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Quantitative analysis of ligand–macromolecule interactions using differential dynamic quenching of the ligand fluorescence to monitor the binding [1]

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Abstract

Quantitative analyses of the thermodynamics and kinetics of ligand–macromolecule interactions in biological systems rely predominately on monitoring changes in the spectroscopic properties of the ligand or macromolecule, particularly fluorescence changes, which accompany the formation of the studied complexes. However, in many instances the interactions do not affect the fluorescence properties of interacting species and do not provide a resolution high enough to perform quantitative and rigorous measurements of the thermodynamic and/or kinetic parameters. In this communication, we describe the theoretical and experimental aspects of a method of studying complex, multiple ligand–macromolecule interactions by the fluorescence titration technique, when the intrinsic fluorescence changes accompanying binding do not provide a resolution necessary to perform quantitative analyses. The method is based on the fact that a fluorescent ligand, or binding sites of the macromolecule, can have different accessibility to the collisional (dynamic) quencher, when involved in the complex, rather than in the free, unbound state. The presence of an external dynamic quencher in solution, i.e., the presence of an extra collisional quenching process, transforms the fluorescence of the ligand or macromolecule, intrinsically independent of the complex formation, into a property which is dramatically different in the free state than in the bound state of the fluorophore. The approach is applicable to any model of noncooperative or cooperative ligand binding to a macromolecule and allows for the optimization of the resolution of the binding or kinetic studies for a given ligand–macromolecule system. The application of the method is illustrated by applying it to the study of the binding of the fluorescent derivative of a nucleotide cofactor, εADP, to the six interacting sites of the *E. coli* primary replicative helicase DnaB protein hexamer.

Keywords: Model-independent isotherms; Macromolecular binding; Helicases; Fluorescence quenching

1. Introduction

Any method used to study quantitatively the equilibrium aspects of ligand binding to a macromolecule must relate the extent of the complex formation to the free ligand concentration in solution. Numerous

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techniques have been developed to study equilibrium properties of specific and nonspecific ligand–macromolecule interactions in which binding is directly monitored, including equilibrium dialysis, ultrafiltration, column chromatography, filter binding assay, and gel electrophoresis [1–5]. These direct methods are very straightforward; however, they are usually time-consuming and some, like filter binding or gel shift assays, are nonequilibrium techniques which require many controls before the reliable equilibrium binding data can be obtained. Therefore, these direct methods are usually applied to systems where the indirect spectroscopic approaches cannot be used due to the lack of suitable signal changes accompanying the formation of the complex.

Using indirect methods, the binding of the ligand is determined by measuring a physicochemical parameter of the macromolecule–ligand mixture, most often a spectroscopic one, e.g., absorbance or fluorescence. The change in the physicochemical parameter is then correlated with the concentration of the free and bound species. The advantages of using spectroscopic measurements are that these can be performed without perturbing the equilibrium and are relatively easy to apply.

Fluorescence spectroscopy appears to be a particularly useful method for studying macromolecular binding in biological systems [3,6–12]. This very sensitive technique allows the experimenter to follow the binding in a range below micromolar concentration, where the application of limiting thermodynamic relationships is fully justified and the requirement for the studied material is very low. Many biologically important compounds are intrinsically fluorescing, e.g., proteins, enzyme substrates, cofactors, etc. Upon forming complexes, these compounds experience strong changes in their fluorescence properties. However, there are many ligand–macromolecule systems where, in spite of strong intrinsic fluorescence, either there is no fluorescence change of the ligand and/or the macromolecule accompanying the binding or the change is too small to be adequate to accurately determine the thermodynamic and kinetic properties of the interacting system.

In this communication, we present a method for studying ligand–macromolecule interactions by a fluorescence titration technique which is applicable when the fluorescence change accompanying the binding is very low or non-existent, and where the fluorescence change originates from the ligand or the macromolecule. The method utilizes the fact that, in spite of a low or no fluorescence change accompanying the binding, fluorescent ligand or fluorescent residues of the macromolecule will have different accessibility to the collisional quencher when engaged in the complex than when in the free state. In such a case, the addition of the collisional quencher to the sample will induce a difference in the fluorescence of the bound and free fluorophore, thus providing the necessary signal change to monitor the binding and perform quantitative analyses of the interactions.

We show that this approach is applicable to any model of multiple cooperative or noncooperative ligand binding to the macromolecule and provides the experimenter with a method to select the optimal conditions to obtain a high-resolution binding isotherm for a given ligand–macromolecule system. We also show that, once such optimal conditions are found, one can apply the same general method of analysis to obtain absolute stoichiometries and interaction parameters, as in the cases where binding is accompanied by a change in the ligand fluorescence, as a result of an intrinsic property of the interacting system [10,13–21].

2. Materials and methods

2.1. Reagents and buffers

All chemicals were reagent grade; εADP, from Sigma, was used without further purification. All solutions were made with distilled and deionized 18 MΩ (Milli-Q) water. The standard buffer (T3) was 50 mM Tris adjusted to pH 8.1 at appropriate temperatures with HCl, 5 mM $MgCl_2$, 25% glycerol. The temperatures and the concentrations of NaCl in the buffer are indicated in the text.

2.2. DnaB protein

The *E. coli* DnaB protein was purified as previously described by us [13]. The concentration of the protein was determined spectrophotometrically, using the extinction coefficient $\varepsilon_{280} = 1.85 \times 10^5\ cm^{-1}\ M^{-1}$ (hexamer) [18,19].

2.3. Steady-state fluorescence measurements and binding analysis

All steady-state fluorescence measurements were performed using the SLM 48000S spectrofluorometer as we previously described [13,16]. Analyses of the isotherms of the εADP binding to the DnaB hexamer have been performed according to the hexagon model which uses only two interaction parameters, the intrinsic binding constant, K, and cooperativity parameter, σ, to describe the nucleotide binding process and is defined by the following [13–15]

$$(\Sigma v_i)_H = \big(6x + 6(3+2\sigma)x^2 + 6(1+6\sigma+3\sigma^2)x^3 + 12(3\sigma^2+2\sigma^3)x^4 + 30\sigma^4x^5 + 6\sigma^6x^6\big)/Z_H \quad (1a)$$

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

where $(\Sigma v_i)_H$ is the average number of the nucleotide bound to DnaB hexamer, and $x = K[L_F]$ and $[L_F]$ is the free nucleotide concentration. The quantity Z_H is the partition function of the DnaB hexamer–nucleotide system [13].

3. Results and discussion.

3.1. General analysis of fluorescence titrations of a ligand with the macromolecule in the presence of a collisional quencher

3.1.1. Effect of the collisional quencher on the fluorescence of a ligand, both free and bound to the macromolecule

When the fluorescent ligand molecule is excited by incident light, it can return to its ground state, via either fluorescence or various radiationless processes (quenching). The steady-state intensity of fluorescence of the molecule, F^o, is a function of the competing rates of these processes and is defined by [22,23]

$$F^o = \frac{k_f}{k_f + \Sigma k_i} = k_f \tau_o \quad (2)$$

where k_f is the rate constant of fluorescence emission, Σk_i is the sum of the rate constants of all radiationless processes which participate in depopulation of the excited state, and $\tau_o = (1/k_f + \Sigma k_i)$ is the excited-state lifetime of the fluorophore.

When a collisional quencher, Q, is present in solution, an additional dynamic quenching process appears and the steady-state fluorescence of the fluorophore, F_q, is described by

$$F_q = \frac{k_f}{k_f + \Sigma k_i + k_q[Q]} \quad (3)$$

where k_q is the bimolecular quenching constant and [Q] is the concentration of the collisional quencher. Dividing Eq. (2) by Eq. (3) one obtains the Stern–Volmer equation [22,24], which is a convenient way of presenting the effect of the added collisional quencher on the fluorescence of the studied molecule

$$\frac{F^o}{F_q} = 1 + k_q\tau_o[Q] = 1 + K_D[Q] \quad (4)$$

where $K_D = k_q\tau_o = \left[\frac{k_q}{k_f + \Sigma k_i}\right]$ is the Stern–Volmer quenching constant [22].

Eqs. (2)–(4) describe the effect of the collisional quencher on the fluorescence of the ligand molecule free in solution. The situation is more complicated when the ligand forms a complex with the macromolecule, where it can exist in multiple bound states, differing in the accessibility to the quencher. Let us consider the situation where the fluorescent ligand binds to the macromolecule (protein or nucleic acid) and the complex formation is not accompanied by any change in the fluorescence of the ligand. However, if the accessibility of the ligand molecule to the added external collisional quencher, Q, is limited in the complex, when compared to the free ligand in solution, the quenching efficiency of the fluorescence of the ligand, at a given quencher concentration, [Q], in the complex with the macromolecule will be lower.

