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Sequence Distribution and Intercooperativity Detection for Two Ligands Simultaneously Binding to DNA

Abstract: *A method for detecting and quantifying the cooperativity in the simultaneous binding of two ligands, A and B, to DNA (intercooperativity; ω_{AB}) is proposed. This involves the determination of an apparent affinity constant* K^{app} *for one of the ligands (A) in the limit of its null saturation ($\nu_A \to 0$), in the presence of the second one (B). A definition for this constant is given and an expression is derived corresponding to a simple model of competitive binding to an unbranched three-state homogeneous polar lattice with nearest-neighbor interactions (Markov chain). The ratio between the apparent and intrinsic affinity constants of one ligand in the maximum saturation limit of the other one becomes ω_{AB}^2, and thus can be graphically obtained from* K_A^{app} vs ν_B *plots. All the frequencies that define the sequence distribution of ligands can be easily calculated by introducing some generalized statistical weights for the free lattice monomer in a standard sequence generating function procedure. A model of fluorescence quenching emission is obtained from such frequencies under the hypothesis of a short-range electron transfer mechanism of the deactivation; it confirms, as suggested by the binding model, an outstanding influence of the intercooperativity on the distribution.* © 2001 John Wiley & Sons, Inc. Biopolymers 58: 562–576, 2001

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INTRODUCTION

The competitive binding of one or several ligands to a one-dimensional polymer has extensively been modeled.[1–10] Several methods are available for obtaining a statistical partition function or a secular equation from which expressions for the binding isotherms can be derived. The matrix method[10–12] is recommended when the size of the lattice representing the biopolymer is finite and the number of states of the lattice is small. Alternatively, nearly infinite chains can be represented the best by the sequence generating function (SGF) method of Lifson.[1–3] Other approaches, such as the conditional probability method, which led to the McGhee–von Hippel isotherm, are

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restricted to particular examples of interaction. For example, in their original study,[4,5] McGhee and von Hippel considered the simultaneous noncooperative binding of several species to the same lattice but cooperativity was beyond its scope, partly because of the requirement of *explicit* analytical expressions for the probabilities. However, this method has the advantage that the statistical distribution of ligands in the lattice is directly obtained from the procedure. On the other hand, the SGF method is easily generalizable to ligands that cover more than one lattice monomer (*n*-mers) or are able to interact in a number of ways with the polymer, with or without cooperativity (see Chen[2]). As far as the weights for a monomer to be in the different possible states are known, the derivation of the secular equation is straightforward. Bujalowski et al.[3] gave an illustrative example for the binding of large ligands to any three-state lattice. The free state of the monomer is common to all of the models, whereas the other two states correspond to very different situations: two different ligands; a single one that binds in two opposed orientations; a ligand capable to bind another molecule itself ("piggyback"); a ligand that transits between two conformations in solution. In turn, the free monomer can exist in two alternative conformations. Extensions of the models to simultaneously include all or some of these features are conceivable but the parameters proliferate in such an excessive manner that prevents their accurate experimental determination.

Certain problems require obtaining the ligand distribution along the polymer, to estimate, for example, mean distances between consecutive bound ligands. This is particularly the case if two or more species bind that undergo cross-reactions, because the reaction yield depends on the distances between the reactants.[13] In addition, the polymer may act as a bridge to keep the chemical potential difference.[14] During the last decade, the question whether DNA is an electron conductor, and if so how efficient, has received much attention.[15–26] Attempts to give an answer include spectroscopic measures of DNA-mediated electron transfer reaction rates between donor–acceptor pairs. Two main approaches have been used. In the first one, reversible and nonspecific binding of the reactants to DNA serves to generate a distribution of ligand.[16–20] An average distance is then calculated, assuming complete binding and random distribution of the ligands. Fluorescence emission quenching, attributable to the electron transfer reaction, is used to calculate the value of a parameter reflecting the dependence of electron transfer on distance. The second approach consists in the employment of reactants tethered to oligonucleotides.[21,27] The determinations performed in these constructs are identical to those accomplished in equilibrated systems; however, the donor–acceptor separation is controlled exactly. Some of the conclusions shed by these alternative approaches are contradictory. There are examples in both sets of experiments indicating the possibility of long-range electron transfer,[18,21] but also others implying a sharp dependence on distance.[20,27] The arguments against long range electron transfer between noncovalently bound species are diverse, but many of them employ some cooperativity reasoning (for a review, see Ref. 25).

This paper is intended to provide a new tool for the determination of cooperativity between different ligands in their binding to linear biopolymers, which may be useful to unify the various experimental results in a coherent view. Whereas the cooperative binding of a single ligand has been studied in a variety of ways, ranging from the simple fit of isotherm equations to specifically designed experiments, such as those proposed by Nechipurenko,[28] the cooperativity between different species has only been tackled by means of model fitting. This frequently leads to lame conclusions, due to the high number of degrees of freedom for the fitting (see Lincoln[8]). Besides, as recognized by Correia and Chaires,[9] the error on the determination of certain parameters propagates on others, which is particularly true for cooperativity and ligand size parameters.

Here we propose a generalization of the three-state secular equation given by Bujalowski et al.,[3] which simplifies the calculation of the sequence frequencies and conditional probabilities by the SGF method. We show that a simple proximity model for electron transfer is highly dependent on a single cooperativity parameter, which explains the fluorescence emission quenching of an intercalating metal complex by another one. An experimental scheme to determine the value of such a cooperativity parameter is suggested on a simple competition basis.

DEFINITION OF THE MODEL

We consider the binding of two different ligands, A and B, to a linear infinite lattice made up of monomers. The ligands are assumed to bind in only one direction. The lattice is homogenous, with nearest-neighbor interactions, i.e., between ligands bound in adjacent elementary units as defined by Chen.[2] Thus, the binding to an elementary unit only depends on the

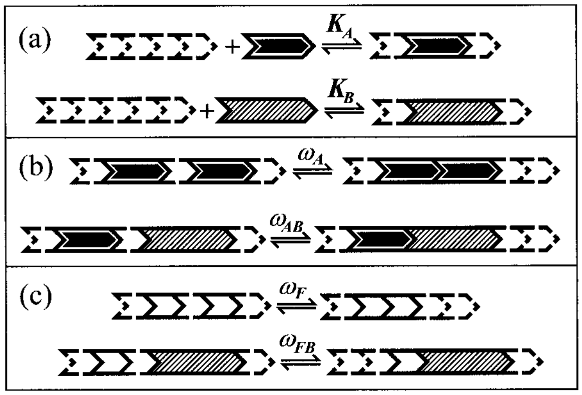

FIGURE 1 One-directional binding model. (a) Binding equilibrium constants of two different ligands to an oriented lattice. The dashed lines identify the lattice monomers. An elementary unit is any uninterrupted fragment of the lattice. In the example, the ligands cover two ($n = 2$) and three ($k = 3$) monomers on binding. (b) Cooperativity constants quantify the predominance of adjacent binding of two ligands over isolated binding. The ligands may be identical (intraspecies cooperativity; upper equilibrium) or differ in nature (interspecies cooperativity or intercooperativity; lower equilibrium). The order of the ligands is essential in the latter case. (c) Hypothetical cooperativity parameters for the free monomers. Ideal labeling of certain free monomers (solid line) permits considering adjacent and isolated binding as for true ligands, and thus defining a set of equilibrium constants that are valid as generalized statistical weights.

one to be occupied and on the previous one, that is, the sequence is a Markov chain.[29] The model here derived includes a "single-binding" condition, which means that any monomer can bind only one molecule, provoking exclusion between occupied elementary units. Three monomer states are possible as a consequence of all these requirements—namely, the unoccupied or free state, and two bound states, with the monomer bound either to the first class of ligands or to the second one. The bound ligands cover n and k monomers, respectively. All these features are shown in Figure 1.

The saturation of the lattice, and thus the distribution of ligands, are functions of the free ligand concentrations (L_A, L_B). This is parameterized by the intrinsic affinity constants for the isolated ligands (K_A and K_B). Consequently, K_AL_A and K_BL_B are the statistical weights for the single occupied elementary units, which will be necessary for applying the SGF method. The interactions between identical ligands are quantified by the parameters ω_A and ω_B, for the two ligands, respectively. Since the lattice is assumed to be polar, and the ligands to bind in only one direction, only *one* intraspecies cooperativity parameter is needed for each ligand. Therefore, there is no difference between the interactions with the preceding and the succeeding ligands in the lattice.

In the following derivation, we introduce a general statistical weight for the free monomer, a_F, instead of the arbitrary value of unity usually chosen. This cannot be interpreted as an affinity parameter of a ligand for the lattice and has no evident physical meaning. In fact, the last step of the derivation will consist in returning to physically sensible conditions by taking the limit to the unitary statistical weight. Therefore, a_F must not be considered as a measurable magnitude, but should be viewed as a mathematical tool that simplifies, as will be shown below, the calculation of some sets of frequencies that constitute the ligand distribution. Every macroscopic magnitude (for instance, a frequency) is related to a weight, so that the later becomes a parameter in the description of the former. Similarly, the statistical relationship between consecutive free monomers may be expressed by the quantity ω_F. The notation refers to the analogy with the cooperativity constants for the ligands (see figure 1 for a definition). However, in a realistic model its value can only be unity, since it represents an equilibrium between two identical situations, and will be treated here in a similar fashion as the weight for the individual free monomer.

