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RELATIONSHIPS BETWEEN STOICHIOMETRIC BINDING CONSTANTS, SITE BINDING CONSTANTS, AND EMPIRICAL BINDING PARAMETERS*

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SUMMARY

The multiple equilibria for the binding of a ligand A by a macromolecule P with n binding sites may be formulated in terms of a stoichiometric analysis or on the basis of a site-oriented scrutiny. The dependence of binding on ligand concentration can always be correlated in terms of n stoichiometric binding constants, K_i, even if there are interactions between sites that accentuate or attenuate binding affinities. A corresponding correlation in terms of site binding constants, k_j, under the most general circumstances depends on the definition of $n2^{n-1}$ different constants of which $2^n - 1$ are independent. If experimental data are correlated in terms of n parameters k_α, $k_\beta \cdots k_\lambda$ in an equation of the site-binding form,

$$r = \sum_{k_T = k_\alpha, k_\beta \cdots, k_\lambda} \frac{k_T(A)}{1 + k_T(A)},$$

then there is no guarantee that the values of k_α, k_β, etc., have any unique relationships to site binding constants. Examples are given to illustrate this point. Equations are derived for relating stoichiometric binding constants to site binding constants, for the general case and for various special circumstances. These equations make it possible to define and analyze binding in systems with interactions and conformational accommodations. Accordingly, a graphical procedure is described (in which iK_i is plotted against i, the stoichiometric binding step) that provides an affinity profile for concise representation of magnitudes of binding constants and for detecting interactions that accentuate or attenuate site binding affinities.

A central aspect of any general study of the interactions of a small molecule with a biological macromolecule is the determination of the distribution of ligand between macromolecule and bulk solvent. This distribution is a manifestation of the stoichiometry of the combinations and the strength of the interactions. The extent of uptake of a small molecule by a macromolecule depends on the number of sites available on the latter, their affinity for the ligand, and the chemical potential or concentration of the nonbound species of the ligand.

Recognizing that a given protein macromolecule, P, may have a multiplet of sites for binding a specific ligand A, we express the mass law relationship in terms of a series of multiple equilibria. Two different formulations may be used for this purpose, one being stoichiometric in its outlook, the other being site-oriented. The binding constants derived by the two approaches are not the same in magnitude nor in the binding step to which they must be assigned. Furthermore, the empirical binding parameters obtained by common graphical methods of analysis may bear no relationship to the site binding constants they superficially resemble. It is the purpose of this exposition to clarify the distinction between stoichiometric binding constants and site binding constants, to illustrate their relationships with empirical binding parameters and with each other, and to discuss the range and limitations in the two modes of analysis of experimental binding data.

STOICHIOMETRIC BINDING CONSTANTS

As has been described in detail recently (1), the stoichiometric formulation focuses on the sequential stoichiometric species PA_1, PA_2, etc., participating in the equilibria between protein P and ligand A,

$$PA_{i-1} + A = PA_i \qquad (1)$$

Stoichiometric equilibrium constants, K_i, are defined by the equation

$$K_i = \frac{(PA_i)}{(PA_{i-1})(A)} \qquad (2)$$

In terms of stoichiometric equilibrium constants, r, the moles of bound A per mole of total protein, may be expressed as (2–4)

$$r = \frac{K_1(A) + 2K_1K_2(A)^2 + \cdots}{1 + K_1(A) + K_1K_2(A)^2 + \cdots} = \frac{\sum_1^n i\left(\prod_{\ell=1}^{i} K_\ell\right)(A)^i}{1 + \sum_1^n \left(\prod_{\ell=1}^{i} K_\ell\right)(A)^i} \qquad (3)$$

where n is the number of binding sites on each protein molecule.

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SITE BINDING CONSTANTS WHEN SITES HAVE FIXED AFFINITIES

Alternatively, if one focuses on the individual sites of binding, the multiple equilibria may be represented by

$$ {}_jP + A = {}_jPA \qquad (4) $$

where the left-hand subscript denotes a particular site. Correspondingly, the site equilibrium constants, k_j, are defined by

$$ k_j = \frac{({}_jPA)}{({}_jP)\,(A)} \qquad (5) $$

When the individual sites have affinities that do not change with the extent of occupancy by ligand, the total moles of bound ligand at all sites is (5–7)

$$ r = \sum_1^n r_j = \sum_1^n \frac{k_j(A)}{1 + k_j(A)} \qquad (6) $$

RELATIONS BETWEEN STOICHIOMETRIC AND SITE BINDING CONSTANTS WHEN SITES HAVE FIXED AFFINITIES

Since r of Equation 3 designates the same experimental quantity as that in Equation 6, the constants K_i must be related to k_j. The relationships between them have been deduced by different procedures (1, 8–11) and are given by Equations 7 to 10.