As we pointed out, in the general case, multiple ligand molecules can bind to a macromolecule. The observed fluorescence of the ligand, F_{obs}, in the presence of the macromolecule but in the absence of the dynamic quencher, has contributions from the free ligand, F_F, and the ligand bound to the macromolecule in all possible j bound states. Thus, the fluorescence of the ligand in the presence of the macromolecule, but in absence of the quencher Q, is described by

$$F^{o}_{obs} = F_F L_F + \Sigma F_j L_j \quad (5)$$

where F^{o}_{obs} is the observed fluorescence of the ligand in solution, in the presence of macromolecule, F_F and L_F are the molar fluorescence and the concentration of the free ligand, F_j and L_j are the molar fluorescence and the concentration of the ligand bound in state j, respectively. Because there is no change in the ligand fluorescence upon complex formation ($F_F = F_j$), the molar fluorescence intensities, F_F and F_j can be described in terms of emission and quenching rate constants by

$$F_F = F_j = \frac{k_f}{k_f + \Sigma k_i} \quad (6)$$

Introducing Eq. (6), for F_F and F_j, into Eq. (5), one obtains

$$F^{o}_{obs} = \left[\frac{k_f}{k_f + \Sigma k_i}\right] L_F + \left[\frac{k_f}{k_f + \Sigma k_i}\right] \Sigma L_j \quad (7)$$

Eq. (7) states that the fluorescence of the ligand is independent of the degree of saturation with the macromolecule and is valid when the molar fluorescence of the ligand in solution is independent of its concentration, e.g., in the absence of the ligand and/or macromolecule aggregation.

If the dynamic quencher is present in solution, it will quench, but in a different manner, the fluorescence of the free ligand and the ligand bound to the macromolecule in all possible j bound states. Therefore, in the presence of the dynamic quencher, at concentration [Q], the fluorescence of the ligand–macromolecule mixture is defined by

$$F_{obs} = F_{Fq} L_F + \Sigma F_{jq} L_j \quad (8a)$$

where

$$F_{Fq} = \frac{k_f}{k_f + \Sigma k_i + k_q[Q]} \quad (8b)$$

and

$$F_{jq} = \frac{k_f}{k_f + \Sigma k_i + k_{jq}[Q]} \quad (8c)$$

The quantities F_{Fq} and F_{jq} are the molar fluorescence of the free ligand and the ligand bound to the protein in a particular state, j, in the presence of the collisional quencher at concentration [Q]. The parameters, k_q and k_{jq}, are the bimolecular quenching constants for the free ligand and the ligand bound to the macromolecule, in a particular state, j.

The resolution of the binding experiment depends on the difference between the observed fluorescence of a free and bound ligand. Thus, the larger the difference between F_{Fq} and F_{jq} in Eqs. (8a), (8b) and (8c), the more accurate the binding isotherms that can be obtained. The effect of the collisional quencher on the fluorescence of the ligand, free and bound to the macromolecule, can be determined using the Stern–Volmer analysis [24]. For simplicity, we consider the binding of the ligand to two independent classes of binding sites on the macromolecule, with each class having three binding sites. The affinities for the first and second classes of binding sites are characterized by intrinsic binding constants, K_1 and K_2, respectively. Thus, in the presence of the macromolecule, the ligand exists in solution in three different states: free, bound to the first class, and bound to the second class. It should be stressed that the analysis presented below is independent of the model and is valid for any cooperative, as well as noncooperative, binding system.

In the most general case, the ligand, free and bound in two different classes of binding sites, will have different Stern–Volmer quenching constants, K_{Df}, K_{Db1}, and K_{Db2}, respectively. The Stern–Volmer relationship for the considered model is given by

$$\frac{F^{o}_{obs}}{F_{obs}} = \frac{L_T(1 + K_{Df}[Q])(1 + K_{Db1}[Q])(1 + K_{Db2}[Q])}{L_F(1 + K_{Db2}[Q])(1 + K_{Db2}[Q]) + L_{B1}(1 + K_{Df}[Q])(1 + K_{Db2}[Q]) + L_{B2}(1 + K_{Df}[Q])(1 + K_{Db2}[Q])} \quad (9)$$

where L_T is the total ligand concentration (the derivation of Eq. (9) is given in Appendix A).

A series of theoretical Stern–Volmer plots for the quenching of the ligand fluorescence at a different degree of saturation of the ligand with the macromolecule is shown in Fig. 1. The degree of the ligand saturation is defined as L_B/L_T, the ratio of the concentration of the bound ligand, L_B, to the total ligand concentration L_T. In the considered case, the Stern–Volmer quenching constants for the ligand bound to macromolecules, K_{Db1} and K_{Db2}, are lower than the quenching constants for the free ligand, K_{Df} ($K_{Df} = 30\ M^{-1}$, $K_{Db1} = 15\ M^{-1}$, $K_{Db2} = 5\ M^{-1}$). Thus, the plot with the highest slope characterizes the quenching of the fluorescence of the ligand at a very low degree of saturation with the macromolecule. As the saturation of the ligand increases, the slope of the Stern–Vomer plot decreases. Finally, the slope reaches the lowest constant value characteristic for the ligand which is virtually completely saturated with the macromolecule. This slope is a function of the quenching constants and the distribution of the ligand molecule on the macromolecule between two classes of binding sites (see below).

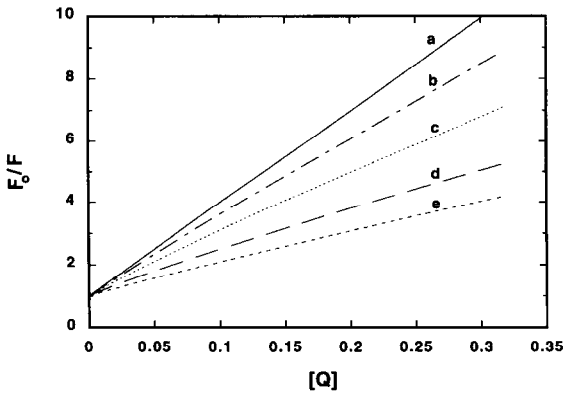

Fig. 1. Theoretical Stern–Volmer plots of the quenching of the fluorescence of the ligand by the collisional quencher, [Q], at a different degree of the ligand saturation with the macromolecule, (L_B/L_T) and (L_B and L_T, are the bound and total ligand concentrations, respectively). The binding of the ligand is described by two independent classes of binding sites, each containing three sites, characterized by two intrinsic binding constants $K_1 = 10^6\ M^{-1}$ and $K_2 = 10^5\ M^{-1}$ (see text for details). The Stern–Volmer quenching constants for the ligand, free and bound to the first and second classes of binding sites, are $K_{Df} = 30\ M^{-1}$, $K_{Db1} = 15\ M^{-1}$, and $K_{Db2} = 5\ M^{-1}$, respectively. The values of the degree of the ligand saturation (L_B/L_T) are: (a) 0.003; (b) 0.076; (c) 0.22; (d) 0.51; (e) 0.99.

Stern–Volmer plots provide an initial estimate of the resolution of the binding experiment. The difference between the fluorescence of the free ligand and of ligand bound to the macromolecule can be increased by increasing the concentration of the collisional quencher in the sample (Fig. 1 and Eqs. (8a), (8b) and (8c)). The computer-simulated fluorescence titrations of the ligand with the macromolecule, in the presence of different concentrations of the collisional quencher, are shown in Fig. 2. The Stern–Volmer quenching constants are the same as in Fig. 1. Progressive addition of the macromolecule to the solution containing the ligand, in the presence of the collisional quencher, results in an increase in the fluorescence of the sample as more ligand molecules are bound in the complex. This fluorescence increase is due to the fact that by increasing the concentration of the macromolecule, the population of the bound ligand molecules, whose fluorescence is quenched to a lower extent, is increasing, while, at the same time, the population of the free ligand molecules, more efficiently quenched by the quencher, is decreasing. Also, at higher quencher concentrations, [Q], a larger relative increase in the fluorescence of the ligand, compared to the free ligand, is observed, resulting from the larger difference between the fluorescence of the free and bound ligand at a higher [Q]. Clearly, titrations which are performed at higher collisional quencher concentrations provide higher resolution binding isotherms.

