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Thermodynamics of local linkage effects

Contracted partition functions and the analysis of site-specific energetics

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A thermodynamic theory is presented for the description of local, site-specific linkage effects in biological macromolecules. The theory is developed from a basic isomorphism involving the intensive quantities of a thermodynamic system at equilibrium. Local linkage effects can be cast within the same mathematical framework as the one used in the statistical thermodynamic theory of global linkage effects involving different ligands. In addition to this parallel, local linkage effects give rise to apparent violations of thermodynamic stability that can be of relevance in energy transduction phenomena. It is also shown that the canonical partition function for the macromolecule as a whole can be expressed in terms of contracted partition functions that greatly simplify calculations of the relevant thermodynamic properties of individual sites. Site-specific Hill plots, partition coefficients and free energies of linkage are introduced and their properties discussed in connection with those of analogous global quantities. Calculation of the free energies of linkage for human hemoglobin yields a minimal phenomenological scheme for the coupling among subunits.

1. Introduction

Linkage expresses reciprocity or interdependence of effects [1]. By its very nature this concept finds an appropriate application to the description of the properties of biological systems at the microscopic level, where coupling and control arise as a consequence of the transducing properties of polyfunctional macromolecules. According to the events being transduced, linkage effects may involve different ligands binding to the macromolecule, with the accompanying volume changes and heat exchange with its environment, or else different portions of the macromolecule within which energy transfer takes place through specific pathways. In the former case, we deal with the properties of the macromolecule as a whole, or *global* linkage effects. In the latter, we are concerned with the transducing properties of individual sites of the macromolecule, or *local* linkage effects.

The description of global linkage effects is based on the fundamental properties of the energy function for a thermodynamic system at equilibrium, whereby formulation of a canonical partition function is straightforward. The salient thermodynamic features arising in this connection have been detailed in outstanding treatments of ligand binding and linkage in biological macromolecules [1–10]. These basic ideas have found application in a wide variety of macromolecular systems and provide most of the theoretical bases of the entire field of biophysical chemistry. However, with the increasing availability of experimental methods and techniques which are capable of measuring

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binding effects at the level of individual sites of a macromolecule, the need for a local thermodynamic description of the facts has become evident [11]. A number of biochemical systems can now be explored by dissecting the underlying energetics at the level of individual sites due to the improved resolution of experimental strategies. As recently shown in the case of human hemoglobin, ligand binding to individual sites of the macromolecule can experimentally be approached with high precision by NMR [12–15], cryogenic quenching of the intermediates [16,17], metal-substituted hemoglobins [18,19], and subunit-assembly techniques [20–22]. Similar techniques have successfully been employed to resolve site-specific redox equilibria in cytochromes [23,24]. In the case of protein-DNA interactions, footprint titration now yields accurate site-specific isotherms [25,26]. All these findings provide a substantial body of information that can be encapsulated within the framework of a phenomenological thermodynamic theory, which is the purpose of this paper to discuss.

2. Thermodynamics of global linkage effects

For a system at equilibrium there are $c+2$ linearly dependent differentials of intensive quantities, or fields [27], entering the definition of the Gibbs-Duhem equation [28] that can be written as

$$S\,\mathrm{d}T - V\,\mathrm{d}P + \sum_{i=1}^{c} n_j\,\mathrm{d}\mu_j = 0 \qquad (1)$$