$$ K_1 = k_1 + k_2 + \ldots + k_n = \sum_{j_1=1}^{n} k_{j_1} \qquad (7) $$

SITE BINDING CONSTANTS WHEN AFFINITIES CHANGE WITH EXTENT OF OCCUPANCY

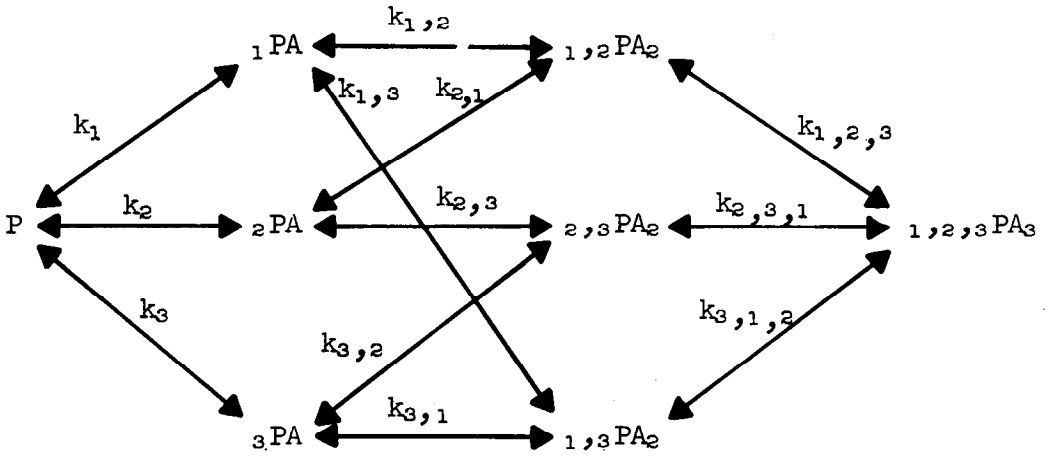
A stoichiometric formulation is always applicable to the correlation of binding data for it is a classical thermodynamic analysis. However, the specific site representation as described above is inadequate (1). The type of difficulty encountered in a constant-site analysis can be illustrated, for example, with aminoethylmercaptide ion, $H_2NCH_2CH_2S^-$. The sequential uptake of two H^+ ions by $H_2NCH_2CH_2S^-$ can be correlated thoroughly by Equation 3 using two stoichiometric equilibrium constants K_1 and K_2. On the other hand, four site binding constants must be assigned (1) to $H_2NCH_2CH_2S^-$. It becomes apparent on reflection that one would encounter difficulties trying to formulate r in terms of Equation 6. If one used a two-term equation, which pair of the four site constants should be inserted for the two k_js? Alternatively, one might extend the number of terms in Equation 6 to four, but that is unacceptable since it implies that r can reach 4 (sites) as (A) $\rightarrow \infty$. One can, of course, arbitrarily fit r and (A) to a two-term form of Equation 6 with empirical parameters k_α and k_β (12). However, that would still leave us with the question of what is the relationship of empirical k_α and k_β to the four site constants. It is of interest, therefore, to examine the relationship between this type of empirically-determined parameter and the site binding constants for a completely general reaction. To formulate this problem, we must first extend the site-oriented approach to systems where the binding affinities depend on the extent of occupancy. Related problems appear in the analysis of multiple dissociations of protons from proteins (9, 10). The approach used here for sequential binding of ligands yields some novel and illuminating insights.

$$ K_1K_2 = k_1k_2 + k_1k_3 + \ldots + k_1k_n + k_2k_3 + k_2k_4 + \ldots = \sum_{j_1=1}^{n-1} \sum_{j_2=j_1+1}^{n} k_{j_1}k_{j_2} \qquad (8) $$

$$ K_1K_2\ldots K_i = \sum_{j_1=1}^{n-i+1} \sum_{j_2=j_1+1}^{n-i+2} \cdots \sum_{j_1=j_{i-1}+1}^{n} k_{j_1}k_{j_2}\ldots k_{j_i} $$

$$ = \sum_{j_1<j_2<\ldots<j_i}^{n} k_{j_1}\,k_{j_2}\ldots k_{j_i} \qquad (9) $$

$$ K_1K_2\ldots K_n = k_1k_2\ldots k_n \qquad (10) $$



(11)

As a bridging step toward our goal we shall examine first a three-site system. The specific site equilibria may be represented as in Equation 11. Consider the specific site species $_1$PA. There is only one path for going from P to $_1$PA; hence we can define a k_1 but can place no constraints on its magnitude. The same is true for $_2$PA and $_3$PA. Thus for stoichiometric uptake of one ligand we get three site binding constants but no constraints. Now consider $_{1,2}$PA$_2$. Since either of the two occupied sites could have been filled in the second step, we can define two new site binding constants for the reactions going from a member of the ensemble of the stoichiometric species PA$_1$ to the specific site species $_{1,2}$PA$_2$. Furthermore, we can combine the two successive equilibria in the upper and lower paths leading to $_{1,2}$PA$_2$ to give

$$(_{1,2}\mathrm{PA}_2) = k_1 k_{1,2} (\mathrm{P})(\mathrm{A})^2 = k_2 k_{2,1} (\mathrm{P})(\mathrm{A})^2 \qquad (12)$$

from which it follows that

$$k_1 k_{1,2} = k_2 k_{2,1} \qquad (13)$$

Thus from the formation of the specific site species $_{1,2}$PA$_2$ we obtain two site binding constants and one constraint. Corresponding arguments for $_{2,3}$PA$_2$ and $_{1,3}$PA$_2$ give additionally two new site binding constants and one new constraint for each species:

$$k_1 k_{1,3} = k_3 k_{3,1} \qquad (14)$$

$$k_2 k_{2,3} = k_3 k_{3,2} \qquad (15)$$

Since there are three such species, we generate a total of $2 \cdot 3 = 6$ site binding constants and $(2 - 1)3 = 3$ constraints from the second stoichiometric step, PA$_1$ adding A to produce PA$_2$. Turning now to $_{1,2,3}$PA$_3$, we see that three new site binding constants are needed since any of the three sites could have been filled in the third step. As for constraints the chart shows that there are six different paths for going from P to $_1$, $_2$, $_3$PA$_3$. The triple product of ks for each of those paths must be equal to that for any other. Hence, in principle we can write five relationships between the respective triple products. Three of these relationships, however, are redundant since they have already been found in our analysis of the formation of PA$_2$. For example we can go from P to $_{1,2,3}$PA$_3$ by the two top paths in the chart by filling the sites in the sequence 1, 2, 3 and 2, 1, 3, respectively. The former path is governed by the product $k_1 k_{1,2} k_{1,2,3}$ and the latter by $k_2 k_{2,1} k_{1,2,3}$. Since both paths have the corresponding initial and final states (sites all empty and all filled, respectively), the values of ΔG° for the two paths are identical and hence

$$k_1 k_{1,2} k_{1,2,3} = k_2 k_{2,1} k_{1,2,3} \qquad (16)$$

TABLE I

Numerical values of various binding parameters for a macromolecule with three binding sites

