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Reformulation of thermodynamic systems with aggregation and theoretical methods for the analysis of ligand binding in proteins with monomer–multimer equilibria

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Abstract

The reformulation of complex thermodynamic systems is a useful tool for their analysis as demonstrated by the theoretical analysis of conformationally mediated cooperativity in a dimeric protein. Many chemical and biochemical systems exhibit monomer–multimer equilibria, behavior not addressed in the original reformulation. A method for reformulating such systems, and the mathematical methods necessary for relating alternative models, are therefore developed. The basic principles of the reformulation are illustrated on homodimeric and heterodimeric systems. The mathematical methods necessary to relate alternative models are then derived from probabilistic considerations. Higher-order models (more interacting subunits) are related to lower-order models (fewer interacting subunits) by a polynomial expansion of the sum of species in the lower-order model to give the sum of species in the higher-order model. Using these methods, the equations describing the ligand binding behavior of a homomeric monomer–dimer system are derived. These methods are also used to relate the two alternative models for cooperativity for a homotetrameric protein; one model where the dimer is the cooperative unit and the other where the tetramer is the cooperative unit.

Key words: Thermodynamic systems; Ligand binding; Proteins

1. Introduction

The reformulation [1] of complex thermodynamic systems has been presented [1] and applied to the analysis of cooperativity in a homodimeric protein [2]. The purpose of this reformulation is to take a system of N complexes, defined by N ΔG^0s of formation, and to redefine it in terms of a new set of N ΔG^0s that reflect the intrinsic ΔG^0s of individual interactions and the hierarchy of potential interactions between them. The advantage of this method of formulation is that the higher-order terms defining a

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[1] This reformulation was originally given the name of the unique and independent parameters (UIP) formulation [1,2]. However, because the full scope of the reformulation, and its potential applications, are as yet unknown, such a name may be premature. Therefore, this method will simply be referred to as the 'reformulation' in this and future work, until a suitably descriptive name can be chosen.

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given model may or may not be significant, and can be set to a null value (no interaction), based upon theoretical or mechanistic considerations for theoretical applications [2], or for the statistical analysis and interpretation of experimental data [3]. The first manuscript on this reformulation [1] extended the concepts of interaction energy and statistical correction factors to the general case of N interacting ligands binding to a fixed template, such as successive ligands binding to a multimeric protein in absence of monomer–multimer equilibria. Many chemical and biochemical systems exhibit monomer–multimer equilibria that are possibly coupled to other thermodynamic events. Examples of such systems include many DNA binding proteins [4,5], human hemoglobin [6–8], and multimeric polyprotic acids such as pyrophosphate. In addition, the mechanism of cooperativity in systems composed of four or more subunits may require that all of the subunits interact with one another, or that some subset of subunits interact either independently of, or in concert with, other subsets. This type of situation is exemplified by the *Limulus polyphemus* hemocyanin which is composed on 48 subunits with 6 subunits acting as a basic functional subset [9].

The thermodynamic formalism of Weber [10] is perhaps the most widely used for protein–ligand systems where monomer–multimer equilibria exist. This formalism however does not use the concept of interaction energy or of statistical correction factors even for a simple monomer–dimer system. These concepts are an essential feature for the formulation of, theoretical analysis of, and statistical analysis of data pertaining to, complex thermodynamic systems, as previously demonstrated [1–3]. Furthermore, mathematical methods for relating alternative thermodynamic models for monomer–multimer systems have not previously been described. Therefore, it is necessary to extend the reformulation to such cases and to develop the mathematical tools required for relating alternative models. Here I describe these methods and apply them to the reformulation of a homodimeric and a heterodimeric protein, to the derivation of the expressions describing ligand binding in a homomeric monomer–dimer system, and to the analysis of the relationship between a cooperative dimer and tetramer model of a hypothetical homotetrameric protein. The method for reformulating homodimeric and heterodimeric systems is described first since this is likely to be of general interest. Mathematical methods for relating alternative models are then developed and demonstrated in several detailed theoretical applications likely to be of interest to a specific audience.

2. Theory

2.1. Reformulation of a homomeric monomer–dimer protein ligand system

Given the reformulation objectives and principles developed previously [1], the model in Fig. 1 can be derived by logical analysis as follows. The standard state in this analysis is S, the free monomer, and L,

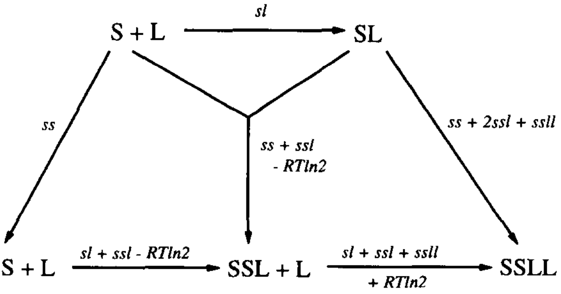

Fig. 1. The ΔG^0 reformulated model for a homomeric monomer–dimer ligand binding protein.

the free ligand. The monomer–dimer step, S + S → SS, is assigned a ΔG^0 of *ss*. The intrinsic affinity of a subunit for ligand, S + L → SL, is assigned a ΔG^0 of *sl*, the ΔG^0 for ligand binding to the dimer, SS + L → SSL, requires a base term, *sl*, plus an interaction term, *ssl*, plus a statistical correction term, $-RT$ ln 2. The pathway drawn from S and SL to SSL, S + SL → SSL, must have a term $ss + ssl - RT \ln 2$ to satisfy the requirement that the ΔG^0 of formation of any complex be path independent. The $-RT$ ln 2 term can be rationalized as the consequence of the fact that SSL can be assembled two ways, S + SL or SL + S. Next consider the path from SSL to SSLL, SSL + L → SSLL. The appropriate term for this step is $sl + ssl + ssll + RT \ln 2$. The appropriate term from SL to SSLL, SL + SL → SSLL, must therefore be $ss + 2ssl + ssll$ to satisfy the requirement that ΔG^0 be path independent. The ΔG^0s of formation for each complex in this system from the standard state of S and L are therefore

$$\Delta G^0_{\mathrm{SL}} = sl, \tag{1a}$$

$$\Delta G^0_{\mathrm{SS}} = ss, \tag{1b}$$

$$\Delta G^0_{\mathrm{SSL}} = ss + sl + ssl - RT \ln 2, \tag{1c}$$

$$\Delta G^0_{\mathrm{SSLL}} = ss + 2sl + 2ssl + ssll. \tag{1d}$$

The four terms, *sl*, *ss*, *ssl*, and *ssll*, are the minimal number necessary to uniquely define the four complexes relative to the standard state of the free components, S and L.