The first step of the SGF method[1] consists in defining the SGF's for all the states of the lattice. This has to be done consistently with the SGF of the complete system, i.e., $\Gamma(x) = \sum_{N=0}^{\infty} Z^{(N)} x^{-N}$, where $Z^{(N)}$ is the partition function of a polymer N monomers long and x is the variable of the SGF. For a single n binder, any state of the polymer can be described as an alternation of homogenous sequences, $i_0, j_1, i_1, \ldots, j_s, i_s, j_0$, i and j being the lengths of sequences of free monomer and bound ligand, respectively. Any i_σ or j_σ must be at least one monomer, except for i_0 or j_0. In fact, $i_0 = 0$ represents a lattice starting with bound ligands, whereas $j_0 = 0$ corresponds to a polymer ending up with free monomers. Therefore, the number of internal sequences is $2s$ and is subject to the following restriction, to accomplish with the total length of the polymer:

$$\sum_{\sigma=0}^{s} (i_\sigma + j_\sigma) = \sum_{\sigma=0}^{s} (i_\sigma + nl_\sigma) = N, \qquad 0 \le s \le \left[\frac{N}{1+n}\right] \tag{1}$$

Since monomers bound to one ligand are lumped in groups of n adjacent monomers, j can be substituted by a different index, l, so that, $j_\sigma = nl_\sigma$. Accordingly, the partition function of the polymer can be expressed as

$$Z^{(N)} = \sum_{s=0}^{[N/(1+n)]} \sum_{\{i_\sigma,l_\sigma\}} \prod_{\sigma=0}^{s} u_{i_\sigma} \upsilon_{l_\sigma}^{n} \tag{2}$$

where$\{i_\sigma,l_\sigma\}$ indicates all possible lengths under the restriction (1), and u_{i_σ} and $\upsilon_{j_\sigma} = \upsilon_{j_\sigma}^{n} = \upsilon_{n_\sigma}^{l}$ are contributions of free (length i) and bound (length $j = nl$) sequences, respectively. Substitution of (2) in the SGF $\Gamma(x)$ using the condition (1), yields, after some calculations,

$$\Gamma(x) = \sum_{s=0}^{\infty} \sum_{i_0=0}^{\infty} u_{i_0} x^{-i_0} \sum_{l_0=0}^{\infty} \upsilon_{l_0}^{n} x^{-nl_0}$$

$$\times \prod_{\sigma=1}^{s} \sum_{i_\sigma=1}^{\infty} u_{i_\sigma} x^{-i_\sigma} \sum_{l_\sigma=1}^{\infty} v_{l_\sigma}^{n} x^{-nl_\sigma} \tag{3}$$

To account for the interactions between consecutive monomers or ligands, the statistical weights should be taken as $u_{i\sigma} = u_i = \omega_F^{i-1} a_F^{i}$ and $\upsilon_{l_\sigma}^{n} = \upsilon_n^{l} = \omega_A^{l-1}(K_\mathrm{A}L_\mathrm{A})^{l}$. Note that in a sequence i long, only $i - 1$ contacts occur, which explains the exponents on the cooperativity factors. The sequence generating functions for each state can be extracted from (3) as follows.[30,31] For the free monomer:

$$U(x) = \frac{1}{\omega_\mathrm{F}} \sum_{i=1}^{\infty} (\omega_\mathrm{F} a_\mathrm{F})^{i} (x)^{-i} = \frac{a_\mathrm{F}}{x - \omega_\mathrm{F} a_\mathrm{F}} \tag{4a}$$

For the monomer bound to ligand A:

$$V(x) = \frac{1}{\omega_\mathrm{A}} \sum_{i=1}^{\infty} (\omega_\mathrm{A} K_\mathrm{A} L_\mathrm{A})^{i} (x^{n})^{-i}$$

$$= \frac{K_\mathrm{A} L_\mathrm{A}}{x^{n} - \omega_\mathrm{A} K_\mathrm{A} L_\mathrm{A}} \tag{4b}$$

Analogously, for the monomer bound to a second ligand, B:

$$W(x) = \frac{1}{\omega_\mathrm{B}} \sum_{i=1}^{\infty} (\omega_\mathrm{B} K_\mathrm{B} L_\mathrm{B})^{i} (x^{k})^{-i}$$

$$= \frac{K_\mathrm{B} L_\mathrm{B}}{x^{k} - \omega_\mathrm{B} K_\mathrm{B} L_\mathrm{B}} \tag{4c}$$

Remarkably, the variables of $V(x)$ and $W(x)$ are raised to the exclusion sizes, indicating that a monomer to a ligand must be succeeded by $n - 1$ or $k - 1$ monomers bound to the same ligand.

Interspecies cooperativity (*intercooperativity*) is appropriately represented by a couple of parameters ω_AB and ω_BA. These are the equilibrium constants associated with the process of moving the two ligands indicated in the subscript to neighbor elementary units from other units initially separated by one free monomer at least. Note that in this case different interactions can exist between the same pair of ligands ordered contrarily. Thus, interactions of this class can be asymmetric. Again, it its reasonable to make use of analogous parameters, ω_FA, ω_AF, ω_FB, and ω_BF, involving the free monomer, as long as they are employed as statistical weights. Obviously, the only possible value for them is unity, since all free monomers are indistinguishable and the equilibrium occurs between identical states. However, we maintain the notation for the reasons given above. To include the parameters of intercooperativity in the model, the procedure established by Lifson[1] involves building a matrix that takes into account all the possible alternations of states.

$$\mathbf{M} = \begin{pmatrix} 0 & \omega_\mathrm{FA} V(x) & \omega_\mathrm{FB} W(x) \\ \omega_\mathrm{AF} U(x) & 0 & \omega_\mathrm{AB} W(x) \\ \omega_\mathrm{BF} U(x) & \omega_\mathrm{BA} V(x) & 0 \end{pmatrix} \tag{5}$$

The secular equation is readily obtained according to $\Phi(x) = |\mathbf{M} - \mathbf{I}| = 0$, where $\mathbf{I}$ is the identity matrix. This equation has three discontinuities, $x = \{\omega_\mathrm{F} a_\mathrm{F}, (\omega_\mathrm{A} K_\mathrm{A} L_\mathrm{A})^{1/n}, (\omega_\mathrm{B} K_\mathrm{B} L_\mathrm{B})^{1/k}\}$. For nearly infinite sequences ($N \rightarrow \infty$, where N is the lattice size), the partition function of the system is $Z = x_1^{N}$. The value x_1 is the greatest root of the secular equation, which only depends on its numerator $\Psi(x)$. Therefore, it is feasible to avoid the discontinuities of $\Phi(x)$ by operating directly on $\Psi(x)$. Its generalized expression becomes:

$$\begin{aligned} \Psi(x) = {} & x^{n+k+1} - \omega_\mathrm{F} a_\mathrm{F} x^{n+k} - \omega_\mathrm{A} K_\mathrm{A} L_\mathrm{A} x^{k+1} \\ & - \omega_\mathrm{B} K_\mathrm{B} L_\mathrm{B} x^{n+1} + (\omega_\mathrm{A}\omega_\mathrm{F} - \omega_\mathrm{AF}\omega_\mathrm{FA}) K_\mathrm{A} L_\mathrm{A} a_\mathrm{F} x^{k} \\ & + (\omega_\mathrm{B}\omega_\mathrm{F} - \omega_\mathrm{BF}\omega_\mathrm{FB}) K_\mathrm{B} L_\mathrm{B} a_\mathrm{F} x^{n} \\ & + (\omega_\mathrm{A}\omega_\mathrm{B} - \omega_\mathrm{AB}\omega_\mathrm{BA}) K_\mathrm{A} K_\mathrm{B} L_\mathrm{A} L_\mathrm{B} x \\ & + (\omega_\mathrm{A}\omega_\mathrm{BF}\omega_\mathrm{FB} + \omega_\mathrm{B}\omega_\mathrm{AF}\omega_\mathrm{FA} - \omega_\mathrm{FA}\omega_\mathrm{AB}\omega_\mathrm{BF} \\ & - \omega_\mathrm{FB}\omega_\mathrm{BA}\omega_\mathrm{AF} - \omega_\mathrm{A}\omega_\mathrm{B}\omega_\mathrm{F} \\ & + \omega_\mathrm{AB}\omega_\mathrm{BA}\omega_\mathrm{F}) K_\mathrm{A} K_\mathrm{B} L_\mathrm{A} L_\mathrm{B} a_\mathrm{F} = 0 \end{aligned} \tag{6}$$

Several particular cases of this equation have been shown to lead to the McGhee–von Hippel isotherms,[3] demonstrating the validity of Eqs. (4).

