ligand molecules, M_i , is a sole function the free ligand concentration, L_F [34–47]. This thermodynamic fact is independent of any binding models. Therefore, for a given L_F , the observed spectroscopic signal (fluorescence intensity), F_{obs} , is the algebraic sum of the

concentrations of all macromolecular states, M_i , weighted by the value of the corresponding intensive (concentration independent) spectroscopic property of that state, F_i . The model-independent, “signal conservation” equation for the observed fluorescence intensity, F_{obs} , at given L_T , and M_T , respectively, is given by [1–6,34–47]

$$F_{\text{obs}} = F_F M_F + \sum F_i M_i \quad (4)$$

where F_F is the molar fluorescence of the free macromolecule and F_i is the molar fluorescence of the complex, M_i , which represents the macromolecule with “i” bound ligand molecules ($i = 1$ to n). Molar fluorescence is defined as the theoretical fluorescence of 1 M solution of the fluorophore times the quantum yield and times the instrumental parameters, which specific for a given experimental setup. A general, model-independent mass conservation equation relates M_F and M_i to M_T by

$$M_T = M_F + \sum M_i \quad (5)$$

The next step is to define the partial degree of binding, Θ_i (“i” moles of ligand bound per mole of macromolecule), corresponding to all complexes with “i” bound ligand molecules as

$$\Theta_i = \frac{i M_i}{M_T} \quad (6)$$

Having Θ_i , we can define the concentration of the macromolecule with “i” ligand molecules bound, M_i , as

$$M_i = \left(\frac{\Theta_i}{i} \right) M_T \quad (7)$$

The general relationship for the observed fluorescence, F_{obs} , is then defined in terms of Θ_i and M_T , by introducing eq. 7 into eq. 4, as

$$F_{\text{obs}} = F_F M_T + \left[\sum (F_i - F_F) \left(\frac{\Theta_i}{i} \right) \right] M_T \quad (8)$$

The experimentally accessible quantity, F_{obs} , *i.e.*, the fractional fluorescence change with respect to the initial fluorescence intensity of the free macromolecule, $F_F M_T$, is obtained by rearranging eq. 8, giving

$$\Delta F_{\text{obs}} = \frac{(F_{\text{obs}} - F_F M_T)}{F_F M_T} \quad (9)$$

and

$$\Delta F_{\text{obs}} = \sum \left(\frac{\Delta F_i}{i} \right) (\Theta_i) \quad (10)$$

For a given total macromolecule concentration, M_T , F_{obs} is the experimentally determined fractional fluorescence change observed at a given total ligand concentration, L_T . The quantity, F_i/i , is the average molar fluorescence change per bound ligand in the complex

containing "i" ligand molecules. Because F_i/i is an intensive molecular property of the macromolecule - ligand complex with "i" ligand molecules bound, *i.e.*, independent of the ligand and macromolecule concentrations, eq. 10 indicates that F_{obs} is only a function of the free ligand concentration through the total average degree of binding, $\Sigma\Theta_i$. Therefore, for a given and specific value of F_{obs} , the total average degree of binding, $\Sigma\Theta_i$, and the free ligand concentration, L_F , must be the same for any value of L_T and M_T . In other words, performing a fluorescence titration of the macromolecule with the ligand, at different total macromolecule concentrations, M_T , the same value of F_{obs} at different M_T 's indicates the same physical state of the macromolecule, *i.e.*, the same $\Sigma\Theta_i$ and L_F [34–47,49].

Expression 10 is thermodynamically rigorous and independent of any binding model [8]. It also indicates a very effective method of transforming the fluorescence titration curve into a quantitative thermodynamic binding isotherm. Often, for the affinity ranges encountered in biochemical systems, only two titrations at two different total concentrations of the macromolecule, M_{T1} and M_{T2} , are enough to obtain a reliable thermodynamic binding isotherm [1–6,34–47]. Figure 2a shows two theoretical fluorescence titration curves for the hypothetical binding process where the ligand binds to two independent and identical binding sites on the macromolecule. The quantity, F_{obs} (eq. 10) is plotted as a function of the logarithm of the total ligand concentration, L_T , for two macromolecule concentrations, M_{T1} and M_{T2} , respectively, with $M_{T2} > M_{T1}$. The binding process is characterized by the intrinsic binding constant, K and the relative fluorescence changes, $F_1 = F_2 = 1$, respectively. The titration curve obtained at high macromolecule concentration, M_{T2} , is shifted toward high ligand concentration, as more ligand is required to saturate the macromolecule at its higher concentration. For the selected "j" value of $(F_{obs})_j$, one draws a horizontal line that intersects both titration curves (Figure 2a). The points of intersections of the horizontal line with each titration curve defines two values of the total ligand concentration, $(L_{T1})_j$ and $(L_{T2})_j$. Because $(F_{obs})_j$ are the same at each titration curve, the values of $(L_F)_j$ and $(\Sigma\Theta_i)_j$ are also the same (eq. 10). One arrives at two mass conservation equations for the total concentrations of the ligand, $(L_{T1})_j$ and $(L_{T2})_j$, as

$$(L_{T1})_j = (L_F)_j + (\Sigma\Theta_i)_j M_{T1} \quad (11)$$

and

$$(L_{T2})_j = (L_F)_j + (\Sigma\Theta_i)_j M_{T2} \quad (12)$$

The total average degree of binding $(\Sigma\Theta_i)_j$, and the free protein concentration, $(L_F)_j$, at a given $(F_{obs})_j$ are then

$$(\Sigma\Theta_i)_j = \frac{((L_{T2})_j - (L_{T1})_j)}{(M_{T2} - M_{T1})} \quad (13)$$

and

$$(L_F)_j = (L_{TX})_j - (\Sigma\Theta_i)_j (M_{TX}) \quad (14)$$

where subscript, X, is 1 or 2 [1–6]. The entire analysis is performed along the titration curves at a selected interval of the observed signal change to obtain model-independent values of $(L_F)_j$ and $(\Sigma\Theta_i)_j$ at any selected “j” value of $(F_{obs})_j$, and for the entire binding process [34–47].