The maximum relative change of the ligand fluorescence, ΔI_{max}, in the presence of a given quencher concentration, [Q], observed at a saturating concentration of the macromolecule is defined by

$$\Delta I_{max} = \frac{\Sigma F_{jq} L_j - F_{Fq} L_T}{F_{Fq} L_T} \qquad (10)$$

where L_T is the total ligand concentration, and F_{Fq} and F_{jq} are defined by Eq. (8b) and Eq. (8c), respectively. Notice that $F_{Fq} L_T$ is the initial fluorescence of the solution of the ligand before addition of the macromolecule, and $\Sigma F_{jq} L_j$ is the fluorescence of the same ligand solution where the ligand molecules are completely saturated with the macromolecule.

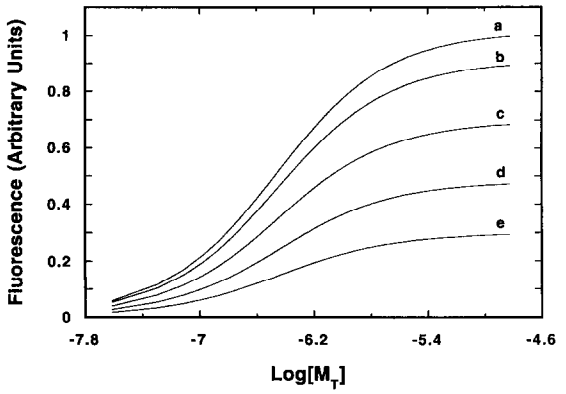

Fig. 2. Computer-simulated titrations of the fluorescent ligand with the macromolecule in the presence of different concentrations of the collisional quencher. The binding of the ligand is described by two independent classes of binding sites (see text for details) with intrinsic binding constants $K_1 = 10^6$ M^{-1} and $K_2 = 10^5$ M^{-1} for the first and second classes, respectively; the quenching constants are as in Fig. 1. The quencher concentrations, [Q], are: (a) 300 mM; (b) 200 mM; (c) 100 mM; (d) 50 mM; (e) 25 mM.

The absolute value of ΔI_{max} at high [Q] depends on the concentration of the quencher in the solution; however, the magnitude of ΔI_{max} is independent of the absolute values of the quenching constants, K_{Df}, K_{Db1}, and K_{Db2}, but is dependent on their ratios. This results from the definition of the ΔI_{max} which is a *relative* increase in the ligand fluorescence upon binding to the macromolecule, with respect to its initial fluorescence in the absence of the macromolecule, as described by Eq. (10). For the model of the ligand binding to the macromolecule, in the two different classes considered here, ΔI_{max} is defined in terms of [Q] and the Stern–Volmer quenching constants by

$$\Delta I_{max} = (1 + K_{Df}[Q]) \times \left(\frac{K_1}{(1 + K_{Db1}[Q])(K_1 + K_2)} + \frac{K_2}{(1 + K_{Db2}[Q])(K_1 + K_2)} - \frac{1}{1 + K_{Df}[Q]} \right) \quad (11)$$

The derivation of Eq. (11) is given in Appendix A). Fig. 3a shows the dependence of the maximum change of the fluorescence, ΔI_{max}, as a function of the quencher concentration for the different values of the dynamic quenching constants, K_{Df}, K_{Db1}, and K_{Db2}, and the same ratio $K_{Df}:K_{Db1};K_{Db2} = 6:2:1$. Because the ratio is the same, all plots reach the same plateau value, i.e., the same ΔI_{max}, at a high quencher concentration. However, for the low values of K_{Df}, K_{Db1}, and K_{Db2}, the same value of ΔI_{max} is reached at a higher concentration of the quencher, which results from the fact that lower values of the

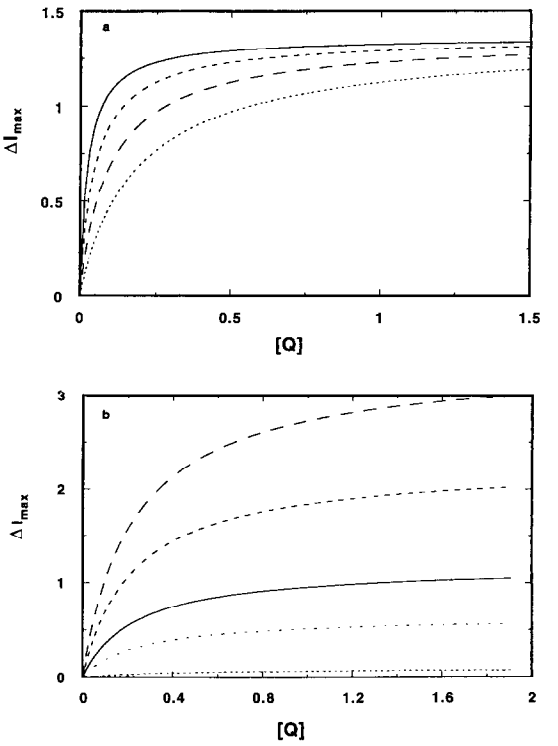

Fig. 3. (a) The dependence of the maximum increase of the ligand fluorescence, ΔI_{max}, in the complex with the macromolecule as a function of the collisional quencher concentration at different values of the dynamic quenching constants, K_{Df}, K_{Db1}, and K_{Db2}, but at the same ratios, $K_{Df}:K_{Db1}:K_{Db2} = 6:2:1$; (· · · · · ·) 15, 7.5, 2.5; (— — —) 30, 15, 5; (— — -) 60, 30, 10; (————) 120, 60, 20. (b) The dependence of the maximum increase of the ligand fluorescence, ΔI_{max}, in the complex with the macromolecule as a function of the collisional quencher concentration at different ratios of the dynamic quenching constants, $K_{Df}:K_{Db1}:K_{Db2}$; (———— ———— ————) 8, 2, 1; (— — —) 6, 2, 1; (————) 4, 2, 1; (– – –) 3, 2, 1; (· · · · ·) 2, 2, 1. The binding of the ligand to the macromolecule is described by two independent classes of binding sites, with three sites in each class (see text for details).

quenching constants indicate lower efficiency of the quenching process [25].

Theoretical plots of the dependence of the maximum increase in the fluorescence of the ligand, ΔI_{max}, as a function of the quencher concentration (Eq. (11)), for different ratios of the collisional quenching constants, $K_{Df}:K_{Db1}:K_{Db2}$, are shown in Fig. 3b. The value of K_{D1} is kept constant (2 M^{-1}) and different values are assigned to K_{Df} and K_{Db2}. Clearly, the higher the $K_{Df}:K_{Db1}:K_{Db2}$ ratio, the higher the maximum increase of the fluorescence ΔI_{max}. As the ratio of $K_{Df}:K_{Db1}:K_{Db2}$ decreases, the possible maximum fluorescence increase is decreased and is lower for the lower ratio at any concentration of the quencher.

3.1.2. Fluorescence titration of the ligand with the macromolecule in the presence of a collisional quencher. Determination of the absolute thermodynamic isotherm for the ligand binding to the macromolecule

In macromolecular binding studies, the titration can be performed in two ways. 1) The ligand is added to the sample containing a constant concentration of the macromolecule. This method is called "normal titration." As the titration proceeds, the degree of binding increases, finally reaching saturation. 2) The macromolecule is added to the sample containing a constant concentration of the ligand [10,11]. As the titration progresses, the degree of binding, or the saturation of the macromolecule with the ligand, decreases. This method is called "reverse titration". If the system is in equilibrium, both methods are completely equivalent. The "normal titration" technique is primarily used when the followed signal originates from the macromolecule, whereas the "reverse titration" technique is used when the signal originates from the ligand. Therefore, in the first case, the apparent saturation of the macromolecule is observed, whereas, in the second case, the apparent fractional saturation of the ligand is followed. In our discussion of the binding analysis in this section, we assume that the data have been collected by the fluorescence reverse titration method.

As we pointed out above, in general, the collisional quencher may have a different effect on the fluorescence of the bound ligand in different classes of binding sites. This generalization has been included in our considerations by applying different quenching constants for the two binding classes, as expressed by Eqs. (6), (7), (8a), (8b) and (8c). As a result, the fractional change of the ligand fluorescence upon addition of the macromolecule will not be equal to the fractional saturation of the ligand [16,17]. In the following section, we show that the general method of analysis used to obtain the thermodynamically rigorous isotherm for the general case of multiple ligand binding, where the fractional change of the ligand fluorescence upon addition of the macromolecule may not be equal to the fractional saturation of the ligand, can be applied when the fluorescence change, used to monitor the binding, results from the differential collisional quenching of the free and bound ligand in the presence of an external quencher [16,17].