2.2. Reformulation of a heteromeric monomer–dimer protein ligand system

For a simple A + B system, where A represents one subunit and B the other of a hypothetical dimeric system where each subunit can bind one equivalent of a ligand L, the complete reformulated thermodynamic model shown in Fig. 2 can be derived. Note that this model is defined by six terms, *al*, *bl*, *ab*, *alb*, *abl*, and *albl*, the minimal number necessary to uniquely relate the six complexes to the standard state of free subunits and ligand. For the association of any two components the term required for the step is simply the italicized lower case name of the complex formed, i.e. for A + B → AB, $\Delta G^0 = ab$. Note that no statistical correction terms are required in Fig. 2 as a consequence of the fact that there are not multiple ways of assembling any complex in this model. For the reaction AL + B → ALB requires the base term for the interaction of B with A, *ab*, plus the interaction term for the effect of B being bound to A on ligand binding to A, *alb*. For the reaction ALB + L → ALBL requires a base term for the

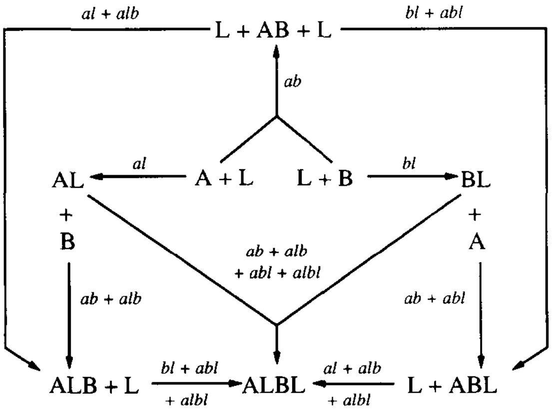

Fig. 2. The ΔG^0 reformulated model for a heteromeric monomer–dimer ligand binding protein.

interaction of L with B, *bl*, plus a second-order interaction term for the effect of A being bound to B on ligand binding to B, *abl*, plus an interaction term for the effect of L being bound to A on the binding of A to BL, or conversely for the effect of L being bound to B on the binding of B to AL, *albl*. (See Ref. [1] for a discussion of the physical significance of reformulated terms.)

2.3. Method for relating a ligand binding homodimer model to a homomeric monomer–dimer model

Relating models using this reformulation begins with expressions which relate the concentrations of the species in one model with those in the other. The question to be addressed in this section is how the behavior of a simple dimer in the absence of monomer–dimer equilibria is related to that of a ligand binding monomer–dimer system, as diagrammed in Fig. 3. The basic strategy used here is identical to that used for the analysis of conformationally mediated cooperativity in a dimeric protein [2], with the exception that a method must be developed to relate a monomer binding ligand, $S + L \rightarrow SL$, to a dimer binding ligand, $SS + L \rightarrow SSL + L \rightarrow SSLL$. To do this, it is necessary to convert S and SL into the apparent concentrations of SS, SSL, and SSLL (SS^{app}, SSL^{app}, and $SSLL^{app}$). The appropriate method is derived from probabilistic considerations. The apparent concentration of SS, SS^{app}, will be proportional to the probability of selecting two equivalents of S from the mixture, i.e. $SS^{app} \propto S \cdot S = S^2$. The apparent concentration of SSL will be proportional to the probability of selecting one equivalent each of S and SL from the mixture, i.e. $SSL^{app} \propto S \cdot SL + SL \cdot S = 2 \cdot S \cdot SL$. Analogously $SSLL^{app} \propto SL \cdot SL = SL^2$. These terms can be combined to give

$$SS^{app} + SSL^{app} + SSLL^{app} \propto S^2 + 2S \cdot SL + SL^2 = (S + SL)^2 = S_T^2, \tag{2}$$

which is equivalent to

$$D_T^{app} \propto S_T^2, \tag{3}$$

where D_T^{app} represents the apparent total concentration of dimer and S_T represents the total concentration of monomer. Using this observation we can normalize Eq. (2) to yield

$$\frac{SS^{app} + SSL^{app} + SSLL^{app}}{D_T^{app}} = \frac{S^2 + 2 + 2S \cdot SL + SL^2}{S_T^2} \tag{4}$$

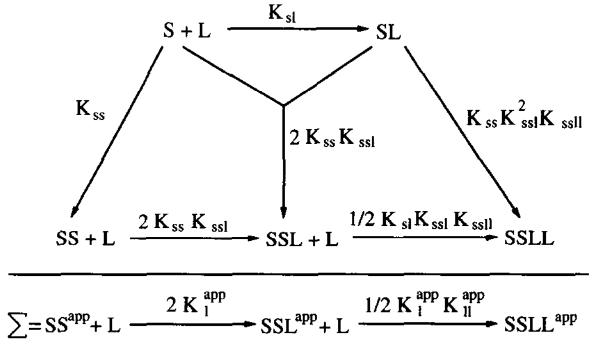

Fig. 3. Top: same as Fig. 1 but using the reformulated equilibrium constant model. Bottom: reformulated equilibrium constant model for a dimeric ligand binding protein where the concentrations and constants are to be related the values given in the top of this figure and are therefore designated as apparent.

D_T^{app} can be expressed as

$$D_T^{app} = \tfrac{1}{2}S_T. \tag{5}$$

Substituting Eq. (5) into Eq. (4), rearranging, and separating terms yields

$$SS^{app} = \tfrac{1}{2}S^2/S_T, \tag{6a}$$

$$SSL^{app} = S \cdot SL/S_T, \tag{6b}$$

$$SSLL^{app} = \tfrac{1}{2}SL^2/S_T. \tag{6c}$$

The situation in Fig. 3 can be analyzed by combining the apparent concentrations of SS, SSL and SSLL due to monomeric species with the true concentrations of SS, SSL, and SSLL. The apparent concentration of a dimeric species will be the sum of the true concentration for that species and the term for the apparent concentration derived above except that the S_T denominator term will be replaced by M_T, the total concentration of monomeric subunits. The following terms therefore define the apparent concentrations of the dimeric states depicted in the bottom of Fig. 3 in terms of the monomer–dimer states depicted in the top of Fig. 3,