Two of them are physical fields, corresponding to temperature and pressure, and the remaining c are chemical fields corresponding to chemical potentials. If a particular chemical field, say μ_c, is selected out and its conjugate intensity, n_c, is used as a scaling factor of the remaining ones [29], then eq. 1 becomes

$$(S/n_c)\,\mathrm{d}T - (V/n_c)\,\mathrm{d}P + \sum_{i=1}^{c-1} (n_j/n_c)\,\mathrm{d}\mu_j = -\mathrm{d}\mu_c \qquad (2)$$

and all extensities are expressed per mol of component c. The field μ_c is an integral of the Gibbs-Duhem equation and is related to the canonical partition function, Z, through [3,28,30]

$$\mu_c = -RT \ln Z(T,P,\mu_1,\ldots\mu_{c-1}) \qquad (3)$$

In the case of a biochemical system composed by a macromolecule and its possible ligands, the field μ_c can conveniently be taken as the chemical potential of the macromolecule [3,31]. The partition function given in eq. 3 then encapsulates all possible ligation intermediates arising from application of mass law. Under these assumptions the ratio $n_j/n_c = X_j$ specifically refers to the number of mols of ligand j *bound* per mol of macromolecule.

Linkage among the various ligands is an expression of the mathematical symmetry of the positive definite matrix, $\boldsymbol{G}$, formed by all second-order partial derivatives of the field μ_c with respect to the independent variables T, P, $\mu_1,\ldots\mu_{c-1}$ [28,32]. At constant T and P each element of the matrix $\boldsymbol{G}$ can be written as

$$g_{ij} = -\left(\partial^2\mu_c/\partial\mu_i\,\partial\mu_j\right) = \partial X_i/\partial\mu_j \qquad (4)$$

from which it results for any i and j that [28,33]

$$g_{ii} > 0 \qquad (5a)$$

$$g_{ij} = g_{ji} \qquad (5b)$$

$$\det \boldsymbol{G} > 0 \qquad (5c)$$

$$\det \boldsymbol{G}' > 0 \qquad (5d)$$

where $\boldsymbol{G}'$ is an arbitrary principal minor of $\boldsymbol{G}$. The above relations provide the thermodynamic background for the description of linkage effects involving different ligands.

The properties of the macromolecule are fully characterized in thermodynamic terms by the $c(c-1)/2$ independent matrix elements g_{ij}. Denoting by $n = c-1$ the number of ligands, at constant T and P one has n possible Legendre transformations of the potential μ_c [34,35], each one mapping into a transformed partition function [36]. From a mathematical point of view, the Legendre transformation simply operates a substitution *field* → *conjugate extensity* as independent variable in the partition function. From a physical point of view, the transformation closes the system to a particular ligand, which is kept at

constant X_j rather than at constant μ_j. In this respect, one should note that the gs reported in eq. 4 refer to the fully open system, and therefore one naturally looks for a method by which the elements gs characterizing the properties of the system after closure to one or more ligands can be arrived at from the gs relative to the fully open system. This method has a deep physical significance, as it will be evident when dealing with local linkage effects, and stems from a simple algorithm.

3. Contractions in the field manifold F^n

Consider the following system of equations

$$dX_1 = g_{11}\, d\mu_1 + g_{12}\, d\mu_2 + g_{13}\, d\mu_3 \quad (6a)$$

$$dX_2 = g_{21}\, d\mu_1 + g_{22}\, d\mu_2 + g_{23}\, d\mu_3 \quad (6b)$$

$$dX_3 = g_{31}\, d\mu_1 + g_{32}\, d\mu_2 + g_{33}\, d\mu_3 \quad (6c)$$

by which each dX can be expressed as a linear function of the $d\mu$s through the matrix $\boldsymbol{G}$. The above transformation and its inverse, which yields each $d\mu$ as a function of the dXs, are made possible by the positive definite nature of $\boldsymbol{G}$ [33]. The rank of $\boldsymbol{G}$ gives the number of independent fields in the system [33] or else, in abstract metric terms [37], the dimension of the manifold F^n spanned by the fields $\mu s \in F^n$. Assume now that the system becomes closed to ligand 1. We wonder how this affects the elements of $\boldsymbol{G}$ in F^{n-1}. Letting $dX_1 = 0$ in eq. 6a one has

$$d\mu_1 = -[g_{12}\, d\mu_2 + g_{13}\, d\mu_3]/g_{11} \quad (7)$$

and defining

$${}^1g_{ij} = [g_{ij}g_{11} - g_{i1}g_{1j}]/g_{11} \quad (i, j = 2, 3) \quad (8)$$