Name of parameter	Numerical value for indicated stoichiometric species			
	P	PA_1	PA_2	PA_3
Number of ligands bound = i	0	1	2	3
Number of different specific site species or configurations with i occupied sites = ν_{PA_i}	1	3	3	1
Number of different paths for going from PA_{i-1} to a specific site species with i occupied sites = i	0	1	2	3
Total number of paths for going from PA_{i-1} to PA_i = $(\nu_{PA_i})i$ = ν_i	0	3	6	3
Total number of site binding constants needed to describe conversions of PA_{i-1} to PA_i = ν_i	0	3	6	3; $\sum$ = 12
Number of new equations relating ks generated by different paths from PA_{i-1} to specific site species with i occupied sites = $(i - 1)$		0	1	2
Total number of new equations relating ks generated from different paths for going from PA_{i-1} to PA_i = $\nu_{PA_i}(i - 1)$		0	3	2; $\sum$ = 5
Number of independent ks generated in going from PA_{i-1} to PA_i = $\nu_{PA_i} i - \nu_{PA_i}(i - 1) = \nu_{PA_i} = F_i$		3	3	1; $\sum$ = 7

But this equation tells us nothing new since (if you cancel $k_{1,2,3}$ from both sides) it is the same as Equation 13. A similar analysis going through each of the intermediates $_{2,3}$PA$_2$ and $_{1,3}$PA$_2$, respectively, generates two additional redundant relationships. Thus it is only the three paths going through the three different specific double-ligand site species that generate the three new product constants and consequently the $(3-1) = 2$ nonredundant relationships between them, *viz.*,

$$k_1 k_{1,2} k_{1,2,3} = k_2 k_{2,3} k_{2,3,1} \qquad (17)$$

$$k_1 k_{1,2} k_{1,2,3} = k_3 k_{3,1} k_{3,1,2} \qquad (18)$$

The characteristics of a three-site system are summarized in Table I.

From the analysis of a three-site system, the general formulation for an n-site multiple complex appears in a straightforward

fashion. Consider one specific site species which is a member of the ensemble constituting the stoichiometric species PA_i. This specific site species has a particular configuration of occupied sites, the total number occupied being i. Since any one of these occupied sites could have been filled in the ith binding step, there are i different paths to reach this specific site species from the set of species with $(i - 1)$ occupied sites. Thus we must designate i site binding constants for each of the specific site species (*i.e.* configurations) with i occupied sites. The total number of different specific site species in the stoichiometric class PA_i is (see Ref. 3)

$$\frac{n(n-1)\cdots(n-i+1)}{i!} = \frac{n!}{(n-i)!\,i!} = \nu_{PA_i} \tag{19}$$

Consequently the total number of site binding constants, ν_i, that are associated with the stoichiometric step from PA_{i-1} to PA_i is

$$\nu_i = (\nu_{PA_i})i = \frac{n!\,i}{(n-i)!\,i!} \tag{20}$$

Furthermore, by analogy with the three-site system, we can also write $(i - 1)$ constraint equations for each specific site species, and hence $\nu_{PA_i}(i - 1)$ such equations for the ensemble PA_i. Consequently the total number of independent site constants, F_i, that are associated with the stoichiometric step from PA_{i-1} to PA_i is

$$F_i = (\nu_{PA_i})i - (\nu_{PA_i})(i-1) = \nu_{PA_i} = \frac{n!}{(n-i)!\,i!} \tag{21}$$

For a complete description of a multisite system we must evaluate summations of Equations 20 and 21. These can be obtained from the following algebraic analysis.

From the binomial theorem we know that

$$P(1+A)^n = \sum_{i=0}^{n} \frac{n!}{(n-i)!\,i!}(P)(A)^i \tag{22}$$

$$= P + \sum_{i=1}^{n} \frac{n!}{(n-i)!\,i!}(P)(A)^i \tag{23}$$

If we set $A = P = 1$ and subtract unity from both sides of Equation 23, we obtain

$$2^n - 1 = \sum_{i=1}^{n} \frac{n!}{(n-i)!\,i!} \tag{24}$$

By straightforward procedures we can also show that

$$A\frac{\partial}{\partial A}[P(1+A)^n] = A\sum_{i=1}^{n} \frac{n!}{(n-i)!\,i!}\,i\,(P)(A)^{i-1} \tag{25}$$

from which it follows (on carrying through the differentiation of the left side) that

$$n(P)(A)(1+A)^{n-1} = \sum_{i=1}^{n} \frac{n!}{(n-i)!\,i!}\,i\,(P)(A)^i \tag{26}$$

Again setting $P = A = 1$, we find that

$$n2^{n-1} = \sum_{i=1}^{n} \frac{n!}{(n-i)!\,i!}\,i \tag{27}$$

The ith term in the summation on the right side of Equation 27 is the number of site binding constants describing the ith stoichiometric steps. The complete summation then is the total number of site binding constants, ν_k, associated with all the site equilibria in the multisite binding system; that is

$$\nu_k = \sum_{i=1}^{n} \nu_i = n2^{n-1} = \frac{n}{2}2^n \tag{28}$$

Furthermore the total number of independent site binding constants, F_k, for a multisite system with n sites, *i.e.* the number of degrees of freedom, can be evaluated using Equations 21 and 24:

$$F_k = \sum_{i=1}^{n} F_i = \sum_{i=1}^{n} \nu_{PA_i} = 2^n - 1 \tag{29}$$

TABLE II

Comparison of numbers of different kinds of binding constants for a macromolecule with n binding sites