In practice, the most accurate estimates of both $(L_F)_j$ and $(\Sigma\Theta_i)_j$ can be obtained in the region of the titration curves where the concentration of the bound ligand, L_B , is comparable to its total concentration, L_T . Therefore, the accurate determination of $\Sigma\Theta_i$ and L_F is limited to the region of the titration curves where the L_B is at least ~10 - 15% of the L_T . The accuracy of the determination of $\Sigma\Theta_i$, but not L_F , is predominantly affected in the region of the high concentrations of the ligand, *i.e.*, where the binding process approaches saturation. On the other hand, the accurate determination of L_F , but not $\Sigma\Theta_i$, is mostly affected in the initial part of the titration curves, where most of the ligand is bound and L_F constitutes a tiny fraction of L_T . Thus, selection of the suitable macromolecule concentrations is crucial for obtaining accurate estimates of $(L_F)_j$ and $(\Sigma\Theta_i)_j$ over the largest possible region of the titration curves. The selection of the macromolecule concentrations is provided by preliminary titrations and guided by the observed behavior of the system and the quality of the observed signal. Too high concentrations will allow the experimenter to obtain $\Sigma\Theta_i$ but not $[L]_F$. Too low concentrations will not provide the separation of the titration curves necessary for the quantitative analysis of the binding reaction and the low resolution of the monitored signal. In other words, if nothing is known about the examined system (usually it is but not always), then the preliminary titrations are the empirical method that will give the initial estimates of the ligand affinity and the total macromolecule concentrations necessary for the complete analysis. For instance, if the affinity is in the range of $\sim 10^6 \text{ M}^{-1}$ than total concentrations of the macromolecule in the range of $\sim 5 \times 10^{-7}$ to $\sim 5 \times 10^{-6}$ can be examined and should provide a proper set of titration curves, with macromolecular concentrations differing by factors ~2 - 3.

2. 2. Example of the Experimental Analysis Where the Fluorescence Change Accompanying the Ligand Binding Originates From the Macromolecule

The polymerase X (pol X) of the African Swine Fever Virus (ASFV) has a molecular weight of ~20000 and is currently the smallest known DNA polymerase [20,21]. The enzyme is involved in repair processes of the viral DNA in response to the host defense mechanism. Pol X is built of the palm domain, which includes the first 105 amino acids from the N-terminus of the protein and the C-terminal domain, composed of the remaining 69 amino acid residues. A puzzling problem in the DNA recognition mechanism by a DNA repair polymerase is the fact that the enzyme must recognize the damaged DNA containing a small ssDNA gap, in the context of the large excess of the dsDNA. The nature of the Pol X - gapped DNA interactions have been addressed using the DNA substrate depicted in insert in Figure 3a [20]. The substrate contains two dsDNA parts each having 10 bps. The dsDNA parts are separated by the ssDNA gap, in which one of the bases is replaced by the fluorescent marker, fluorescein. The marker provides the signal to monitor the interaction.

Fluorescence titrations of the DNA substrate, having a ssDNA gap with five nucleotides, with the ASFV pol X at two different nucleic acid concentrations, are shown in Figure 3a.

The relative quenching of the nucleic acid fluorescence, F_{obs} , reaches the plateau value, $F_{\text{max}} = 0.58 \pm 0.03$ at saturation. Nevertheless, the fluorescence titration curves alone do not provide any indication as to the maximum stoichiometry and the nature of the enzyme - gapped DNA recognition process. By the same token, the structure of the DNA substrate does not allow the experimenter to make an educated guess about the maximum stoichiometry. The total average degree of binding, $\Sigma\Theta_i$, independent of any assumption about the relationship between the observed signal and $\Sigma\Theta_i$, have been analyzed using the approach discussed above (eqs. 11 – 14) [20]. Figure 3b shows the dependence of the observed relative fluorescence quenching, F_{obs} , as a function of $\Sigma\Theta_i$ on the considered DNA substrate. The plot is, within experimental accuracy, linear. The value of $\Sigma\Theta_i$ could be determined up to ~ 1.6 , *i.e.*, in the region of the titration curves, where the concentration of the bound enzyme constitutes a significant ($\sim 10 - 15\%$) part of the total polymerase concentration (see above). However, the maximum value of the observed relative fluorescence quenching, F_{max} , is provided by the original titration curves (Figure 3a). A short extrapolation of $\Sigma\Theta_i$ to $F_{\text{max}} = 0.58 \pm 0.03$, provides a value of $\Sigma\Theta_i = 2 \pm 0.2$. Therefore, at saturation, two ASFV pol X molecules bind to the gapped DNA substrate containing 5 nucleotides in the gap [20].