LIMIT APPARENT AFFINITY CONSTANT AS AN INDICATOR OF INTERCOOPERATIVITY

Binding experiments are those intended to measure the equilibrium macroscopic saturation fractions ν defined as bound ligand concentration over macromolecule concentration (monomer concentration in the case of linear polymers), and the free concentrations L for every ligand. Intercooperativity is the only binding property that cannot be considered in individual experiments of one ligand interacting with a linear polymer. This fact suggests that its determination requires specifically designed schemes. However, currently available methods for evaluating its magnitude involve fitting the experimental data, collected in Scatchard plots (ν/L vs ν), to the equations derived from the model. This is in any case cumbersome, since for two species the Scatchard surface to be fitted is a tetradimensional one (ν_A/L_A and ν_B/L_B vs ν_A and ν_B). Although previous knowledge of other parameters of the interaction, such as affinities and exclusion sizes, may be helpful to unambiguously estimate the value of ω_{AB}, no obvious features of the plots point out the qualitative presence of intercooperativity on the binding. This qualitative information would be very valuable. Nechipurenko[28] developed a challenging methodology to specifically ascertain the cooperativity effects in the binding of a single ligand to DNA. He demonstrated that the extrapolation of a cooperative isotherm to a noncooperative one with an effective binding constant K_{eff} gives an accurate approximation of the first at high levels of lattice occupancy. The value of the effective constant makes it possible to distinguish between dimeric formations ($K_{\text{eff}} \approx \omega^{1/2}$) and aggregates of unrestricted size ($K_{\text{eff}} \approx \omega^{n+1}$). The aim of this section is to extend this approach to systems with intercooperativity.

The intrinsic binding constant of any ligand to DNA is $K = L_b/(L \cdot [\text{free sites}])$, where L_b and L are the bound and free ligand concentrations, respectively, and [free sites] is the concentration of unoccupied sites. When two ligands simultaneously interact with DNA, [free sites] is a function of both ν_A and ν_B, although an analytical expression relating them cannot be found. However, it is possible to ideally decompose the lattice into two parts: those fractions bound to one of the ligands (say B) and all the remaining sequences, that are found either unoccupied or bound to the other ligand. Let us assume that this second fraction were completely free ($\nu_A \to 0$). One can define, for every value of ν_B, a total concentration of sites available for the binding of a small concentration of ligand A, denoted as $[\Theta]$, and then a new affinity constant:

$$K_A^{\text{app}} = \lim_{\nu_A \to 0} \frac{L_{Ab}}{L_A \cdot [\Theta]} \tag{7}$$

There is one reason to consider K_A^{app} as an apparent constant: the affinity of A for all the sites is supposed to be identical. No objections apply in a nonintercooperative situation but as soon as the binding occurs with $\omega_{AB} \neq 1$, the interactions between both kinds of ligands provoke an increase (positive cooperativity) or a decrease (negative cooperativity) of the affinity with respect to the intrinsic constant. A concomitant effect takes place on the bound concentration of the entering ligand. This last point makes K_A^{app} a promising candidate for detecting intercooperativity independently of other interaction parameters.

The procedure to calculate $[\Theta]$ is closely related to the derivation originally employed by McGhee and von Hippel in the development of their model.[4] Here we make it the base and keep the same notation. The amount of available sites for the binding of A depends on the concentration of free monomers at a saturation ν_B. Besides, due to exclusion phenomena, the gap distribution is also influential. Since the sites correspond to ligand A, they cover n monomers. The average number of sites per gap, $\bar{s}$, is the arithmetic mean of the number of sites associated to a gap of length g, weighted with the probability of finding such a gap P_g. No sites are formed for gaps smaller than n residues. The mentioned averaging is expressed by the series

$$\begin{aligned} \bar{s} &= \sum_{g=n}^{\infty} (g - n + 1) P_g \\ &= (b_k f)(f b_1) \sum_{g=n}^{\infty} (g - n + 1)(ff)^{g-1} \end{aligned} \tag{8}$$

The factors $(b_k f)$, (fb_1), and (ff) are conditional probabilities. The subscripts denote the number of monomer to be considered. Thus, f is a free monomer, b_k is the last monomer covered by a certain bound B ligand, k being the number of monomers covered by the species B on binding, and b_1 is the first one occupied by any B ligand. The probability $(b_k f)$ reads as "probability of finding a free residue once a bound B ligand has already been found." The preceding summation can be decomposed in a geometric series and in a derivative of the geometric series, subtracting the terms omitted in (8) for exclusion reasons ($g < n$).

$$\sum_{g=n}^{\infty}(g-n+1)(ff)^{g-1} = \sum_{g=1}^{\infty} g(ff)^{g-1}$$

$$- (n-1)\sum_{g'=0}^{\infty}(ff)^{g'-1} - \sum_{g=1}^{n-1}(g-n+1)(ff)^{g-1}$$

$$= \frac{(ff)^{n-1}}{(1-(ff))^2}, \quad \text{where } g' = g-1 \tag{9}$$

Bearing in mind that, in the limit of $\nu_{\mathrm{A}} \to 0$, $(ff) + (fb_1) \approx 1$, substitution of (9) in (8) yields $\bar{s} = (b_k f)(ff)^{n-1}(fb_1)^{-1}$. To calculate the concentration of sites it is necessary to multiply by the number of gaps of any size ($g \geq 0$), which is proportional to the concentration of bound ligand B for infinitely long lattices. The conditional probabilities may be obtained from the frequency equations given in this paper (see next section). However, for the simple model here presented, analytical expressions for $(b_k f)$ and (ff) can be found in the original paper by McGhee and von Hippel (Ref. 4, equation 13). For the probability (ff), we use the formulation derived by Tsuchiya and Szabo,[12] which is valid for any ω_{B}. To conclude, the concentration of free sites left free by the bound B molecules for the binding of ligand A becomes

$$[\Theta] = \mathrm{DNA}(1 - k\nu_{\mathrm{B}})x_1^{1-n} \tag{10a}$$

$$x_1 = \left(\frac{(2\omega_{\mathrm{B}} - 1)(1 - k\nu_{\mathrm{B}}) + \nu_{\mathrm{B}} + R}{2\omega_{\mathrm{B}}(1 - k\nu_{\mathrm{B}})}\right) = (ff)^{-1} \tag{10b}$$

$$R = ((1 - (k+1)\nu_{\mathrm{B}})^2 + 4\omega_{\mathrm{B}}\nu_{\mathrm{B}}(1 - k\nu_{\mathrm{B}}))^{1/2} \tag{10c}$$

The expressions (10), where *DNA* is the polymer concentration in base pairs, closely resemble the McGhee–von Hippel equations for the binding of a single species to DNA. However, attention must be paid to the exponent $1 - n$, which reflects the fact that the entering ligand is A, although the available space is left free by the B species. Besides, the quadratic factor in the original equation (Ref. 4, equation 15), which weights the affinity for isolated and nonisolated sites, is missing in (10). Obviously, this must not appear in the expression of the concentration of sites.

Measurements of L_{A}, $L_{\mathrm{A}b}$, and ν_{B} are necessary for experimentally estimating the apparent affinity constant [Eqs. (7) and (10)]. Besides, some binding parameters must be previously known. These are the site sizes for the ligands, n and k, and the cooperativity of the second ligand ω_{B}. In principle, all of them may be obtained from independent experiments. With the purpose of understanding the kind of information that K_A^{app} supplies concerning the intercooperativity, simulated experiments were carried out. A simple bracketing and bisection algorithm[32] proved robust enough for finding the maximum root of the secular Eq. (6) by iteration to convergence of the saturation fractions, which were used to calculate the apparent affinity constant in each case. For a given set of parameters, a constant ratio of DNA to ligand B total concentrations r was chosen, and then ligand A concentration was reduced until convergence to the value of the apparent constant. The procedure was repeated for increasing ratios r and the resulting data were compiled in K_A^{app} vs ν_B plots (Figure 2). Such limit curves proved to have very interesting characteristics. First, ligand A exclusion size and cooperativity are not influential on the shape of the curves, consistent with a vanishing saturation for this species. [It may seem from Eq. (10a) that the apparent constant depends on n. However, such a dependence is eliminated by the factor $L_{\mathrm{Ab}}/L_{\mathrm{A}}$ when $\nu_{\mathrm{A}} \to 0$, as shown in the appendix.] Moreover, as expected, the apparent and intrinsic affinity constants coincide under no intercooperative binding conditions, regardless of the values of any other parameter, including all other types of cooperativity, site sizes, and even affinity and saturation of the second ligand (horizontal line in Figure 2).

