A General Method of Analysis of Ligand Binding to Competing Macromolecules Using the Spectroscopic Signal Originating from a Reference Macromolecule. Application to *Escherichia coli* Replicative Helicase DnaB Protein−Nucleic Acid Interactions[†,‡]

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ABSTRACT: Quantitative and accurate analyses of protein−nucleic acid interactions in solution are greatly facilitated if the formation of the complex is accompanied by a large change of the spectroscopic signal (e.g., fluorescence) originating from the protein or nucleic acid. However, there are many instances when protein−nucleic acid interactions do not induce adequate changes in spectroscopic properties of the interacting macromolecules. We describe the theoretical and experimental aspects of a general method to analyze such protein−nucleic acid interactions. The method is based on quantitative titrations of a reference nucleic acid with the protein in the presence of a competing nucleic acid whose interaction parameters with the protein are to be determined. The Macromolecule Competition Titration (MCT) method allows for the determination of the absolute average binding density and the free protein ligand concentration over a large binding density range, unavailable by other methods, and construction of a model-independent true binding isotherm. Moreover, the determination of the absolute binding density of the ligand on nonfluorescent nucleic acid is independent of *a priori* knowledge of the binding characteristics of the protein to the reference fluorescent nucleic acid. Although the MCT method is applicable to any type of physicochemical signal that can be used to monitor the binding, we discuss the details of the method as it applies to the analysis monitored by a change in the nucleic acid fluorescence intensity and anisotropy upon binding a ligand. Moreover, the interaction parameters for a given nucleic acid can be determined by using as a reference the long polymer nucleic acid as well as short oligomers. In particular, the analysis is greatly simplified if the short fluorescent nucleic acid fragment, spanning the exact site-size of the complex and binding with only a 1:1 stoichiometry to the protein, is used as a reference macromolecule. We have illustrated the MCT method by applying it to the binding of the *Escherichia coli* DnaB helicase to unmodified, nonfluorescent single-stranded nucleic acids where the interactions are not accompanied by any adequate spectroscopic signal changes. In order to analyze simultaneous binding of a ligand to different competing nucleic acid lattices, we introduced the combined application of the McGhee−von Hippel theory and the Epstein combinatorial approach for the binding of a large ligand to a linear, homogeneous nucleic acid lattice. Our approach allows one to perform a direct fit of the entire experimental isotherm for the protein binding to two competing nucleic acid lattices without resorting to complex numerical calculations.

Protein−nucleic acid interactions play a fundamental role in the transmission of genetic information of the cell from one generation to the next. In order to understand the forces that drive the interactions between proteins and nucleic acids and the functionally relevant conformational changes that occur in interactions between proteins and nucleic acids, the energetics of the complex formation must be determined. This can be achieved by measuring equilibrium binding isotherms over a range of protein binding densities (protein bound per nucleotide). Equilibrium binding isotherms for protein binding to a nucleic acid and, in general, ligand binding to a macromolecule represent the relationship between the extent of protein binding and free protein concentrations. A true thermodynamic isotherm reflects only this relationship and does not depend upon any particular binding models or any particular assumptions.

Many techniques have been developed to study protein−nucleic acid interactions. Some of these approaches directly monitor the binding (Riggs et al., 1970; Garner & Revzin, 1981; Fried & Crothers, 1981; Revzin & von Hippel, 1977; deHaseth et al., 1977; Brenowitz et al., 1986), whereas others indirectly measure the equilibrium distribution of free and bound ligands, utilizing a change of spectroscopic property of either the protein or the nucleic acid resulting from the formation of the complex (Jensen & von Hippel, 1976; Kelly et al., 1976; Boschelli, 1982; Kowalczykowski et al., 1981; Draper & von Hippel, 1978; Lohman & Bujalowski, 1991). We previously developed a systematic approach to study macromolecular binding using spectroscopic signals to monitor the interactions and to obtain true thermodynamically rigorous binding isotherms (Bujalowski & Lohman, 1987; Lohman & Bujalowski, 1991).

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Spectroscopic methods, particularly fluorescence, are widely used in studying ligand−nucleic acid interactions (Alma et al., 1983; Revet et al., 1971; Bontemps & Fredericq, 1974; Jensen & von Hippel, 1976; Kelly et al., 1976; Boschelli, 1982; Kansy et al., 1986; Porschke & Rauh, 1983; Bujalowski & Lohman, 1987). This is because the spectroscopic methods are relatively easy to perform, do not require large quantities of material, allow one to perform solution experiments without perturbing the studied equilibrium, and can be applied at very low, submicromolar concentrations of interacting macromolecules, thus, eliminating any correction for nonideality that may be necessary when high concentrations are required, e.g., in transport or chromatographic methods.

The primary limitation of spectroscopic methods for studying many protein−nucleic acid interactions is the lack of sufficient spectroscopic signal changes accompanying the complex formation. Sometimes, the lack of spectroscopic signals can be overcome by introducing a fluorescence label into the nucleic acid or the protein. However, the nonspecific nature of labeling a protein or a polymer nucleic acid limits these approaches to some specific applications, e.g., use of short nucleic acids, *a priori* knowledge of the labeling site on the protein, and no perturbation of the protein or the nucleic acid conformation by the fluorescent marker, which may affect the interactions. Lack of adequate changes in spectroscopic properties of the interacting macromolecules in many protein−nucleic acid systems has hindered the rigorous quantitative analyses of such systems.

In this communication, we present a Macromolecular Competition Titration (MCT) method to analyze protein−nucleic acid interactions where the formation of the studied complexes is not accompanied by any adequate spectroscopic signal change that can be used to monitor the interactions. The MCT method is based on quantitative titrations of the reference fluorescent nucleic acid with a protein in the presence of a competing, nonfluorescent nucleic acid whose interaction parameters with the protein are to be determined. This approach allows the determination of the absolute average binding density and the free protein ligand concentration over a large binding density range, unavailable by other methods, and construction of a model-independent, true binding isotherm. Moreover, the determination of the absolute binding density is independent of the *a priori* knowledge of the binding characteristics of the protein to the reference fluorescent nucleic acid. We illustrate the method by applying it to the binding of the *Escherichia coli* primary replicative helicase DnaB protein to unmodified, nonfluorescent single-stranded nucleic acid where the interactions are not accompanied by adequate spectroscopic signal changes.

Existing theoretical analyses of nonspecific protein−nucleic acid interactions have primarily focused on the interactions of the protein with a single type of nucleic acid (Crothers, 1968; McGhee & von Hippel, 1974; Epstein, 1978, Bujalowski et al., 1989). We introduced the combined application of the McGhee−von Hippel theory and the Epstein combinatorial approach for the binding of a large ligand to a linear, homogeneous nucleic acid lattice, which enabled us to analyze the simultaneous ligand binding to different competing nucleic acids, using the entire experimental isotherm and without resorting to complex numerical calculations.

MATERIALS AND METHODS

Reagents and Buffers. All solutions were made with distilled and deionized >18 MΩ (Milli-Q Plus) water. All chemicals were reagent grade. Buffer T2 is 50 mM Tris adjusted to pH 8.1 with HCl, 5 mM $MgCl_2$, and 10% glycerol.

DnaB Protein and Nucleic Acids. The *E. coli* DnaB protein was purified as previously described by us (Bujalowski & Klonowska, 1993; 1994a). Poly(dA) and dA-$(pA)_{19}$ were purchased from Midland Certified Reagents (Midland, TX). Etheno derivatives of the nucleic acids were obtained by modification with chloroacetaldehyde (Secrist et al., 1972; Bujalowski & Jezewska, 1995).

Fluorescence Measurements. All steady-state fluorescence measurements were performed using the SLM-AMINCO 48000S spectrofluorimeter as previously described by us and in the accompanying paper (Bujalowski & Klonowska, 1993, 1994a,b; Bujalowski et al., 1994). Computer fits were performed using KaleidaGraph software (Synergy Software, PA) and Mathematica (Wolfram Research, IL).

RESULTS AND DISCUSSION

Determination of the Thermodynamically Rigorous Binding Isotherm for Ligand Binding to the Macromolecule Using the Macromolecular Competition Titration Method. Consider the multiple-equilibrium binding of a ligand to a macromolecule, such as protein binding to a long polymer nucleic acid, which is accompanied by a fluorescence change of the macromolecule. In general, the ligand−nucleic acid complexes can exist in different "*i*" states, and in each state the fluorescence properties of the complex may be different from each other as well as from the free macromolecule. In the cooperative binding of a large protein molecule to a linear nucleic acid, with *n* nucleotides occluded by the protein in the complex, different binding states may result from different conformational states of the bound protein molecules, the existence of different binding modes, cooperative interactions between bound protein molecules, etc.