$$SS^{app} = SS + \tfrac{1}{2}S^2/M_T, \tag{7a}$$

$$SSL^{app} = SSL + S \cdot SL/M_T, \tag{7b}$$

$$SSLL^{app} = SSLL + \tfrac{1}{2}SL^2/M_T. \tag{7c}$$

2.4. Substitution of equilibrium terms and algebraic solution for K_I^{app} and K_{II}^{app}

Using the equilibrium constant reformulated model shown in the top of Fig. 3, the following terms relate the concentration of each complex to the standard state of free subunit and ligand

$$SS = K_{ss}S^2, \tag{8a}$$

$$SL = K_{sl}S \cdot L, \tag{8b}$$

$$SSL = 2K_{ss}K_{sl}K_{ssl}S^2 \cdot L, \tag{8c}$$

$$SSLL = K_{ss}K_{sl}^2K_{ssl}^2K_{ssll}S^2 \cdot L^2 \tag{8d}$$

The expression for M_T gives

$$M_T = S + SL = S + K_{sl}S \cdot L = S \cdot (1 + K_{sl}L) \tag{9}$$

Substituting the expressions for SS, SL, SSL, and SSLL (Eqs. (8a)–(8d) and M_T (Eq. (9)) into the expressions for SS^{app}, SSL^{app}, and $SSLL^{app}$ (Eqs. (7a)–(7c)) gives

$$SS^{app} = K_{ss}S^2 + \tfrac{1}{2}S^2/M_T = K_{ss}S^2 + \tfrac{1}{2}S^2/[S(1 + K_{sl}L)] = S^2\left(K_{ss} + \tfrac{1}{2}/[S(1 + K_{sl}L)]\right) \tag{10a}$$

$$\begin{aligned} SSL^{app} &= 2K_{ss}K_{sl}K_{ssl}S^2 \cdot L + S \cdot SL/M_T = 2K_{ss}K_{sl}K_{ssl}S^2 \cdot L + K_{sl}S^2 \cdot L/[S \cdot (1 + K_{sl}L)] \\ &= 2K_{sl}S^2 \cdot L\{K_{ss}K_{ssl} + \tfrac{1}{2}/[S \cdot (1 + K_{sl}L)]\}, \end{aligned} \tag{10b}$$

$$\begin{aligned} SSLL^{app} &= K_{ss}K_{sl}^2K_{ssl}^2K_{ssll}S^2 \cdot L^2 + \tfrac{1}{2}SL^2/M_T = K_{ss}K_{sl}^2K_{ssl}^2K_{ssll}S^2 \cdot L^2 + \tfrac{1}{2}K_{sl}^2S^2 \cdot L^2/[S \cdot (1 + K_{sl}L)] \\ &= K_{sl}^2S^2 \cdot L^2\left(K_{ss}K_{ssl}^2K_{ssll} + \tfrac{1}{2}/[S \cdot (1 + K_{sl}L)]\right). \end{aligned} \tag{10c}$$

Using the definition of K_{l} for a homodimeric protein (Ref. [1], Eq. (21)), the following expression can be obtained for $K_{\mathrm{l}}^{\mathrm{app}}$

$$K_{\mathrm{l}}^{\mathrm{app}} = \frac{1}{2}\frac{\mathrm{SSL}^{\mathrm{app}}}{\mathrm{SS}^{\mathrm{app}}\cdot\mathrm{L}} = \frac{1}{2}\frac{2K_{\mathrm{sl}}\mathrm{S}^2\cdot\mathrm{L}\left(K_{\mathrm{ss}}K_{\mathrm{ssl}} + \frac{1}{2}/[\mathrm{S}\cdot(1+K_{\mathrm{sl}}\mathrm{L})]\right)}{\mathrm{S}^2\cdot\mathrm{L}\cdot\left(K_{\mathrm{ss}} + \frac{1}{2}/[\mathrm{S}\cdot(1+K_{\mathrm{sl}}\mathrm{L})]\right)}$$

$$= K_{\mathrm{l}}\frac{K_{\mathrm{ss}}K_{\mathrm{ssl}}\mathrm{S}\cdot(1+K_{\mathrm{sl}}\mathrm{L}) + \frac{1}{2}}{K_{\mathrm{ss}}\mathrm{S}\cdot(1+K_{\mathrm{sl}}\mathrm{L}) + \frac{1}{2}} = K_{\mathrm{l}}\frac{2K_{\mathrm{ss}}K_{\mathrm{ssl}}\mathrm{S}\cdot(1+K_{\mathrm{sl}}\mathrm{L}) + 1}{2K_{\mathrm{ss}}\mathrm{S}\cdot(1+K_{\mathrm{sl}}\mathrm{L}) + 1}. \tag{11}$$

Using the definition of K_{ll} for a homodimeric protein (Ref. [1], Eq. (22)), the following expression can be obtained for $K_{\mathrm{ll}}^{\mathrm{app}}$

$$K_{\mathrm{ll}}^{\mathrm{app}} = 4\frac{\mathrm{SSLL}^{\mathrm{app}}\cdot\mathrm{SS}^{\mathrm{app}}}{\mathrm{SSL}^{\mathrm{app}\,2}}$$

$$= 4\frac{\left(K_{\mathrm{sl}}^2\mathrm{S}^2\cdot\mathrm{L}^2\{K_{\mathrm{ss}}K_{\mathrm{ssl}}^2K_{\mathrm{ssll}} + \frac{1}{2}/[\mathrm{S}(1+K_{\mathrm{sl}}\mathrm{L})]\}\right)\left(\mathrm{S}^2\{K_{\mathrm{ss}} + \frac{1}{2}/[\mathrm{S}(1+K_{\mathrm{sl}}\mathrm{L})]\}\right)}{\left(2K_{\mathrm{sl}}\mathrm{S}^2\cdot\mathrm{L}\{K_{\mathrm{ss}}K_{\mathrm{ssl}} + \frac{1}{2}/[\mathrm{S}(1+K_{\mathrm{sl}}\mathrm{L})]\}\right)^2}$$

$$+\frac{\left[K_{\mathrm{ss}}K_{\mathrm{ssl}}^2K_{\mathrm{ssll}}\mathrm{S}\cdot(1+K_{\mathrm{sl}}\mathrm{L}) + \frac{1}{2}\right]\left[K_{\mathrm{ss}}\mathrm{S}\cdot(1+K_{\mathrm{sl}}\mathrm{L}) + \frac{1}{2}\right]}{\left[K_{\mathrm{ss}}K_{\mathrm{ssl}}\mathrm{S}\cdot(1+K_{\mathrm{sl}}\mathrm{L}) + \frac{1}{2}\right]^2}$$

$$= \frac{\left[2K_{\mathrm{ss}}K_{\mathrm{ssl}}^2K_{\mathrm{ssll}}\cdot\mathrm{S}\cdot(1+K_{\mathrm{sl}}\mathrm{L}) + 1\right]\left[2K_{\mathrm{ss}}\mathrm{S}\cdot(1+K_{\mathrm{sl}}\mathrm{L}) + 1\right]}{\left[2K_{\mathrm{ss}}K_{\mathrm{ssl}}\mathrm{S}\cdot(1+K_{\mathrm{sl}}\mathrm{L}) + 1\right]^2}. \tag{12}$$