one has

$$dX_2 = {}^1g_{22}\, d\mu_2 + {}^1g_{23}\, d\mu_3 \quad (9a)$$

$$dX_3 = {}^1g_{32}\, d\mu_2 + {}^1g_{33}\, d\mu_3 \quad (9b)$$

The algorithm whereby the system of eq. 6 is reduced to the system of eq. 9 is the familiar elimination algorithm introduced by Gauss [33]. The ${}^1g$s in eq. 9 can be derived from the gs of the fully open system according to eq. 8. One also sees from eqs 6 and 9 that

$$\mathrm{rank}({}^1\boldsymbol{G}) = \mathrm{rank}(\boldsymbol{G}) - 1 \quad (10)$$

which of course holds for an arbitrary number of ligands n. Therefore, in a system of n linearly dependent fields only $n-1$ of them can still be linearly independent upon closure of the system with respect to a given ligand. This can readily be verified from the example given in eqs 6–9. Closure of the system to a particular ligand thus corresponds exactly to a 'contraction' of the field manifold F^n as implied by eq. 10.

Further contraction of eqs 9 is obtained by closing the system to ligand 2, so that

$$d\mu_2 = -{}^1g_{33}\, d\mu_2/{}^1g_{22} \quad (11)$$

Hence,

$$dX_3 = {}^{12}g_{33}\, d\mu_3 \quad (12)$$

with

$${}^{12}g_{33} = \left[{}^1g_{33}{}^1g_{22} - {}^1g_{32}{}^1g_{23}\right]/{}^1g_{22} \quad (13)$$

and after substituting eq. 8 one obtains ${}^{12}g_{33}$ as a function of the gs. The new matrix ${}^{12}\boldsymbol{G}$ associated with eq. 12 is composed by a single element, ${}^{12}g_{33}$, and its rank is such that

$$\mathrm{rank}({}^{12}\boldsymbol{G}) = \mathrm{rank}({}^1\boldsymbol{G}) - 1 = \mathrm{rank}(\boldsymbol{G}) - 2 \quad (14)$$

Generalization of eqs 10 and 14 leads to consideration of a *contraction domain*, σ, which contains all ligands to which the system is closed. The dimension of σ, $\dim(\sigma)$, equals the number of ligands for which $dX = 0$. The remaining $n - \dim(\sigma)$ fields are linearly independent and give the rank of the matrix $\boldsymbol{G}$ contracted over σ, so that

$$\mathrm{rank}({}^\sigma\boldsymbol{G}) = \mathrm{rank}(\boldsymbol{G}) - \dim(\sigma) \quad (15)$$

which includes eqs 10 and 14 as particular cases. The equation above also implies that the contraction domain σ and the manifold spanned by the $n - \dim(\sigma)$ linearly independent fields are orthogonal subspaces in F^n. This is a consequence of the fact that the system cannot be open and closed to a given ligand at the same time. Hence,

any field in F^n necessarily belongs either to the manifold of linearly independent fields, τ, or to σ, so that

$$\tau \cup \sigma = F^n \tag{16a}$$

$$\tau \cap \sigma = \emptyset \tag{16b}$$

$$\dim(\tau) + \dim(\sigma) = \dim(F^n) = n \tag{16c}$$

which are the abstract metric forms corresponding to eq. 15.