The observed fluorescence of the nucleic acid in the presence of the ligand will have contributions from each possible "*i*" complex as well as from free nucleic acid, and in the absence of ligand or macromolecule aggregation it can be formally described by the signal conservation equation

$$F = F_F N_F + \sum F_i N_{b_i} \tag{1}$$

where F_F and N_F are the molar fluorescence and concentration (Nucleotide) of the free nucleic acid, and F_i and N_{b_i} are molar fluorescence and concentration of the nucleic acid, respectively, in a given "*i*" state. The binding density, ν_i, of the protein in a given "*i*" state is defined as

$$\nu_i = \frac{L_{b_i}}{N_T} \tag{2}$$

where L_{b_i} is the concentration of the protein bound in state "*i*" and N_T is the total nucleic acid concentration. Therefore, the fraction of the nucleic acid with the protein bound in an "*i*" state can be defined as

$$\frac{N_{b_i}}{N_T} = n_i \nu_i \tag{3a}$$

and

$$N_{b_i} = N_T n_i \nu_i \tag{3b}$$

where n_i is the number of nucleotides occluded by the protein bound in state "*i*". Introducing eq 3b into eq 1 gives

$$F_{obs} = F_F N_F + N_T \sum F_i n_i \nu_i \tag{4}$$

The quantity that is usually experimentally determined in fluorescence titrations is the relative change, ΔF_{obs}, of the monitored fluorescence upon the increasing protein ligand concentration in solution with respect to the initial fluorescence of the sample, $F_F N_T$, defined as

$$\Delta F_{obs} = \frac{(F_{obs} - F_F N_T)}{F_F N_T} \tag{5}$$

Rearranging eq 4 and dividing by $F_F N_T$ provides

$$\Delta F_{obs} = \frac{(F_{obs} - F_F N_T)}{F_F N_T} = \sum \left[\frac{(F_i - F_F)}{F_F} \right] n_i \nu_i \tag{6a}$$

and furthermore,

$$\Delta F_{obs} = \sum \Delta F_{mi} n_i \nu_i \tag{6b}$$

where $\Delta F_{mi} = (F_i - F_F)/F_F$ is the maximum relative change of the nucleic acid fluorescence in the given "*i*" complex.

Equation 6b shows that the observed fluorescence change ΔF_{obs} is equal to $\sum \Delta F_{mi} n_i \nu_i$, the sum of the fractional nucleic acid lattice saturation in all "*i*" states with the bound ligand weighted by the maximum fluorescence change, ΔF_{mi}, characterizing a given "*i*" state.

The ΔF_{mi} parameters are molecular quantities, constant in a given set of solution conditions. Thus, the observed ΔF_{obs} strictly reflects changes in $n_i \nu_i$ which result from changes in the ligand concentration during titration. Because the fractional nucleic acid lattice saturation in a given "*i*" state, $n_i \nu_i$ is a sole, unique function of the free ligand concentration, P_F, ΔF_{obs} must be the same at a given P_F, independent of fluorescent nucleic acid concentration (Bujalowski & Klonowska, 1993, 1994a). Stated in other words, if the binding of a protein to a nucleic acid is accompanied by the spectroscopic signal change (e.g., fluorescence) originating from the nucleic acid, then the same value of ΔF_{obs}, reached at different fluorescent nucleic acid concentrations in identical solution conditions, indicates the same free protein ligand concentration, P_F, in the samples, the same ligand binding density, $\sum \nu_i$, and the degree of nucleic acid lattice saturation, $\sum n_i \nu_i$. Therefore, for the same value of ΔF_{obs} obtained at two different total nucleic acid concentrations, N_{T_1} and N_{T_2}, the degree of binding, $\sum \nu_i$, and the free protein ligand concentration, P_F, must be the same. The values of $\sum \nu_i$ and P_F are then related to the known total protein concentrations, P_{T_1} and P_{T_2}, and the total nucleic acid concentrations, N_{T_1} and N_{T_2}, at which the same ΔF_{obs} is obtained, by the formulae

$$\sum \nu_i = \frac{(P_{T_2} - P_{T_1})}{(N_{T_2} - N_{T_1})} \tag{7a}$$

and

$$P_F = P_{T_x} - \sum \nu_i (N_{T_x}) \tag{7b}$$

where $x = 1$ or 2 (Bujalowski & Klonowska, 1993, 1994a,b).

Thus, if the ligand binding to a macromolecule is accompanied by a spectroscopic signal change originating from the macromolecule, then the analysis of the titration curves obtained for two or more different concentrations of the macromolecule using eqs 7a and 7b allows one to generate a thermodynamically rigorous isotherm free of any assumption about the relationship between the observed signal and the absolute degree of binding (Bujalowski & Klonowska, 1994a,b; Bujalowski & Jezewska, 1995).

We would like to stress that *the same thermodynamic argument leading to eqs 7a,b applies to situations where titration of a fluorescent nucleic acid with a ligand is performed in the presence of the second competing, nonfluorescent nucleic acid lattice*. Here, the ligand binds to two different nucleic acids present in solution, but the observed signal originates only from the fluorescent "reference" lattice. As the titration progresses, the saturation of both nucleic acid lattices increases as the free ligand concentration increases in the sample. In order to illustrate the general behavior of such isotherms, a series of theoretical titration curves of a reference fluorescent nucleic acid with the ligand at a constant reference fluorescent nucleic acid concentration but in the presence of different concentrations of a nonfluorescent, competing nucleic acid is shown in Figure 1. The binding isotherms of the protein to both nucleic acid lattices have been generated using the combined application of the generalized McGhee−von Hippel approach and the Epstein combinatorial theory for large ligand binding to a linear, homogeneous nucleic acid described below (McGhee & von Hippel, 1974; Bujalowski et al., 1989; Epstein, 1978). For simplicity, the protein−lattice complexes for both nucleic acids have been assumed to have a site-size of $n = 20$, a cooperativity parameter of $\omega = 1$, and intrinsic binding constants of $K = 10^5$ and 10^6 M^{-1} for the fluorescent reference nucleic acid and the nonfluorescent, competing lattice, respectively; however, the analysis is independent of any particular binding model for both the reference and the studied nucleic acid. Because the measured relative fluorescence increase, ΔF, monitors exclusively ligand binding to the reference fluorescence nucleic acid, all curves span the same range of the fluorescence change and reach the same plateau value ($\Delta F_{max} = 3.5$). On the other hand, at higher concentrations of the competing nucleic acid the titration curves are shifted toward higher total ligand concentrations resulting from the excess of the ligand required to saturate the competing, nonfluorescent nucleic acid lattice. Recall that the same value of the relative fluorescence change of the reference lattice in the presence of the ligand means the same degree of lattice saturation, $\sum n_i \nu_i$, and the same ligand binding density, $\sum \nu_i$, (eq 6b, see above) on the lattice and the same free ligand concentration, P_F. Therefore, in the presence of different concentrations of a competing nucleic acid and a constant concentration of the reference fluorescence lattice, at the same value of ΔF,

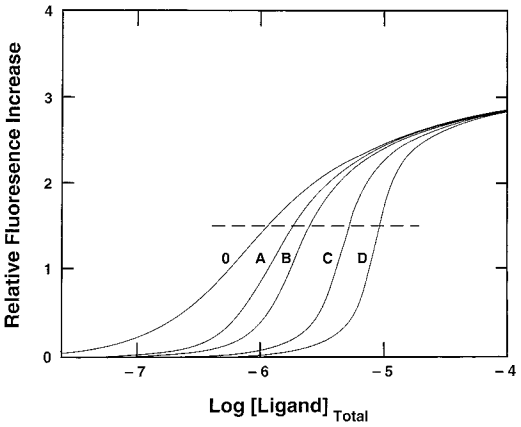

FIGURE 1: Theoretical fluorescence titration curves of the reference fluorescent nucleic acid with the protein ligand in the presence of different concentrations of the competing, nonfluorescent nucleic acid lattice. Binding of the ligand to the reference lattice is described by the McGhee−von Hippel model for large ligand binding to a linear, homogeneous nucleic acid using the intrinsic binding constant $K = 10^5$ M^{-1}, the cooperativity parameter $\omega = 1$, and the site-size $n = 20$. The maximum increase of the nucleic acid fluorescence intensity upon saturation with the ligand is $\Delta F_{max} = 3.5$. Binding of the ligand to competing, nonfluorescent nucleic acid is described by the Epstein combinatorial theory using the intrinsic binding constant $K = 10^5$ M^{-1}, the cooperativity parameter $\omega = 1$, and the site-size $n = 20$. The selected length of the nucleic acid is 1600 nucleotides. The concentration of the competing nucleic acid (Nucleotide) is (**0**) 0; (**A**) 2×10^{-5} M; (**B**) 4×10^{-5} M; (**C**) 1.2×10^{-4} M; (**D**) 2.4×10^{-4} M. The concentration of the reference fluorescent nucleic acid is 2×10^{-5} M (Nucleotide). The horizontal dashed line connects points on all titration curves characterized by the same value of the relative fluorescence increase, ΔF_i.

the concentration of the free ligand, P_F, must be the same and independent of the competing nucleic acid concentration. Because the binding density of the ligand on the nonfluorescent, competing nucleic acid, $(\sum \nu_i)_S$, is also a unique function of P_F, at a given value of ΔF corresponding with the same value of P_F the binding density, $(\sum \nu_i)_S$, must be the same and independent of the total concentration of the competing lattice, N_T. Hence, one can obtain absolute measurements of the ligand binding density, $(\sum \nu_i)_S$, on the nonfluorescent, competing nucleic acid and of the free protein ligand concentration, P_F, from titrations of samples containing constant concentrations of reference fluorescent nucleic acid, N_{T_R}, with the ligand in the presence of two or more concentrations of the nonfluorescent, competing nucleic acid lattice (Figure 1). This can be accomplished by solving a set of mass conservation equations for the total ligand concentration in solution. In the presence of two different competing nucleic acid concentrations, N_{T_1} and N_{T_2}, the total ligand concentrations, P_{T_1} and P_{T_2}, at which the same relative fluorescence change, ΔF, is observed, are defined as

$$P_{T_1} = (\sum \nu_i)_S N_{T_1} + (\sum \nu_i)_R N_{T_R} + P_F \qquad (8a)$$

and

$$P_{T_2} = (\sum \nu_i)_S N_{T_2} + (\sum \nu_i)_R N_{T_R} + P_F \qquad (8b)$$

where $(\sum \nu_i)_R$ is the ligand binding density on the fluorescent reference lattice and $(\sum \nu_i)_S$ is the ligand binding density on the nonfluorescent, competing lattice. Because the binding density, $(\sum \nu_i)_R$, of the protein on the reference nucleic acid at any value of ΔF can be easily and independently