Intercooperativity affects the value of the calculated K_A^{app}, which deviates from the intrinsic constant as the saturation of the ligand B increases. In this case, ω_{B} does modify the dependence of the isotherm on ν_{B}. However, in the extreme of total saturation of the polymer, the apparent constant takes a significant limiting value of $K_{\mathrm{A}}\omega_{\mathrm{AB}}\omega_{\mathrm{BA}}$. The rationale of this result is a consequence of the proximity to total saturation. As ν_{B} increases, the available sites for the binding of the other ligand tend to be of a minimum size and thus are flanked by two bound molecules of the other species (smaller gaps do not form an accessible site). In this situation, the effective constant is the intrinsic constant corrected by the two cooperativity parameters, because of the alternate sequence

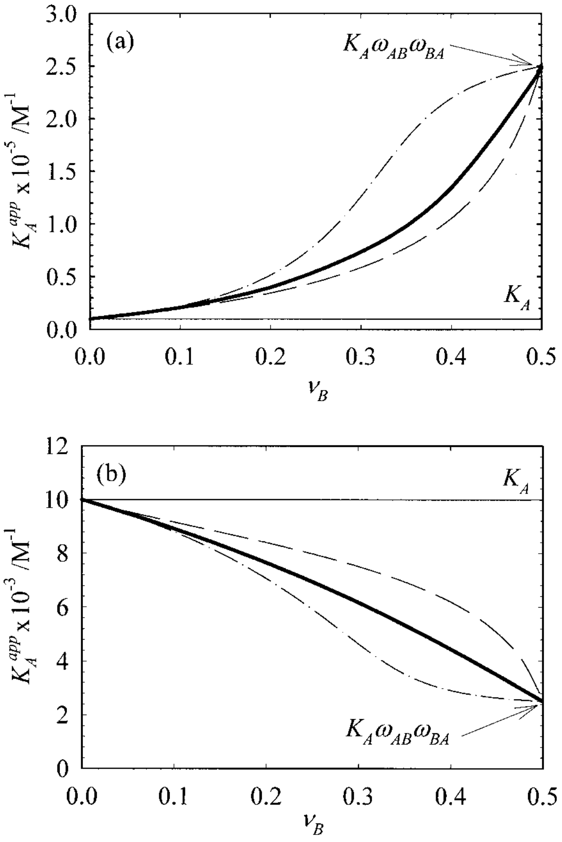

FIGURE 2 Apparent binding constant of one ligand as a function of the saturation of the other one. (a) Positive intercooperativity ($\omega_{AB} = \omega_{BA} = 5.0$) deviates the curves from the intrinsic binding constant. The ordinate at maximum saturation is explained in the text. (b) Negative intercooperativity ($\omega_{AB} = \omega_{BA} = 0.5$) induces a symmetrical effect on the curves. The binding parameters used were $K_A = 10^4 M^{-1}$ and $k = 2$ base pairs (bp). The curves are unaffected by the values of K_B, the exclusion size *n* and the cooperativity ω_A. The constant ω_B was 0.1 (dashed-dotted lines), 1.0 (solid lines), and 2.0 [dashed line, panel (a)] or 5.0 [(dashed line, panel (b)]. Any calculation performed for $\omega_{AB} = \omega_{BA} = 1.0$ produces a horizontal straight line at the intrinsic constant value.

generated. An analytical demonstration is given in the appendix. Thus, K_A^{app} vs ν_B plots are convenient to graphically determine the value of the ω_{AB} parameter. They also provide good qualitative information on the presence of intercooperativity as a characteristic of the interaction.

DISTRIBUTION OF LIGANDS IN THE SEQUENCE

Interpretation of the experiments of electron transfer commented on in the introduction is not possible unless the distribution of ligands in the sequence is known. Furthermore, assessing the relevance of intercooperativity on the distances between bound ligands also requires this information. In this section, a method is presented for the derivation of all the probabilities needed for the calculation of the distribution, as functions of macroscopic magnitudes. Any mean macroscopic magnitude, $\bar{Y}$ can be obtained from the partition function by derivation with respect to the appropriate statistical weight *y* according to the expression $\bar{Y} = \partial \ln x_1^N / \partial \ln y$. Implicit derivation leads to the same result from the secular equation:

$$\bar{Y}/N = -((\partial\Psi(x)/\partial \ln y)/(\partial\Psi(x)/\partial \ln x))_{x=x_1} \quad (11)$$

According to the presented model, interactions are limited to nearest-neighbor contacts. By this hypothesis, the probability to find any randomly chosen subsequence only depends on two kinds of frequencies: those to encounter any given single elementary unit, and those at which double-unit sequences appear. In what follows, we will denote the former as f_A, f_B, and f_F, and the later as f_{AB}, f_{BA}, f_{AA}, f_{BB}, f_{FF}, f_{AF}, f_{FA}, f_{BF}, and f_{FB}.

The first set of frequencies relates to the macroscopic saturation fractions ν_A, ν_B, and ν_F. These magnitudes are defined as bound ligand concentrations over lattice concentration (in monomers). Of course, the bound concentration must be substituted by the total unoccupied monomer concentration in the last saturation fraction. As far as the frequencies of double-unit sequences are concerned, there is no obvious relevant macroscopic magnitude related to them. Nevertheless, an analogous line of reasoning suggests the introduction of new fractions ν_{XY}. X and Y are elementary units and ν_{XY} quantifies the occurrences of the sequence XY in the lattice over the monomer concentration. Overlapping must be borne in mind in the calculation of these frequencies. For example, the sequence AFB contains two double unit sequences, AF and FB. The free monomer belongs to both.

To derive the frequencies from the secular equation, the appropriate statistical weights need to be selected. For single unit sequences, they have been described previously. In fact, L_A and L_B are suitable, since K_A and K_B are constants that appear both in numerator and denominator in expression (11). The predominance of certain double-unit sequences over others is a consequence of the cooperativity parameters, that are, accordingly, the most convenient weights to obtain the second set of frequencies. This is the reason why it was necessary to introduce cooperativity parameters for the free-monomer-containing alternations of sequence in the model previously described. Let us now define the matrix of single unit saturation fractions as

$$\mathbf{N}^{\mathrm{I}} = \begin{pmatrix} \nu_{\mathrm{F}} & 0 & 0 \\ 0 & \nu_{\mathrm{A}} & 0 \\ 0 & 0 & \nu_{\mathrm{B}} \end{pmatrix} = \lim_{\substack{a_{\mathrm{F}}\to 1 \\ \omega_f \to 1}} \left. \begin{pmatrix} \dfrac{\partial \ln x}{\partial \ln a_{\mathrm{F}}} & 0 & 0 \\ 0 & \dfrac{\partial \ln x}{\partial \ln L_{\mathrm{A}}} & 0 \\ 0 & 0 & \dfrac{\partial \ln x}{\partial \ln L_{\mathrm{B}}} \end{pmatrix} \right|_{x=x_1} \tag{12a}$$

The matrix of double-unit saturation fractions is, in a similar way:

$$\mathbf{N}^{\mathrm{II}} = \begin{pmatrix} \nu_{\mathrm{FF}} & \nu_{\mathrm{AF}} & \nu_{\mathrm{BF}} \\ \nu_{\mathrm{FA}} & \nu_{\mathrm{AA}} & \nu_{\mathrm{BA}} \\ \nu_{\mathrm{FB}} & \nu_{\mathrm{AB}} & \nu_{\mathrm{BB}} \end{pmatrix} = \lim_{\substack{a_{\mathrm{F}}\to 1 \\ \omega_f \to 1}} \left. \begin{pmatrix} \dfrac{\partial \ln x}{\partial \ln \omega_{\mathrm{F}}} & \dfrac{\partial \ln x}{\partial \ln \omega_{\mathrm{AF}}} & \dfrac{\partial \ln x}{\partial \ln \omega_{\mathrm{BF}}} \\ \dfrac{\partial \ln x}{\partial \ln \omega_{\mathrm{FA}}} & \dfrac{\partial \ln x}{\partial \ln \omega_{\mathrm{A}}} & \dfrac{\partial \ln x}{\partial \ln \omega_{\mathrm{BA}}} \\ \dfrac{\partial \ln x}{\partial \ln \omega_{\mathrm{FB}}} & \dfrac{\partial \ln x}{\partial \ln \omega_{\mathrm{AB}}} & \dfrac{\partial \ln x}{\partial \ln \omega_{\mathrm{B}}} \end{pmatrix} \right|_{x=x_1} \tag{12b}$$

The limit is taken to achieve physical meaning for the free monomer state, as exposed in the definition of the model. The variable ω_{f} represents all the cooperativity constants involving the free monomer. The meaningful partition function is for a value of the SGF variable equal to the highest root of the secular equation and hence the substitution in the matrices. Table I compiles a comprehensive list of the saturation fractions corresponding to this model. Observe that only two intercooperativity parameters ω_{AB} and ω_{BA}, remain in the final equations. Assuming symmetrical interspecies interactions allows reducing them to a single one ($\omega_{\mathrm{AB}} = \omega_{\mathrm{BA}}$). In this case, the identities $\nu_{\mathrm{AF}} = \nu_{\mathrm{FA}}$, $\nu_{\mathrm{BF}} = \nu_{\mathrm{FB}}$, and $\nu_{\mathrm{AB}} = \nu_{\mathrm{BA}}$ must hold, which is easy to verify.