Number of binding sites, n	Total number of site binding constants	Number of independent site binding constants	Number of stoichiometric binding constants
2	4	3	2
3	12	7	3
4	32	15	4
6	192	63	6
8	1024	255	8
12	24576	4095	12

RELATIONS OF STOICHIOMETRIC BINDING CONSTANTS WITH OCCUPANCY-DEPENDENT SITE BINDING CONSTANTS

At this stage we might compare first the total number of each type of constant for a multisite system. For a macromolecule with n sites, the stepwise uptake of ligand can be correlated by n stoichiometric binding constants. On the other hand a very much larger number of site binding constants are needed to define the equilibria between all configurations of occupied sites. A comparison between the number of stoichiometric and independent site binding constants for a few simple cases, typical of those frequently encountered in proteins constituted of subunits, is illustrated in Table II.

It becomes immediately obvious that an experimental binding study that leads to a full determination of the stoichiometric binding constants K is always inadequate for a complete specification of all of the site binding constants k.

The complementary problem, however, is one that can be completely resolved, at least in principle. That is, if all the site constants are known, the stoichiometric binding constants can be specified. The necessary relationships can be derived as follows.

Consider a protein with individual sites numbered. In the ensemble of specific site species constituting the stoichiometric class PA_i, let us focus on one of the site species with a specified configuration of occupied sites, which we will designate as $_{j_1,j_2,\cdots j_i}PA_i$, where the indices j_1, j_2, etc. are listed in the order starting with the lowest-numbered site and increasing in ordinal fashion. One reaction pathway for generating this species by successive addition of ligands is shown in Equation 30. That is the lowest numbered site is filled first, the next higher numbered site second, etc. The concentration of the site species formed can be expressed by Equation 31. The concentration of the stoichio-

$$P \overset{k_{j_1}}{\rightleftarrows} {}_{j_1}PA_1 \overset{k_{j_1,j_2}}{\rightleftarrows} {}_{j_1,j_2}PA_2 \rightleftarrows \cdots \overset{k_{j_1,j_2\cdots j_i}}{\rightleftarrows} {}_{j_1,j_2,\cdots j_i}PA_i \tag{30}$$

$$(_{j_1,j_2,\ldots j_i}PA_i) = k_{j_1} k_{j_1,j_2} \cdots k_{j_1,j_2,\ldots j_i} (P)(A)^i \tag{31}$$

$$(PA_i) = \sum_{j_1<j_2<\ldots<j_i}^{n} k_{j_1} k_{j_1,j_2} \cdots k_{j_1,j_2,\ldots j_i} (P)(A)^i \tag{32}$$

metric species, (PA_i) is then given by the sum of concentrations of each of the specific site species that are within that stoichiometric ensemble, Equation 32.

Now let us turn our attention to the ith stoichiometric step:

$$PA_{i-1} + A \overset{K_i}{\rightleftarrows} PA_i \tag{33}$$

for which

$$K_i = \frac{(PA_i)}{(PA_{i-1})(A)} \tag{34}$$

Taking account of Equation 32, with suitable indexing to distinguish the stoichiometric species, we obtain

$$K_i = \frac{\sum_{j_1<j_2<\ldots<j_i}^{n} (k_{j_1} k_{j_1,j_2} \cdots k_{j_1,j_2\ldots j_i})}{\sum_{j_1'<j_2'<\ldots<j_{i-1}'}^{n} (k_{j_1'} k_{j_1',j_2'} \cdots k_{j_1',j_2',\ldots j_{i-1}'})} \tag{35}$$

Through Equation 35 the stoichiometric binding constants can be linked to a set of $(2^n - 1)$ independent site binding constants.

Equation 35 is necessary for the most general situation, that in which each site changes its affinity as ligand is bound at any other site. When the interactions between sites are not so all-pervasive, Equation 35 can be reduced to simpler forms.

If the sites are independent, unchanging in affinity as other sites are occupied, then the site equilibrium constants fit the following constraints;

$$\begin{aligned} k_{j_1} &= k_{j_1} \\ k_{j_1,j_2} &= k_{j_2} \\ &\cdots \\ k_{j_1,j_2,\ldots j_i} &= k_{j_i} \end{aligned} \tag{36}$$

Under these circumstances Equation 35 can be reduced to

$$K_i \text{ (independent sites)} = \frac{\sum_{j_1<j_2<\ldots j_i}^{n} (k_{j_1} k_{j_2} \cdots k_{j_i})}{\sum_{j_1'<j_2'<\ldots j_{i-1}'}^{n} (k_{j_1'} k_{j_2'} \cdots k_{j_{i-1}'})} \tag{37}$$

Equation 37 is equivalent to Equations 7 to 10.