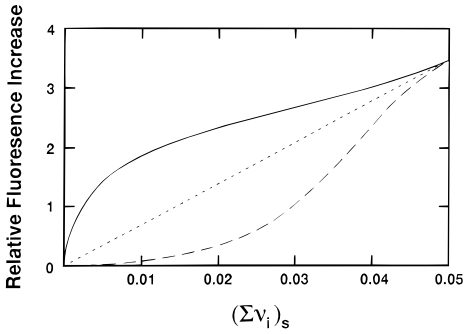

FIGURE 2: Computer simulation of the dependence of the relative fluorescence increase of the reference fluorescent nucleic acid, ΔF, upon the binding density, $(\sum \nu_i)_S$, of the ligand on the competing, nonfluorescent lattice where the competing, nonfluorescent lattice has higher (- - -) and lower (—) affinity toward the ligand. The binding of the ligand to the reference lattice is described by the McGhee−von Hippel model of large ligand binding to a linear, homogeneous nucleic acid, using the intrinsic binding constant $K = 10^5$ M^{-1}, the cooperativity parameter $\omega = 1$, and the site-size $n = 20$. The maximum increase of the nucleic acid fluorescence intensity upon saturation with the ligand is $\Delta F_{max} = 3.5$. Binding of the ligand to competing, nonfluorescent nucleic acid is described by the Epstein combinatorial theory using $\omega = 1$ and n = 20, with $K = 10^4$ and 10^6 M^{-1} for lower and higher affinity cases, respectively. The selected length of the nucleic acid is 1600 nucleotides.

determined, subtracting eq 8a from eq 8b and rearranging provides the expression for the absolute binding density on the competing, nonfluorescent lattice, $(\sum \nu_i)_S$, and the free protein concentration, P_F, in terms of known quantities, $(\sum \nu_i)_R$, total ligand, and nucleic acid concentrations (Bujalowski & Jezewska, 1995)

$$(\sum \nu_i)_S = \frac{(P_{T_2} - P_{T_1})}{(N_{T_2} - N_{T_1})} \qquad (9a)$$

and

$$P_F = P_{T_x} - (\sum \nu_i)_S N_{T_x} - (\sum \nu_i)_R N_{T_R} \qquad (9b)$$

where x is 1 or 2.

Although determination of P_F requires $(\sum \nu_i)_R$, notice that $(\sum \nu_i)_S$ is independent of any *a priori* knowledge of the mechanism and the degree of protein binding to the reference fluorescent nucleic acid. Plotting the observed fluorescence change, ΔF, as a function of $(\sum \nu_i)_S$ allows one to obtain the absolute stoichiometry of the protein−nonfluorescent nucleic acid complex (see below). Calculations of $(\sum \nu_i)_S$ and subsequently P_F can be performed at any value of the observed fluorescence change along the titration curves (Figure 1), generating a thermodynamically rigorous, model-independent binding isotherm for ligand binding to the competing nucleic acid. These procedures are demonstrated in the next section, using the experimental data for the binding of *E. coli* replicative helicase DnaB protein to nonfluorescent poly(dA) in the presence of the reference fluorescent etheno derivative poly(dϵA).

It should be noted that if the ligand affinity for the reference fluorescent nucleic acid and the competing, nonfluorescent lattice is different, then the binding density, $\sum \nu_i$, will be different for both nucleic acids at the same value of the measured relative fluorescence change of ΔF. Figure 2 shows the theoretical dependence of the observed ΔF upon

the binding density of the ligand on the competing lattice, for two different intrinsic affinities of the ligand for the competing nucleic acid. Ligand binding to the reference fluorescent nucleic acid is characterized by $K = 10^5\ M^{-1}$, $\omega = 1$, and $\Delta F_{max} = 3.5$; the ligand binding to the competing lattice is characterized by $K = 10^6\ M^{-1}$, $\omega = 1$ and by $K = 10^4\ M^{-1}$, $\omega = 1$, for high- and low-affinity cases, respectively. For simplicity, the dependence of ΔF upon $(\sum \nu_i)_R$ for the reference lattice was selected to be strictly proportional (straight, dotted line).

In the case where the macroscopic ligand affinity is higher for the competing lattice than for the reference lattice (dashed line), the ligand binding density, $(\sum \nu_i)_S$, on the competing, nonfluorescent lattice is higher when compared to the reference lattice at any value of the observed relative fluorescence change, ΔF. The opposite is true in the case where the ligand affinity is lower for the competing lattice when compared to the reference fluorescent nucleic acid (solid line). The plot rises sharply at the initial values of the the binding density and levels off at the intermediate range of $(\sum \nu_i)_S$, slowly approaching the maximum possible value of ΔF. This behavior results from the fact that ΔF solely reflects the binding density on the reference lattice, $(\sum \nu_i)_R$, which, due to the higher affinity of the reference lattice for the ligand, saturates with the ligand in advance of the competing lattice. The plots in Figure 2 indicate that, in order to obtain the most accurate estimation of the stoichiometry of a protein−competing nucleic acid complex, the affinity of the competing lattice for the protein should be similar to or higher than that of the reference lattice.

Application of the MCT Method to the Binding of the E. coli Replicative Helicase DnaB Protein to Single-Stranded Polynucleotides. In order to illustrate the MCT method for studying the interactions between proteins and nucleic acids, we applied this method to the binding of the *E. coli* replicative helicase DnaB protein with ss polydeoxynucleotide, poly(dA). Binding of poly(dA) and other polydeoxynucleotides to the DnaB helicase does not cause any significant change in the protein fluorescence (M. J. Jezewska, U.-S. Kim, and W. M. Bujalowski, accompanying paper). On the other hand, we recently discovered that binding of the DnaB helicase to the fluorescent etheno derivative of poly(dA), poly(dϵA), induces a strong, ~3.5-fold relative fluorescence increase of the nucleic acid which allows the precise estimation of the stoichiometry and interaction parameters of the DnaB protein−poly(dϵA) complex (Bujalowski & Jezewska, 1995). Thus, poly(dϵA) can serve as a reference fluorescent nucleic acid in the MCT method.

Figure 3 shows the fluorescence titration curves of poly(dϵA) with the DnaB protein in buffer T2 (pH 8.1, 10 °C) containing 50 mM NaCl and 1 mM AMP-PNP[1] in the absence and presence of two different concentrations of poly(dA). A strong shift of the titration curves toward higher $[DnaB]_{Total}$ at higher poly(dA) concentrations indicates significant competition between the two nucleic acids for the protein. Recall that at the same value of relative fluorescence increase the average binding density, $(\sum \nu_i)_S$, on the competing lattice, poly(dA), and the free DnaB

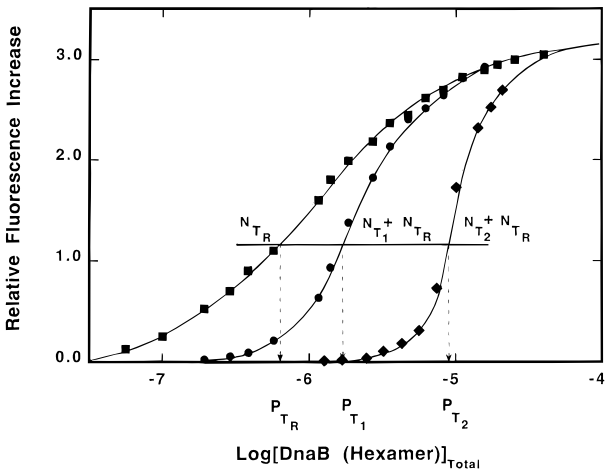

FIGURE 3: Fluorescence titrations ($\lambda_{ex} = 325$ nm, $\lambda_{em} = 410$ nm) of poly(dϵA) with the DnaB helicase in buffer T2 (pH 8.1, 10 °C) containing 50 mM NaCl and 1 mM AMP-PNP, in the presence of different concentrations of poly(dA) (Nucleotide): (■) 0; (●) 2×10^{-5} M; (◆) 1.2×10^{-4} M. The concentration of the poly(dϵA) is 2×10^{-5} M (Nucleotide). Solid lines separate the sets of data points and do not have theoretical bases. The horizontal solid line connects points on all titration curves characterized by the same value of the relative fluorescence increase, ΔF_i. The intersection points of the solid horizontal line with the titration curves, in the presence of poly(dA), define the total DnaB concentrations, P_{T_1} and P_{T_2}, at which the binding density on poly(dϵA), $(\sum \nu_i)_R$, the binding density on poly(dA), $(\sum \nu_i)_S$, and the free helicase concentration, P_F, are constant (see eq 8). The total concentrations of nucleic acids at each titration are included in the figure (see text for details).

concentration, $[DnaB]_F$, must be the same, independent of the concentration of poly(dA) (eq 9a,b). Therefore, from this set of titration curves, one can obtain a set of total DnaB concentrations, P_{T_1} and P_{T_2}, which are determined by the intersection of the horizontal line with either titration curve at which the value of the observed relative fluorescence increase is the same (Figure 3). Since the total concentrations of poly(dA) are known, one can calculate the absolute binding density, $(\sum \nu_i)_S$, of the DnaB protein on poly(dA) and the free DnaB concentration, $[DnaB]_F$, using eq 9a,b. This procedure is then repeated over the entire range of ΔF, providing $(\sum \nu_i)_S$ as a function of $[DnaB]_F$ and thus enabling the construction of a thermodynamically rigorous binding isotherm for DnaB helicase interactions with poly(dA), although the signal used to monitor the binding originates from the reference fluorescent lattice, poly(dϵA) (see below).