At this point we are in the condition of deriving a general expression for the elements ${}^{\sigma}g$s of ${}^{\sigma}\boldsymbol{G}$ as a function of the elements gs of $\boldsymbol{G}$. Consider the matrix $\boldsymbol{G}$ in F^n

$$\boldsymbol{G} = \begin{pmatrix} g_{11} & g_{12} & \cdots & g_{1n} \\ g_{21} & g_{22} & \cdots & g_{2n} \\ \cdot & \cdot & \cdots & \cdot \\ \cdot & \cdot & \cdots & \cdot \\ \cdot & \cdot & \cdots & \cdot \\ g_{n1} & g_{n2} & \cdots & g_{nn} \end{pmatrix} \tag{17}$$

Since σ is a subspace of F^n, then the matrix associated with σ is a principal minor of $\boldsymbol{G}$, $\boldsymbol{G}_{\sigma}$, its order being equal to $\dim(\sigma) = \operatorname{rank}(\boldsymbol{G}_{\sigma})$. The submatrix $\boldsymbol{G}_{\sigma}$ can be written as

$$\boldsymbol{G}_{\sigma} = \begin{pmatrix} g_{11} & g_{12} & \cdots & g_{1k} \\ g_{21} & g_{22} & \cdots & g_{2k} \\ \cdot & \cdot & \cdots & \cdot \\ \cdot & \cdot & \cdots & \cdot \\ \cdot & \cdot & \cdots & \cdot \\ g_{k1} & g_{k2} & \cdots & g_{kk} \end{pmatrix} \tag{18}$$

and can always be cast in the form given in eq. 18, with $\boldsymbol{G}$ given as in eq. 17, by properly numbering (or renumbering) the particular ligands belonging to the contraction domain. Consider now any two linearly independent fields μ_i and μ_j $(k < i, j \leq n) \in \tau$, and the associated matrix element ${}^{12\ldots k}g_{ij} = {}^{\sigma}g_{ij}$ of ${}^{\sigma}\boldsymbol{G}$. One has from eq. 8

$${}^{1}g_{ij} = \begin{vmatrix} g_{11} & g_{1j} \\ g_{i1} & g_{ij} \end{vmatrix} / g_{11} \tag{19}$$

along with

$$\begin{aligned} {}^{12}g_{ij} &= \begin{vmatrix} {}^{1}g_{22} & {}^{1}g_{2j} \\ {}^{1}g_{i2} & {}^{1}g_{ij} \end{vmatrix} / {}^{1}g_{22} \\ &= \begin{vmatrix} g_{11} & g_{12} & g_{1j} \\ g_{21} & g_{22} & g_{2j} \\ g_{i1} & g_{i2} & g_{ij} \end{vmatrix} \Bigg/ \begin{vmatrix} g_{11} & g_{12} \\ g_{21} & g_{22} \end{vmatrix} \end{aligned} \tag{20}$$

Hence, in general one has

$${}^{\sigma}g_{ij} = \det \boldsymbol{G}_{\sigma,ij} / \det \boldsymbol{G}_{\sigma} \tag{21}$$

where

$$\boldsymbol{G}_{\sigma,ij} = \begin{pmatrix} g_{11} & g_{12} & \cdots & g_{1k} & g_{1j} \\ g_{21} & g_{22} & \cdots & g_{2k} & g_{2j} \\ \cdot & \cdot & \cdots & \cdot & \cdot \\ \cdot & \cdot & \cdots & \cdot & \cdot \\ \cdot & \cdot & \cdots & \cdot & \cdot \\ g_{k1} & g_{k2} & \cdots & g_{kk} & g_{kj} \\ g_{i1} & g_{i2} & \cdots & g_{ik} & g_{ij} \end{pmatrix} \tag{22}$$

is the matrix $\boldsymbol{G}_{\sigma}$ bordered by the element g_{ij} [33].