Once the maximum stoichiometry is determined, further analysis of the binding reaction requires application of the statistical thermodynamic model to extract intrinsic binding parameters [1–6,20,21]. The ASFV pol X can form a high affinity gap complex and the enzyme can also associate with the dsDNA parts of the gapped DNA substrate [20]. The simplest partition function, Z_G , of the system, which describes such behavior and takes into account the determined maximum stoichiometry, is defined by

$$Z_G = 1 + (K_G + K_D)L_F + \sigma K_D^2 L_F^2 \quad (15)$$

where K_G and K_D are intrinsic binding constants characterizing the formation of the gap complex and the complex of the enzyme with the dsDNA parts of the gapped DNA substrate, respectively (Table 1). The quantity, σ , is the cooperative interactions parameter, which characterizes possible cooperative interactions between bound polymerase molecules (Table 1). The total average degree of binding, $\Sigma\Theta_i$, is then defined as

$$\Sigma\Theta_i = \frac{(K_G + K_D)L_F + 2\sigma K_D^2 L_F^2}{Z_G} \quad (16)$$

The observed relative fluorescence quenching, F_{obs} , is then

$$\Delta F_{\text{obs}} = \frac{\Delta F_G K_G L_F + \Delta F_D K_D L_F + 2\Delta F_D \sigma K_D^2 L_F^2}{Z_G} \quad (17)$$

where, F_G , and F_D are the relative molar fluorescence quenchings of the DNA emission, *i.e.*, fluorescence quenching parameters referring to the hypothetical molar fluorescence and concentrations of the considered species, characterizing the pol X binding in the gap complex and the complex with the dsDNA part [20].

There is a large number of independent parameters, F_G , F_D , K_G , K_D , and s in eq. 17. Nevertheless, some of them can be independently determined using both the parental titration curves and the plot of F as a function of $\Sigma\Theta_i$ in Figure 3b. The values of F_G can be obtained from the plot in Figures 3b, *i.e.*, $F_G = dF/d(\Sigma\Theta_i)$. The linear character of the plot in Figure 3b indicates that $F_G = F_D$. The value of F_D can be determined from the relationship, $F_{\max} = 2 \times F_D$. The solid lines in Figure 3a are nonlinear least squares fits of the experimental titration curves to eq. 17 with three fitting parameters, which provide, $K_G = (6.5 \pm 1.6) \times 10^8 \text{ M}^{-1}$, $K_D = (6.5 \pm 1.6) \times 10^8 \text{ M}^{-1}$, and $s = 1$, for the gapped DNA substrate with 5 nucleotides in the ssDNA gap. The data indicate that binding of each of the two ASFV pol X molecules to the DNA substrate containing five nucleotides in the ssDNA gap is characterized by virtually the same affinity, independent of the presumed nature of the formed complexes (see above). The profound meaning of these data is that they indicate that, with the substrate containing the ssDNA gap with five nucleotides, the ASFV pol X is not capable of forming the high-affinity gap complex to any detectable extent. The reader is referred to the original work to consult the full discussion of the biological importance of these results [20,21].

For completeness, we include the corresponding thermodynamic binding isotherm for the binding of ASFV pol X to the DNA substrate in Figure 3c. In the examined concentration range of DNA substrate the values of $\Sigma\Theta_i$ could be reliably determined from ~0.14 to ~1.6 (see above). This comprises from ~10% to ~80% of the entire range of the maximum value of $\Sigma\Theta_i = 2$ of the polymerase - DNA complex. The solid line is a theoretical plot of the statistical thermodynamic model defined by eqs. 15 – 17. The values of the binding constants, K_G , K_D , and s are, $K_G \approx 6.5 \times 10^8 \text{ M}^{-1}$, $K_D \approx 6.5 \times 10^8 \text{ M}^{-1}$, and $s \approx 1$, and within experimental accuracy, the same as those independently determined, using the quantitative fluorescence titration method described above.

3. Quantitative Equilibrium Spectroscopic Titrations Using the Spectroscopic Signal Generated From the Ligand

3. 1. Theory

Analysis of the binding processes by following the signal from the macromolecule, known as “normal” titrations, is intuitively easier than the situation where the binding reaction is monitored using the signal from the ligand. This is because in a natural way, the saturation of the macromolecule increases during the titration, as the chemical potential of the ligand increases. In a “reverse” titration, some spectroscopic property (*e.g.* fluorescence) of the ligand changes upon binding to the macromolecule. Therefore, the observed spectroscopic signal changes monitor the apparent degree of saturation of the ligand with the macromolecule. For instance, several protein - nucleic acid systems have been analyzed by examining the changes of the fluorescence of a protein titrated with a nucleic acid [2,6,7,9,10,12,13,31,33]. The quantitative approach that allowed the experimenter to transform spectroscopic titration curves into thermodynamic binding isotherms has been first developed for the “reverse” titration method in fluorescence titration studies of *E. coli* SSB protein - nucleic acid interactions [4].

For multiple-ligand binding to a macromolecule, there can be "i" states of the bound ligand, with each state possessing a different molar fluorescence, F_i . The observed fluorescence, F_{obs} , from the ligand, at the total concentration, L_T , in the presence of the macromolecule, at the total concentration, M_T , has contributions from the free ligand and the ligand bound in any of its "i" possible bound states and is defined as

$$F_{\text{obs}} = F_F L_F + \sum F_i L_i \quad (18)$$

where F_F and L_F are the molar fluorescence and concentration of the free ligand, respectively, and F_i and L_i are the molar fluorescence and concentration of the ligand bound in state "i", respectively. The mass conservation equation relates the concentrations of the free and bound ligand as

$$L_T = L_F + \sum L_i \quad (19a)$$

where

$$L_i = \Theta_i M_T \quad (19b)$$

where Θ_i , is the partial degree of binding of the ligand in the "i" state. Combining eqs. 18, 19a, and 19b, one obtains that

$$F_{\text{obs}} - F_F L_T = M_T \sum (F_i - F_F) \Theta_i \quad (20)$$

It should be noted that the product, $F_F L_T$, is the initial fluorescence of the ligand solution before titration with the macromolecule. Dividing both sides of eq. 20 by $F_F L_T$ and

multiplying by the ratio, $\left(\frac{L_T}{M_T}\right)$, provides

$$\left[\frac{(F_{\text{obs}} - F_F L_T)}{F_F L_T} \right] \left(\frac{L_T}{M_T} \right) = \sum \left[\frac{(F_i - F_F)}{F_F} \right] \Theta_i \quad (21)$$

and

$$\frac{(F_{\text{obs}} - F_F L_T)}{F_F L_T} \left(\frac{L_T}{M_T} \right) = \sum \Delta F_i \Theta_i \quad (22)$$