Figure 4 shows the dependence of the observed relative fluorescence change, ΔF, upon the binding density, $(\sum \nu_i)_S$, of the DnaB helicase on poly(dA). For comparison, the dependence of ΔF upon the binding density, $(\sum \nu_i)_R$, of the DnaB helicase on the reference lattice, poly(dϵA), is also included (straight dashed line; Bujalowski & Jezewska, 1995). The binding density, $(\sum \nu_i)_S$, could be determined up to the value of ~0.044, which corresponds with $\Delta F \approx 2.6$. Extrapolation to the maximum possible value of $\Delta F = 3.6 \pm 0.3$ gives the maximum value of $(\sum \nu_i)_S = 0.05 \pm 0.005$ and the estimation of the site-size poly(dA)−DnaB helicase complex, $n = 20 \pm 3$. This value is the same as the estimated $n = 20 \pm 3$ in identical solution conditions for the poly(dϵA)−DnaB complex (Bujalowski & Jezewska, 1995; M. Jezewska, U.-S. Kim, and W. Bujalowski, accompanying paper).

[1] Abbreviations: AMP-PNP, β,γ-imidoadenosine 5′-triphosphate; Tris, tris(hydroxymethyl)aminomethane.

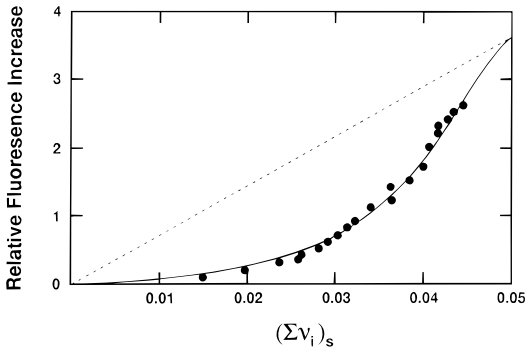

FIGURE 4: The dependence of the relative fluorescence increase of poly(dϵA) upon the binding density, $(\sum\nu_i)_S$, of the DnaB helicase on competing poly(dA) in buffer T2 (pH 8.1, 10 °C) containing 50 mM NaCl and 1 mM AMP-PNP. The dependence of the relative fluorescence increase of poly(dϵA) upon the binding density, $(\sum\nu_i)_R$, of the DnaB helicase on poly(dϵA), in the same buffer conditions, is also included (dashed, straight line). The solid line is the computer fit, using an approach based on the combined McGhee−von Hippel model and the Epstein combinatorial theory for binding a large ligand to two different, competing homogeneous lattices (see below) for the simultaneous binding of the DnaB helicase to poly(dϵA) and poly(dA). The binding of the helicase to the reference poly(dϵA) is described by the McGhee−von Hippel model, using the independently determined intrinsic binding constant $K = 1.2 \times 10^5$ M^{-1}, cooperativity parameter $\omega = 3$, and site-size $n = 20$ (Bujalowski & Jezewska, 1995). Binding of the enzyme to the competing poly(dA) is described by the Epstein combinatorial theory, using cooperativity parameter $\omega = 4.8$, site-size n = 20, and intrinsic binding constant $K = 1.4 \times 10^6$ M^{-1}.

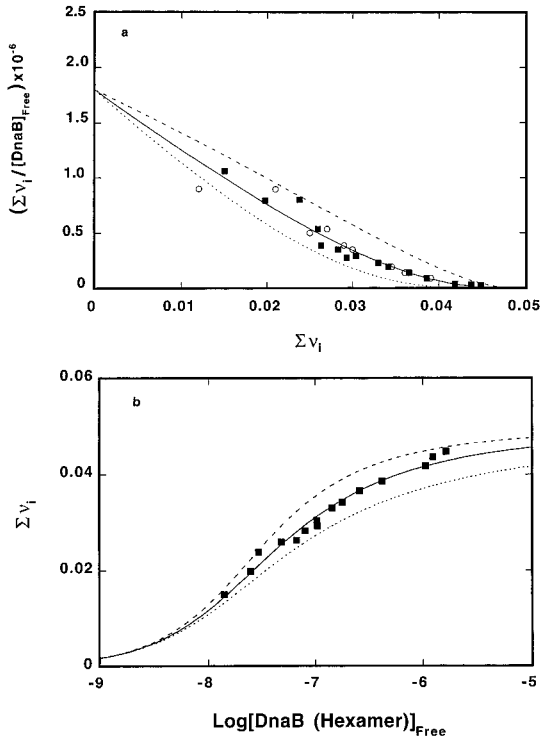

FIGURE 5: a. Scatchard plot of the binding of the DnaB helicase to poly(dA) in buffer T2 (pH 8.1, 10 °C) containing 50 mM NaCl and 1 mM AMP-PNP. The plot was constructed using the absolute binding density, $(\sum\nu_i)_S$, and the free DnaB concentration, $[DnaB]_{Free}$, obtained by applying the MCT method to the titration curves shown in Figure 3. The solid line is the computer fit of the isotherm using the generalized McGhee−von Hippel equation (eq 11a,b) for large ligand binding to the linear, homogeneous lattice, with intrinsic binding constant $K = 1.8 \times 10^6$ M^{-1}, cooperativity parameter $\omega = 6$, and site-size $n = 20$. The computer simulations of the binding isotherms using the same intrinsic binding constant $K = 1.8 \times 10^6$ M^{-1} but different values of ω are also included: (- - -) $\omega = 10$; (⋯) $\omega = 1$. b. Dependence of the average binding density of the DnaB helicase on poly(dA) upon free enzyme concentration in buffer T2 (pH 8.1, 10 °C) containing 50 mM NaCl and 1 mM AMP-PNP. The solid line is the computer fit of the binding isotherm, according to the McGhee−von Hippel model, using intrinsic binding constant $K = 1.8 \times 10^6$ M^{-1}, cooperativity parameter $\omega = 4.8$, and site-size $n = 20$. The computer simulations of the binding isotherms using the same intrinsic binding constant $K = 1.8 \times 10^6$ M^{-1} but with different values of ω are also included: (- - -) $\omega = 10$; (⋯) $\omega = 1$.

Figure 5a shows the binding isotherm in the form of a Scatchard plot (Scatchard, 1949) constructed as described-above (using the titrations shown in Figure 3) for binding the DnaB helicase to poly(dA) in buffer T2 (pH 8.1, 10 °C) containing 50 mM NaCl and 1 mM AMP-PNP. The same binding isotherm in the form of a direct plot of $(\sum\nu_i)_S$ versus $\log[DnaB]_F$ is shown in Figure 5b. Because the site-size of the DnaB−poly(dA) complex has been independently estimated ($n = 20$, Figure 4), the intrinsic binding constant K and the cooperativity parameter ω are the only two parameters left to be determined. The solid lines in Figure 5a,b are computer fits using the generalized McGhee−von Hippel equation (see below) with $K = 1.8 \pm 0.8 \times 10^6$ M^{-1} and $\omega = 6 \pm 3$. The Scatchard plot (Figure 5a) is concave down because the effect of a positive but very low value of ω is overwhelmed by the entropy factor, resulting from the difficulty in finding a gap for free sites which is long enough to accommodate the DnaB protein with the site-size of 20 nucleotides at higher binding densities (McGhee & von Hippel, 1974; Bujalowski et al., 1989). For comparison, theoretical binding isotherms with the same intrinsic binding constant but with different values of ω are also included.

Competition Titration Method Using a Single Concentration of Nonfluorescent, Competing Nucleic Acid. The method described above allows the determination of absolute binding parameters for ligand binding to nonfluorescent nucleic acid by performing fluorescence titrations of reference nucleic acid solutions with the ligand at constant reference nucleic acid concentrations in the presence of two or more concentrations of the competing nucleic acid. It should be noted that the same absolute value of the binding density, $(\sum\nu_i)_S$, and the free ligand concentration, P_F, can be obtained by using a single titration of fluorescent nucleic acid at a single concentration of a competing nonfluorescent nucleic acid in direct reference to the titration curve of the fluorescent nucleic acid alone (Figure 3). This results from the fact that, independently of the presence of a competing, nonfluorescent nucleic acid, the same value of the relative fluorescent change, ΔF, reflects the same value of the ligand binding density on the fluorescent reference lattice, $(\sum\nu_i)_R$, and in turn the same free ligand concentration, P_F. Thus, in Figure 3, the same fluorescence increase of poly(dϵA) upon binding the DnaB helicase corresponds with the same helicase binding density on poly(dϵA) in the presence and absence of the competing poly(dA). Therefore, one can obtain $(\sum\nu_i)_S$ and $[DnaB]_F$ by simultaneously analyzing titration curves 1 and 2 or 1 and 3 instead of 2 and 3 (Figure 3). This approach can be particularly useful if the amount of the competing nucleic acid is limited.