The contraction $F^n \rightarrow F^{n-\dim(\sigma)}$ due to closure of the system to a number $\dim(\sigma) = \operatorname{rank}(\boldsymbol{G}_{\sigma})$ of ligands is accompanied by a 'physical' contraction of each coordinate in the manifold. When $i = j$ in eq. 20 the bordered matrix $\boldsymbol{G}_{\sigma,ii}$ becomes a principal minor of $\boldsymbol{G}$ just as $\boldsymbol{G}_{\sigma}$ itself and therefore, by Hadamard's inequality involving principal minors [33], one has the important inequality

$$g_{ii} \det \boldsymbol{G}_{\sigma} > \det \boldsymbol{G}_{\sigma,ii} \tag{23}$$

Hence

$$g_{ii} > {}^{\sigma}g_{ii} \tag{24}$$

for arbitrary σ. The relationship above expresses the important stability principle that a diagonal element of $\boldsymbol{G}$, i.e., the binding capacity [38] of the macromolecule with respect to a particular ligand, is always less when the system is closed to some other linked ligands [38,39].

The foregoing treatment has outlined the relevant relationships in a global thermodynamic description of a macromolecular system at equilibrium. Linkage among the various ligands binding to the macromolecule is approached within this framework by studying the system along different coordinates in the field manifold F^n. Here our attention is concentrated on the properties of the macromolecular system as a whole and on the effect that binding of one ligand has on binding of another ligand. When we consider a particular ligand, or coordinate, of the field manifold F^n we can look at linkage effects that occur *locally* and involve sites of the macromolecule binding that particular ligand. The basic question arising is

whether these effects can themselves be described in terms of a field manifold along the lines discussed above.

4. Thermodynamics of local linkage effects: a basic isomorphism

We start from consideration of a basic isomorphism involving the thermodynamic variables of a macromolecular system at equilibrium. At constant T and P * the general expression for the chemical potential of the macromolecule, μ_M, in the presence of n ligands is given by

$$\sum_{j=1}^{n} X_j \, d\mu_j = -d\mu_M \tag{25}$$

with

$$\mu_M = -RT \ln Z(\{\mu_j\}) \tag{26}$$

where the various quantities have already been defined in the previous sections, and $\{\mu_j\}$ is a shorthand notation for the set of fields μ_1, $\mu_2 \ldots \mu_n$. Consider now a particular ligand, whose chemical potential is μ. There exists a form, $d\Phi$, isomorphic with eq. 25 that can be written as [40,41]

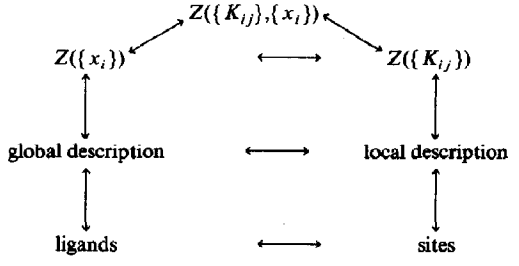
$$\sum_{j=1}^{t} X_j \, d \ln K_j = d\Phi \tag{27}$$

where t is the number of sites binding that ligand, X_j the fractional saturation of the j-th site, and K_j the apparent equilibrium constant for ligand binding to the j-th site. The form Φ is given by

$$\Phi = \ln Z(\{K_j\}) \tag{28}$$

where it should be pointed out that $Z(\{K_j\})$ is the canonical partition function of the system in the presence of only one ligand, $Z(\{K_j\},x)$, where x is the ligand activity. One sees from eq. 28 that the description of the local properties of the macromolecule is focussed on the fields Ks, while the global description is focussed on the fields μs, or the equivalent xs. These two sets of fields are isomorphic by virtue of eqs 25 and 27, which means that the metric of the field manifold F^n outlined in section 3 can be mapped with a 1:1 correspondence with the metric of the field manifold F^t constructed from the t fields Ks [41]. The choice of the manifold depends solely on the description, global or local, of the facts. In the former case we give priority to the μs because we are interested in linkage effects among ligands. In the latter case we deal with the Ks and linkage effects among binding sites of the macromolecule. In terms of the canonical partition function, $Z(\{K_{ij}\},\{x_i\})$, the global description makes use of $Z(\{x_i\})$ and neglects the Ks assumed to be constant, while the local description makes use of $Z(\{K_{ij}\})$ and neglects the xs assumed to be constant. Here K_{ij} indicates the apparent association constant of the i-th ligand ($i = 1, 2 \ldots n$) to the j-th site ($j = 1, 2 \ldots t$). A value of $K_{ij} = 0$ indicates that ligand j does not bind to site i, i.e., site i is a binding site for another ligand. If two ligands, i and h, bind competitively to site j, then both K_{ij} and $K_{hj} \neq 0$. In the case of only one ligand ($n = 1$) the index i can be omitted and K_j gives the apparent association constant for the j-th site.