Notice that the quantity, $\frac{(F_{\text{obs}} - F_F L_T)}{F_F L_T} = \Delta F_{\text{obs}}$ is the experimentally observed fractional molar change in the spectroscopic signal from the ligand in the presence of the

macromolecule. The quantity, $(\Delta F)_i = \frac{(F_i - F_F)}{F_F}$, is the molar signal change characterizing the ligand bound to the macromolecule in a state "i". Therefore, eq. 22 can be written as

$$\Delta F_{\text{obs}} \left(\frac{L_T}{M_T} \right) = \sum \Delta F_i \Theta_i \quad (23)$$

Expression 23 is general and independent of the spectroscopic signal used to monitor the interactions [1,4,6,36]. It indicates that the quantity, $\Delta F_{\text{obs}} \left(\frac{L_T}{M_T} \right)$, is equal to $\sum F_i \Theta_i$, the sum of all partial degrees of binding for "i" states of the bound ligand, weighted by the molar fluorescence change for the ligand in each bound state. Furthermore, the weighting factors F_i are molecular intensive quantities, *i.e.*, independent of the ligand and macromolecule concentrations. They are constant for a particular binding state "i". Therefore, the quantity, $\sum F_i \Theta_i$, is constant for a given distribution of the ligand among different possible bound states, *i.e.*, for the given value of the total average degree of binding, $\sum \Theta_i$. Therefore, the values of L_F and $\sum \Theta_i$ are constant for a given value of

$\Delta F_{\text{obs}} \left(\frac{L_T}{M_T} \right)$, independent of L_T and M_T . Hence, one can obtain thermodynamically

rigorous estimates of $\sum \Theta_i$ and L_F from plots of $\Delta F_{\text{obs}} \left(\frac{L_T}{M_T} \right)$ vs. M_T for a series of titrations performed at different total ligand concentrations, L_T . The plots provide a set of

concentrations (L_T , M_T), from each titration, for which the quantity of $\Delta F_{\text{obs}} \left(\frac{L_T}{M_T} \right)$ is

constant. For a given "p" value of $\Delta F_{\text{obs}} \left(\frac{L_T}{M_T} \right)$, the resulting linear function is

$$(L_{Tx})_p = (L_F)_p - (\sum \Theta_i)_p (M_{Tx})_p \quad (24)$$

where the subscript $\times$ refers to the titration obtained at a given total concentration of the ligand, L_{Tx} [4,6,31,33]. Plotting the linear function defined by eq. 24 allows the

experimenter to solve for $\sum \Theta_i$ and L_F , for a given value of $\Delta F_{\text{obs}} \left(\frac{L_T}{M_T} \right)$, which is referred to as the Ligand Binding Density Function (LBDF) [4,6,31,33].

3. 2. Example of the Experimental Analysis Where the Fluorescence Change Accompanying the Ligand Binding Originates From the Ligand

The DnaB protein is the primary replicative helicase of *E. coli* [18,32–38]. The protein is a homo-hexamer, which binds six nucleotide cofactors [33,50]. We will illustrate the analysis of a multiple-ligand binding system, where the formation of the complex is monitored by the signal from the ligand, using the DnaB hexamer - etheno-ADP (eADP, etheno-adenosine 5'-diphosphate) system [33,50]. Because the fluorescence of e-ADP only increases by ~21% upon binding to the DnaB protein, acrylamide has been added to the solution to increase the signal change and the resolution of the titration experiments [33]. The induced dynamic quenching process, which does not affect the energetics of the nucleotide - protein interactions, is much less efficient for the bound than for the free nucleotide, leading to a much larger change in the e-ADP fluorescence upon formation of the complex with the DnaB protein. The reader is referred to the original work where the application of the differential dynamic quenching to increase the resolution of the binding experiments is thoroughly discussed [33].

Fluorescence titrations of ϵ ADP with the DnaB hexamer, obtained at different nucleotide concentrations, are shown in Figure 4. Once again, the spectroscopic titration curves alone do not provide any information as to the maximum stoichiometry of the formed complex, or the nature of the binding process. Moreover, the fractional change of the e-ADP fluorescence upon the DnaB protein concentration does not necessarily strictly correspond to the fractional e-ADP saturation. As pointed out above, this is never *a priori* known for any multiple-ligand binding system. The curves are shifted toward higher concentrations of the DnaB protein at higher nucleotide concentrations, as more of the enzyme is necessary to saturate the increased amount of ϵ ADP in the sample.

Figure 5 shows the plot of LBDF function, $\Delta F_{\text{obs}} \left(\frac{L_T}{M_T} \right)$, as a function of the DnaB hexamer concentration, obtained as described above (eqs. 23 and 24) using the original fluorescence titration curves included in Figure 4. It is important to notice that the reverse titrations in Figure 4 do not span the same range of the degree of binding, as seen from the LBDF plots in Figure 5. Thus, in the quantitative analysis of spectroscopic reverse titrations, multiple titrations at different values of the total ligand concentrations, L_T , are always necessary to span the full range of the total average degree of binding (Figure 4) [4,6,33]. The LBDF plots should cover as wide a range of ligand concentrations as possible. However, large changes in the total ligand concentration between two successive titrations may bias the determination of L_F and $\Sigma\Theta_i$ [4,6,33]. In our experience, 6 to 8 reverse titrations, using successive total ligand concentrations that differ by a factor of 1.5 - 2, generate an accurate set of data, *i.e.*, the lowest and the highest total macromolecule concentrations must be different by at least one order of magnitude. Such a macromolecule concentration difference is enough to obtain the binding isotherm spanning ~70–80% of the complete binding isotherm [3,4,30].