If the ligand binds to the macromolecule in different multiple classes of binding sites, the concentrations of the free ligand, L_F, and the concentration of the ligand bound in all different states, ΣL_j, are related to the total ligand concentration by the mass conservation equation

$$L_T = L_F + \Sigma L_j \tag{12}$$

Also, for every L_j there is the relationship

$$L_j = M_T \upsilon_j \tag{13}$$

where υ_j is the binding density or degree of binding (moles of ligand bound per mole of macromolecule) for the j state, and M_T is the total concentration of the macromolecule. In the presence of the collisional quencher, the fluorescence of the ligand, in the presence of a macromolecule, is defined by Eqs. (8a), (8b) and (8c). Introducing Eq. (12) and Eq. (13) into Eq. (8a) gives

$$F_{obs} - F_{Fq} L_T = M_T \Sigma (F_{jq} - F_{Fq}) \upsilon_j \tag{14}$$

After dividing both sides of Eq. (14) by $F_{Fq} L_T$ (the initial fluorescence of the sample in the presence of the quencher [Q], but before the addition of the macromolecule) then multiplying by $\left(\frac{L_T}{M_T}\right)$, one obtains

$$\left[\frac{F_{obs} - F_{Fq} L_T}{F_{Fq} L_T}\right]\left(\frac{L_T}{M_T}\right) = \Sigma\left[\frac{F_{jq} - F_{Fq}}{F_{Fq}}\right]\upsilon_j \tag{15}$$

or

$$\Delta I_{obs}\left(\frac{L_T}{M_T}\right) = \Sigma(\Delta I_{max})_j \upsilon_j \qquad (16)$$

where $\Delta I_{obs} = (F_{obs} - F_{Fq}L_T/F_{Fq}L_T)$is the observed change of the protein fluorescence for the particular values of L_T and M_T, and $(\Delta I_{max})_j = (F_{jq} - F_{Fq}/F_{Fq})$ is the maximum fluorescence change of the protein fluorescence when bound in state j, in the presence of the collisional quencher at concentration [Q].

Eqs. (15) and (16) indicate that the quantity $\Delta I_{obs}\left(\frac{L_T}{M_T}\right)$ is equal to $\Sigma(\Delta I_{max})_j \upsilon_j$, the net binding density for all j states of ligand binding, weighted by the extent of the fluorescence change of each bound state. We refer to the quantity, $\Delta I_{obs}\left(\frac{L_T}{M_T}\right)$ as the "binding density function" [10]. For titrations carried out under a given set of constant experimental conditions and the same total quencher concentration [Q], the weighting factors, $(\Delta I_{max})_j$, are intensive molecular quantities which are characteristic for a particular binding state, j, and are the sole function of the structure of the ligand–macromolecule complex j and the collisional quencher concentration [Q]. Therefore, for a given binding density distribution, $\Sigma\upsilon_j$, the function, $\Delta I_{obs}\left(\frac{L_F}{M_T}\right)$, has the same value, independent of the macromolecule concentration M_T. In other words, the binding density distribution, $\Sigma\upsilon_j$, depends solely on the free ligand concentration L_F. Therefore, at the same value of the binding density function, at different macromolecule concentrations, M_T, the total ligand concentrations, L_T, are related to L_F and $\Sigma\upsilon_j$ by the linear mass conservation equation

$$L_T = L_F + (\Sigma\upsilon_j)M_T \qquad (17)$$

Thus, one can obtain absolute measurements of $\Sigma\upsilon_j$ and L_F by performing two or more titrations at different total ligand concentrations and plotting $\Delta I_{obs}\left(\frac{L_T}{M_T}\right)$ vs. $\log[M_T]$. A series of theoretical "binding density function" plots, $\Delta I_{obs}\left(\frac{L_T}{M_T}\right)$ vs. $\log[M_T]$, at different total ligand concentrations, and at a given constant concentration of the quencher, [Q], are shown in Fig. 4a. These plots show the general behavior of the binding density functions [10]. As the total ligand concentration increases, the attainable binding density also increases. Because the ordinate $\Delta I_{obs}\left(\frac{L_T}{M_T}\right)$ is a relative binding density scale weighted by constant factors $(\Delta I_{max})_j$, the

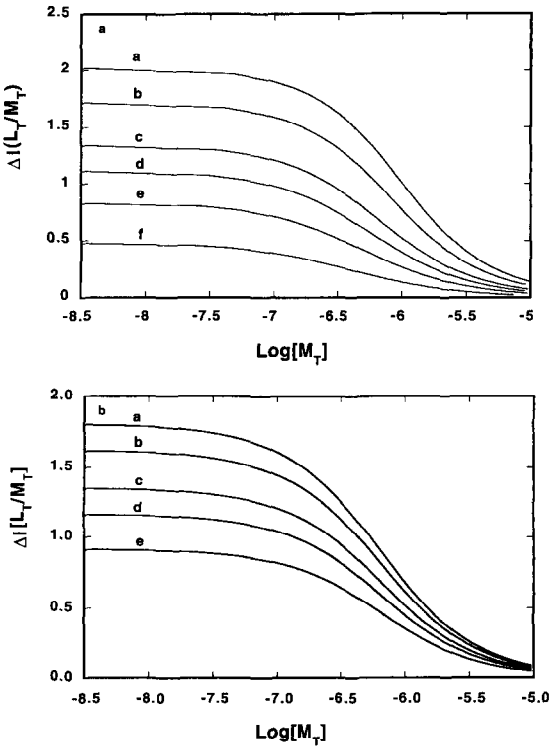

Fig. 4. (a) Plots of the binding density functions, $\Delta I_{obs}\left(\frac{L_T}{M_T}\right)$, as a function of the logarithm of the total macromolecule concentration, $\text{Log}[M_T]$, at the single collisional quencher concentration, [Q] = 100 mM. The curves have been generated for the case of the reverse titrations of the ligand which binds to the macromolecule in two independent classes of binding sites, each having three sites, with intrinsic binding constants, $K_1 = 10^6\ M^{-1}$ and $K_2 = 10^5\ M^{-1}$ for the first and second classes, respectively. The total ligand concentrations are: (a) 2×10^{-6} M; (b) 1.5×10^{-6} M; (c) 1×10^{-6} M; (d) 7.5×10^{-7} M; (e) 5×10^{-7} M; (f) 2.5×10^{-7} M. The Stern–Volmer quenching constants for the ligand free and bound in the first and second classes are $K_{Df} = 30\ M^{-1}$, $K_{Db1} = 15\ M^{-1}$, and $K_{Db2} = 5\ M^{-1}$, respectively. (b) Plots of the binding density functions, $\Delta I_{obs}\left(\frac{L_T}{M_T}\right)$, as a function of the logarithm of the total macromolecule concentration $\text{Log}[M_T]$ at the single total ligand concentration (1×10^{-6} M), but with different collisional quencher concentrations, [Q]: (a) 200 mM; (b) 150 mM; (c) 100 mM; (d) 75 mM; (e) 50 mM. The intrinsic binding constants and the Stern–Volmer quenching constants are the same as in (a).

plateau of the binding density function appears at higher values with an increasing L_T. This is a simple consequence of the fact that binding density, Σv_j, is a sole function of the free ligand concentration, L_F. Therefore, at a very low macromolecule concentration, where $L_F - L_T$, the value of Σv_j is limited by the value of L_T. An important consequence of this fact is that binding density functions determined at different L_T do not span the same region of the degree of binding, Σv_j, at all concentrations of the macromolecule (see Fig. 4a). In order to generate a binding isotherm covering a full range of Σv_j, it is necessary to generate several overlapping binding density curves. From these plots, the set of concentrations, L_T and M_T, for each titration, is obtained for which the binding density function is constant (as shown in Fig. 4a) and, subsequently, one obtains Σv_j and L_F, using the mass conservation equation Eq. (17).