Alternatively, the interactions between each pair of sites may be expressed in terms of an interaction parameter $v_{j,j'}$. Thus if only interactions between pairs of sites are present (13), we may write[1]

$$\begin{aligned} k_{j_1} &= k_{j_1} \\ k_{j_1,j_2} &= k_{j_2} v_{j_1,j_2} \\ k_{j_1,j_2,j_3} &= k_{j_3} v_{j_1,j_3} v_{j_2,j_3} \\ &\cdots \\ k_{j_1,j_2,\ldots j_i} &= k_{j_i} v_{j_1,j_i} v_{j_2,j_i} \cdots v_{j_{i-1},j_i} \end{aligned} \tag{38}$$

If all of the interaction parameters are equal then

$$k_{j_1,j_2,\ldots j_i} = k_{j_i} v^{i-1} \tag{39}$$

and Equation 35 can be reduced to

$$K_i(\text{equal interaction}) = v^{i-1} K_i(\text{independent site}) \tag{40}$$

Finally we might return to the very simple situation, all sites independent, unchanging, and equivalent in affinity for ligand. Under these circumstances all of the site binding constants are equal.

$$\begin{aligned} k_{j_1} &= k \\ k_{j_1,j_2} &= k_{j_2} = k \\ &\cdots \\ k_{j_1,j_2,\ldots j_i} &= k_{j_i} = k \end{aligned} \tag{41}$$

Consequently each term in the summation in the numerator of Equation 35 becomes k^i. The number of terms in the numerator is $n!/[(n-i)!i!]$. The denominator can be reduced in a corresponding manner. Consequently Equation 35 becomes

$$K_i(\text{independent, equivalent sites}) = \frac{\frac{n!}{(n-i)!\,i!} k^i}{\frac{n!}{[n-(i-1)]!\,(i-1)!} k^{i-1}} = \frac{n-i+1}{i} k \tag{42}$$

a result well known from earlier considerations (2, 3).

RELATIONSHIPS BETWEEN BINDING CONSTANTS AND EMPIRICAL PARAMETERS WHEN AFFINITIES ARE OCCUPANCY-DEPENDENT

We begin by writing the complete equation for r, Equation 8, in the alternative form (2, 3, 13),

$$r = \frac{(A)}{Z} \frac{dZ}{d(A)} \tag{43}$$

where

$$Z = 1 + \sum_{i=1}^{n} \left(\prod_{\ell=1}^{i} K_\ell \right) (A)^i \tag{44}$$

that is, Z is the denominator of Equation 3. If we use Equation 35 to replace the K_i factors in Equation 44 we obtain Equation

[1] It might be of interest to note that the total number of constraints in Equation 38 is n for the k values and $n(n-1)/2$ for the v values, or a total of $n(n+1)/2$. This number may be compared with the third column of Table II:

n	$n(n+1)/2$	2^n-1
2	3	3
3	6	7
4	10	15

$$Z = 1 + \sum_{j_1}^{n} k_{j_1}(A) + \ldots + \sum_{j_1<j_2<\ldots<j_i}^{n} k_{j_1} k_{j_1,j_2} \ldots k_{j_1,j_2,\ldots j_i}(A)^i + \ldots + k_1 k_{1,2} \ldots k_{1,2\ldots n}(A)^n \quad (45)$$

45. Clearly in the general case this yields a very unwieldy result if Equation 45 is inserted into Equation 43 to give a detailed explicit expression for r. Nevertheless, some useful insights are generated from this approach when it is applied to special cases of common interest.

Consider a situation in which a three-site macromolecule has two classes of independent sites of unchanging affinities. These might be designated as follows:

$$k_1 = k_\alpha \qquad k_2 = k_\alpha \qquad k_3 = k_\beta \quad (46)$$

Under the constraints specified we can also write

$$k_{1,2} = k_\alpha \qquad k_{1,2,3} = k_\beta \qquad k_{1,3} = k_\beta \qquad k_{2,3} = k_\beta \quad (47)$$

With these specifications Equation 45 becomes

$$Z = 1 + (2k_\alpha + k_\beta)(A) + (k_\alpha^2 + 2k_\alpha k_\beta)(A)^2 + k_\alpha^2 k_\beta (A)^3 \quad (48)$$

$$= [1 + k_\beta(A)]\,[1 + k_\alpha(A)]^2$$

From this it follows that

$$\frac{dZ}{d(A)} = k_\beta[1 + k_\alpha(A)]^2 + [1 + k_\beta(A)]2[1 + k_\alpha(A)]k_\alpha \quad (49)$$

Consequently a simple explicit equation for r can be obtained by insertion of Equations 48 and 49 into Equation 43, *viz.*

$$r = \frac{2k_\alpha(A)}{1 + k_\alpha(A)} + \frac{k_\beta(A)}{1 + k_\beta(A)} \quad (50)$$

This result is formally the same as would be obtained from Equation 6, as indeed it must be, since $k_1 = k_2 = k_\alpha$ and $k_3 = k_\beta$. Consequently curve fitting with Equation 6 will yield the site-binding parameters. In fact, as one might expect, curve fitting with Equation 6 is always proper when the sites are independent.

Now let us turn to a different set of constraints on a three-site system. Suppose that at the outset we have three identical sites (*e.g.* three identical protomers in a quaternary ensemble). Under these circumstances we may write

$$k_1 = k_2 = k_3 = k_I \quad (51)$$

Let us assume, furthermore, that the uptake of a second ligand at any one of the open sites occurs with the same affinity as would the uptake of the first, but that after that the affinity for the third ligand of any site species is altered (accentuated or attenuated). Under these conditions, which would correspond to an interacting system, we may state that

$$k_{1,2} = k_{1,3} = k_{2,3} = k_I \quad (52)$$

$$k_{1,2,3} = k_{II} \quad (53)$$

The constraints of Equations 51 to 53 lead to the following relations for Z and r, respectively:

$$Z = 1 + 3k_I(A) + 3k_I^2(A)^2 + k_I^2 k_{II}(A)^3 \quad (54)$$

$$r = \frac{3k_I(A) + 6k_I^2(A)^2 + 3k_I^2 k_{II}(A)^3}{1 + 3k_I(A) + 3k_I^2(A)^2 + k_I^2 k_{II}(A)^3} \quad (55)$$

If we were to describe this interacting 3-site system as consisting of one class of two sites of affinity k_α and of one class with one site of affinity k_β and then apply the algebraic format of Equation 6, we would obtain

$$r = \frac{2k_\alpha(A)}{1 + k_\alpha(A)} + \frac{k_\beta(A)}{1 + k_\beta(A)} \quad (50)$$

For an interacting system, however, there is no unique relationship between the algebraic parameters k_α and k_β and the site binding constants k_I and k_{II}. For example, if at the outset we have three identical sites so that

$$k_1 = k_2 = k_3 = k_I \quad (51)$$

then it is not possible to have simultaneously

$$k_1 = k_2 = k_\alpha \text{ and } k_3 = k_\beta \quad (56)$$

This impossibility may also be stated in more general terms. Equations 50 and 55 are two different functions. If Equation 55 fits a given set of data exactly, Equation 50 could not do so. Equation 55 arises from a general stoichiometric treatment valid for interacting, as well as noninteracting, systems.