The experimenter can choose an interval of the observed titration curve, which he/she would use to construct the LBDF plot. In our practice, we initially select 5–10% intervals of the observed spectroscopic signal change [3,4,30]. If more data are necessary, then other intervals can be selected from the original titration curves. The LBDF plots are smooth functions and the experimental points can be interpolated using any suitable computer software [4,6,30]. A horizontal line that intersects the LBDF curves is drawn, defining a

given “p” constant value of $\Delta F_{\text{obs}} \left(\frac{L_T}{M_T} \right)$ (horizontal dashed line in Figure 5). The points of intersection of the horizontal line with each LBDF curve determine the set of values $((M_{Tx})_p, (L_{Tx})_p)$ for which $(L_F)_p$ and $(\Sigma\Theta_i)_p$ are constant. Using eq. 24, the values of $(\Sigma\Theta_i)_p$ and $(L_F)_p$ respectively, are then determined from the slope and intercept of a plot of $(L_{Tx})_p$ vs. $(M_{Tx})_p$. The procedure is repeated for a series of values of $\Delta F_{\text{obs}} \left(\frac{L_T}{M_T} \right)$, providing the average total degree of binding $\Sigma\Theta_i$ as a function of L_F .

The thermodynamic binding isotherm for the binding of ϵ ADP to the DnaB hexamer is shown in Figure 6. In the examined concentration range of ϵ ADP, the values of $\Sigma\Theta_i$ could be reliably determined from ~0.5 to up to the value of ~4.5. This comprises from ~10% to ~75% of the entire range of the maximum value of $\Sigma\Theta_i = 6$ of the DnaB hexamer -

nucleotide complex [33,50]. The solid line is a theoretical plot of the statistical thermodynamic model, which describes the binding of six ligands to a short circular lattice of six identical discrete sites, characterized by the intrinsic binding constant, K , and cooperativity parameter, σ (see below) (Table 1) [33]. The values of K and s are, within experimental accuracy, the same as those independently determined, using the quantitative fluorescence titration method in which the quenching of the DnaB fluorescence has been used to monitor the complex formation [50].

4. Complex Titration Curves Analyzed Using the Empirical Function Approach

A multiple-ligand binding thermodynamic isotherm can be fitted to extract binding parameters using a statistical thermodynamic model without any considerations as to the specific values of molar spectroscopic signals originating from various complexes [1–7,9,10,12,34–47]. In the case of the parental spectroscopic titration curves, the situation is much more difficult because spectroscopic parameters of all possible complexes enter the binding model. The reason for fitting the parental spectroscopic titration curve is that it spans the entire range of the total average degree of binding, while a reliable determination of $\Sigma\Theta_i$ is usually limited to the range between ~10% to 85% of the maximum stoichiometry of the complex (see above). In other words, fitting the spectroscopic titration curve provides more accurate estimate of the binding parameters. Moreover, it is valuable to have a quantitative method which would allow us to address original titration curves without resorting every time to the time-consuming, general thermodynamic analysis. Below, we discuss a method that allows the experimenter to fit any spectroscopic titration curves for a given statistical thermodynamic binding model, for the situations where the monitored signal originates from the macromolecule. Using this approach, the impossible task of determining all spectroscopic parameters characterizing the complex interacting system is avoided [1,48].

To address the direct fitting of the spectroscopic titration curves, one needs both original titration curves and the plot of the observed relative change of the spectroscopic signal (e.g., fluorescence) as a function of the total average degree of binding. We will illustrate the method based on the binding of the ATP analog, TNP-ATP (2',3'-O-(2,4,6-trinitrophenyl)adenosine 5'-triphosphate) to the *E. coli* primary replicative helicase, the DnaB protein homo-hexamer [48]. Fluorescence titrations of the DnaB hexamer with TNP-ATP, at two different protein concentrations are shown in Figure 7a. The maximum quenching of the protein fluorescence at saturation is 0.76 ± 0.03 . The quantitative thermodynamic approach as outlined above (eqs. 12 - 14), allowed us to determine the total average degree of the binding, $\Sigma\Theta_i$, and the free TNP-ATP concentration, L_F (see above) [48]. The dependence of the observed fluorescence quenching, F_{obs} , upon the total average degree of binding, $\Sigma\Theta_i$, of TNP-ATP on the DnaB hexamer is shown in Figure 7b. The separation of the binding isotherms allows us to obtain the values of $\Sigma\Theta_i$ up to ~5.1 of the TNP-ATP molecules per DnaB hexamer. The plot of $F_{obs}(\Sigma\Theta_i)$ in Figure 7b is clearly nonlinear, with the binding of the first three nucleotide cofactors inducing a significantly larger relative quenching of the protein fluorescence than the remaining three cofactor

molecules. Any fitting of the parental titration curves with the assumption of the strict proportionality between F_{obs} and $\Sigma\Theta_i$ would result in a significant error in the determined binding parameters. For comparison, we have included the dashed line, which represents the hypothetical case when a strict proportionality between $\Sigma\Theta_i$ and the quenching of the DnaB protein fluorescence, F_{obs} , would exist.