The total DnaB concentrations at which the same value of the relative fluorescence increase, ΔF, of poly(dϵA) is

observed in the absence of poly(dA), P_{T_R}, and in the presence of poly(dA), P_{T_x}, are defined as

$$P_{T_R} = (\sum \nu_i)_R N_{T_R} + P_F \quad (10a)$$

$$P_{T_x} = (\sum \nu_i)_R N_{T_R} + (\sum \nu_i)_S N_{T_x} + P_F \quad (10b)$$

where x can be 1 or 2 (Figure 3).

Solving the set of equations (10a,b) for $(\sum \nu_i)_S$ provides

$$(\sum \nu_i)_S = \frac{(P_{T_x} - P_{T_R})}{(N_{T_x})} \quad (10c)$$

and

$$P_F = P_{T_x} - (\sum \nu_i)_S N_{T_R} - (\sum \nu_i)_R N_{T_x} \quad (10d)$$

The isotherm for the DnaB binding to poly(dA) generated by using the titration at a single poly(dA) concentration in reference to the titration of poly(dϵA) (curves 1 and 3 in Figure 3) is shown in Figure 5a in the form of a Scatchard plot (open circles).

Direct Analysis of the Experimental Isotherm of Large Ligand Binding to Two Competing Nucleic Acid Lattices. The Scatchard analysis provides a way to obtain all interaction parameters for cooperative binding of a large protein ligand to nucleic acid. However, this approach, although very useful, can be applied only to a limited region of the experimental binding isotherm for which absolute binding density and free ligand concentration can be determined (Figure 5). Moreover, the required major transformation of the data may lead to the amplification of errors, particularly at the high and low binding density regions. Therefore, an approach which would allow a direct fit of the experimental binding isotherm using an appropriate physical binding model is of great advantage and provides a method of estimating the binding parameters using all points of the experimental isotherm.

For binding to a single type of nucleic acid lattice, McGhee and von Hippel (1974) derived two equations for noncooperative and cooperative binding of a large ligand to an infinite one-dimensional, homogeneous lattice with overlapping potential binding sites. Using the sequence-generation function approach (Lifson, 1964), we previously derived a single, generalized equation for the McGhee−von Hippel binding model that can be applied to both cooperative and noncooperative binding (Bujalowski et al., 1989). By virtue of eliminating the necessity of using multiple equations, the generalized McGhee−von Hippel equation is particularly useful in any computer fitting or simulation of large ligand binding to nucleic acid for $0 < \omega < \infty$. Using the generalized equation, the binding density, $\sum \nu_i$, and free ligand concentrations, P_F, are described by the following expressions:

$$\sum \nu_i = K(1 - n\sum \nu_i) \left\{\frac{[2\omega(1 - n\sum \nu_i)]}{[(2\omega - 1)(1 - n\sum \nu_i) + \sum \nu_i + R]}\right\}^{(n-1)} \left\{\frac{[1 - (n+1)\sum \nu_i + R)}{2(n - \sum \nu_i)}\right\}^2 P_F \quad (11a)$$

and

$$P_F = \sum \nu_i / \left\{K(1 - n\sum \nu_i) \left\{\frac{[2\omega(1 - n\sum \nu_i)]}{[(2\omega - 1)(1 - n\sum \nu_i) + \sum \nu_i + R]}\right\}^{(n-1)} \left\{\frac{[1 - (n+1)\sum \nu_i + R]}{2(n - \sum \nu_i)}\right\}^2\right\} \quad (11b)$$

where K is the intrinsic binding constant, n is the number of nucleotides covered by the protein in the complex (site-size), ω is the cooperativity parameter, and $R = \{[1 - (n+1)\sum \nu_i]^2 + 4\omega \sum \nu_i(1 - n\sum \nu_i)\}^{0.5}$.

In the case of ligand binding to two competing nucleic acid lattices, the ligand interactions with each nucleic acid are described by an equation in the form of expressions 11a and 11b; thus the whole interacting system is described by two independent isotherms. Any attempt to simultaneously use two isotherms of the type given by 11a is hindered by the fact that they are complex, polynomial, implicit functions of the binding density, $\sum \nu_i$, and free ligand concentration, $\sum \nu_i[F(\sum \nu_i, P_F)]$. Thus, in order to simulate or fit the competition titration isotherms of large ligand binding to two competing nucleic acid lattices, complex and cumbersome numerical calculations are required (Kowalczykowski et al., 1986; McSwingen et al., 1988). To overcome this problem and to obtain a general method to analyze simultaneous binding of a ligand to two or more nucleic acids, without resorting to complex numerical calculations we develop here an approach based on the combined application of the generalized McGhee−von Hippel theory, as defined by eq 11a, and the exact combinatorial theory of Epstein for large ligand binding to a linear, homogeneous lattice (Epstein, 1978). In the Epstein combinatorial approach for binding a large ligand which covers n nucleotide residues to a linear nucleic acid, the partition function of the ligand−nucleic acid system, Z, is defined by eq 12a

$$Z = \sum_{k=0}^{g} \sum_{j=0}^{k-1} P_M(k, j)(KP_F)^k \omega^j \quad (12a)$$

where g is the maximum number of ligand molecules that may bind to the finite nucleic acid lattice (for the nucleic acid lattice M residues long, $g = M/n$; Epstein, 1978), K is the intrinsic binding constant, ω is the cooperative interaction parameter, k is the number of ligand molecules bound, and j is the number of cooperative contacts between k bound ligand molecules in a particular configuration on the lattice. The combinatorial factor $P_M(k,j)$ is the number of distinct ways that k ligands bind to a lattice with j cooperative contacts and is defined by eq 12b (Epstein, 1978).

$$P_M(k, j) = \frac{[(M - nk + 1)!(k - 1)!]}{[(M - nk + j + 1)!(k - j)!(k - j - 1)!]} \quad (12b)$$

The binding density, $\sum \nu_i$, is then obtained by using the standard statistical thermodynamic expression, $\sum \nu_i = \partial \ln Z / \partial \ln P_F$ (Hill, 1985).

$$(\sum \nu_i)_S = \frac{\sum_{k=1}^{g}\sum_{j=0}^{k-1} kP_M(k,j)(KP_F)^k\omega^j}{\sum_{k=0}^{g}\sum_{j=0}^{k-1} P_M(k,j)(KP_F)^k\omega^j} \qquad (12c)$$

Equations 12a−c describe the binding of a large ligand to a finite, linear homogeneous lattice. For a long enough lattice, the isotherm obtained will be, within experimental accuracy, indistinguishable from the isotherm generated using the generalized equation 11a for binding the large ligand to the infinite lattice. We found that in the case of a protein, like the DnaB helicase ($n = 20$), a lattice that can accommodate >40 protein molecules (800 nucleotides) represents an "infinite" lattice for any practical purpose (Epstein, 1978). Contrary to the generalized McGhee−von Hippel equation (11a), expression 12c is an explicit function of the free ligand concentration which allows us to calculate the binding density directly for known K, ω, n, and P_F. Because free ligand concentrations can be explicitly calculated using an infinite lattice model through eq 11b, combining both equations offers a simple way of fitting the simultaneous binding of a large ligand to two (or more) competing, different linear lattices, e.g., a reference fluorescent nucleic acid in the presence of a competing, nonfluorescent nucleic acid. This is accomplished by first applying eq 11a,b to the reference fluorescent nucleic acid, calculating the free ligand concentration of P_F for given values of the parameters K, ω, and n, with $(\sum \nu_i)_R$ as a variable. Subsequently, the obtained value of P_F is introduced into eq 12c which is used to describe the protein binding to a competing, nonfluorescent nucleic acid, and the binding density, $(\sum \nu_i)_S$, is calculated for the given K_S, ω_S, and n_S which characterize the binding of the protein to the competing lattice. The calculations are repeated for the entire range of the $(\sum \nu_i)_R$, generating the required $(\sum \nu_i)_S$ for the competing nucleic acid lattice, as a function of P_F. The experimental binding isotherm, which is the observed fluorescence change ΔF as a function of the total protein concentration P_T, is then obtained by calculating for each value of ΔF the total protein concentration P_T, by introducing $(\sum \nu_i)_S$, $(\sum \nu_i)_R$, and P_F into eq 8a or eq 8b.