For the frequencies to be consistent, they should satisfy the following relationships:

$$f_{\mathrm{A}} + f_{\mathrm{B}} + f_{\mathrm{F}} = 1 \tag{13a}$$

Table I List of Saturation Fractions for the Model of Competitive Binding of Two Ligands to a Homogeneous Infinitely Long Linear Polymer[a]

Single-Unit Saturation Fractions[b]

$$\nu_{\mathrm{F}} = \frac{1}{\gamma(x_1)}[x_1^{n+k} - (\omega_{\mathrm{A}} - 1)K_{\mathrm{A}}L_{\mathrm{A}}x_1^{k} - (\omega_{\mathrm{B}} - 1)K_{\mathrm{B}}L_{\mathrm{B}}x_1^{n} - (\omega_{\mathrm{A}} + \omega_{\mathrm{B}} - \omega_{\mathrm{AB}} - \omega_{\mathrm{BA}} - \omega_{\mathrm{A}}\omega_{\mathrm{B}} + \omega_{\mathrm{AB}}\omega_{\mathrm{BA}})K_{\mathrm{A}}K_{\mathrm{B}}L_{\mathrm{A}}L_{\mathrm{B}}]$$

$$\nu_{L1} = \frac{1}{\gamma(x_1)}[x_1^{n+k+1} - x_1^{n+k} - \omega_{L2}K_{L2}L_{L2}x_1^{n1+1} + (\omega_{L2} - 1)K_{L2}L_{L2}x_1^{n1}]$$

Double-Unit Saturation Fractions[b]

$$\nu_{\mathrm{FF}} = \frac{1}{\gamma(x_1)}[x_1^{n+k} - \omega_{\mathrm{A}}K_{\mathrm{A}}L_{\mathrm{A}}x_1^{k} - \omega_{\mathrm{B}}K_{\mathrm{B}}L_{\mathrm{B}}x_1^{n} + (\omega_{\mathrm{A}}\omega_{\mathrm{B}} - \omega_{\mathrm{AB}}\omega_{\mathrm{BA}})K_{\mathrm{A}}K_{\mathrm{B}}L_{\mathrm{A}}L_{\mathrm{B}}]$$

$$\nu_{L1\text{-}L1} = \frac{\omega_{L1}}{\gamma(x_1)}[K_{L1}L_{L1}x_1^{n2+1} - K_{L1}L_{L1}x_1^{n2} - \omega_{L2}K_{\mathrm{A}}K_{\mathrm{B}}L_{\mathrm{A}}L_{\mathrm{B}}x_1 + (\omega_{L2} - 1)K_{\mathrm{A}}K_{\mathrm{B}}L_{\mathrm{A}}L_{\mathrm{B}}]$$

$$\nu_{\mathrm{F}\text{-}L1} = \frac{1}{\gamma(x_1)}[K_{L1}L_{L1}x_1^{n2} + (\omega_{L1\text{-}L2} - \omega_{L2})K_{\mathrm{A}}K_{\mathrm{B}}L_{\mathrm{A}}L_{\mathrm{B}}]$$

$$\nu_{L1\text{-}\mathrm{F}} = \frac{1}{\gamma(x_1)}[K_{L1}L_{L1}x_1^{n2} + (\omega_{L2\text{-}L1} - \omega_{L2})K_{\mathrm{A}}K_{\mathrm{B}}L_{\mathrm{A}}L_{\mathrm{B}}]$$

$$\nu_{L1\text{-}L2} = \frac{\omega_{L1\text{-}L2}}{\gamma(x_1)}[\omega_{L2\text{-}L1}K_{\mathrm{A}}K_{\mathrm{B}}L_{\mathrm{A}}L_{\mathrm{B}}x_1 - (\omega_{L2\text{-}L1} - 1)K_{\mathrm{A}}K_{\mathrm{B}}L_{\mathrm{A}}L_{\mathrm{B}}]$$

[a] The denominator is common to every saturation fraction, that is

$$\gamma(x_1) = (n + k + 1)x_1^{n+k+1} - (n + k)x_1^{n+k} - (n + 1)\omega_{\mathrm{B}}K_{\mathrm{B}}L_{\mathrm{B}}x_1^{n+1} - (k + 1)\omega_{\mathrm{A}}K_{\mathrm{A}}L_{\mathrm{A}}x_1^{k+1} + (\omega_{\mathrm{B}} - 1)K_{\mathrm{B}}L_{\mathrm{B}}nx_1^{n} + (\omega_{\mathrm{A}} - 1)K_{\mathrm{A}}L_{\mathrm{A}}kx_1^{k} + (\omega_{\mathrm{A}}\omega_{\mathrm{B}} - \omega_{\mathrm{AB}}\omega_{\mathrm{BA}})K_{\mathrm{A}}K_{\mathrm{B}}L_{\mathrm{A}}L_{\mathrm{B}}x_1$$

[b] The subscript $L1$ represents either ligand A or B. The exclusion sizes $n1$ and $n2$ represent n and k or vice versa, when $L1$ is A or B, respectively. $L1$-$L1$ means either AA or BB, $L1$-$L2$ means either AB and BA, and so on.

$$f_{\mathrm{AA}} + f_{\mathrm{AB}} + f_{\mathrm{AF}} + f_{\mathrm{BA}} + f_{\mathrm{BB}} + f_{\mathrm{BF}} + f_{\mathrm{FA}} + f_{\mathrm{FB}} + f_{\mathrm{FF}} = 1 \tag{13b}$$

Obviously, similar equations do not hold for saturation fractions. The reason is that they are normalized by monomers and not by elementary units. A conversion to the later is essential, in order to obtain probabilities. The required ratio between the total number of elementary units in the lattice to the number of monomers can be derived as follows. The number of elementary units of the class A is proportional to the bound concentration of this ligand, which in turn is proportional to the saturation fraction ν_A. Similarly, the ligand B appears in the lattice in an amount related to ν_B. Last, the number of unoccupied residues (monomers) refers to the remaining elementary units of the sequence. Since the maximum value a saturation fraction can reach corresponds to the inverse of the exclusion size of the ligand (n or k), the total fraction of occupied monomers is $n\nu_A + k\nu_B$. Therefore, the ratio between elementary units and monomers, may be defined as

$$N(\nu_A, \nu_B) = \nu_A + \nu_B + (1 - n\nu_A - k\nu_B) \qquad (14)$$

which is always smaller than or equal to unity. Note that this normalization factor is valid both for single-unit and double-unit sequences. Recall that the later ones overlap and that, for nearly infinite lattices, the extreme effects can be neglected. In this way, the matrices of frequencies result from dividing the matrices of saturations (12) by Eq. (14).

$$\mathbf{P^I} = \frac{\mathbf{N^I}}{N(\nu_A, \nu_B)} = \begin{pmatrix} f_F & 0 & 0 \\ 0 & f_A & 0 \\ 0 & 0 & f_B \end{pmatrix} \qquad (15a)$$

$$\mathbf{P^{II}} = \frac{\mathbf{N^{II}}}{N(\nu_A, \nu_B)} = \begin{pmatrix} f_{FF} & f_{AF} & f_{BF} \\ f_{FA} & f_{AA} & f_{BA} \\ f_{FB} & f_{AB} & f_{BB} \end{pmatrix} \qquad (15b)$$

In the absolutely noncooperative case, i.e. when all the ω parameters equal one, the frequencies of double-unit sequences coincide with the product of the corresponding single-unit frequencies, which is $f_{XY} = f_X f_Y$. No interactions at all occur between bound ligands, and the lattice can be described by means of the frequencies for each species. From the matrices in Eq. (15), a matrix of conditional probabilities is defined as $\mathbf{\Pi} = \mathbf{P^{II}}(\mathbf{P^I})^{-1}$. The probability of finding any sequence may be calculated either from Eq. (15) or from $\mathbf{P^I}$ and $\mathbf{\Pi}$. For multiple-unit sequences, it is necessary to employ the properties of event intersection, formulated in probability theory. For example, the frequency with which the sequence AFB appears in the lattice is

$$f_{AFB} = f_A(a_n f)(fb_1) = f_A \frac{f_{AF}}{f_A}\frac{f_{FB}}{f_F} = \frac{f_{AF}f_{FB}}{f_F} \qquad (16)$$

The factors $(a_n f)$ and (fb_1) are conditional probabilities in the notation employed by McGhee and von Hippel[4] (see above).

From an experimental viewpoint, the saturation fractions ν_A and ν_B can be empirically determined, together with the two free ligand concentrations. Hence, the matrix $\mathbf{P^I}$ can be estimated from measurements of ν_A and ν_B and the exclusion sizes. However, giving a calculation of $\mathbf{P^{II}}$ implies obtaining the value of the maximum root x_1. As a result, an iteration cycle for solving the secular equation for each set of observations must be run and an adequate nonlinear algorithm needs to be implemented.

A SHORT-RANGE MODEL OF FLUORESCENCE EMISSION QUENCHING

Cooperative behavior between noncovalently bound metal complexes has been alleged to refute the arguments in favor of long-range electron transfer in DNA.[8,20,33] The presumed intercooperativity needed to explain the fluorescence quenching experiments in the simultaneous binding of the paradigmatic pair $[Ru(phen)_2dppz]^{2+}/[Rh(phi)_2phen]^{3+}$ (phen, phenantroline; dppz, dipyridophenazine; phi, phenanthrenequinone diimine) is smaller than $\omega_{RU\text{-}RH} \approx 100$. However, even the existence of cooperativity is a controversial matter[20,34] and it is difficult to reach an agreement on the basis of simple fitting to a model. Nevertheless, it is always interesting to check the sensitivity of a model to a parameter by comparison with reported experiments. This is the motivation of the current section. Here we propose an equation in terms of frequencies, instead of conditional probabilities, for the probability to find an emitter nearest-neighboring an active quencher.

First, we assume that all the emitter molecules (B) bound to any site adjacent to that of an effective quencher (A) become quenched, but no others. Every bound A molecule is supposed to be effective, but it can only act on one adjacent emitter, i.e., in the sequence FBABF one of the B molecules is still able to emit, since the quencher gets inactivated after quenching the other emitter. This is so because the mechanism for deactivation is electron transfer, with slow recombination (back-reaction; see Bixon[35] for biologically significant examples of this fact), and only one A electron is available for reaction. These assumptions implicitly mean a high quantum yield for the quenching.