A vast number of proteins are constituted of 4 to 12 protomeric subunits (14, 15). Conformational adaptations frequently are linked with the binding of one or more ligands, and these structural accommodations may accentuate or attenuate binding affinities at individual sites. Furthermore, as soon as even the first site is occupied, the others may no longer be in strictly symmetrical environments. Unless one has additional probes, such as spectroscopic or magnetic molecular sensors, it is not feasible to specify the behavioral features of individual sites. Nevertheless, it has often been the custom to distinguish between sites of an ensemble of n identical subunits and to classify a certain number, x in one group (*e.g.* strong binders) and $n - x$ in another category (*e.g.* weak binders). For example, in aspartate transcarbamoylase, with six originally identical sites, the observed binding of nucleotides has been rationalized (16–18) in terms of one group of three sites with a strong binding affinity and a second group of three sites with a weak binding affinity. In actual calculational practice this means that the binding data are fitted to an algebraic format that is a special case of Equation 6, that is,

$$r = \frac{3k_\alpha(A)}{1 + k_\alpha(A)} + \frac{3k_\beta(A)}{1 + k_\beta(A)} \quad (57)$$

in which k_α and k_β are empirical parameters. There is no doubt that the observations can be fitted to an equation with two adjustable parameters k_α and k_β (in addition to the choice of 3 and 3 for the coefficients in the two terms of Equation 57). However,

k_α is not the site binding constant, k_1, for uptake of the first ligand at any one of the six sites. In fact, in an interacting system there is no unique relationship between k_α and the site binding constants. *The parameters obtained by a statistical best fitting of binding data to an equation of the algebraic form of Equation 6 are not site binding constants if there are conformational or other accommodations that change the binding affinity of sites with progressive occupancy of ligands.* Consequently it is necessary to develop alternative methods for assessing the contributions of site-site interactions and conformational changes in multiple ligand binding.

TRENDS IN STOICHIOMETRIC BINDING CONSTANTS WHEN THERE ARE INTERACTIONS BETWEEN SITES

A correlation of equilibrium binding data in terms of stoichiometric binding constants provides the most economical algebraic description of experimental results. For a macromolecule with n binding sites the number of stoichiometric binding constants is simply n. Of course from K_is alone one obtains no direct insight into the behavior of specific individual sites. A thermodynamic analysis in itself cannot reveal a molecular perception. On the other hand, interactions between sites due to conformational accommodations or other causes, do manifest themselves in K_i since the stoichiometric equilibrium constant implicitly reflects the properties of its constituent site equilibrium constants (see Equation 35) once we introduce and define them.

Thus in the limiting situation of identical independent binding sites, K_i obeys Equation 42a

$$K_i = \frac{n - i + 1}{i} k \qquad (42a)$$

From this it follows that

$$iK_i = k(n+1) - ki \qquad (58)$$

In other words, iK_i should be a linear function of i. Thus, if the values of K_i that fit the experimental observations have been established, iK_i should be linear in a graph *versus* i (Fig. 1). Such a graph can be made without any knowledge of n, the total number of binding sites, which is usually very difficult to determine with confidence from binding observations alone (1). If the binding data very clearly fit a linear graph of iK_i *versus* i, then, as Fig. 1 shows, n can be established by a straightforward linear extrapolation to the intercept on the i axis.

When there are interactions between sites that accentuate or attenuate binding affinity, the data for iK_i, the affinity profile, will depart from the linear ideal relation shown in Fig. 1. At first glance one would expect accentuating interactions ("positive cooperativity") to lead to points above the line, attenuating interactions ("negative cooperativity") to place them below the line. A more careful examination of what we mean by such interactions shows, however, that the experimental trends are manifestations of more complicated behavior.

To define an interaction precisely and quantitatively we must specify two things: (*a*) the specific reaction that is being considered, and (*b*) the reference or noninteracting reaction that is the basis of comparison. The reaction to be studied can be either a stoichiometric or site binding step and can involve the addition of one or more ligands. As the reference base, we will use the constant (or constants) for binding of the first ligand on the grounds that there will be no interactions when the other sites are not occupied (a second possible choice will be discussed later). For site binding reactions the definition of an interaction con-