Figure 6 shows the fluorescence titration curves of poly(dϵA) with the DnaB helicase in buffer T2 (pH 8.1, 10 °C) containing 50 mM NaCl and 1 mM AMP-PNP in the presence of different concentrations of poly(dA). The solid lines are computer fits of the simultaneous binding of the helicase to two competing nucleic acids using the procedure outlined above for fitting the binding of a large ligand to two competing nucleic acids. The binding of the DnaB helicase to poly(dϵA) has been described using the generalized McGhee−von Hippel equation (eq 11a) for an infinite, homogeneous lattice and using independently determined $K = 1.2 \times 10^5$ M^{-1}, $\omega = 3$, and $n = 20$ (Bujalowski & Jezewska, 1995). The binding of the helicase to competing poly(dA) has been modeled using the Epstein combinatorial theory as described by eqs 12a−c. Because the site-size of the DnaB−poly(dA) complex, $n = 20 \pm 3$, has been independently estimated (see above), there are only two parameters to be determined: the intrinsic binding constant, K, and the cooperativity parameter, ω. The best fit is obtained with $K = (1.4 \pm 0.5) \times 10^6$ M^{-1} and $\omega = 6 \pm 3$,

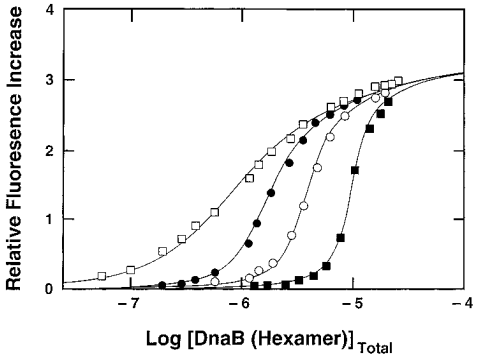

FIGURE 6: Direct computer fit of the fluorescence titrations of poly(dϵA) with the DnaB helicase in buffer T2 (pH 8.1, 10 °C) containing 50 mM NaCl and 1 mM AMP-PNP, in the presence of different concentrations of poly(dA), (data from Figure 3 included), using the combined application of the McGhee−von Hippel and Epstein theories to analyze the binding of a large ligand to two competing linear, homogeneous lattices, as described in the text. Binding of the helicase to the reference nucleic acid, poly(dϵA), is described by the McGhee−von Hippel model, using the independently determined intrinsic binding constant $K = 1.2 \times 10^5$ M^{-1}, cooperativity parameter $\omega = 3$, and site-size $n = 20$ (Bujalowski & Jezewska, 1995). Binding the enzyme to the competing poly(dA) is described by the Epstein combinatorial theory using cooperativity parameter $\omega = 6$, site-size $n = 20$, and intrinsic binding constant $K = 1.4 \times 10^6$ M^{-1}; the selected length of the nucleic acid is 1600 nucleotides. The concentrations of poly(dA) (Nucleotide) are as follows: (□) 0; (●) 2.5×10^{-5} M; (○) 7.5×10^{-5} M; (■) 2.22×10^{-4} M.

which, within experimental accuracy, are in excellent agreement with the values obtained using the Scatchard analysis or the direct binding isotherm (Figure 5a,b).

It should be pointed out that the analysis of the simultaneous binding of a large ligand to competing nucleic acid lattices can also be performed by applying eqs 12a−c of the combinatorial theory to both the reference and the competing nucleic acids. This approach is completely equivalent to the combined application of the McGhee−von Hippel and Epstein models described above. However, the advantage of using the combined McGhee−von Hippel and the Epstein combinatorial theories lies in the tremendously decreased computational time to model the nucleic acid, particularly if a very long lattice is used.

Fluorescence Titrations of Short, Fluorescent Oligonucleotides in the Presence of a Competing Polymer Nucleic Acid. In the analysis described above, we have focused on the simultaneous, competitive binding of the protein to two different nucleic acids with the signal originating from a single reference polymer nucleic acid. However, the analysis does not have to be confined to a polymer reference lattice, and in some cases it can be simplified in terms of mathematics necessary to analyze the isotherms as well as in terms of experimental procedures by using short fragments of nucleic acid as a reference lattice. Such an approach may also serve as an auxiliary test for the interaction parameters of the ligand−nucleic acid complex which have been previously determined, using a polymer reference lattice.

A short nucleic acid fragment, optimal for such analysis, would be an oligomer that forms a simple 1:1 complex with the protein. The obvious choice for a short, reference fluorescent nucleic acid lattice is a nucleic acid fragment long enough to span the site-size of the protein−nucleic acid complex. Such an oligomer binds to the same binding site as a polymer lattice and occupies the entire site.

A selection of the length of the fluorescent reference fragment can be based on the initial estimation of the stoichiometry of the protein−nucleic acid complex obtained using a modified fluorescent polymer nucleic acid.

The degree of saturation of the nucleic acid oligomer with the protein ligand, q_R, and the fluorescence change, ΔF_{oligo}, accompanying the formation of the complex, are described by equations

$$q_R = \frac{K_o P_F}{(1 + K_o P_F)} \tag{13a}$$

and

$$\Delta F_{oligo} = \Delta F_{max}\left[\frac{K_o P_F}{(1 + K_o P_F)}\right] \tag{13b}$$

where ΔF_{max} is the maximum observed fluorescence change of the short nucleic acid lattice upon saturation with the protein and K_o is the binding constant of the oligomer to the protein.

In the presence of a competing, polymer nucleic acid, the observed fluorescence change of the short nucleic acid fragment is thermodynamically linked with the protein binding density, $(\sum \nu_i)_S$, on the competing polymer through the free protein ligand concentration, P_F. However, as in the case of the reference polymer nucleic acid, the same value of ΔF_{oligo} (eq 13b) at different competing nonfluorescent nucleic acid concentrations corresponds with the same degree of saturation of the short nucleic acid fragment and the same free concentration of the protein, P_F, i.e., the same $(\sum \nu_i)_S$. Therefore, performing two fluorescence titrations of a short oligomer nucleic acid, with the protein at the same total oligomer concentrations, O_{T_R}, but in the presence of two different total concentrations of the competing nucleic acid, N_{T_1} and N_{T_2}, allows the determination of the binding density, $(\sum \nu_i)_S$, and free protein concentration, P_F, by applying the same method as described above for a polymer reference lattice. In the case of the short reference lattice, eq 8a and eq 8b take the form of

$$P_{T_1} = (\sum \nu_i)_S N_{T_1} + q_R O_{T_R} + P_F \tag{14a}$$

$$P_{T_2} = (\sum \nu_i)_S N_{T_2} + q_R O_{T_R} + P_F \tag{14b}$$

The absolute binding density, $(\sum \nu_i)_S$, is then defined by eq 9a,b with $(\sum \nu_i)_R$ replaced by q_R.

Analogously, if a single fluorescence titration of a reference oligomer with the protein in the presence of a competing polymer lattice at the concentration N_{T_x}, is used in direct reference to the fluorescence titration of the reference oligomer alone with the protein, eq 10a and eq 10b become

$$P_{T_R} = q_R O_{T_R} + P_F \tag{15a}$$

$$P_{T_x} = q_R O_{T_R} + (\sum \nu_i)_S N_{T_x} + P_F \tag{15b}$$

The absolute binding density, $(\sum \nu_i)_S$, and the free protein ligand concentration, P_F, are then defined by eq 10c and eq 10d with $(\sum \nu_i)_R$ replaced by q_R. To illustrate this simplified procedure, we performed competition titrations of dϵA(pϵA)$_{19}$ with the DnaB protein in the presence of the competing poly(dA). Figure 7a shows the fluorescence titrations of dϵA(pϵA)$_{19}$ with the DnaB protein in buffer T2 (pH 8.1, 10 °C) containing 100 mM NaCl and 1 mM AMP-PNP, in the presence of two different concentrations of poly(dA). The length of dϵA(pϵA)$_{19}$ corresponds exactly with the length of the site-size of the DnaB−ssDNA complex (see accompanying paper), and the oligomer forms a 1:1 complex with the helicase (Bujalowski & Jezewska, 1995).