From a statistical thermodynamic point, the DnaB homo-hexamer with the ring-like structure is a short circular lattice of six identical binding sites and can be described using a hexagon model with only two interaction parameters, the intrinsic binding constant, K , and the nearest-neighbor cooperativity parameter, σ for the nucleotide binding (Table 1) [48]. The partition function (binding polynomial) for the system (Table 1) is

$$Z_H = 1 + 6x + 3(3 + 2\sigma)x^2 + 2(1 + 6\sigma + 3\sigma^2)x^3 + 3(3\sigma^2 + 2\sigma^3)x^4 + 6\sigma^4x^5 + \sigma^6x^6 \quad (25)$$

The total average degree of binding, $\Sigma\Theta_i$, is

$$\Sigma\Theta_i = \frac{6x + 6(3 + 2\sigma)x^2 + 6(1 + 6\sigma + 3\sigma^2)x^3 + 12(3\sigma^2 + 2\sigma^3)x^4 + 30\sigma^4x^5 + 6\sigma^6x^6}{Z_H} \quad (26)$$

where $x = KL_F$, stands for the product of the intrinsic binding constant, K , and the free nucleotide concentration, L_F . The dependence of $\Sigma\Theta_i$ upon the logarithm of the free TNP-ATP concentration, *i.e.*, the thermodynamic isotherm of the examined system, is shown in figure 8 [48]. The solid line is the nonlinear least squares fit the isotherm to the hexagon model (eq. 26), with the intrinsic binding constant $K = (5.9 \pm 1) \times 10^5 \text{ M}^{-1}$ and $\sigma = 0.55 \pm 0.05$. As discussed above, the fit did not require any considerations of the spectroscopic parameters of different DnaB - TNP-ATP complexes.

In the case of the parental titration curves, one must consider that the DnaB hexamer with a given number of nucleotide molecules bound, can exist in multiple configurations, *e.g.*, the hexamer with four ligands bound has sixty possible configurations (eq. 25). Although some of these configurations are physically indistinguishable, resulting from simple statistical effects, there are configurations which differ by the number of cooperative interactions and they may also differ by the values of the individual molecular spectroscopic parameters, F_{ij} (Figure 9). For example, the minimum analytical relationship between F_{obs} and the molar spectroscopic parameters, F_{ij} , and the interaction parameters, K and σ , is

$$\begin{aligned} \Delta F_{\text{obs}} = & (6(\Delta F_{11}x \\ & + 6(3\Delta F_{21} \\ & + 2\sigma\Delta F_{22})x^2 + 6(\Delta F_{31} \\ & + 6\sigma\Delta F_{32} \\ & + 3\sigma^2\Delta F_{33})x^3 \\ & + 12(3\sigma^2\Delta F_{41} \\ & + 2\sigma^3\Delta F_{42})x^4 \\ & + 30\sigma^4\Delta F_{51}x^5 \\ & + 6\sigma^6\Delta F_{61}x^6)/Z_H \end{aligned} \quad (27)$$

Expression 27 contains ten spectroscopic parameters. To obtain interaction parameters, K and σ , from a single spectroscopic titration curve, all ten optical constants, F_{ij} , must be known. In practice, this is a hopeless task. The empirical function approach circumvents this problem.

The approach is based on using the numerical representation of the observed change in the spectroscopic signal from the macromolecule, F_{obs} , as a function of the total average degree of binding, $\Sigma\Theta_i$, via an empirical function [48,50]. High resolution, quantitative determination of $\Sigma\Theta_i$ is essential for this approach to succeed. The simplest empirical function is a polynomial that relates the experimentally determined dependence of the spectroscopic parameter, F_{obs} , to the experimentally determined, total average degree of binding, $\Sigma\Theta_i$, defined as

$$\Delta F_{obs} = \sum_{j=0}^n a_j (\Sigma\Theta_i)^j \quad (28)$$

where a_j are the fitting constants. This function is then used to generate/fit a theoretical spectroscopic titration curve for the selected, statistical thermodynamic binding model and to extract intrinsic binding parameters for that model, from the experimental single titration curve. The procedure is the following. First, one calculates the value of $\Sigma\Theta_i$ for a given free ligand concentration and initial estimates of the binding parameters of the statistical thermodynamic model. Second, the obtained $\Sigma\Theta_i$ is introduced into eq. 28 and F_{obs} is calculated, corresponding to a given value of $\Sigma\Theta_i$. The analysis is then performed for the entire titration curve.

For the TNP-ATP binding to the DnaB hexamer, the plot of the observed relative fluorescence quenching, F_{obs} , as a function of $\Sigma\Theta_i$ in Figure 7b is described by a third-degree polynomial with the coefficients $a_1 = 0.286$ and $a_2 = 0.0458$, and $a_3 = 3.194 \times 10^{-3}$, as

$$\Delta F_{obs} = 0.286(\Sigma\Theta_i) + 0.0458(\Sigma\Theta_i)^2 + 3.194 \times 10^{-3}(\Sigma\Theta_i)^3 \quad (29)$$

This function is then used to fit and/or generate theoretical titration curves of the TNP-ATP binding to the DnaB protein and to extract intrinsic binding constant, K , and cooperativity parameter, σ (eqs. 25 and 26). The solid lines in Figure 7a are the nonlinear least squares fits of the experimental fluorescence titration curves for TNP-ATP binding to the DnaB hexamer, using eqs. 25, 26, and eq. 29, with a single set of binding parameters, that provide the intrinsic binding constant $K = (5.9 \pm 1) \times 10^5 \text{ M}^{-1}$ and $\sigma = 0.55 \pm 0.05$, i.e., as expected, the binding parameters are identical to those obtained using the true binding isotherm in Figure 8 [48,50].