As we pointed out above, the weighting factors, $(\Delta I_{max})_j$, are intensive molecular quantities which characterize a ligand molecule in a particular binding state, j. These quantities are not only the sole functions of the structure of the given ligand–macromolecule complex, j, but also the collisional quencher concentration, [Q]. The key element of the analysis presented in this work is that the dependence of $(\Delta I_{max})_j$ upon [Q] gives the possibility of modulating the observed change in the ligand fluorescence upon binding to the macromolecule by changing [Q] to a level where the observed signal is suitable for measurement, hence, increasing the resolution of the titration experiments. The effect of changing the weighting factors $(\Delta I_{max})_j$, at the constant distribution, Σv_j, on the binding density function is illustrated in Fig. 4b, where the binding density function is plotted for the same constant concentration of the ligand (and the same binding parameters), but for different concentrations of the quencher. Because at a higher [Q] the difference between the fluorescence of the free and bound ligand is higher, the values of $(\Delta I_{max})_j$ are also higher (Eqs. (8a), (8b) and (8c) and Eq. (15)). As a result of the increased $(\Delta I_{max})_j$, the binding density function, $\Delta I_{obs}\left(\frac{L_T}{M_T}\right)$, is shifted to higher values at the same macromolecule concentration and the same $\left(\frac{L_T}{M_T}\right)$ ratio, hence increasing the resolution of the binding isotherms.

3.2. Application of the differential dynamic quenching method to the studies of εADP binding to the DnaB hexamer

To illustrate the method described in the previous section, we will apply it to the analysis of the ADP fluorescent analog, εADP, binding to six nucleotide binding sites of the DnaB helicase hexamer. This system provides an excellent opportunity to demonstrate all experimental aspects of the analysis.

Acrylamide is an efficient dynamic quencher of the etheno-nucleotide fluorescence [15]. Moreover, the neutral character of the quencher indicates that, even at higher concentrations, acrylamide should not affect the ligand–macromolecule interactions to any significant degree (see below). A series of Stern–Volmer plots of the εADP fluorescence quenching with acrylamide, in the presence of different concentrations of the DnaB protein, is shown in Fig. 5. As expected, the largest slope and the largest Stern–Volmer quenching constant are observed in the case of the free εADP in solution, where the quencher

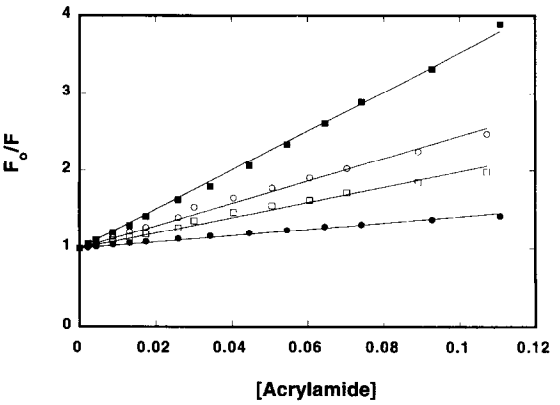

Fig. 5. Stern–Volmer plots of acrylamide quenching of the fluorescence of εADP, free and in the presence of different concentrations of the DnaB helicase in buffer T3 (100 mM NaCl, pH 8.1, 10°C); $\lambda_{ex} = 325$ nm, $\lambda_{em} = 410$ nm. Solid lines are linear least squares fits of the Stern–Volmer equation (Eq. (4) in text) to the data points using the following values of K_D (M^{-1}): (■) 25.3, [DnaB] (hexamer) = 0; (○) 14.5, [DnaB] = 5.4×10^{-7}; (□) 9.9, [DnaB] = 1.08×10^{-6}; (●) 4.5, DnaB = 5.4×10^{-6}.

has full accessibility to the fluorophore. At increasing concentrations of the DnaB protein, i.e., the increasing degree of εADP saturation with the enzyme, the slope decreases indicating the significantly diminished accessibility of the bound fluorescent nucleotide to the quencher [15,19]. The plots are linear in the studied range of [acrylamide] (0– ~ 115 mM), indicating that the fluorescence of the free and bound εADP is predominantly quenched by the dynamic quenching process [15]. Because the efficiency of the quenching of the free εADP fluorescence is higher than the bound nucleotide, the difference between the fluorescence intensity of the free εADP and when bound to the helicase is higher at a higher [acrylamide] (Fig. 5). The effect of the quencher concentration on the resolution of the binding isotherms is demonstrated in Fig. 6 which shows fluorescence titrations of εADP with the DnaB protein in the absence and presence of different acrylamide concentrations in buffer T3 (pH 8.1, 100 mM NaC], 10°C). The difference in the observed fluorescence increase accompanying the binding of εADP to the enzyme is dramatic. While in the absence of acrylamide, the relative increase in the nucleotide

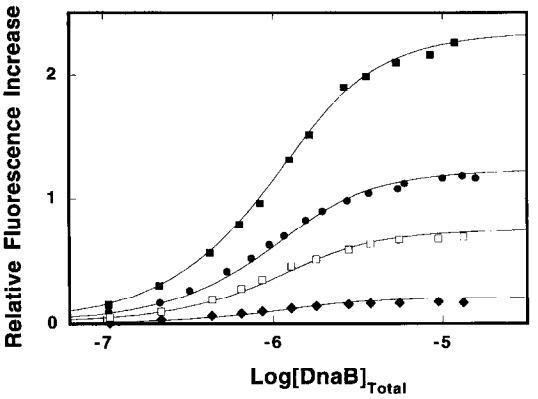

Fig. 6. Fluorescence titrations of εADP, at the same concentration of the nucleotide, with the DnaB helicase in buffer T3 (100 mM NaCl, pH 8.1, 10°C) containing different concentrations of acrylamide: (◆) 0; (□) 25 mM; (●) 50 mM; (■) 100 mM (λ_{ex} = 325 nm, λ_{em} = 410 nm). The concentration of ϵADP is 4×10^{-6} M. Solid lines are computer fits of the binding isotherms, according to the hexagon model (Eqs. (1a) and (1b)), using the following values of the intrinsic binding constant, K, and cooperativity parameter, σ: (◆) $K = 4\times10^5$ M^{-1}, $\sigma = 0.31$; (□) $K = 4\times10^5$ M^{-1}, $\sigma = 0.35$; (●) $K = 4.3\times10^5$ M^{-1}, $\sigma = 0.35$; (■) $K = 4\times10^5$ M^{-1}, $\sigma = 0.4$.

fluorescence reaches a plateau at ~ 21% of its initial value, the relative fluorescence change increases to the value of ~ 125% and ~ 235% of the initial fluorescence intensity of the free εADP in the presence of 50 and 100 mM acrylamide. Thus, in the presence of the 100 mM quencher, the recorded relative fluorescence change accompanying the binding is amplified by approximately an order of magnitude. This dramatic increase provides the required resolution to perform quantitative analyses of the binding process.

A series of fluorescence titration curves of εADP with the DnaB protein, in buffer T3 (pH 8.1, 100 mM NaCl, 10°C), at different nucleotide concentrations and in the presence of 100 mM acrylamide, is shown in Fig. 7. At higher nucleotide concentrations, the curves are shifted toward higher concentrations of the DnaB helicase as more enzyme is necessary to saturate the increased amount of εADP in the sample. All curves reach the same plateau of the relative fluorescence increase, ΔF, at saturating concentrations of the DnaB helicase. As we pointed out above, in general, the fractional change in the ligand fluorescence upon the macromolecule concentration does not necessarily strictly correspond to the fractional ligand saturation [16,18]. This is never *a priori* known for any multiple ligand binding system. However, the estimate of the degree of binding and free ligand concentrations can be obtained by using the binding density function approach, as discussed above. Fig. 8 shows the binding density function plots for the εADP–DnaB helicase system obtained using fluorescence titrations presented in Fig. 7. For the different total concentrations of εADP, at the same value of binding density function, the absolute degree of binding, Σv_j, and free εADP concentrations $[\varepsilon\text{ADP}]_{\text{free}}$ must be the same, allowing their determination using Eq. (17).

The dependence of the fractional fluorescence increase as a function of the fraction of ligand molecules $L_B/L_T = [\Sigma v_j(M_T/L_T)]$ is shown in Fig. 9. The value of L_B/L_T has been determined using the binding density function plots shown in Fig. 8. It is clear that in the studied case, binding of εADP to the DnaB hexamer, there is a linear correspondence between the relative increase in the nucleotide fluorescence, ΔI, and the fraction of ligand bound, L_B/L_T, in the examined range of the degree of

binding. Thus, application of the binding density function approach to determine the absolute degree of binding, Σv_j, and the free ligand concentration, L_F, free of any assumptions concerning the relationship between the observed signal and the fractional ligand saturation, allows us to verify this relationship and to construct a true thermodynamic binding isotherm. Moreover, short extrapolation (dashed line) to $L_B/L_T = 1$ gives the value of the maximum relative increase $\Delta I_{max} = 2.35$ of the εADP fluorescence upon saturation with the DnaB protein.