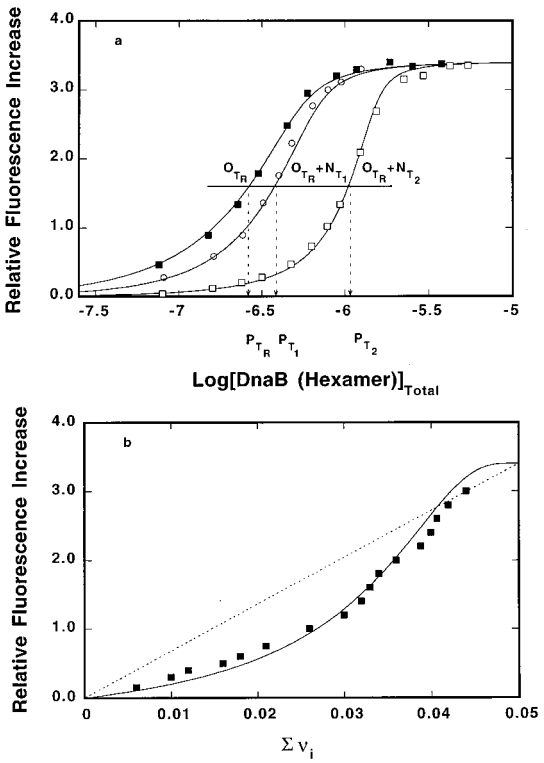

FIGURE 7: a. Fluorescence titrations (λ_{ex} = 325 nm, λ_{em} = 410 nm) of dϵA(pϵA)$_{19}$ with the DnaB helicase in buffer T2 (pH 8.1, 10 °C) containing 100 mM NaCl and 1 mM AMP-PNP, in the presence of different concentrations of poly(dA): (■) 0; (○) 3.5 × 10^{-6} M (Nucleotide); (□) 2.4 × 10^{-5} M (Nucleotide). The concentration of dϵA(pϵA)$_{19}$ is 2 × 10^{-5} M (Oligomer). The solid horizontal line connects points on the titration curves which are characterized by the same value of the relative fluorescence increase, ΔF_{oligo}. The intersection points of the solid horizontal line with the titration curves in the presence of poly(dA) define the total DnaB concentrations, P_{T_1} and P_{T_2}, at which the degree of enzyme binding on dϵA(pϵA)$_{19}$, q_R, the binding density on poly(dA), $(\sum \nu_i)_S$, and free helicase, P_F, concentrations are constant (see eqs 13 and 14). Solid lines are direct computer fits of the binding of the DnaB helicase to dϵA(pϵA)$_{19}$ in the presence of a competing, polymer nucleic acid, poly(dA), using a single-site binding isotherm for the DnaB binding to dϵA(pϵA)$_{19}$ (K_o = 3 × 10^7 M^{-1}, ΔF_{max} = 3.4) and the generalized McGhee−von Hippel equation to describe binding of the helicase to poly(dA) (eq 11a,b) with intrinsic binding constant K = 6.7 × 10^6 M^{-1}, cooperativity parameter ω = 6, and site-size n = 20. b. The dependence of the relative fluorescence increase of dϵA(pϵA)$_{19}$ upon the absolute binding density, $(\sum \nu_i)_S$, of the DnaB helicase on the competing poly(dA) in buffer T2 (pH 8.1, 10 °C) containing 100 mM NaCl and 1 mM AMP-PNP. The plot is constructed by using the absolute binding density values obtained by applying the MCT method to the titrations shown in Figure 7a. The dependence of the relative fluorescence increase of dϵA(pϵA)$_{19}$ upon the degree of binding of the DnaB helicase on dϵA(pϵA)$_{19}$, in the same buffer conditions, is also included (dashed, straight line). Solid line is the computer simulation of the binding of the DnaB helicase to dϵA(pϵA)$_{19}$, in the presence of the competing poly(dA), using a single-site binding isotherm for the DnaB binding to dϵA(pϵA)$_{19}$ (K_o = 3 × 10^7 M^{-1}, ΔF_{max} = 3.4) and the generalized McGhee−von Hippel equation to describe the binding of the helicase to poly(dA) (eq 11a,b) with intrinsic binding constant K = 6.7 × 10^6 M^{-1}, cooperativity parameter ω = 6, and site-size n = 20.

The dependence of the observed relative fluorescence increase, ΔF_{oligo}, upon the absolute binding density, $(\sum \nu_i)_S$, of DnaB on poly(dA) is shown in Figure 7b. The dependence of ΔF_{oligo} upon the degree of binding of DnaB on dϵA(pϵA)$_{19}$, expressed as the DnaB hexamer per nucleotide of dϵA(pϵA)$_{19}$, is also included (dashed, straight line; M. Jezewska, U.-S. Kim, and W. M. Bujalowski, , accompanying paper). The separation of the binding isotherms allowed us to obtain $(\sum \nu_i)_S$ up to the value of $\sim$0.045 ($\sim$22 nucleotides per the DnaB helicase hexamer) and up to $\Delta F_{oligo} \approx 3$. Extrapolation through the last $\sim$10% of the fluorescence change to $\Delta F_{max} = 3.4$ provides an estimation of the stoichiometry of the complex at saturation, $(\sum \nu_i)_S = 0.047 \pm 0.005$ ($n = 21 \pm 4$). The plot is concave down, showing that the degree of saturation of poly(dA) exceeds the degree of dϵA(pϵA)$_{19}$ saturation with the helicase at the same value of ΔF_{oligo} and indicating a higher macroscopic affinity of DnaB for the polymer when compared with dϵA(pϵA)$_{19}$. However, the computer simulation (solid line in Figure 7b) using binding parameters for the DnaB−poly(dA) complex (see below) indicates that at very high values of $(\sum \nu_i)_S$ (>0.046; very close to the maximum saturation of the lattice), the macroscopic affinity for the poly(dA) becomes weaker when compared with the oligomer due to the entropy factor resulting from the difficulty of finding a free nucleotide gap large enough to accommodate the helicase. The solid lines in Figure 7a are direct computer fits of the experimental binding isotherms for the simultaneous binding of the protein to the short oligomer (eq 13b) and the polymer nucleic acid (eq 11a,b), using $K = 6.7 \times 10^6$ M^{-1}, $\omega = 6$, and $n = 20$ for poly(dA). The binding parameters determined for the DnaB−poly(dA) system are, within experimental accuracy, the same as the parameters obtained using the MCT method with the polymer reference lattice, poly(dϵA) (Jezewska et al., accompanying paper).

An interesting feature of the competition titrations of the short oligomer, with the protein in the presence of a competing long polymer nucleic acid, is that it may provide *qualitative* information about the cooperativity of the binding of a protein to the polymer nucleic acid by merely inspecting the shape of the fluorescence titration curve. Figure 8a shows computer simulations of the fluorescence titrations of the oligomer with a protein ligand in the presence of long polymer nucleic acids which differ by the value of the cooperativity parameter ω. The oligomer binds to the protein with a 1:1 stoichiometry, and its affinity is characterized by the binding constant $K = 1 \times 10^7$ M^{-1}. The maximum fluorescence increase upon binding is $\Delta F_{max} = 3.5$. The intrinsic binding constant of the protein for the polymer nucleic acid and the site-size of the complex are 10^5 M^{-1} and 20, respectively. Increasing the value of ω by two orders of magnitude (from 1 to 100) at the same intrinsic binding constant, has little effect on the initial part of the binding curve. However, as ω increases, the upper part of the titration curve shifts significantly, approaching the stoichiometric curve at high ω values (see Figure 8a). This behavior results from the fact that at a low value of binding density, $(\sum \nu_i)_S$, with only a few protein molecules bound, the macroscopic affinity of the protein for polymer lattices is dominated by the intrinsic binding constant, K, with little influence of ω, while at a high value of binding density the cooperative interactions become a significant part of the ligand macroscopic affinity. Thus, larger separation in the upper parts of the titration curves of the fluorescent oligomer with the protein in the presence of competing, nonfluorescent polymer nucleic acid, indicates significant, positive cooperativity in the protein binding to the polymer lattice. The situation is different if the protein binds noncooperatively to different nucleic acids with different intrinsic binding constants (Figure 8b). As the intrinsic binding constant increases, the whole titration curve is shifted, approaching the stoichiometric curve at a very high binding affinity. This results from the fact that, contrary to the cooperative binding, at any value of the binding density, the macroscopic affinity of the protein ligand is mainly determined by the intrinsic binding constant. In this context, inspection of the titration curves of dϵA(pϵA)$_{19}$, with DnaB in the presence of poly(dA) (Figure 7), qualitatively indicates that the macroscopic

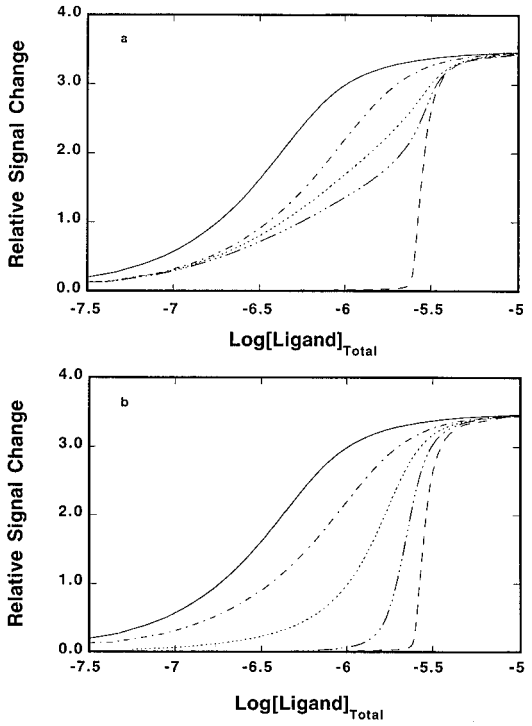

FIGURE 8: a. Theoretical fluorescence titrations of a fluorescent nucleic acid oligomer with a protein which form a complex with a 1:1 stoichiometry in the presence of different, competing polymer nucleic acids at the same concentration of the polymer [5×10^{-5} M (Nucleotide)]. The interactions of the protein with polymer nucleic acids are characterized by the same intrinsic binding constant $K = 1 \times 10^5$ M^{-1}, site-size $n = 20$, but by a different cooperativity parameter ω; nucleic acid oligomer alone (—); $\omega = 1$ (-•-); $\omega = 50$ (- - -); $\omega = 100$ (-•••-). The concentration of the oligomer is 5×10^{-7} M, its affinity toward the protein is characterized by the equilibrium constant $K = 1 \times 10^7$ M^{-1} and the maximum fluorescence increase upon protein binding $\Delta F_{max} = 3.5$. b. Theoretical fluorescence titrations of a fluorescent nucleic acid oligomer with a protein which form a complex with a 1:1 stoichiometry in the presence of different, competing polymer nucleic acids at the same concentration of the polymer [5×10^{-5} M (Nucleotide)]. The interactions of the protein with polymer nucleic acids are characterized by the same cooperativity parameter $\omega = 1$, site-size $n = 20$, but by different intrinsic binding constants, K, nucleic acid oligomer alone (—); 1×10^5 M^{-1} (-•-); 1×10^6 M^{-1} (- - -); 1×10^7 M^{-1} (-•••-). The concentration of the oligomer is 5×10^{-7} M, its affinity toward the protein is characterized by equilibrium constant $K = 1 \times 10^7$ M^{-1} and the maximum fluorescence increase upon protein binding, $\Delta F_{max} = 3.5$. Stoichiometric binding curves in Figure 8a,b (— — —) have been obtained using $K = 1 \times 10^7$ M^{-1} and $\omega = 150$ for the ligand binding to the polymer lattice.

affinity of the helicase to poly(dA) is dominated by the intrinsic binding constant with very weak or nonexistent cooperative interactions, as quantitatively determined by the rigorous analysis described in this work and in the accompanying paper. Similar, simple qualitative evaluations of the cooperativity in ligand binding to a long, competing nucleic acid, using a polymer reference lattice, may be obscured by the effects of the cooperativity and by the entropy factor resulting from the overlapping nature of the binding sites occuring on both polymers.