These expressions all contain terms for S. S is a function of $\mathrm{S_T}$ and L, and can be solved as follows. By mass balance and Eqs. (8a)–(8d)

$$\mathrm{S_T} = \mathrm{S} + \mathrm{SL} + 2\mathrm{SS} + 2\mathrm{SSL} + 2\mathrm{SSLL} = \mathrm{S} + K_{\mathrm{sl}}\mathrm{S}\cdot\mathrm{L} + 2K_{\mathrm{ss}}\cdot\mathrm{S}^2 + 4K_{\mathrm{ss}}K_{\mathrm{sl}}K_{\mathrm{ssl}}\mathrm{S}^2\cdot\mathrm{L}$$

$$+ 2K_{\mathrm{ss}}K_{\mathrm{sl}}^2K_{\mathrm{ssl}}^2K_{\mathrm{ssll}}\cdot\mathrm{S}^2\cdot\mathrm{L}^2$$

$$= \mathrm{S}(1+K_{\mathrm{sl}}\mathrm{L}) + 2\mathrm{S}^2\left(K_{\mathrm{ss}} + 2K_{\mathrm{ss}}K_{\mathrm{sl}}K_{\mathrm{ssl}}\mathrm{L} + K_{\mathrm{ss}}K_{\mathrm{sl}}^2K_{\mathrm{ssl}}^2K_{\mathrm{ssll}}\cdot\mathrm{L}^2\right) \tag{13}$$

This can be rearranged to

$$2\cdot\mathrm{S}^2\cdot K_{\mathrm{ss}}\left(1 + 2K_{\mathrm{sl}}K_{\mathrm{ssl}}\mathrm{L} + K_{\mathrm{sl}}^2K_{\mathrm{ssl}}^2K_{\mathrm{ssll}}\mathrm{L}^2\right) + \mathrm{S}\cdot(1+K_{\mathrm{sl}}\mathrm{L}) - \mathrm{S_T} = 0. \tag{14}$$

Defining

$$A = 2K_{\mathrm{ss}}\left(1 + 2K_{\mathrm{sl}}K_{\mathrm{ssl}}\mathrm{L} + K_{\mathrm{sl}}^2K_{\mathrm{ssl}}^2K_{\mathrm{ssll}}\mathrm{L}^2\right), \tag{15}$$

$$B = 1 + K_{\mathrm{sl}}\mathrm{L}, \tag{16}$$

$$C = \mathrm{S_T}, \tag{17}$$

we can solve for S with the quadratic equation (note that C used here is $-C$ in the traditional quadratic equation)

$$\mathrm{S} = \frac{-B + \sqrt{(B^2 + 4AC)}}{2A}. \tag{18}$$

Defining

$$Q = -B + \sqrt{(B^2 + 4AC)}, \tag{19}$$

and substituting these expressions back into Eqs. (11) and (12) and rearranging gives

$$K_{\mathrm{I}}^{\mathrm{app}} = K_{\mathrm{sI}} \frac{2K_{\mathrm{ss}}K_{\mathrm{ssI}}BQ + A}{2K_{\mathrm{ss}}BQ + A} \tag{20}$$

$$K_{\mathrm{II}}^{\mathrm{app}} = \frac{(2K_{\mathrm{ss}}K_{\mathrm{ssI}}^{2}K_{\mathrm{ssII}}BQ + A)(2K_{\mathrm{ss}}BQ + A)}{(2K_{\mathrm{ss}}K_{\mathrm{ssI}}BQ + A)^2}. \tag{21}$$

These expressions define $K_{\mathrm{I}}^{\mathrm{app}}$ and $K_{\mathrm{II}}^{\mathrm{app}}$ in terms of the physical parameters K_{sI}, K_{ss}, K_{ssI}, and K_{ssII}, and the experimental parameters $\mathrm{S_T}$ and L.

2.5. Algebraic analysis of K_{I}^{app} and K_{II}^{app}

The expressions derived for $K_{\mathrm{I}}^{\mathrm{app}}$ and $K_{\mathrm{II}}^{\mathrm{app}}$ (Eqs. (20) and (21)) have a dependence on L and will therefore not be constant as a function of L except under certain conditions. One such set of conditions is at high $\mathrm{S_T}$ (i.e. $\mathrm{S_T} \gg K_{\mathrm{ss}}$), where dimer dominates the subunit equilibria, or low $\mathrm{S_T}$ (i.e. $\mathrm{S_T} \ll K_{\mathrm{ss}}$), where the monomer dominates the subunit equilibria. The following relations can be derived

As $\mathrm{S_T} \to 0$,

$$C \to 0, \tag{22a}$$

$$K_{\mathrm{I}}^{\mathrm{app}} \to K_{\mathrm{sI}}, \tag{22b}$$

$$K_{\mathrm{II}}^{\mathrm{app}} \to 1. \tag{22c}$$

As $\mathrm{S_T} \to \infty$,

$$C \to \infty, \tag{23a}$$

$$K_{\mathrm{I}}^{\mathrm{app}} \to K_{\mathrm{sI}} \cdot K_{\mathrm{ssI}}, \tag{23b}$$

$$K_{\mathrm{II}}^{\mathrm{app}} \to K_{\mathrm{ssII}}. \tag{23c}$$

These results are expected from inspection of Fig. 3. If K_{ssI} and K_{ssII} both equal 1 there will be no interaction between ligand binding and subunit–subunit binding and no interaction between ligands binding on the dimer. Then the following relations can then be derived

If $K_{\mathrm{ssI}} = 1$ and $K_{\mathrm{ssII}} = 1$

$$K_{\mathrm{I}}^{\mathrm{app}} = K_{\mathrm{sI}}, \tag{24a}$$

$$K_{\mathrm{II}}^{\mathrm{app}} = 1, \tag{24b}$$

which is also expected.