The dependence of the average degree of binding, Σv_j, as a function of the logarithms of the free ADP concentration, determined using the binding density method, is shown in Fig. 10. The isotherm has been superimposed on the solid line which is a theoretical isotherm according to the hexagon model, constructed using the intrinsic binding constant $K = 4 \pm 1 \times 10^5$ M^{-1} and the cooperativity parameter $\sigma = 0.4 \pm 0.1$ (Eqs. (1a), (1b) and (13)). These values of K and σ have been obtained independently using the rigorous fluorescence titration method in which the quenching of the protein fluorescence was used to monitor the binding (data not shown) [13–15,19]. Clearly, there is excellent agreement between the

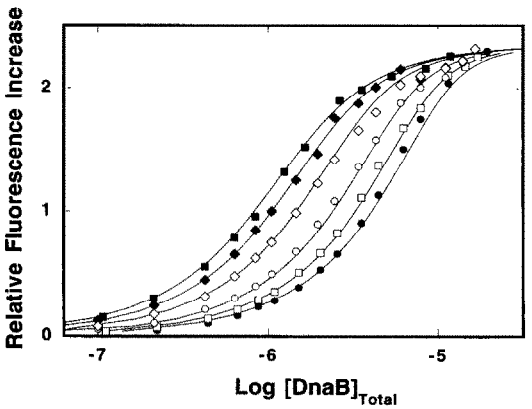

Fig. 7. Fluorescence titrations ("reverse titrations") of εADP, at different concetrations of the nucleotide, with the DnaB helicase in buffer T3 (100 mM NaC], pH 8.1, 10°C) containing 100 mM acrylamide ($\lambda_{ex} = 325$ nm, $\lambda_{em} = 410$ nm). The concentrations of the nucleotide are: (■) 4×10^{-6} M; (◆) 6×10^{-6} M; (◇) 1×10^{-5} M; (○) 2×10^{-5} M; (□) 3×10^{-5}; (●) 4×10^{-5} M. Solid lines are computer fits of the experimental binding isotherms, according to the hexagon model, using a single set of binding parameters, $K = 4 \times 10^5$ M^{-1}, $\sigma = 0.4$, and $\Delta I_{max} = 2.35$.

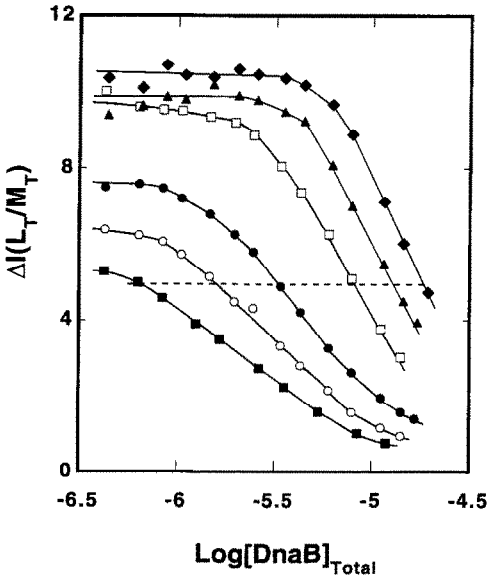

Fig. 8. Dependence of the binding density function, $\Delta I_{obs}(L_T/M_T)$, on the logarithm of the total DnaB protein concentration at different concentrations of the εADP in buffer T3 (100 mM NaCl, pH 8.1, 10°C): (■) 4×10^{-6} M; (○) 6×10^{-6} M; (●) 1×10^{-5} M; (□) 2×10^{-5} M; (▲) 3×10^{-5}; (◆) 4×10^{-5} M. Solid lines separate different data sets and do not have theoretical basis. The horizontal dashed line connects points at the same value of the binding density function, at different εADP concentrations, at which $[\varepsilon ADP]_{Free}$ and the degree of binding, Σv_j, of the nucleotide on the DnaB hexamer are the same (see text for details).

binding isotherms obtained using the two quantitative methods.

It should be pointed out that once the relation between the observed signal and the fractional ligand saturation, as well as the maximum relative change of the ligand fluorescence upon saturation with the macromolecule, ΔI_{max}, are known, the binding parameters can be determined using direct computer fits of the experimental fluorescence titration curves of the ligand with a macromolecule. The solid lines in Fig. 7 are computer fits of the fluorescence titration curves of εADP with the DnaB helicase using a single set of the binding parameters, $K = 4 \times 10^5$ M^{-1}, $\sigma = 0.4$ and $\Delta I_{max} = 2.35$. It is evident that this single set of binding parameters provides an excellent description of the binding process at different total concentrations of the ligand, εADP.

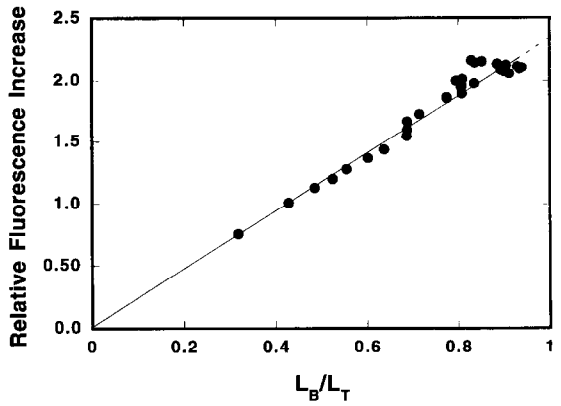

Fig. 9. Relation between the relative fluorescence increase and the fractional saturation of the nucleotide for εADP binding to the DnaB helicase in buffer T3 (100 mM NaCl, pH 8.1, 10°C). The selected concentration of εADP is 2×10^{-5} M. The concentration of the bound nucletide has been calculated from $[\varepsilon ADP]_{Bound} = (\Sigma v_j)[DnaB]_{Total}$, where the average degree of binding of εADP on the DnaB protein, Σv_j, has been determined using the binding density function approach described in the text.

3.3. Analysis of the ligand–macromolecule binding in the case of one type of binding site

So far, we have considered the most general case in which a ligand binds to a macromolecule, in different classes of binding sites, characterized by different fluorescence properties. However, in the simplest case, the ligand binds to the macromolecule in only one class of binding sites. In such a model, the ligand exists only in two states, free and bound with the macromolecule. In the case of a single class of binding sites, the equations developed for the general case (see above) are greatly simplified. The fluorescence of the ligand solution, in the presence of the macromolecule and without the collisional quencher, is described by

$$F_{obs} = F_F L_F + F_F L_B \tag{18}$$

where L_B is the concentration of the bound ligand. In the presence of the quencher, Q, the fluorescence of the sample is described by

$$F_{obs} = F_{Fq} L_F + F_{qb} L_B \tag{19}$$

where

$$F_{Fq} = \frac{k_f}{k_f + \Sigma k_i + k_q[Q]} \tag{20a}$$

and

$$F_{qb} = \frac{k_f}{k_f + \Sigma k_i + k_{qb}[Q]} \tag{20b}$$

The observed maximum change of the ligand fluorescence upon saturation with macromolecule ($L_B = L_T$) is a function of the quencher concentration and is defined as

$$\Delta I_{max} = \frac{F_{qb} - F_{Fq}}{F_{Fq}} \tag{21a}$$

and

$$\Delta I_{max} = \frac{k_f + \Sigma k_i + k_q[Q]}{k_f + \Sigma k_i + k_{qb}[Q]} - 1 \tag{21b}$$

Using the Stern–Volmer quenching constants for free and bound ligands, (K_{Df} and K_{Db}), Eq. (21b) can be written as

$$\Delta I_{max} = \frac{1 + K_{Df}[Q]}{1 + K_{Db}[Q]} - 1 \tag{22}$$

At a very high quencher concentration, ΔI_{max} reaches a constant value defined as

$$\Delta I_{max} = \frac{K_{Df}}{K_{Db}} - 1 \tag{23}$$

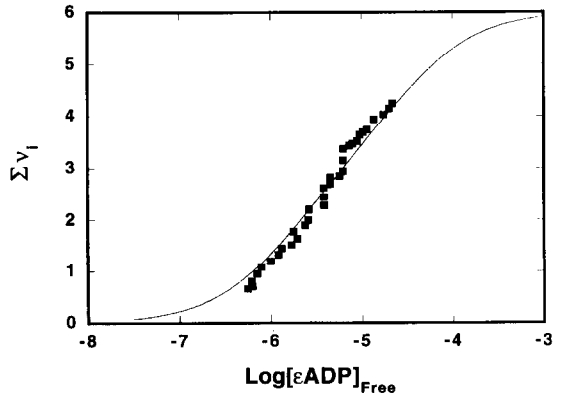

Fig. 10. The dependence of the average degree of binding of εADP on the DnaB helicase hexamer, as a function of the logarithm of the free nucleotide concentrations $[\varepsilon ADP]_{Free}$ in buffer T3 (100 mM NaCl, pH 8.1, 10°C). The solid line is the theoretical binding isotherm, according to the hexagon model (Eqs. (1a) and (1b)), using intrinsic binding constant $K = 4\times10^5$ M^{-1} and cooperativity parameter $\sigma = 0.4$.