The topology of the isomorphism between global and local descriptions can be illustrated in the scheme below



(29)

Linkage effects in the local description can be cast within the framework of a thermodynamic treatment isomorphic with the one given in the case of global linkage effects. The matrix G in the local description contains t^2 elements, $g_{ij} = \partial X_i /$

* This assumption is made solely for the sake of simplicity. The isomorphism in fact involves only the chemical fields μs and applies irrespective of whether T and P are held constant.

$\partial \ln K_j$, that give the effect of field K_j on X_i in the fully open system. As in the case of the global description, $\boldsymbol{G}$ is symmetric and positive definite. The *contraction domain*, σ, contains all fields the system is closed to and $\dim(\sigma)$ equals the number of sites for which $\mathrm{d}X = 0$. The remaining $t - \dim(\sigma)$ Ks are linearly independent and give the rank of ${}^{\sigma}\boldsymbol{G}$, so that

$$\tau \cup \sigma = F^t \tag{30a}$$

$$\tau \cap \sigma = \varnothing \tag{30b}$$

$$\dim(\tau) + \dim(\sigma) = \dim(F^t) = t \tag{30c}$$

The relationships above parallel those given in eq. 16. The elements ${}^{\sigma}g$s of ${}^{\sigma}\boldsymbol{G}$ can in turn be derived from the elements of $\boldsymbol{G}$ as shown in eq. 21.

The isomorphism ligands ↔ sites clearly points out the physical parallel involving chemical potentials and apparent equilibrium constants. By virtue of this isomorphism, the contraction $F^t \rightarrow F^{t-\dim(\sigma)}$ corresponds to closure of the system to $\dim(\sigma)$ sites and draws attention to the properties of a 'contracted macromolecule' where the binding sites belonging to the contraction domain σ drop out of the picture. The new scenario disclosed by the contraction raises a number of questions regarding the thermodynamic properties of these contracted macromolecules, the way they can be experimentally approached and the relation they have with the 'uncontracted' macromolecule. It is here that the concept of 'contracted partition functions' comes into the picture.

5. Contracted partition functions

Consider a macromolecule M containing t binding sites for a ligand X, whose activity is x. The canonical partition function is given by [11,41]

$$\begin{aligned} Z(\{K_j\},x) &= 1 + (K_1 + K_2 + \dots K_t)x \\ &\quad + (c_{12}K_1K_2 + \dots c_{t-1t}K_{t-1}K_t)x^2 \\ &\quad + \dots c_{12\dots t}K_1K_2\dots K_t x^t \end{aligned} \tag{31}$$

where K_j is the apparent association constant for ligand binding to site j, and the cs are interaction constants. Each term of Z is proportional to the equilibrium concentration of a particular, site-specific configuration of the macromolecule. The standard free energy level of any configuration is given by the sum of individual (standard) free energies of binding, $\Delta G_j = -RT \ln K_j$, plus a cooperative free energy term, $\Delta G^c_{ij\dots} = -RT \ln c_{ij\dots}$, that quantifies the energetics of interaction among the ligated sites. For example, the free energy level of the configuration with sites i and j ligated is given by

$$\Delta G_{ij} = \Delta G_i + \Delta G_j + \Delta G^c_{ij} = -RT \ln c_{ij}K_iK_j \tag{32}$$