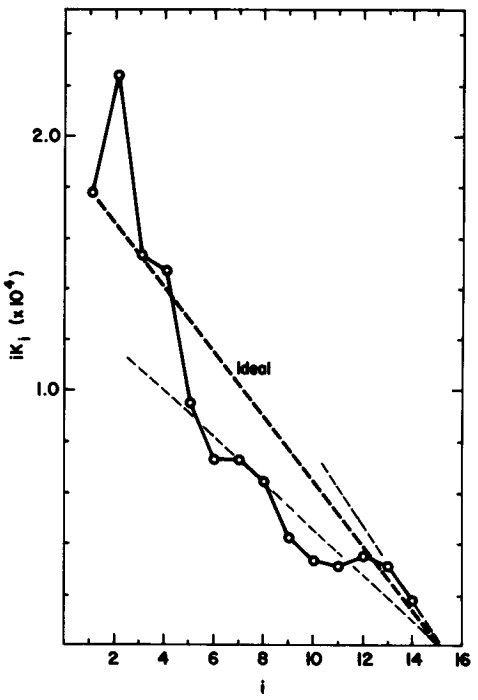


FIG. 1. Affinity profile showing variation of the weighted stoichiometric binding constant, iK_i, with stoichiometric binding step, i. *Dashed line* shows linear dependence when binding sites are identical and unchanging in affinity; the intercept on the x-axis is $(n + 1)$, as required by Equation 58. The *circles* represent stoichiometric binding constants for binding of cupric ion by bovine serum albumin (19). The values of K_i illustrated were computer best fits determined by Dr. John E. Fletcher, Division of Computer Research and Technology, National Institutes of Health.

$$j_1,j_2,j_3 P + 3A \rightleftarrows j_1,j_2,j_3 PA_3 \qquad (59)$$

stant, I, might be given as

$$I = k_{j_1} k_{j_1,j_2} k_{j_1,j_2,j_3} / k_{j_1} k_{j_2} k_{j_3} \qquad (60)$$

For a stoichiometric reaction an appropriate definition is

$$PA + 2A \rightleftarrows PA_3 \qquad (61)$$

$$I = K_2 K_3 / K_{2(\mathrm{Ind})} K_{3(\mathrm{Ind})} \qquad (62)$$

where (Ind) indicates "independent sites" and $K_{i(\mathrm{Ind})}$ is given by Equation 37.

Now consider how these definitions can be simplified for the special case where all of the sites are equivalent but not independent. Let κ_i be the site binding constant for the addition of a ligand to any open site on a molecule with $(i - 1)$ bound ligands. Thus

$$\kappa_i = k_{j_1,j_2,\ldots j_i} \text{ for any set of i different j's} \qquad (63)$$

For reaction (Equation 59), we can now describe I as

$$I = \kappa_1 \kappa_2 \kappa_3 / \kappa_1^3 = I_1 I_2 I_3 \qquad (64)$$

where I_i is the contribution of the ith single-ligand addition step:

$$I_i = \kappa_i / \kappa_1 \qquad (65)$$

Turning to stoichiometric reaction (Equation 61), we can obtain an expression for K_i by substituting Equation 63 into

Equation 35:

$$K_i = \frac{\sum_{j_1<\ldots<j_i} \kappa_1\kappa_2\ldots\kappa_i}{\sum_{j_1'<\ldots<j_{i-1}'} \kappa_1\kappa_2\ldots\kappa_{i-1}} \quad (66)$$

Each term in the numerator is the same and there are $(n - i + 1)!/i!$ such terms. Similar statements can be made for the denominator and thus

$$K_i = \frac{n - i + 1}{i} \kappa_i \quad (67)$$

Clearly the stoichiometric constant is made up of two parts: (*a*) a statistical factor indicating the number of possible site reactions in the ith stoichiometric step, and (*b*) an affinity factor indicating the site binding affinity of the unoccupied sites when $(i - 1)$ are occupied.

We can now define a reference constant as

$$K_{i(\mathrm{Ind})} = \frac{n - i + 1}{i} \kappa_1 \quad (68)$$

The interaction constant for reaction (Equation 61) becomes, therefore,

$$I = \kappa_2\kappa_3/\kappa_1^2 \quad (69)$$

It is interesting to note that the interaction parameter for the ith stoichiometric step is also the interaction parameter for the site binding reaction in which the ith ligand is added:

$$I_i = K_i/K_{i(\mathrm{Ind})} = \kappa_i/\kappa_1 \quad (70)$$

Now consider a graph of iK_i *versus* i for a system with equivalent sites. The ideal line for independent sites is, from Equation 68,

$$iK_{i(\mathrm{Ind})} = (n + 1)\kappa_1 - i\kappa_1 \quad (71)$$

which is a straight line with slope κ_1 and x and y intercepts of $(n + 1)$ and $(n + 1)\kappa_1$. The actual relationship, Equation 67, is

$$iK_i = (n + 1)\kappa_i - i\kappa_i \quad (72)$$

This is not a straight line since κ_i is not constant with varying i; nevertheless, the x intercept is still $(n + 1)$. The difference between an actual point and the ideal line is

$$iK_{i(\mathrm{Ind})} - iK_i = (n - i + 1)(\kappa_1 - \kappa_i) \quad (73)$$

Thus if the ith point is below the ideal line shown in Fig. 1, $\kappa_1 > \kappa_i$ and the interaction for the ith step is attenuating. Conversely if the ith point is above the ideal line, $\kappa_1 < \kappa_i$ and the interaction is accentuating. This is true for both the stoichiometric and site reactions. Thus the iK_i *versus* i graph is an ideal means to evaluate such interactions when all of the sites are equivalent.

We can also define the free energy of interaction as

$$\Delta G_i^\circ = RT \ln[K_{i(\mathrm{Ind})}/K_i] \quad (74)$$

Using equation 70 we find

$$\Delta G_i^\circ = RT \ln[\kappa_1/\kappa_i] \quad (75)$$

To isolate the interactions that are introduced in the ith step we simply write

$$\Delta G_i^\circ - \Delta G_{i-1}^\circ = RT \ln[\kappa_{i-1}/\kappa_i] \quad (76)$$

It is pertinent to note that this relationship is valid no matter what is taken as the reference reaction so long as it is used as reference for calculating both ΔG_i° and ΔG_{i-1}°.