Therefore, as we discussed above, for the general case, the maximum possible relative change in the ligand fluorescence, in the presence of the high dynamic quencher concentration [Q], is solely dependent upon the ratio of the two Stern–Volmer quenching constants for the free and bound protein, K_{Db} and K_{Df}, respectively.

For the simple case of a single class of binding sites, Eq. (16) reduces to

$$\frac{\Delta I_{obs}}{\Delta I_{max}} = \frac{L_B}{L_T} \tag{24}$$

and it follows that

$$L_f = \left(1 - \frac{\Delta I_{obs}}{\Delta I_{max}}\right) L_T \tag{25}$$

The average degree of binding is then defined by

$$\upsilon = \left(\frac{\Delta I_{obs}}{\Delta I_{max}}\right)\left(\frac{L_T}{M_T}\right) \tag{26}$$

3.4. General analysis of the multiple ligand–macromolecule binding when fluorescence originates from the macromolecule

The analysis presented in the previous sections applies when the binding of the ligand to the macromolecule is followed by the changes in the fluorescence originating from the ligand and resulting from the differential dynamic quenching of the free and bound ligand. An analogous approach can be developed where the binding of the ligand to the macromolecule is followed by the fluorescence changes of the macromolecule, resulting from the differential quenching of the macromolecule free and bound with the ligand. The observed fluorescence $F^M_{o_{obs}}$ of the macromolecule in the presence of the ligand and in the absence of the dynamic quencher, Q, is described by

$$F^M_{o_{obs}} = F^M_F M_F + \Sigma F^M_j M_j \tag{27}$$

where F^M_F and M_F are the molar fluorescence and the concentration of the free macromolecule, F^M_j and M_j are the molar fluorescence and the concentration of the macromolecule bound in state, j, respectively.

In the presence of [Q], the fluorescence of the ligand–macromolecule mixture is defined by

$$F^M_{obs} = F^M_{Fq} M_F + \Sigma F^M_{jq} M_j \tag{28}$$

where F^M_{Fq} and F^M_{jq} are the molar fluorescence of the macromolecule free and bound with the ligand in state, j, in the presence of the dynamic quencher at concentration, [Q].

Analogously to Eq. (8b) and Eq. (8c), the quantities F^M_{Fq} and F^M_{jq} are defined in terms of the corresponding rate constants for emission and radiationless processes as

$$F^M_{Fq} = \frac{k^M_f}{k^M_f + \Sigma k^M_i + k^M_q[Q]} \tag{29a}$$

$$F^M_{jq} = \frac{k^M_f}{k^M_f + \Sigma k^M_i + k^M_{jq}[Q]} \tag{29b}$$

The mass conservation equation relates the total concentration of the macromolecule to M_F and M_j

$$M_T = M_F + \Sigma M_j \tag{30}$$

From the definition of the binding density, υ_j, one obtains

$$\upsilon_j = \frac{jM_j}{M_T} \tag{31}$$

Introducing Eq. (30) and Eq. (31) into Eq. (28) provides

$$F^M_{obs} = F^M_{Fq} M_T + \Sigma\left(F^M_{jq} - F^M_j\right)\frac{\upsilon_j}{j} \tag{32}$$

Dividing by the initial fluorescence of the free macromolecule, $F^M_{Fq} M_T$, and after rearranging, the obtained macromolecule binding density function is

$$\left[\frac{F^M_{obs} - F^M_{Fq} M_T}{F^M_{Fq} M_T}\right] = \Sigma\left[\frac{F^M_{jq} - F^M_{Fq}}{F^M_{Fq}}\right]\upsilon_j \tag{33}$$

or

$$\Delta I^M_{obs} = \Sigma \frac{\left(\Delta I^M_{max}\right)_j}{j}\upsilon_j \tag{34}$$

where $\Delta I^M_{obs} = \left[\frac{F^M_{obs} - F^M_{Fq} M_T}{F^M_{Fq} M_T}\right]$ is the measured change of the macromolecule fluorescence upon binding the ligand in the presence of the collisional

quencher, [Q], and $\frac{(\Delta I^{M}_{max})_j}{j}$ is the average molecular fluorescence change per bound ligand in the complex, M_j, containing j bound ligand molecules.

3.5. Effect of the collisional quencher on the ligand–macromolecule interactions

A true collisional quenching process, caused by the presence of a nonionic quenchor, will not perturb the thermodynamic and kinetic parameters of the studied ligand–macromolecule interactions to any significant degree, provided that the concentration of the quencher is low (−0.3 M) [27–29]. Higher quencher concentrations may affect the activity of the principal solvent (water) and, thus, cause an indirect effect on the complex formation. However, there is a possibility that, besides participating in collisional quenching, the quencher also binds to the macromolecule. On the other hand, this additional binding of a dynamic quencher like acrylamide is usually very weak or nonexistent [25,26]. However, since the concentration [Q] is most often in the range of 0.05–0.3 M, some effect of the quencher on the binding of the ligand to the macromolecule may appear, in spite of very low binding affinity.

A very direct control to test whether or not there is an effect of Q on the determined intrinsic binding parameters would be to perform titrations at different concentrations of the quencher. Because the binding of the quencher, if any, is very weak, the correction for the effect of the weak binding of Q on the determined binding parameters can be performed using the linear free energy model. This approach is the most suitable for the weak interactions in solutions. The excess free energy of the interaction can be represented by the Taylor series in the component concentrations [27–29]. In the limit of weak interactions and the low concentration of [Q], the intrinsic free energy of binding ΔG_{intr} is defined as

$$\Delta G_{intr} = \Delta G^{o}_{intr} + RT\Delta b_1[Q] \quad (35)$$

where ΔG^{o}_{intr} is the intrinsic free energy independent of the quencher concentration [Q], Δb_1 is a parameter which is a function of temperature, pressure, and R and T are the gas constant and temperature, respectively [27]. Introducing intrinsic binding constant K one obtains

$$\ln K = \ln K^{o} - \Delta b_1[Q] \quad (36)$$

Thus, the dependence of the intrinsic binding constant, K, versus the quencher concentration, should be a straight line and the intercept at [Q] = 0 equals the required value of $\ln K^{o}$. If the binding of the studied ligand to the macromolecule is cooperative and the cooperative interactions are characterized by a cooperativity factor, σ, then the same relation can be derived, providing

$$\ln \sigma = \ln \sigma^{o} - \Delta b_{\sigma 1}[Q] \quad (37)$$

where σ^{o} is the cooperativity factor in the absence of [Q] and $\Delta b_{\sigma 1}$ is a constant, dependent only on temperature and pressure.

The dependence of the intrinsic binding constant, K, and the cooperativity parameter, σ, upon the acrylamide concentration, for the binding of εADP to the DnaB protein, is shown in Fig. 11. In the studied range of [acrylamide] (= 0 − 100 mM), both binding parameters show very little dependence on [acrylamide] with a slightly larger effect on σ, which is ~ 30% lower at 100 mM acrylamide than in the absence of the quencher (see the legend to Fig. 6). Thus, the data indicate a lack of a significant effect of the neutral quencher, acrylamide, on the nucleotide–DnaB protein interactions.

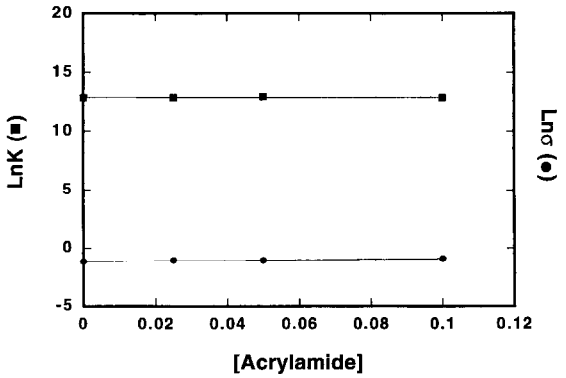

Fig. 11. The dependence of $\ln K$ and $\ln \sigma$ for the binding of εADP to the DnaB hexamer on the acrylamide concentration in buffer T3 (100 mM NaCl, pH 8.1, 10°C). Solid lines are least squares fits of Eq. (36) and Eq. (37) with Δb_1 and $\Delta b_{\sigma 1}$ equal to 0.089 and 2.3, respectively.