Conclusions

Elucidation of ligand - macromolecule interactions requires detailed knowledge of the energetics and kinetics of the formed complexes and spectroscopic methods are the most widely used in characterizing the ligand - macromolecule interactions in solution. However, spectroscopic methods are in general indirect and address the complex formation through monitoring changes of some physico-chemical (spectroscopic) parameter. Therefore, it is

imperative that the relationship between the observed signal and the degree of binding is determined in order to obtain thermodynamically and kinetically meaningful parameters. The discussed methods address general quantitative approaches, which allow an experimenter to quantitatively determine the degree of binding and the free ligand concentration and to obtain the *thermodynamically rigorous* binding isotherms. Once the thermodynamically isotherm is constructed, it can be addressed using statistical thermodynamic models, which incorporate molecular aspects of the ligand - macromolecule interactions, like cooperativity, allosteric conformational changes, overlap of potential binding sites, *etc.* The methods have been discussed, in the context of protein - nucleic acid interactions, using the fluorescence intensity to monitor the binding. However, these methodologies can generally be applied to any ligand - macromolecule system, studied using the spectroscopic signal originating from a macromolecule or a ligand.

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Highlights

1. Spectroscopic methods rely on spectroscopic property responses to complex formations.
2. In general, spectroscopic titration curves are not thermodynamic binding isotherms.
3. The examined spectroscopic property should be related to the true degree of binding.
4. Methods to obtain true degree of bindings in spectroscopic titrations are discussed.
5. Described methodologies are particularly useful for multiple-ligand binding processes.

Figure 1.

a. Theoretical fluorescence titration curve of a macromolecule (points), containing four identical independent binding sites, with the ligand, where the monitored fluorescence change originates from the macromolecule. Macromolecule concentration is 5×10^{-7} M (tetramer). The partition function of the binding system, used to generate the curve, is $Z_T = 1 + 4KL_F + 6K^2L_F^2 + 4K^3L_F^3 + K^4L_F^4$. The binding is accompanied by the same relative fluorescence increase $F_{\max} = 0.5$ for each site, *i.e.*, the observed spectroscopic signal is proportional to the total average degree of binding, $\Sigma\Theta_i$. The solid line is the continuous

simulation of the same titration curve with the intrinsic binding constant, $K = 1 \times 10^6 \text{ M}^{-1}$. The dashed line is the computer fit of the same theoretical titration curve using the single-site binding model (eq. 2), with $K = 4.8 \times 10^5 \text{ M}^{-1}$ (details in text). **b.** The same theoretical fluorescence titration curve (points) of a macromolecule containing four identical independent binding sites with the ligand, where the monitored fluorescence change originates from the macromolecule as in panel a, but recorded at the macromolecule concentration of $4 \times 10^{-6} \text{ M}$ (tetramer). The solid line is the simulation of the titration curve with the intrinsic binding constant, $K = 1 \times 10^6 \text{ M}^{-1}$. The dashed line is the computer fit of the titration curve using the binding model of two identical cooperative sites, with $K = 1.27 \times 10^5 \text{ M}^{-1}$ and cooperativity parameter $s = 2.7$ (eq. 3) (details in text).

Figure 2.

Theoretical fluorescence titrations of a macromolecule with a ligand, obtained at two different macromolecule concentrations, (■) $M_1 = 5 \times 10^{-7} \text{ M}$; (□) and $M_2 = 4 \times 10^{-6} \text{ M}$, respectively. The macromolecule has two identical independent binding sites characterized by the intrinsic binding constant, $K_1 = 1 \times 10^6 \text{ M}^{-1}$. The partition of the system, Z_D , is then $Z_D = 1 + 2KL_F + K^2L_F^2$. The relative fluorescence change accompanying binding of each ligand molecule is $F_1 = F_2 = 1$. The arrows indicates the total ligand concentrations, L_{T1} and L_{T2} , at the selected value of the observed relative fluorescence change, $(F_{\text{obs}})_j$, marked by the dashed horizontal line, at which the total average degree of binding, $\Sigma\Theta_i$, and the free ligand concentration, L_F , are the same for both titration curves.

Figure 3.

a. Fluorescence titrations ($\lambda_{\text{ex}} = 495 \text{ nm}$, $\lambda_{\text{em}} = 520 \text{ nm}$) of the gapped DNA substrate with five nucleotides in the ssDNA gap (insert) with the ASFV pol X at two different concentrations of the nucleic acid, $5 \times 10^{-9} \text{ M}$ (■) and $1.2 \times 10^{-8} \text{ M}$ (□), respectively [20]. The solid lines are the nonlinear least squares fits of the experimental titration curves to the model of two distinct cooperative binding sites (eq. 17), using a single set of binding and spectroscopic parameters, $K_G = 6.5 \times 10^8 \text{ M}^{-1}$, $K_D = 6.5 \times 10^8 \text{ M}^{-1}$, $s = 1$, $F_G = 0.29$, and $F_D = 0.29$ (details in text). **b.** The dependence of the observed relative fluorescence

quenching, F_{obs} , upon the total average degree of binding, $\Sigma\Theta_i$, of the ASFV pol X on the DNA substrate with five nucleotide residues in the ssDNA gap. The solid line follows the experimental points and does not have a theoretical basis. The dashed line is an extrapolation of the plot to the maximum observed fluorescence quenching, F_{max} , marked by the solid horizontal line. Reprinted with permission from ref. 20 (details in text) [48]. **c.** The dependence of the total average degree of the binding, $\Sigma\Theta_i$, upon the logarithm of the free ASFV pol X, *i.e.*, the true thermodynamic binding isotherm of the ASFV- gapped DNA system (details in text).

Figure 4.