These equations demonstrate that if data is obtained at high $\mathrm{S_T}$, then $K_{\mathrm{I}}^{\mathrm{app}}$ determined by statistical analysis would be equal to $K_{\mathrm{sI}} \cdot K_{\mathrm{ssI}}$ and $K_{\mathrm{II}}^{\mathrm{app}}$ would be equal to K_{ssII}. If the data is obtained at low $\mathrm{S_T}$, $K_{\mathrm{I}}^{\mathrm{app}}$ would be equal to K_{sI} and $K_{\mathrm{II}}^{\mathrm{app}}$ would be equal to 1, i.e. no cooperativity.

2.6. Application to relating lower-order to higher-order models. The monomer–trimer case

The method presented above for relating a monomer–dimer model with a simple dimer model also allows a monomer model to be related to a dimer model, and by extension to allow any N-mer model to be related to any M-mer model with the condition that $N < M$ and that M be an integer multiple of N. The terms describing the higher-order (more complex) model can be derived from the terms describing the lower-order (less complex) model by a polynomial expansion of the terms describing the lower-order model by the M/N power and normalization as described above for the monomer–dimer case. Consider the monomer and trimer models

$$\mathrm{M}+\mathrm{L}\xrightarrow{K_{\mathrm{I}}^{\mathrm{m}}}\mathrm{ML} \tag{25}$$

$$\mathrm{T}+\mathrm{L}\xrightarrow{3K_{\mathrm{I}}^{\mathrm{t}}}\mathrm{TL}+\mathrm{L}\xrightarrow{K_{\mathrm{I}}^{\mathrm{t}}K_{\mathrm{II}}}\mathrm{TL}_2+\mathrm{L}\xrightarrow{\frac{1}{3}K_{\mathrm{I}}^{\mathrm{t}}K_{\mathrm{II}}^{2}K_{\mathrm{III}}}\mathrm{TL}_3 \tag{26}$$

where the superscript m refers to the monomer model and t refers to the trimer model. Note that superscripted t and m denote monomer and trimer only with terms that are potentially ambiguous. T can only be assembled one way from monomer

$$\mathrm{T}\propto\mathrm{M}\cdot\mathrm{M}\cdot\mathrm{M}=\mathrm{M}^3. \tag{27}$$

TL can be assembled three ways

$$\mathrm{TL}\propto\mathrm{M}\cdot\mathrm{M}\cdot\mathrm{ML}+\mathrm{M}\cdot\mathrm{ML}\cdot\mathrm{M}+\mathrm{ML}\cdot\mathrm{M}\cdot\mathrm{M}=3\mathrm{M}^2\cdot\mathrm{ML}. \tag{28}$$

TL_2 can also be assembled three ways

$$\mathrm{TL}_2\propto\mathrm{M}\cdot\mathrm{ML}\cdot\mathrm{ML}+\mathrm{ML}\cdot\mathrm{M}\cdot\mathrm{ML}+\mathrm{ML}\cdot\mathrm{ML}\cdot\mathrm{M}=3\mathrm{M}\cdot\mathrm{ML}^2. \tag{29}$$

TL_3 can be assembled one way

$$\mathrm{TL}_3\propto\mathrm{ML}\cdot\mathrm{ML}\cdot\mathrm{ML}=\mathrm{ML}^3. \tag{30}$$

Exact relationships can be established as described above by normalizing with the relationships

$$\mathrm{T}+\mathrm{TL}+\mathrm{TL}_2+\mathrm{TL}_3=\mathrm{T}_\mathrm{T}, \tag{31a}$$

$$\mathrm{M}^3+3\mathrm{M}^2\cdot\mathrm{ML}+3\mathrm{M}\cdot\mathrm{ML}^2+\mathrm{ML}^3=(\mathrm{M}+\mathrm{ML})^3=\mathrm{M}_\mathrm{T}^3, \tag{31b}$$

to give

$$\mathrm{T}/\mathrm{T}_\mathrm{T}=\mathrm{M}^3/\mathrm{M}_\mathrm{T}^3, \tag{32a}$$

$$\mathrm{TL}/\mathrm{T}_\mathrm{T}=3\mathrm{M}^2\cdot\mathrm{ML}/\mathrm{M}_\mathrm{T}^3, \tag{32b}$$

$$\mathrm{TL}_2/\mathrm{T}_\mathrm{T}=3\mathrm{M}\cdot\mathrm{ML}^2/\mathrm{M}_\mathrm{T}^3, \tag{32c}$$

$$\mathrm{TL}_3/\mathrm{T}_\mathrm{T}=\mathrm{ML}^3/\mathrm{M}_\mathrm{T}^3. \tag{32d}$$

The inverse relationships, describing Ms as a function of Ts can also be derived

$$\mathrm{M}^3/\mathrm{M}_\mathrm{T}^3=\mathrm{T}/\mathrm{T}_\mathrm{T};\quad \mathrm{M}/\mathrm{M}_\mathrm{T}=(\mathrm{T}/\mathrm{T}_\mathrm{T})^{1/3}, \tag{33a}$$

$$\mathrm{ML}^3/\mathrm{M}_\mathrm{T}^3=\mathrm{TL}_3/\mathrm{T}_\mathrm{T};\quad \mathrm{ML}/\mathrm{M}_\mathrm{T}=(\mathrm{TL}_3/\mathrm{T}_\mathrm{T})^{1/3}. \tag{33b}$$

From equilibrium relationships

$$\mathrm{ML}=\mathrm{M}\cdot\mathrm{L}\cdot K_\mathrm{I}^\mathrm{m}, \tag{34a}$$

$$\mathrm{TL}=\mathrm{T}\cdot\mathrm{L}\cdot 3K_\mathrm{I}^\mathrm{t}. \tag{34b}$$

Substituting the equilibrium relations for ML and TL (Eqs. (34a) and (34b)) into the definition of TL in terms of M and ML (Eq. (32b)) gives

$$\mathrm{TL}/\mathrm{T}_\mathrm{T}=3\mathrm{M}^2\cdot\mathrm{ML}/\mathrm{M}_\mathrm{T}^3 \tag{35a}$$

$$\mathrm{T}\cdot\mathrm{L}\cdot 3K_\mathrm{I}^\mathrm{t}/\mathrm{T}_\mathrm{T}=3\mathrm{M}^2\cdot\mathrm{M}\cdot\mathrm{L}\cdot K_\mathrm{I}^\mathrm{m}/\mathrm{M}_\mathrm{T}^3 \tag{35b}$$