4. Conclusions

The fluorescence titration technique is one of the most often used spectroscopic approaches to the quantitative study of the thermodynamics and kinetics of the ligand–macromolecule interactions in biological systems. This technique is almost universally applicable because proteins and many ligands are highly fluorescent substances, and the binding is accompanied by a significant change in the intensity of the fluorescence. However, there are many interacting systems where very little fluorescence change accompanies the complex formation. The theoretical and experimental bases of an alternative method to study ligand–macromolecule interactions, using the fluorescence titration technique, have been proposed in this communication. The method is based on induced differential collisional quenching of the intrinsic fluorescence of a ligand or a macromolecule, by the presence of an efficient, preferably nonionic, collisional quencher. A true collisional quenching process simply transforms the fluorescence of the ligand, or macromolecule, intrinsically independent of the complex formation, into a property which is different for the free and bound fluorophores. This is a unique property of fluorescence and results from the fact that an *extra radiationless process* can be introduced without affecting, to any significant degree, the thermodynamic equilibrium and kinetic properties of the interacting system. If the collisional quenching efficiency is different for the free and bound fluorophore, the binding will be manifested by a change in the fluorescence of the ligand or macromolecule, and the thermodynamically rigorous binding isotherm can be determined.

We illustrated the application of the method to quantitative studies of the binding of the fluorescence nucleotide analog, εADP, to the *E. coli* DnaB helicase hexamer. Binding of ϵADP to the enzyme is accompanied by a very modest nucleotide fluorescence increase ($\sim 21\%$). Although such a change in the fluorescence intensity could be used to determine a single binding constant in the case of a simple system characterized by a 1:1 stoichiometry, however, it is not adequate to perform quantitative analyses of the complex binding to six interacting sites of the hexameric enzyme. This results from the fact that common, small errors in the signal measurement constitute much larger errors of the total signal observed, and this error can be additionally increased when the data are transformed to construct a binding density function. However, in the presence of 100 mM acrylamide, this modest fluorescence increase is amplified ~ 10 times, providing the high resolution necessary for rigorous analyses of this complex, cooperative binding process [13].

In principle, there are two main criteria in the selection of a quencher. First, it must have a different effect on the fluorescence of the free and bound ligand or macromolecule. Different quenchers may demonstrate different capabilities in differential quenching. The efficiency of the quenching process will certainly depend upon the electronic structure of the quencher; however, in some cases quenchers with a larger molecular size may be preferred. Larger quencher molecules may be prevented from colliding with the fluorescent part of the ligand, or macromolecule, to a greater extent than smaller molecules, and this effect may be further amplified when the fluorophore is engaged in the complex [30]. Second, the presence of the quencher should introduce very weak, if any, perturbation to the thermodynamics or kinetics of the studied interactions. This can be achieved by using a nonionic quencher, e.g., acrylamide, H_2^2O, H_2O_2 etc. [30]. In this context, acrylamide is the dynamic quencher of choice. The quenching characteristics of acrylamide, with different biological molecules, are well studied and documented [25,26]. There are also several studies indicating that acrylamide binds very weakly, if at all, to the protein with the association constants around 0.5–1.0 M^{-1} and the effect of acrylamide on the enzyme activity is very low, or negligible [25,26]. As we have shown in this work, acrylamide does not exert any significant effect on the nucleotide binding to the DnaB helicase (Fig. 11). Nevertheless, it is important to perform necessary controls to test for any effect of the quencher on the determined binding or kinetic parameters and to introduce, if necessary, corrections which can be obtained by using Eq. (35) and Eq. (36).

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Appendix A

Derivation of Eq. (9)

Consider the situation where the fluorescent ligand binds to the macromolecule in two different binding classes, each having multiple binding sites, and the binding process is not accompanied by any change of the fluorescence of the ligand. The fluorescence of the ligand in solution, in the presence of the macromolecule, but in the absence of the collisional quencher, Q, is then described by

$$F^{o}_{obs} = F_F(L_F + L_{B1} + L_{B1}) \quad (A1)$$

where F_F is the molar fluorescence of the free ligand and $L_T = L_{B1} + L_{B2}$ is the total ligand concentration. In the presence of the quencher, the collisional quenching process is characterized by different Stern–Volmer quenching constants, K_{Df}, K_{Db1}, and K_{Db2}, for the free and bound ligand in the first and second binding classes, respectively. Thus, in the presence of the quencher and the macromolecule, the fluorescence of the ligand is described by

$$F_{obs} = F_F\left(\frac{L_F}{1 + K_{Df}[Q]} + \frac{L_{B1}}{1 + K_{Db1}[Q]} + \frac{L_{B2}}{1 + K_{Db2}[Q]}\right) \quad (A2)$$

where [Q] is the molar concentration of the quencher. The Stern–Volmer relationship is then obtained by dividing Eq. (A1) by Eq. (A2). After rearrangement, Eq. (A3) is obtained

$$\frac{F^{o}_{obs}}{F_{obs}} = \frac{L_T(1 + K_{Df}[Q])(1 + K_{Db1}[Q])(1 + K_{Db2}[Q])}{L_F(1 + K_{Db1}[Q])(1 + K_{Db2}[Q]) + L_{B1}(1 + K_{Df}[Q])(1 + K_{Db2}[Q]) + L_{B2}(1 + K_{Df}[Q])(1 + K_{Db2}[Q])]} \quad (A3)$$

Appendix B

Derivation of Eq. (11)

Consider the fluorescent ligand binding to the macromolecule which has two different classes of binding sites, each site characterized by the intrinsic binding constants, K_1 and K_2, and the binding is not accompanied by any change in the fluorescence of the ligand. The molar fluorescence of the ligand free and bound in two independent classes of binding sites, in the absence of a collisional quencher [Q], is F_{Fo}. In a general case, the presence of the quencher will have a different effect on the fluorescence of each species in solution, i.e., each of the possible states of the ligand in solution (free and bound to the sites in the first and second binding classes) will have a different Stern–Volmer quenching constant. Eqs. (A4), (A5) and (A6) give the relationships for the fluorescence of the free and bound ligand in the presence of a collisional quencher

$$F_F = \frac{F_{Fo}}{1 + K_{Df}[Q]} \quad (A4)$$

$$F_{B_1} = \frac{F_{Fo}}{1 + K_{Db_1}[Q]} \quad (A5)$$

$$F_{B_2} = \frac{F_{Fo}}{1 + K_{Db_2}[Q]} \quad (A6)$$

where K_{Df} K_{Db1}, and K_{Db2} are the Stern–Volmer quenching constants for the free ligand and bound in the first and second classes, respectively. In the absence of the macromolecule, and in the presence of the quencher, the fluorescence of the ligand solution, F_o, is

$$F_o = F_F L_T \quad (A7)$$

In the presence of the collisional quencher and the macromolecule, the fluorescence of the ligand, F, is described by

$$F = F_F L_F + F_{B1} L_{B1} + F_{B2} L_{B2} \quad (A8)$$

where L_{B1} and L_{B2} are the concentrations of the ligand bound in the first and second classes, respectively. The relative change of fluorescence, ΔI, at a given titration point, is defined by

$$\Delta I = \frac{F - F_o}{F_o} \quad (A9)$$

The maximum possible change in the fluorescence of the ligand, in the presence of the saturating concentration of the macromolecule, is described by Eq. (A10) which simply takes into account that at a

very high macromolecule concentration the binding density is very low, $L_F = 0$, and the distribution of the bound ligand between two classes of binding sites is solely the function of the intrinsic binding constants, K_1 and K_2.

$$\Delta I_{max} = (1 + K_{Df}[Q]) \times \left(\frac{K_1}{(1 + K_{Db1}[Q])(K_1 + K_2)} + \frac{K_2}{(1 + K_{Db2}[Q])(K_1 + K_2)} - \frac{1}{1 + K_{Df}[Q]} \right) \quad \text{(A10)}$$

It should be noted that at a very high quencher concentration, ($[Q] \rightarrow \infty$), the maximum change in the fluorescence of the ligand, at the saturating concentration of the macromolecule, becomes independent of [Q] and Eq. (A10) reduces to

$$\Delta I_{max} = \left[\frac{K_1 K_{Db2} K_{Df} + K_2 K_{Db1} K_{Df}}{K_{Db1} K_{Db2} (K_1 + K_2)} \right] - 1 \quad \text{(A11)}$$

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