Hence, the partition function can also be written as

$$Z(\{K_j\},x) = \sum_{j=0}^{t} \sum_{w(j)} \exp(-\Delta G_{w(j)}/RT)x^j \tag{33}$$

where summation over w means summation over all possible configurations. The fractional saturation of site j is derived from eq. 33 by differentiation as follows [40,41]

$$X_j = \partial \ln Z(\{K_j\},x)/\partial \ln K_j \tag{34}$$

and the number of ligated sites is given by

$$X = \partial \ln Z(\{K_j\},x)/\partial \ln x = X_1 + X_2 + \dots X_t \tag{35}$$

From the relationships above, the interplay between local $(X_1, X_2, \dots X_t)$ and global (X) thermodynamic variables is evident, as well as the use of $\{K_j\}$ or x as the relevant fields in either case.

The mathematical form of Z written as in eq. 33 deserves particular attention. Partial differentiation of Z with respect to $\ln K_j$ yields a first-order homogeneous function of K_j that will be invariant upon further differentiation with respect to $\ln K_j$. In view of this fact one has

$$\partial Z(\{K_i\})/\partial \ln K_j = {}^1Z(\{K_{i\neq j}\})K_j x \tag{36}$$

where the variable x in the definition of the partition functions has been dropped for the sake of simplicity, and ${}^1Z(\{K_{i\neq j}\})$ is a function of all

Ks but K_j. Since the right-hand side of eq. 36 is a first-order homogeneous function of K_j we can replace it with any of its partial derivatives with respect to ln K_j. Substitution of the first-order partial derivative leads to

$$\partial\left[Z(\{K_i\}) - {}^1Z(\{K_{i\neq j}\})K_j x\right]/\partial \ln K_j = 0 \tag{37}$$

and integration yields

$$Z(\{K_i\}) = {}^1Z(\{K_{i\neq j}\})K_j x + {}^0Z(\{K_{i\neq j}\}) \tag{38}$$

The partition function Z can thus be expressed as the sum of two terms, one being a first-order homogeneous function of K_j and the other being a function of all Ks but K_j. The physical relevance of these two functions stems from the fact that they are linearly independent and therefore do not share redundant information. The first term on the right-hand side of eq. 38 gives the sum of all site-specific configurations of the macromolecule with site j ligated. This accounts for factorization of the term $K_j x$. The function ${}^1Z(\{K_{i\neq j}\})$ is the partition function for the remaining $t-1$ sites, with site j ligated. The second term on the right-hand side of eq. 38 is simply the partition function of the remaining $t-1$ sites, with site j unligated. Both ${}^1Z(\{K_{i\neq j}\})$ and ${}^0Z(\{K_{i\neq j}\})$ are 'contracted partition functions' (CPFs), as they encapsulate the configurations of a 'contracted macromolecule' with $t-1$ sites. Site j represents the domain of contraction, σ, so that the field K_j does not enter the definition of a CPF of the form ${}^\sigma Z$. The set $\{K_{i\neq j}\}$, on the other hand, represents the domain of independent fields, τ. The connection with arguments developed in section 3 now becomes evident.

As there are two discrete ligation states for each site, so one has two different CPFs corresponding to any given contraction. Furthermore, since a CPF is itself a partition function, then eq. 38 must hold for any CPF as well, so that further contraction over site m yields

$${}^1Z(\{K_{i\neq j}\}) = {}^{11}Z(\{K_{i\neq j,m}\})K_m x + {}^{10}Z(\{K_{i\neq j,m}\}) \tag{39a}$$

$${}^0Z(\{K_{i\neq j}\}) = {}^{01}Z(\{K_{i\neq j,m}\})K_m x + {}^{00}Z(\{K_{i\neq j,m}\}) \tag{39b}$$