Canceling common terms and using Eq. (32a) gives

$$K_\mathrm{I}^\mathrm{t}=K_\mathrm{I}^\mathrm{m}. \tag{36}$$

This result is as expected from the definition of $K_{\rm I}^{\rm t}$ as the intrinsic binding constant for a binding site. Applying the same analysis to the expressions for $\rm TL_2$ and $\rm TL_3$ yields

$$K_{\rm II} = 1, \tag{37a}$$

$$K_{\rm III} = 1. \tag{37b}$$

These results demonstrate that for a trimer to exhibit the identical behavior as a monomer requires that the interaction equilibrium constants $K_{\rm II}$ and $K_{\rm III}$ must be equal to 1 (i.e., *II* and *III* equal to 0). Conversely these results show that any truly monomeric model which is formulated as a trimeric model will not exhibit any cooperativity. Although these results are intuitively obvious they are proven in this analysis.

2.7. Application to the homomeric dimer–tetramer case

In the previous analyses intuitively obvious situations were analyzed to develop and demonstrate the necessary methods. Next consider what behavior would be expected for a tetrameric protein that was really a pair of cooperative dimers. The question to be addressed is if a tetrameric protein was a pair of cooperative dimers, would fitting a tetrameric model to the data provide any clue that this was the case from the fitted parameter values or would the tetrameric and dimeric models have to be fit separately to distinguish between them? In this case we are dealing with a dimer (D) and a tetramer (T). The reformulated model for the dimeric protein is (Ref. [1], Fig. 2)

$$\rm D + L \xrightarrow{2K_{I}^{d}} DL + L \xrightarrow{\frac{1}{2}K_{I}^{d}K_{II}^{d}} DL_2. \tag{38}$$

The reformulated model for the tetramer is (Ref. [1], Eq. (28) converted into the equilibrium constant form)

$$\rm T + L \xrightarrow{4K_{I}^{t}} TL + L \xrightarrow{\frac{3}{2}K_{I}^{t}K_{II}^{t}} TL_2 + L \xrightarrow{\frac{2}{3}K_{I}^{t}K_{II}^{t2}K_{III}} TL_3 + L \xrightarrow{\frac{1}{4}K_{I}^{t}K_{II}^{t3}K_{III}^{3}K_{IIII}^{3}} TL_4. \tag{39}$$

Note that the terms for $K_{\rm I}$ and $K_{\rm II}$ bear a superscript to distinguish these terms for the two models. The third- and fourth-order terms ($K_{\rm III}$ and $K_{\rm IIII}$) are unique to the model for the tetramer. Using the principles established in Section 2.6 we can relate these two models as follows:

$$\rm T/T_T = D^2/D_T^2, \tag{40a}$$

$$\rm TL/T_T = 2 \cdot D \cdot DL/D_T^2, \tag{40b}$$

$$\rm TL_2/T_T = (2 \cdot D \cdot DL_2 + DL^2)/D_T^2, \tag{40c}$$

$$\rm TL_3/T_T = 2 \cdot DL \cdot DL_2/D_T^2, \tag{40d}$$

$$\rm TL_4/T_T = DL_2^2/D_T^2. \tag{40e}$$

Note that the term for $\rm TL_2$ is composed of two individual terms reflecting both ways of assembling this complex. If we substitute the equilibrium relationships, defined by Eq. (38) and Eq. (39), into these expressions we get a new set of expressions. Eq. (40a) is irreducible and is essentially an identity. Expanding Eq. (40b) gives

$$\rm T \cdot L \cdot 4K_I^t/T_T = 2 \cdot D \cdot D \cdot L \cdot 2K_I^d/D_T^2, \tag{41}$$

which can be reduced, using Eq. (40a), to give

$$K_{\rm I}^{\rm t} = K_{\rm I}^{\rm d}. \tag{42}$$

This expression confirms that K_{I} in both models is identical. K_{I} will denote this constant for the remainder of this discussion. Eq. (40c) can be expanded and reduced as follows:

$$\mathrm{T}\cdot\mathrm{L}^2\cdot 4K_{\mathrm{I}}\cdot\tfrac{3}{2}K_{\mathrm{I}}K^{\mathrm{t}}_{\mathrm{II}}/\mathrm{T}_{\mathrm{T}}=\left[2\mathrm{D}\cdot\mathrm{D}\cdot\mathrm{L}^2\cdot K^2_{\mathrm{I}}K^{\mathrm{d}}_{\mathrm{II}}+(\mathrm{D}\cdot\mathrm{L}\cdot 2K_{\mathrm{I}})^2\right]/\mathrm{D}^2_{\mathrm{T}}, \tag{43a}$$

$$4\tfrac{3}{2}K^{\mathrm{t}}_{\mathrm{II}}=2K^{\mathrm{d}}_{\mathrm{II}}+(2)^2, \tag{43b}$$

$$K^{\mathrm{t}}_{\mathrm{II}}=\tfrac{1}{3}K^{\mathrm{d}}_{\mathrm{II}}+\tfrac{2}{3}. \tag{43c}$$

Note that if $K^{\mathrm{d}}_{\mathrm{II}}=1$, then $K^{\mathrm{t}}_{\mathrm{II}}=1$. Eq. (40d) can be treated similarly

$$\mathrm{T}\cdot\mathrm{L}^3\cdot 4K_{\mathrm{I}}\tfrac{3}{2}K_{\mathrm{I}}K^{\mathrm{t}}_{\mathrm{II}}\tfrac{2}{3}K_{\mathrm{I}}K^{\mathrm{t}2}_{\mathrm{II}}K_{\mathrm{III}}/\mathrm{T}_{\mathrm{T}}=2\mathrm{D}\cdot\mathrm{L}\cdot 2K_{\mathrm{I}}\mathrm{D}\cdot\mathrm{L}^2\cdot K^2_{\mathrm{I}}K^{\mathrm{d}}_{\mathrm{II}}/\mathrm{D}^2_{\mathrm{T}}, \tag{44a}$$

$$K^{\mathrm{t}3}_{\mathrm{II}}K_{\mathrm{III}}=K^{\mathrm{d}}_{\mathrm{II}}. \tag{44b}$$

Substituting the expression for $K^{\mathrm{t}}_{\mathrm{II}}$ (Eq. (43c)) into Eq. (44b) gives

$$K_{\mathrm{III}}=K^{\mathrm{d}}_{\mathrm{II}}/\left(\tfrac{1}{3}K^{\mathrm{d}}_{\mathrm{II}}+\tfrac{2}{3}\right)^3. \tag{45}$$