With the foregoing interaction analysis as a foundation, we can examine alternative choices of the basic reference reaction that may be advantageous for revealing the type of interaction occurring in individual binding steps. For example, the binding of the first ligand may be accompanied by a conformational accommodation of the macromolecule that alters the site binding affinity of the residual open sites. After the addition of this first ligand, successive uptake of all additional ligands could occur with affinities following a simple statistical pattern. For such a system the affinity profile would follow a dashed *line* from the point at $i = 2$ to that at $i = 15$ in Fig. 1. The weighted stoichiometric constants, iK_i, for $2 \leq i \leq n$ would fall on a straight line that follows the algebraic equation

$$iK_i = (n+1)\kappa_2 - i\kappa_2 \quad (77)$$

Clearly the slope of this line is not the same as that of the "ideal," but the intercept on the abscissa is the same $(n + 1)$.

This analysis can be generalized to any consecutive experimental points in an affinity profile. Whenever two or more consecutive points fall on a straight line with an intercept at $(n + 1)$ on the x-axis, then the corresponding ligand binding steps have binding constants that are related purely statistically; the slope of this line is $-\kappa_i$, the negative of the site binding constant. Thus for any point i on the affinity profile, ideal behavior is described by a line connecting the points at $(i - 1)$, i, and $(n + 1)$ on the abscissa. If interactions do appear in the ith binding step, then the observed value of iK_i will fall above or below the line connecting the $(i - 1)$th point with $(n + 1)$ on the abscissa. Furthermore, if we use the $(i - 1)$th point to define $K_{i(\mathrm{Ind})}$,

$$K_{i(\mathrm{Ind})} = \frac{n-i+1}{i} \kappa_{i-1} \quad (78)$$

and insert this into Equation 74, the free energy of interaction becomes

$$\Delta G_i^\circ = RT \ln(\kappa_{i-1}/\kappa_i) \quad (79)$$

In essence then, if the $(i - 1)$ step is the reference base, Equation 76 is replaced by Equation 79.

CONCLUSION

It has long been recognized (8, 9) from an analysis of the interactions of polyvalent acids and bases that the number of ("microscopic") site binding constants that must be defined, for an interacting system, will exceed the total number of binding sites, n (Table II). Thus a full description of individual sites at each stage of occupancy requires additional sources of experimental input (or *ad hoc* assumptions) besides stoichiometric binding data. On the other hand, it has not been realized that a consequence of a detailed analysis of the relationships of stoichiometric ("macroscopic") and site ("microscopic") binding constants is that it is not feasible to use an expression of the form of Equation 6 with n terms in it to obtain site binding constants. The empirical parameters, k_α, $k_\beta \cdots k_\lambda$, so obtained are not uniquely related to the site binding constants. It is important to recognize that this principle applies to the binding of substrates and of effectors by multimeric proteins constituted of identical protomeric subunits. Such binding has often been analyzed by algebraic relations with the format of Equation 6 and the k_λ parameters so obtained have been mistakenly assigned to particular sites of the quaternary ensemble.

On the other hand, the distribution of a ligand between bulk

solvent and a binding macromolecule can be described economically and definitively in terms of stepwise stoichiometric equilibria. For a molecule with n binding sites, the number of stoichiometric binding constants, K_i, required is n. Furthermore, interactions between sites, whether they accentuate or attenuate binding affinities, manifest themselves in K_i. With careful definitions of interaction energies one can even isolate and characterize the type of interaction associated with individual stoichiometric binding steps. It seems clear, therefore, that a stoichiometric formulation provides the most versatile format for a thermodynamic analysis of the interactions of small molecules with a biological macromolecule.

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REFERENCES

1. Klotz, I. M. (1974) *Accts. Chem. Res.* **7**, 162–168
2. Klotz, I. M. (1946) *Arch. Biochem.* **9**, 109–117
3. Klotz, I. M. (1953) in *The Proteins* (Neurath, H. ed) 1 B, pp. 727–806, Academic Press, New York
4. Klotz, I. M., Walker, F. M., and Pivan, R. B. (1946) *J. Am. Chem. Soc.* **68**, 1486–1490
5. Scatchard, G. (1949) *Ann. N. Y. Acad. Sci.* **51**, 660–672
6. Scatchard, G., Scheinberg, I. H., and Armstrong, S. H., Jr. (1950) *J. Am. Chem. Soc.* **72**, 535–540
7. Karush, F. (1950) *J. Am. Chem. Soc.* **72**, 2705–2713
8. Adams, E. Q. (1916) *J. Am. Chem. Soc.* **38**, 1503–1510
9. Simms, H. S. (1926) *J. Am. Chem. Soc.* **48**, 1239–1261
10. Wyman, J. (1948) *Adv. Protein Chem.* **4**, 407–531
11. Fletcher, J. E., Spector, A. A., and Ashbrook, J. D. (1970) *Biochemistry* **9**, 4580–4587
12. Klotz, I. M., and Hunston, D. L. (1971) *Biochemistry* **10**, 3065–3069
13. Thompson, C. J., and Klotz, I. M. (1971) *Arch. Biochem. Biophys.* **147**, 178–185
14. Klotz, I. M., Langerman, N. R., and Darnall, D. W. (1970) *Annu. Rev. Biochem.* **39**, 25–62
15. Klotz, I. M., Darnall, D. W., and Langerman, N. R. (1975) in *The Proteins* (Neurath, H., and Hill, R. eds) 3rd Ed, Academic Press, New York, in press
16. Winlund, C. C., and Chamberlin, M. J. (1970) *Biochem. Biophys. Res. Commun.* **40**, 43–49
17. Cook, R. A. (1972) *Biochemistry* **11**, 3792–3797
18. Matsumoto, S., and Hammes, G. G. (1973) *Biochemistry* **12**, 1388–1394
19. Klotz, I. M., and Curme, H. G. (1948) *J. Am. Chem. Soc.* **70**, 939–943