The probability ***Q*** for a bound ligand B to be adjacent to *any effective* ligand A, and thus to become inactive, is related to the probability of finding it taking part in the sequence AB or in its symmetrical BA. Then, both frequencies add. However, for saturations high enough, these two sequences can overlap and therefore, by the mechanistic conditions enumerated in the previous paragraph, only one emitter is quenched, no matter that the overlap is ABA or BAB. As a consequence, ***Q*** diminishes as the number of such sequences increases. To summarize, the pursued frequency expression is, up to now, the summation of all double-unit alternate-ligand sequence frequencies and the subtraction of every triple-unit alternate-ligand sequence frequency (i.e., $f_{\mathrm{AB}} + f_{\mathrm{BA}} - f_{\mathrm{ABA}} - f_{\mathrm{BAB}}$). The same argument applies for triple-unit sequences, which can also overlap to form ABAB or BABA. In this case, no discount should have been made, because certainly two B molecules are subject to quenching. Hence, the frequencies of the alternate four member sequences must be added. This procedure can be extended ad infinitum, varying the signs in accordance with the sequence length. The frequencies may be calculated from analogous expressions to Eq. (16). Finally, because we have defined ***Q*** for each bound B molecule, it is necessary to normalize by the frequency of B in the lattice.

$$\begin{aligned}\mathbf{Q}f_{\mathrm{B}} &= f_{\mathrm{AB}} + f_{\mathrm{BA}} - (f_{\mathrm{ABA}} + f_{\mathrm{BAB}} - (f_{\mathrm{ABAB}} + f_{\mathrm{BABA}} \\ &\qquad - (f_{\mathrm{ABABA}} + f_{\mathrm{BABAB}} - (\cdots)))) \\ &= f_{\mathrm{AB}} + f_{\mathrm{BA}} - \frac{f_{\mathrm{AB}}f_{\mathrm{BA}}}{f_{\mathrm{B}}} - \frac{f_{\mathrm{BA}}f_{\mathrm{AB}}}{f_{\mathrm{A}}} \\ &\quad + \frac{f_{\mathrm{AB}}^2 f_{\mathrm{BA}}}{f_{\mathrm{A}}f_{\mathrm{B}}} + \frac{f_{\mathrm{AB}} f_{\mathrm{BA}}^2}{f_{\mathrm{A}}f_{\mathrm{B}}} - \frac{f_{\mathrm{AB}}^2 f_{\mathrm{BA}}^2}{f_{\mathrm{A}}f_{\mathrm{B}}^2} \\ &\quad - \frac{f_{\mathrm{AB}}^2 f_{\mathrm{BA}}^2}{f_{\mathrm{A}}^2 f_{\mathrm{B}}} + \cdots \end{aligned} \tag{17}$$

The general expression of the series is as follows:

$$\begin{aligned}\mathbf{Q} = \frac{1}{f_{\mathrm{B}}} \sum_{i=1}^{\infty} \Bigg[&\frac{f_{\mathrm{AB}}^{i} f_{\mathrm{BA}}^{i-1}}{(f_{\mathrm{A}}f_{\mathrm{B}})^{i-1}} + \frac{f_{\mathrm{AB}}^{i-1} f_{\mathrm{BA}}^{i}}{(f_{\mathrm{A}}f_{\mathrm{B}})^{i-1}} \\ &- \frac{(f_{\mathrm{AB}}f_{\mathrm{BA}})^{i}}{f_{\mathrm{A}}^{i} f_{\mathrm{B}}^{i-1}} - \frac{(f_{\mathrm{AB}}f_{\mathrm{BA}})^{i}}{f_{\mathrm{A}}^{i-1} f_{\mathrm{B}}^{i}} \Bigg] \end{aligned} \tag{18a}$$

$$\begin{aligned}\mathbf{Q} = \frac{1}{f_{\mathrm{B}}} \left(f_{\mathrm{AB}} + f_{\mathrm{BA}} - \frac{f_{\mathrm{AB}}f_{\mathrm{BA}}}{f_{\mathrm{A}}} - \frac{f_{\mathrm{AB}}f_{\mathrm{BA}}}{f_{\mathrm{B}}} \right) \\ \times \sum_{j=0}^{\infty} \left(\frac{f_{\mathrm{AB}}f_{\mathrm{BA}}}{f_{\mathrm{A}}f_{\mathrm{B}}} \right)^{j} \end{aligned} \tag{18b}$$

The product $f_{\mathrm{AB}}f_{\mathrm{BA}}$ is always smaller than or equal to $f_{\mathrm{A}}f_{\mathrm{B}}$. They are coincident only for completely alternate sequences of A and B ligands. In such an extreme, all four frequencies equal 0.5 and the ratio under the summation in Eq. (18b) becomes unity. The overall equation is undetermined, although it is easy to realize that it must solve to total quenching (***Q*** $= 1$). But in most cases ($f_{\mathrm{AB}}f_{\mathrm{BA}} < f_{\mathrm{A}}f_{\mathrm{B}}$) the series in (18b) is a geometric one and can be summed to the fairly symmetrical expression (19).

$$\mathbf{Q} = \frac{1}{f_{\mathrm{B}}} \frac{f_{\mathrm{A}}f_{\mathrm{B}}(f_{\mathrm{AB}} + f_{\mathrm{BA}}) - f_{\mathrm{AB}}f_{\mathrm{BA}}(f_{\mathrm{A}} + f_{\mathrm{B}})}{f_{\mathrm{A}}f_{\mathrm{B}} - f_{\mathrm{AB}}f_{\mathrm{BA}}} \tag{19}$$

If the binding of ligands to the lattice is absolutely noncooperative, two-unit sequence frequencies are functions of f_{A} and f_{B}. This further simplification leads to the compact Eq. (20):

$$\mathbf{Q} = f_{\mathrm{A}} \frac{(2 - f_{\mathrm{A}} - f_{\mathrm{B}})}{1 - f_{\mathrm{A}}f_{\mathrm{B}}} \tag{20}$$

Note that complete saturation of the lattice with an equal number of both ligands in highly intercooperative situations (i.e., AB and BA sequences predominate over AA and BB) means absolute quenching in this model, although this is not the case in the noncooperative example. In fact, in this case alternation is not favored over homogeneous sequences and only two thirds of the emitter result quenched (see Figure 3).

Simulations of fluorescence quenching for the donor–acceptor pair $[\mathrm{Ru(phen)_2dppz}]^{2+}/[\mathrm{Rh(phi)_2phen}]^{3+}$ were performed, using binding parameters compiled from bibliographic sources[18,20] and a single intercooperativity constant ($\omega_{\mathrm{AB}} = \omega_{\mathrm{BA}}$) to be fitted. Good qualitative and quantitative agreement was obtained between experimental data (reported in Ref. 18) and the model (Figure 4), with little requirement for intercooperativity ($\omega_{\mathrm{AB}} \approx 5$). Noticeably, high sensitivity to this parameter was observed in every conducted simulation (compare the cooperative model with the noncooperative one; see also Figure A1, in the appendix). However, it must be recognized that this is a minimum estimate, as real imperfect quenching (i.e., quenching quantum yield lower than unity) compels

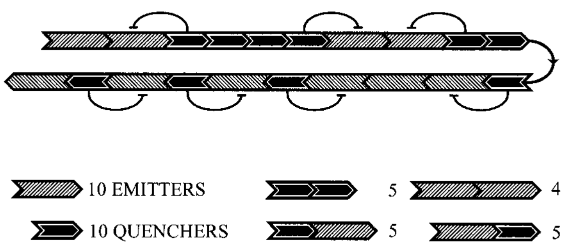

FIGURE 3 Fluorescence quenching model. Any quencher is able to act on one and only one adjacent emitter (curly interrupted arrows on the lattice). In a lattice completely saturated by an equal number of randomly distributed quencher and emitter molecules, 67% of the emitters become deactivated. The random distribution implies frequencies of 0.5 for each elementary unit and of 0.25 for pairs of bound ligands. Note that the last emitter in the depicted sequence is not deactivated because the adjacent quencher operates on the preceding molecule.

to increasing the cooperativity to get a satisfactory fit. This could be included in the models as a multiplicative factor of the equations.

DISCUSSION

Models of nonspecific interaction of ligands with infinitely long lattices relate some macroscopic magnitudes with others and implicitly contain information concerning the distribution of ligands in the sequence. Whereas the binding densities provide a direct indication of the frequencies with which every class of elementary unit appears in the sequence, frequencies of two or more units are not obvious and must be indirectly ascertained. The probabilistic approach of McGhee and von Hippel[4] gives a convenient set of functionalities between the binding densities and the conditional probabilities of finding successive pairs of units for the cooperative binding of a single ligand to DNA, but is not extensible to a higher number of species. On the other hand, the SGF method facilitates the derivation of statistical magnitudes for arbitrarily complex systems. However, the common practice of taking the free monomer as the reference state makes it difficult to realize which are the statistical weights that represent the higher order sequences. Consequently, the available procedures involve obtaining expressions for all the frequencies for which the weights are know, i.e., those referring to the ligands and not to the free monomers, and then adjust the other frequencies until the conditions of certain event [Equations (13)] are verified.