Note that if $K^{\mathrm{d}}_{\mathrm{II}}=1$, then $K_{\mathrm{III}}=1$. Finally for Eq. (40e)

$$\mathrm{T}\cdot\mathrm{L}^4\cdot K^4_{\mathrm{I}}K^{\mathrm{t}6}_{\mathrm{II}}K^4_{\mathrm{III}}K_{\mathrm{IIII}}/\mathrm{T}_{\mathrm{T}}=\left(\mathrm{D}\cdot\mathrm{L}^2\cdot K^2_{\mathrm{I}}K^{\mathrm{d}}_{\mathrm{II}}\right)^2/\mathrm{D}^2_{\mathrm{T}}, \tag{46a}$$

$$K^{\mathrm{t}6}_{\mathrm{II}}K^4_{\mathrm{III}}K_{\mathrm{IIII}}=K^{\mathrm{d}2}_{\mathrm{II}}. \tag{46b}$$

Substituting the expression for $K^{\mathrm{t}}_{\mathrm{II}}$ (Eq. (43c)) into Eq. (46b) gives

$$K^{\mathrm{t}6}_{\mathrm{II}}K^4_{\mathrm{III}}K_{\mathrm{IIII}}=K^{\mathrm{t}6}_{\mathrm{II}}K^2_{\mathrm{III}}, \tag{47a}$$

$$K^2_{\mathrm{III}}K_{\mathrm{IIII}}=1. \tag{47b}$$

Substituting the expression for K_{III} (Eq. (45)) into Eq. (47b) gives

$$\begin{aligned}K_{\mathrm{IIII}}&=1/K^2_{\mathrm{III}}\\&=\left(\tfrac{1}{3}K^{\mathrm{d}}_{\mathrm{II}}+\tfrac{2}{3}\right)^6/K^{\mathrm{d}2}_{\mathrm{II}}.\end{aligned} \tag{48}$$

Note that if $K^{\mathrm{d}}_{\mathrm{II}}=1$, then $K_{\mathrm{IIII}}=1$. These results are summarized in Table 1.

These results show that for a tetrameric protein which really behaves as a dimer of cooperatively interacting dimers, fitting to a tetrameric model will provide values for the base term (K_{I}) and for the three interaction terms. To determine the suitability of the cooperative dimer model, it would be necessary to explicitly fit data with such a model.

Table 1
Relationship between values of parameters in a cooperative dimer model and the apparent values in a tetramer model

Parameter in the tetramer model	Full expression relating models	Limiting condition		
		if $K^{\mathrm{d}}_{\mathrm{II}}=1$	if $K^{\mathrm{d}}_{\mathrm{II}}\gg 1$	if $K^{\mathrm{d}}_{\mathrm{II}}\ll 1$
$K^{\mathrm{t}}_{\mathrm{I}}$	$=K^{\mathrm{d}}_{\mathrm{I}}$	$K^{\mathrm{d}}_{\mathrm{I}}$	$K^{\mathrm{d}}_{\mathrm{I}}$	$K^{\mathrm{d}}_{\mathrm{I}}$
$K^{\mathrm{t}}_{\mathrm{II}}$	$=\frac{1}{3}K^{\mathrm{d}}_{\mathrm{II}}+\frac{2}{3}$	1	$\frac{1}{3}K^{\mathrm{d}}_{\mathrm{II}}$	$\frac{2}{3}$
$K^{\mathrm{t}}_{\mathrm{III}}$	$=K^{\mathrm{d}}_{\mathrm{II}}/(\frac{1}{3}K^{\mathrm{d}}_{\mathrm{II}}+\frac{2}{3})^3$	1	$3^3/K^{\mathrm{d}2}_{\mathrm{II}}$	$(\frac{3}{2})^3K^{\mathrm{d}}_{\mathrm{II}}$
$K^{\mathrm{t}}_{\mathrm{IIII}}$	$=(\frac{1}{3}K^{\mathrm{d}}_{\mathrm{II}}+\frac{2}{3})^6/K^{\mathrm{d}2}_{\mathrm{II}}$	1	$3^6K^{\mathrm{d}4}_{\mathrm{II}}$	$(\frac{2}{3})^6/K^{\mathrm{d}2}_{\mathrm{II}}$

2.8. Method for relating heteromeric monomer–dimer models

The preceding treatment was for homomeric systems. It is of interest to develop the basic principles of this type of analysis for heteromeric systems using probabilistic arguments as described above for the homomeric case. Consider the case of a mixture of monomers, A and B, which can each bind one equivalent of a ligand L. Given A_T and B_T in equilibrium with a concentration of free ligand L, a distribution of A, AL, B, and BL will exist in the absence of multimerization. The apparent concentrations of the hypothetical dimers will be described by

$$AB^{app} \propto A \cdot B, \tag{49a}$$

$$ALB^{app} \propto AL \cdot B, \tag{49b}$$

$$ABL^{app} \propto A \cdot BL, \tag{49c}$$

$$ALBL^{app} \propto AL \cdot BL \tag{49d}$$

Summing both sides of these expressions gives

$$\begin{aligned} AB^{app} + ALB^{app} + ABL^{app} + ALBL^{app} &\propto A \cdot B + AL \cdot B + A \cdot BL + AL \cdot BL \\ &\propto (A + AL) \cdot (B + BL). \end{aligned} \tag{50}$$

This can also be expressed as

$$AB_T \propto (A_T) \cdot (B_T). \tag{51}$$

If $A_T \neq B_T$ there will be some excess of one monomer. The amount of apparent complex, AB_T^{app}, will be equal to the minimum of A_T and B_T (the limiting reagent), $\min(A_T, B_T)$. Using this relationship to normalize the above equation gives

$$\frac{AB^{app} + ALB^{app} + ABL^{app} + ALBL^{app}}{\min(A_T, B_T)} = \frac{A \cdot B + AL \cdot B + A \cdot BL + AL \cdot BL}{A_T B_T}. \tag{52}$$

Note that $\min(A_T, B_T)/A_T B_T = 1/\max(A_T, B_T)$. Therefore

$$AB^{app} = A \cdot B/\max(A_T, B_T), \tag{53a}$$

$$ALB^{app} = AL \cdot B/\max(A_T, B_T), \tag{53b}$$

$$ABL^{app} = A \cdot BL/\max(A_T, B_T), \tag{53c}$$

$$ALBL^{app} = AL \cdot BL/\max(A_T, B_T). \tag{53d}$$

The excess subunit will appear to remain unassociated in this analysis.