Application of Fluorescence Anisotropy Using the MCT Method. In some protein−nucleic acid systems, in spite of the fact that a labeled nucleic acid is fluorescent, the changes in its fluorescence intensity upon the complex formation may not be adequate enough to obtain reliable stoichiometry or interaction parameters, although the affinity can still be high. In such cases, fluorescence anisotropy of the labeled nucleic acid may be used to monitor the binding, provided the complex formation leads to a substantial decrease of the mobility of the fluorescent marker on the nucleic acid (Heyduk & Lee, 1990, 1992; LeTilly & Royer, 1993). The binding of large protein molecules to a short fragment of labeled nucleic acid will always decrease rotational mobility of the fluorophore and lead to a substantial increase of the fluorescence anisotropy, providing an adequate spectroscopic signal to monitor the binding. On the other hand, interactions of the protein with a long fluorescent polymer lattice may not, and most probably will not, cause significant changes of the nucleic acid fluorescence anisotropy. This is also one of the reasons the use of fluorescence anisotropy of a fluorescent-labeled nucleic acid is usually limited to studying protein interactions with short oligomers. However, the binding of the protein to long polymer nucleic acids can be rigorously determined by using the MCT method described here when the binding of the protein to the short fluorescent fragment of nucleic acid is followed by monitoring the increase of its fluorescence anisotropy in the presence of a competing, nonfluorescent polymer nucleic acid. This approach is completely analogous to the one described in the previous section, the difference being the type of spectroscopic signal used to monitor the binding.

An example of the application of this approach is presented in Figure 9, showing the fluorescence titrations of dϵA(pϵA)$_{19}$ with the DnaB helicase in the absence and presence of poly(dA) and using the fluorescence anisotropy of dϵA(pϵA)$_{19}$ to monitor the binding. Binding of the large DnaB hexamer to the 20-mer causes a significant (~2.5-fold) increase of the fluorescence anisotropy of the nucleic acid from 0.05 to 0.128. The obtained binding constant, $K_o = (3 \pm 0.5) \times 10^7$ M^{-1}, is the same (as expected) as the one determined by measuring the increase of the fluorescence intensity of the 20-mer upon binding to the curve. In the presence of poly(dA), the titration curve is shifted toward higher DnaB concentrations, indicating competition for the helicase between the oligomer and the polymer. Analysis of the titration curves, using the method described above for the titrations where the binding was followed by the fluorescence intensity of the oligomer (eq 14a,b), yields the estimation of the absolute binding density, $(\sum \nu_i)_S$, and the free DnaB concentration over a large range of titration curves. Within experimental accuracy, these estimates are identical to the ones obtained by using fluorescence intensity to monitor the binding (data not shown). The solid lines in Figure 9 are computer fits using the same binding parameters, $K = 6.7 \times 10^6$ M^{-1}, $\omega = 6$, and $n = 20$, as previously determined and using the fluorescence intensity of dϵA(pϵA)$_{19}$ to monitor the binding.

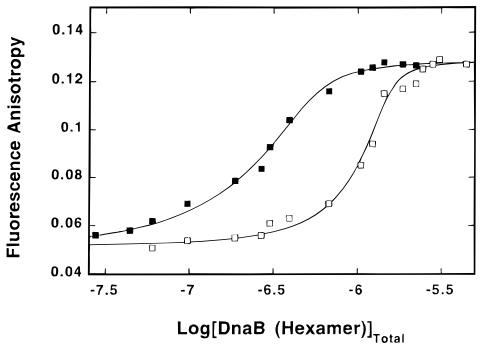

FIGURE 9: Fluorescence anisotropy titrations ($\lambda_{ex} = 325$ nm, $\lambda_{em} = 410$ nm) of dϵA(pϵA)$_{19}$ with the DnaB helicase in buffer T2 (pH 8.1, 10 °C) containing 100 mM NaCl and 1 mM AMP-PNP, in the absence and presence of poly(dA): (■) 0; (□) 2.4×10^{-5} M (Nucleotide). The concentration of dϵA(pϵA)$_{19}$ is 5×10^{-7} M (Oligomer). Solid lines are the direct computer fit of the binding of the DnaB helicase to dϵA(pϵA)$_{19}$ in the absence and presence of competing poly(dA), using a single-site binding isotherm for the DnaB binding to dϵA(pϵA)$_{19}$ ($K_o = 3 \times 10^7$ M^{-1}, $r_{min} = 0.052$, $r_{max} = 0.128$) and the generalized McGhee−von Hippel equation to describe binding of the helicase to poly(dA) (eq 11a,b) with intrinsic binding constant $K = 6.7 \times 10^6$ M^{-1}, cooperativity parameter $\omega = 6$, and site-size $n = 20$.

CONCLUSION

Understanding macromolecular interactions, such as those involving proteins and nucleic acids, requires detailed knowledge of the energetics and kinetics of the formed complexes. Spectroscopic methods are widely used in characterizing the energetics (thermodynamics) of ligand−macromolecule interactions in solution. However, spectroscopic methods can only be applied if a change of spectroscopic property of the macromolecule (or ligand) occurs upon formation of a complex. The approach presented in this work describes a general method of analysis of macromolecular binding for the protein−nucleic acid systems in which there is insufficient spectroscopic signal change directly accompanying the binding. This can be achieved through competition titrations of a suitable fluorescent derivative of a nucleic acid with the protein in the presence of a competing, nonfluorescent nucleic acid, whose interaction parameters (site-size, intrinsic affinity, cooperativity) with the protein are to be determined. The method enables one to obtain the absolute binding density, $(\sum \nu_i)_S$, and the free protein ligand concentration, P_F, hence, to construct the entire, model-independent binding isotherm. Once the thermodynamically rigorous isotherm is obtained, it can be analyzed by using the statistical thermodynamic models that incorporate known molecular aspects of the protein−nucleic acid interactions, like cooperativity, overlap of potential binding sites, etc. (Crothers, 1968; McGhee & von Hippel, 1974; Bujalowski et al., 1989).

We illustrated the application of the MCT method for the *E. coli* replicative helicase DnaB protein binding to a single-stranded nucleic acid, poly(dA), using the fluorescent etheno derivative, poly(dϵA), as the reference fluorescent nucleic acid (Bujalowski & Jezewska, 1995). Fluorescence titrations of

poly(dεA) with the DnaB helicase in the presence of competing poly(dA) allowed us to determine the site-size of the poly(dA)−DnaB complex and the true, thermodynamically rigorous binding isotherm (Figure 5a,b).

Because the expression for the isotherm for large ligand binding to an infinite homogeneous lattice is a complex, implicit polynomial, the analysis of the simultaneous ligand binding to two different competing nucleic acids has so far relied on complex numerical calculations (Kowalczykowski et al., 1986). The approach developed in this work is based on the combined McGhee−von Hippel and Epstein theories for the binding of large ligands to two competing, linear lattices and allows a precise, direct fit of the entire experimental isotherm and the extraction of the binding parameters. Using our approach, the complexity of fitting a simultaneous binding of a large ligand to two or more competing nucleic acid lattices is reduced to the same level as fitting ligand binding to a single type of nucleic acid.

The Macromolecular Competition Titration method described in this paper enables one to obtain, rigorously and quantitatively, the interaction parameters of protein−nucleic acid interactions, which may not be available by other methods, particularly for a long polymer lattice, if the binding is not accompanied by adequate spectroscopic signal changes. The method can be applied using different fluorescent nucleic acids or fluorophores, although the etheno derivatives of nucleic acid are especially suitable. These derivatives are relatively easy to prepare, they have significant blue fluorescence, their excitation band lies far from the protein absorption spectrum, and the modification eliminates the possibility of base pairing with other nucleic acids. The method is not limited to the particular size of the reference nucleic acid. The interaction parameters for a given unmodified nucleic acid can be determined using a fluorescent polymer nucleic acid as well as short nucleic acid oligomers. Particularly, a simple analysis of the competition titration experiments is described in which the fluorescent, short fragment of a nucleic acid, spanning the exact site-size of the protein−nucleic acid complex and binding with only a 1:1 stoichiometry to the protein, is used as a reference macromolecule.

We have discussed the MCT method as applied to studying protein−nucleic acid interactions using the fluorescence of the reference nucleic acid to monitor the binding. However, the MCT method can generally be applied to any ligand−macromolecule system by monitoring the binding using the spectroscopic signal originating from a reference macromolecule in the presence of the competing macromolecule whose interaction parameters with the ligand are to be determined and whose spectroscopic properties do not change upon complex formation with the ligand.

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