Fluorescence titrations of ϵ ADP at different concentrations of the nucleotide with the DnaB protein ($\lambda_{\text{ex}} = 325 \text{ nm}$, $\lambda_{\text{em}} = 410 \text{ nm}$) [33]. The concentrations of the nucleotide are: (■) $4 \times 10^{-6} \text{ M}$; (◆) $6 \times 10^{-6} \text{ M}$; (○) $1 \times 10^{-5} \text{ M}$; (○) $2 \times 10^{-5} \text{ M}$; (□) 3×10^{-5} ; (●) $4 \times 10^{-5} \text{ M}$. Solid lines are nonlinear least squares fits of the experimental titration curves, according to the hexagon model, using a single set of binding parameters, $K = 4 \times 10^5 \text{ M}^{-1}$, $\sigma = 0.4$, and $F_{\text{max}} = 2.35$ (details in text). Reprinted with permission from ref. 33.

Figure 5.

Dependence of the binding density function, $F_{obs}(L_T/M_T)$, on the logarithm of the total DnaB protein concentration at different concentrations of ϵ ADP: (■) 4×10^{-6} M; (○) 6×10^{-6} M; (●) 1×10^{-5} M; (□) 2×10^{-5} M; (◐) 3×10^{-5} ; (◆) 4×10^{-5} M [33]. Solid lines separate different data sets and do not have theoretical basis. Horizontal dashed line connects points at the same value of the binding density function, at different ϵ ADP concentrations, at which $[\epsilon \text{ ADP}]_{Free}$ and the total average degree of binding, $\Sigma \Theta_i$, of the nucleotide on the DnaB hexamer are the same (details in text). Reprinted with permission from ref. 33.

Figure 6.

The dependence of the total average degree of binding of ϵ ADP on the DnaB hexamer, upon the logarithm of the free nucleotide concentration, $\log[\epsilon \text{ ADP}]_{\text{Free}}$ [33]. The solid line is the theoretical binding isotherm, according to the hexagon model, using intrinsic binding constant $K = 4 \times 10^5 \text{ M}^{-1}$ and cooperativity parameter $\sigma = 0.4$. Reprinted with permission from ref. 33.

Figure 7.

a. Fluorescence titration of the DnaB hexamer ($\lambda_{\text{ex}} = 300 \text{ nm}$, $\lambda_{\text{em}} = 345 \text{ nm}$) with TNP-ATP, monitored by the quenching of the intrinsic protein fluorescence at two different concentrations of the DnaB hexamer: (●) $5.38 \times 10^{-7} \text{ M}$; (○) $3.17 \times 10^{-6} \text{ M}$ [48]. Solid lines are the nonlinear least squares fits of the experimental titration curves to the hexagon model, using the empirical function method (details in text). **b.** The dependence of the relative fluorescence quenching of the DnaB protein fluorescence, F_{obs} , upon the average number of TNP-ATP molecules bound per DnaB hexamer, $\Sigma \Theta_i$. The values of $\Sigma \Theta_i$ have been

determined using two titrations shown in figures 7a. The dashed line represents the theoretical situation when the strict proportionality between $\Sigma\Theta_i$ and the quenching of the DnaB protein fluorescence existed. Solid line is generated using the empirical polynomial function as defined by eq. 29. Reprinted with permission from ref. 48.

Figure 8.

The dependence of the total average degree of the binding, $\Sigma\Theta_i$, upon the logarithm of the free TNP-ATP, *i.e.*, the true thermodynamic binding isotherm of the TNP-ATP - DnaB hexamer system (details in text) [48]. Reprinted with permission from ref. 48.

Figure 9.

Schematic representation of the DnaB hexamer with three sites occupied by the ligand molecules and with different numbers of cooperative ligand-ligand interactions in different protein - ligand configurations; (a) configuration where ligand molecules are separated by the hexamer subunits and do not engage in cooperative interactions, (b) configuration where two ligand molecules are engaged in cooperative interactions, (c) configuration where three ligand molecules engaged in cooperative interactions.

Table 1

Elementary Definitions

Macroscopic Binding Constant	binding constant obtained without considering possible statistical and cooperativity effects in the examined binding process.
Intrinsic Binding Constant	binding constant characterizing interactions between the ligand and the binding site after all statistical or cooperative interactions have been accounted for.
Allosteric Equilibrium Constant	equilibrium constant characterizing conformational changes of the macromolecule existing <i>prior</i> to the ligand binding and affecting the ligand-binding process
Independent Binding Sites	binding sites without any cooperative interactions among them
Discrete Binding Sites	binding sites constituting separate chemical entities and interacting with one ligand molecule at a time
Overlapping Potential Binding Sites	binding sites, which do not constitute a separate chemical entity, <i>e.g.</i> , on a linear nucleic acid polymer, and appear or disappear depending on the available number of monomers of the polymer
Cooperativity	interactions between or among bound ligand molecules, or their binding sites, or both on the macromolecule
Direct Binding Methods	methods that rely on direct determination of the thermodynamic degree of binding
Indirect Binding Methods	methods like spectroscopic techniques, which examined the behavior of a physico-chemical parameter in response to the ligand binding process
Different Binding Modes	possible different conformational states of the ligand bound to the macromolecule with different thermodynamic properties
Binding Isotherm	direct dependence of the thermodynamic degree of binding upon the ligand concentration
Spectroscopic Titration Curve	dependence of the observed spectroscopic signal upon the ligand or macromolecule concentration
Normal Titration	titration where the macromolecule saturation with the ligand is monitored.
Reverse Titration	titration where the ligand saturation by the macromolecule is monitored
Partition function	a sum of thermodynamic terms (statistical weights), which in binding studies are functions of binding parameters and the free ligand concentration, and which are proportional to the probability of a given macromolecular states associated with the ligand
Binding polynomial	the partition function of the macromolecule - ligand system