We have shown in this paper that assigning an additional nonunitary statistical weight to the free monomer simplifies the characterization of the distribution and no probabilistic assumptions need be expressed, except for those necessary when defining the model (SGF's). This allows for verification of the correctness of the results through independent assessment of Eqs. (13), which are not included in the treatment, and of other relevant relations. For example, according to the definition of the intercooperativity constant, it may be calculated as $\omega_{AB} = (a_n b_1)(ff)[(a_n f)(f b_1)]^{-1}$. We illustrate the procedure for a three-state model: the competitive binding of two ligands to a homogenous one-dimensional lattice with nearest-neighbor interactions. The necessary corrections to pass from lattice residues to elementary units are simple manipulations of the frequencies that take into account the sizes of the ligands and the binding densities. It is clear that the model is readily generalizable to more complex situations and the limiting step is the definition of the SGF's.

The motivation for obtaining the ligand distribution is to be able to justify certain properties of some

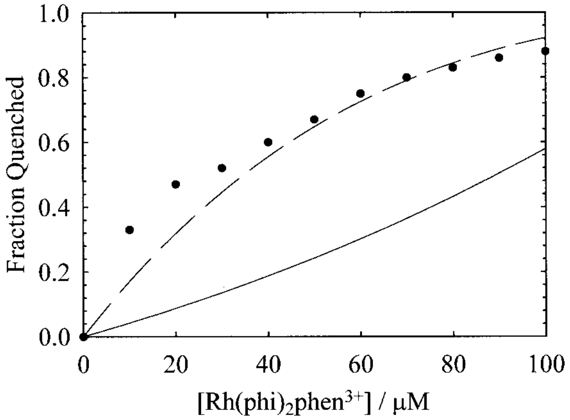

FIGURE 4 Fraction of $Ru(phen)_2dppz^{2+}$ quenched by increasing concentrations of $Rh(phi)_2phen^{3+}$ as reported by Murphy et al.[18] (dots). The experimental conditions were 0.5 m*M* long chain calf thymus DNA (bp) and 10 μM $Ru(phen)_2dppz^{2+}$ in buffer (5 m*M* Tris · HCl/50 m*M* NaCl, pH 7.2) at ambient temperature. The model described in the text was used to fit the experimental data. The binding constants were taken $K_{Ru} = 10^6\ M^{-1}$ and $K_{Rh} = 10^7\ M^{-1}$ as in Ref. 20. Both ligands were assumed to cover 3 bp. The absolutely noncooperative model (solid line) proved unsatisfactory. However, inclusion of symmetric intercooperativity ($\omega_{Ru\text{-}Rh} = \omega_{Rh\text{-}Ru}$) is highly influential. A value as low as $\omega_{Ru\text{-}Rh} = 5$ gives a good agreement between the model (dashed line) and the data. Varying the intracooperativity constant $\omega_{Ru\text{-}Ru}$ up to 10, as suggested by Lincoln et al.,[20] has little effect (results not shown).

observable phenomena, such as fluorescence emission quenching,[18] reactivity at a distance,[26] or DNA cleavage by photosensitizers.[33] The separation between bound ligands and their order in the sequence constitutes an aspect of all these problems that should be brought to consideration when interpreting the data.[20,22] The controversy arisen from electron transfer studies in DNA is partly a consequence of different criteria of interpretation, rather than alternative experimental results.[25] In many instances, the objections are not directed to the particular model being employed. In fact, there is general agreement on details like homogeneity of the lattice and total binding of the ligands, and so repetitive DNA sequences and low concentrations of DNA-avid ligands are used to avoid misleading effects that are not essential as to whether the polymer is conducting. Considerations on cooperative behavior are more common in the arguments against and for long-range electron transfer.[8,19,20,34]

The elementary model of fluorescence quenching developed in this paper confirms once more that the most influential binding parameter on the increase of quenching facilitated by DNA is ω_{AB}. The effect of other cooperativity parameters is less marked, particularly at low saturations (results not shown; but see for example Figure 2). This further proves the validity of our approach for calculating the sequence frequencies (note that the quenching model is independent from the SGF procedure) and prompts to the proposal of a new methodology for the experimental assessment of intercooperativity.

We demonstrate that the intercooperative behavior in the binding of two competing ligands to a lattice can be separated from the rest of the interaction characteristics by comparing an apparent affinity constant with the intrinsic one for any of the ligands. The former constant [Eq. (7), with Eq. (10)] is here defined in the limit of null saturation for the species being considered (A) and is a function of the saturation fraction for the other ligand (B). The exclusion sizes and the intraspecies cooperativity of the second ligand are needed for the calculation. As a consequence, the experiments oriented to the detection of intercooperativity must be preceded by fine characterization of the individual binding properties. In general, this is a requisite easy to accomplish.

A more difficult detail is how to achieve sufficiently low and accurately measurable concentrations of one of the ligands. The most obvious procedure consists in constructing “perpendicular” isotherms by progressively reducing its concentration while the other’s remains constant and then extrapolating to null saturation. This can be repeated a number of times for different total concentrations of the second ligand to produce the complete K_A^{app} vs ν_{B} plot. Nevertheless, the relevant point in this plot is at maximum saturation of the polymer with ligand B. This suggests an alternative procedure: a saturating initial concentration of this ligand may be progressively displaced by increasing quantities of ligand A. Again, extrapolation to the limit is unavoidable. However, in this way the amount of employed material is greatly reduced since only a few points are required to estimate the limiting value of the apparent constant ($K_{\mathrm{A}}\omega_{\mathrm{AB}}\omega_{\mathrm{BA}}$). An important remark is the arbitrary choice of the ligand whose K^{app} is going to be determined. Consequently, two different sets of assays can be designed that produce two experimentally different evaluations of the product $\omega_{\mathrm{AB}}\omega_{\mathrm{BA}}$ (from K_A^{app} and K_B^{app}). These can be compared to gain confidence on the conclusions cast by the analysis.

The main advantage of this new procedure for the determination of an apparent constant is the oriented design of the experiments to obtain one and only one type of parameter. A noncooperative situation is unambiguous since the apparent constant becomes invariant with the saturation of the tunable ligand. On the other hand, imperfect knowledge of other binding parameters (n, k, ω_{B}) may affect the credibility of the conclusions, since deviated apparent constant would be obtained. Fortunately, the scheme admits refinement by fitting to the analytical expression of the curve (see appendix) and iteratively recalculating the apparent constant. Stress must be made in that such a fitting *is not* a step of the procedure, but only a tool for its verification.

APPENDIX

Analytical Expression for $\nu_{\mathrm{A}}/L_{\mathrm{A}}$ in the Lower Limit $\nu_{\mathrm{A}} \to 0$ as a Function of ν_{B}

In the limit of unity for every free monomer parameter and null saturation for one ligand, which is equivalent to take its free concentration $L_{\mathrm{A}} = 0$, the general secular Eq. (6) reduces to the corresponding for the binding of a single ligand to DNA:

$$\Psi(x) = x^{n+k+1} - x^{n+k} - \omega_{\mathrm{B}}K_{\mathrm{B}}L_{\mathrm{B}}x^{n+1} + (\omega_{\mathrm{B}} - 1)K_{\mathrm{B}}L_{\mathrm{B}}x^{n} = 0 \quad \text{(A1)}$$

The saturation fraction ν_{A} (Table I) admits conversion to terms proportional to L_{A} by means of Eq. (6) and thus can be directly transformed into $\nu_\mathrm{A}/L_\mathrm{A}$. In the limit situation,

$$\begin{aligned}\left(\frac{\nu_\mathrm{A}}{L_\mathrm{A}}\right)_{\nu_\mathrm{A}\to 0} = \frac{K_\mathrm{A}}{\gamma(x_1, L_\mathrm{A}=0)}&[\omega_\mathrm{A}x_1^{k+1} - (\omega_\mathrm{A}-1)x_1^k \\ &- (\omega_\mathrm{A}\omega_\mathrm{B} - \omega_\mathrm{AB}\omega_\mathrm{BA})K_\mathrm{B}L_\mathrm{B}x_1 \\ &- (\omega_\mathrm{A} + \omega_\mathrm{B} - \omega_\mathrm{AB} - \omega_\mathrm{BA} \\ &- \omega_\mathrm{A}\omega_\mathrm{B} + \omega_\mathrm{AB}\omega_\mathrm{BA})K_\mathrm{B}L_\mathrm{B}] \qquad \text{(A2)}\end{aligned}$$

The denominator $\gamma(x_1)$ is as in Table I, except for the substitution $L_\mathrm{A} = 0$. The numerator of (A2) contains all the terms of the secular Eq. (A1). Therefore, it can be simplified to

$$\begin{aligned}K_\mathrm{A}[x_1^k - (\omega_\mathrm{AB}\omega_\mathrm{BA}x_1 + \omega_\mathrm{AB} + \omega_\mathrm{BA} \\ - \omega_\mathrm{B} - \omega_\mathrm{AB}\omega_\mathrm{BA})K_\mathrm{B}L_\mathrm{B}] \qquad \text{(A3)}\end{aligned}$$