3. Discussion

The nomenclature for the terms used for systems exhibiting monomer–multimer equilibria is somewhat more complicated than that previously described for fixed template equilibria [1]. On a fixed template, for example the PABC system analyzed previously (Ref. [1], Fig. 5), all the complexes have P as a component, and it is therefore not necessary that the reformulated terms contain the letter "p" for the number of terms to be equal to the number of complexes. In this case the base terms are one letter, the second-order terms are two letters etc., i.e. omitting the letter "p". However, for systems exhibiting monomer–multimer equilibria, the template changes upon multimerization and this requires the use of two letter terms for the base terms, three letters for the second-order terms, etc. This appears to be a rational nomenclature at this point since it is the most efficient in terms of space and allows the terms required in either case to be designated simply by inspection of the model being reformulated.

The reformulation is dictated by the requirement that each complex be uniquely defined, that the number of terms used in formulating a model be equal to the number of states other than the standard state, and by the requirement of path independence. The rules previously developed to reformulate models for ligand binding on a fixed template [1] will provide nonsensical results if applied blindly to monomer–multimer systems. By a slight modification of these rules, however, a consistent method for formulating monomer–multimer systems can be proposed. Consider the step ALB + L → ALBL shown in Fig. 2. The original rules for reformulating thermodynamic models [1] might suggest the appropriate term should be $bl + al + ll + alb + abl + all + bll + albl$, which is not correct. In this case L binding to B cannot be perturbed by L bound to A unless A and B are bound as implied in the model. From a physical point of view it appears rational that the rule for formulating models should be that the term for a step be composed of terms for all possible associations of the incoming component with components in the complex that can be assembled without breaking the connection between the incoming component and the components in the complex. Note that because we are using the lower case italicized names of our complexes as reformulated ΔG^0s then this is the same as saying that terms must correspond to a real complex in the model, i.e. for *albl* to be an acceptable term the complex ALBL must exist in the model. Consider as an example the term for the step ALB + L → ALBL. Since L is binding to B we need the base term *bl*. There is no *al* term for this step since this would omit B which provides the link between A, and L binding to B. However, the term *abl* is a valid term. The term *ll* is not valid because it omits A and B which provide the link between L bound to A and L bound to B, i.e. because there is no LL state. The term *albl* is required for this step since all the intervening components between L binding to B and the other components are included in this term. This rule for formulating a step can be extended to the derivation of the term for the ΔG^0 of formation of a complex from its components simply by taking all possible combinations of the components subject to the limitation that no intervening components be omitted. For example, the term for the ΔG^0 of formation of the ALBL complex requires the base terms $al + bl + ab$, corresponding to all of the pairwise ways of associating the components of ALBL, the second order interaction terms $alb + abl$, corresponding to the ways of associating three components without omitting intervening components, and the third order interaction term for this complex of *albl*. Combined these terms gives $\Delta G^0_{\mathrm{ALBL}} = al + bl + ab + alb + abl + albl$ which can be verified by inspection of Fig. 2. The purpose of developing rules for reformulating systems is to facilitate the reformulation of complex systems. The rules reflect the physical–mathematical requirements of the reformulation and are not an end in themselves but simply an aid.

Systems exhibiting monomer–multimer equilibria are relatively common and include many DNA binding proteins [4,5], human hemoglobin [6–8], and a large number of chemical systems including polyprotic acids. Here I have developed the general principles for the application of the reformulation to systems where components may form aggregates. The thermodynamic behavior of the system shown in Fig. 1 was analyzed by algebraically relating the parameters describing this system to $K_{\mathrm{I}}^{\mathrm{app}}$ and $K_{\mathrm{II}}^{\mathrm{app}}$, the parameters describing the behavior of a simple dimer [1,2]. This requires a method for relating the thermodynamic behavior of a system of monomers to that of multimers and such a method is derived. This analysis demonstrates that the homomeric monomer–dimer system; (1) will be noncooperative (i.e. $K_{\mathrm{II}}^{\mathrm{app}} = 1$) at dilute monomer concentrations where dimer formation is negligible, (2) will act as a dimer at high monomer concentrations where essentially all monomer will be present in the form of dimer, and (3) may show systematic deviations from simple dimer behavior at intermediate monomer concentrations where significant monomer–dimer equilibria exists.

This approach was extended to relate a cooperative dimer and cooperative tetramer model for cooperativity in a homomeric tetrameric protein. These results demonstrate that no simple relationship exists between the higher-order parameters in the tetramer model and the second-order term in the dimer model, although the first-order term in both models is identical. Therefore data pertaining to such a system would have to be fit with both models to ascertain the best model. Note that if data were fit with a tetramer model, for example hemoglobin oxygen binding data, and the third- and fourth-order interaction terms were found to be not statistically different than zero, one could not conclude that a dimer model was more appropriate. These results together suggest that it will be necessary to fit data pertaining to multimeric systems with lower and higher-order models to identify the most appropriate model.

A homodimeric ligand binding protein system has previously been considered by Weber [10] and this method of formulation (the classical formulation) serves as the foundation for the analyses of dimer–tetramer dependent hemoglobin oxygen binding data [6–10]. Several points need to be made in comparing this method with the reformulation described here. First, failure to include statistical correction factors in the classical formulation of such systems creates difficulties for the interpretation of parameter values and can lead to errors in the analysis and interpretation of such parameter values, a problem which has been recognized in some analyses [6,7], but was overlooked in the original description of this formulation (Ref. [10], Fig. 13 and Eq. (3.4)) as well as in a subsequent application of this method (Ref. [11], Table 1). Second, although the classical formulation uses the concept of interaction energy when treating two ligands binding to a fixed template [10], this concept was not used when treating a simple monomer–dimer system (Ref. [10], Fig. 13). It is clear that the extension of the concept of interaction energy to monomer–multimer equilibria is a necessity for the mathematical analysis of these types of systems. This concept is also essential to theoretical and statistical applications of the reformulation as established in previous applications [2,3]. Third, the reformulation allows rigorous mathematical relationships to be derived using the methods developed here and in the previous applications of this method [1,2]. Although intuitively obviously conclusions concerning the behavior of a monomer–dimer system have been suggested [10], it is only by using the methods developed above that these conclusions can be proven mathematically.

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