In a similar way, $\nu_\mathrm{B}(\nu_\mathrm{A} \to 0)$ results, from the expression in Table I and (A1):

$$\begin{aligned}\nu_\mathrm{B}(\nu_\mathrm{A} \to 0) = \frac{K_\mathrm{B}L_\mathrm{B}}{\gamma(x_1, L_\mathrm{A}=0)} \\ \times [\omega_\mathrm{B}x_1^{n+1} - (\omega_\mathrm{B}-1)x_1^n] \qquad \text{(A4)}\end{aligned}$$

Additionally, from the secular Eq. (A1), the following identity holds:

$$\begin{aligned}L_\mathrm{B}\frac{\partial\Psi(x_1)}{\partial L_\mathrm{B}} - (k+1)\nu_\mathrm{B}\Psi(x_1) = \nu_\mathrm{B}x_1^k \\ + (k\nu_\mathrm{B} - 1)\omega_\mathrm{B}K_\mathrm{B}L_\mathrm{B}x_1 + (1 - k\nu_\mathrm{B} - \nu_\mathrm{B}) \\ \times (\omega_\mathrm{B} - 1)K_\mathrm{B}L_\mathrm{B} = 0 \qquad \text{(A5)}\end{aligned}$$

from which x_l^k may be implicitly expressed as

$$\begin{aligned}x_1^k = \frac{K_\mathrm{B}L_\mathrm{B}}{\nu_\mathrm{B}}[(1 - k\nu_\mathrm{B})\omega_\mathrm{B}x_1 \\ - (1 - k\nu_\mathrm{B} - \nu_\mathrm{B})(\omega_\mathrm{B} - 1)] \qquad \text{(A6)}\end{aligned}$$

Dividing (A2), with (A3), by (A4) and substituting (A6), all dependencies with K_B and L_B disappear to yield the equation of a binding isotherm (*Cross Limit Isotherm*, CLI):

$$\begin{aligned}&\frac{\nu_\mathrm{A}}{L_\mathrm{A}}(\nu_\mathrm{A} \to 0) \\ &= K_\mathrm{A}\frac{\begin{array}{c}(1 - k\nu_\mathrm{B})(\omega_\mathrm{B}(x_1 - 1) + 1) \\ + \nu_\mathrm{B}\omega_\mathrm{AB}\omega_\mathrm{BA}(x_1 - 1) + \omega_\mathrm{AB}\nu_\mathrm{B} + \omega_\mathrm{BA}\nu_\mathrm{B} - \nu_\mathrm{B}\end{array}}{x_1^n[\omega_\mathrm{B}(x_1 - 1) + 1]} \qquad \text{(A7)}\end{aligned}$$

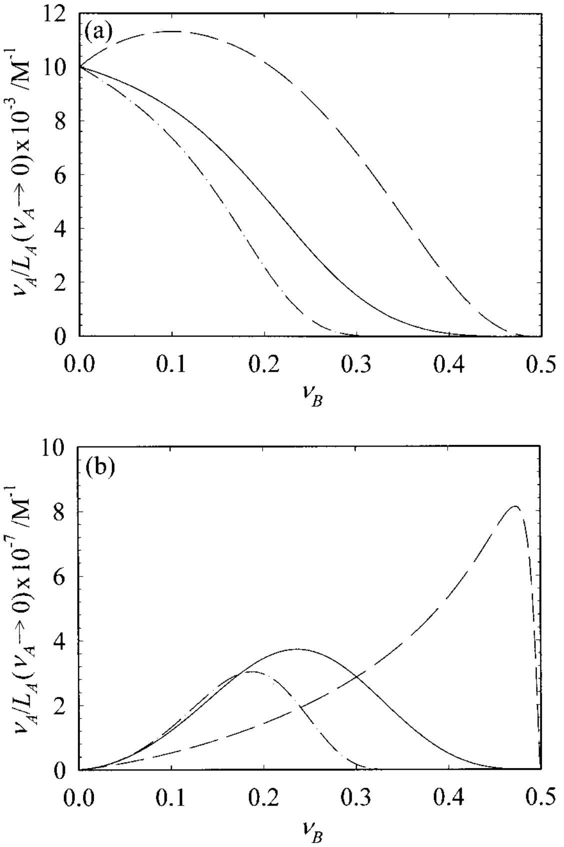

FIGURE A1 CLI ($\nu_\mathrm{A}/L_\mathrm{A}(\nu_\mathrm{A} \to 0)$ vs ν_B) for the same parameters as in Figure 2. The several values of ω_B considered were 0.01 (dashed-dotted line), 1.0 (solid line), and 5.0 [dashed line, panel (a)] or 100.0 [dashed line, panel (b)]. (a) Nonintercooperative isotherms ($\omega_\mathrm{AB} = \omega_\mathrm{BA} = 1.0$) depend on the cooperativity of the ligand B, in contrast to the apparent binding constant. (b) The intercooperativity ($\omega_\mathrm{AB} = \omega_\mathrm{BA} = 100$) markedly affects the shape of the CLI.

This is the intersection of the surface in $\nu_\mathrm{A}/L_\mathrm{A}$ vs ν_A and ν_B with the plane $\nu_\mathrm{A}/L_\mathrm{A}$ vs ν_B. Note that K_A^{app} differs from (A7) by a factor of $(1- k\nu_\mathrm{B})x_l^{1-n}$, where x_1 is as in Eq. (10b), meaning that, whereas K_A^{app} is normalized by available free sites, the normalization of the CLI is by the DNA concentration. Therefore, it provides the $\lim_{\nu\mathrm{A}\to 0}(L_{\mathrm{A}b}/L_\mathrm{A})$ factor necessary to evaluate (7) (see also below). The CLI is a one-variable (ν_B) closed form equation, suitable for nonlinear least-square fitting of the parameters. Figure A1 depicts the CLI for some exemplifying cooperative cases. It is remarkable that, although the isotherm (A7) is an explicit function of the binding exclusion size of the ligand A, this relationship is eliminated in the apparent constant, in accordance with the simulations. Also, no influence of ω_A is possible from (A7).

Constancy of $K_{\mathrm{A}}^{\mathrm{app}}$ in Nonintercooperative Situations

Let us start from the simplified expression of $K_{\mathrm{A}}^{\mathrm{app}}$ when $\omega_{\mathrm{AB}} = \omega_{\mathrm{BA}} = 1$:

$$K_{\mathrm{A}}^{\mathrm{app}} = K_{\mathrm{A}} \frac{(1 - k\nu_{\mathrm{B}})(\omega_{\mathrm{B}}(x_1 - 1) + 1) + \nu_{\mathrm{B}} x_1}{x_1(1 - k\nu_{\mathrm{B}})(\omega_{\mathrm{B}}(x_1 - 1) + 1)} \tag{A8}$$

Demonstrating $K_{\mathrm{A}}^{\mathrm{app}}/K_{\mathrm{A}} = 1$ that implies giving a proof that

$$\nu_{\mathrm{B}} x_1 = (1 - k\nu_{\mathrm{B}})(\omega_{\mathrm{B}}(x - 1) + 1)(x_1 - 1) \tag{A9}$$

Substituting x_1 by its value [Eq. (10b)], the right member of the previous identity becomes

$$\frac{\begin{array}{l}[\nu_{\mathrm{B}} + R - (1 - k\nu_{\mathrm{B}})] \\ \quad \times [\nu_{\mathrm{B}} + R + (1 - k\nu_{\mathrm{B}})]\end{array}}{4\omega_{\mathrm{B}}(1 - k\nu_{\mathrm{B}})} \tag{A10}$$

Simple algebraic manipulations show that (A10) is exactly the left member of (A9).

Limit Value of $K_{\mathrm{A}}^{\mathrm{aap}}$ at $\nu_{\mathrm{A}} \to 0$ and Maximum Saturation of B

From (A7), and from the expression of $x_1(1 - k\nu_{\mathrm{B}})$, readily found from (10b), the apparent constant can be rearranged as

$$K_{\mathrm{A}}^{\mathrm{app}} = 2K_{\mathrm{A}}\omega_{\mathrm{B}} \times \frac{\begin{array}{r}(1 - k\nu_{\mathrm{B}})(1 - \omega_{\mathrm{B}}) + X/2 + \nu_{\mathrm{B}}\omega_{\mathrm{AB}}\omega_{\mathrm{BA}}(x_1 - 1) \\ + \omega_{\mathrm{AB}}\nu_{\mathrm{B}} + \omega_{\mathrm{BA}}\nu_{\mathrm{B}} - \nu_{\mathrm{B}}\end{array}}{X[\omega_{\mathrm{B}}(x_1 - 1) + 1]} \tag{A11a}$$

$$X = [(2\omega_{\mathrm{B}} - 1)(1 - k\nu_{\mathrm{B}}) + \nu_{\mathrm{B}} + R] \tag{A11b}$$

Taking the limit of maximum saturation ($\nu_{\mathrm{B}} \to 1/k$) in (10b) and (10c), one obtains infinity and $1/k$, respectively. This leads to indetermination (∞/∞) in the apparent constant. Applying the L'Hôpital rule in the equivalent limit $x_1 \to \infty$ (taking the derivatives for x_1), with $\nu_{\mathrm{B}} = 1/k$, the extreme value $K_{\mathrm{A}}\omega_{\mathrm{AB}}\omega_{\mathrm{BA}}$ results, which is the same as the one inferred from numerical calculations.

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