Hence,

$$\begin{aligned} Z(\{K_i\}) = {}&{}^{11}Z(\{K_{i\neq j,m}\})K_j K_m x^2 \\ &+ {}^{10}Z(\{K_{i\neq j,m}\})K_j x \\ &+ {}^{01}Z(\{K_{i\neq j,m}\})K_m x \\ &+ {}^{00}Z(\{K_{i\neq j,m}\}) \end{aligned} \tag{40}$$

and so forth. The number of CPFs of the form ${}^\sigma Z$ defining the 'uncontracted' Z is set by the dimension of the contraction domain and is equal to $2^{\dim(\sigma)}$, for any $0 \le \dim(\sigma) \le t$. The number of terms defining any of the $2^{\dim(\sigma)}$ CPFs of the form ${}^\sigma Z$ is given by $2^{t-\dim(\sigma)}$, since ${}^\sigma Z$ is a $[t-\dim(\sigma)]$ − degree polynomial in the ligand activity x. Hence, the number of terms defining Z over the domain of contraction σ is $2^{\dim(\sigma)}2^{t-\dim(\sigma)} = 2^t$, and is independent of σ as one would expect.

Generalization of eqs 38 and 40 is now straightforward and leads to

$$Z(\{K_i\}) = \sum_\sigma {}^\sigma Z(\tau) w(\sigma) \tag{41}$$

where summation is over all configurations of σ, and τ is the domain of independent fields complementary to σ by virtue of eq. 30. The terms $w(\sigma)$ are 'weighting factors' for the CPFs obtained by factorization of the fields entering the definition of a particular configuration of σ. Accordingly, generalization of eqs 34 and 35 yields

$$\partial \ln {}^\sigma Z(\tau)/\partial \ln K_j = {}^\sigma X_j \tag{42}$$

$$\partial \ln {}^\sigma Z(\tau)/\partial \ln x = {}^\sigma X \tag{43}$$

The first relation gives the fractional saturation of the j-th site of the macromolecule contracted over σ, with $K_j \in \tau$. The second relation gives the number of ligated sites of the macromolecule contracted over σ, which is given by the sum of $t - \dim(\sigma)$ terms of the form given in eq. 42.

The complete set of CPFs for the case of a trimer is shown in table 1. The use of CPFs casts the analysis of local linkage effects within a new and more comprehensive framework. It also broadens our interpretation of the interplay be-

Table 1

Contracted partition functions for a trimeric macromolecule ($t = 3$)

CPF	$w(\sigma)$
$\dim(\sigma) = 0$, $\dim(\tau) = 3$	
$Z(K_1, K_2, K_3) = 1 + (K_1 + K_2 + K_3)x + (c_{12}K_1K_2 + c_{13}K_1K_3 + c_{23}K_2K_3)x^2 + c_{123}K_1K_2K_3x^3$	1
$\dim(\sigma) = 1$, $\dim(\tau) = 2$	
${}^1Z(K_2, K_3) = 1 + (c_{12}K_2 + c_{13}K_3)x + c_{123}K_2K_3x^2$	K_1x
${}^0Z(K_2, K_3) = 1 + (K_2 + K_3)x + c_{23}K_2K_3x^2$	1
${}^1Z(K_1, K_3) = 1 + (c_{12}K_1 + c_{23}K_3)x + c_{123}K_1K_3x^2$	K_2x
${}^0Z(K_1, K_3) = 1 + (K_1 + K_3)x + c_{13}K_{13}x^2$	1
${}^1Z(K_1, K_2) = 1 + (c_{13}K_1 + c_{23}K_2)x + c_{123}K_1K_2x^2$	K_3x
${}^0Z(K_1, K_2) = 1 + (K_1 + K_2)x + c_{12}K_1K_2x^2$	1
$\dim(\sigma) = 2$, $\dim(\tau) = 1$	
${}^{11}Z(K_3) = c_{12} + c_{123}K_3x$	$K_1K_2x^2$
${}^{01}Z(K_3) = 1 + c_{23}K_3x$	K_2x
${}^{10}Z(K_3) = 1 + c_{13}K_3x$	K_1x
${}^{00}Z(K_3) = 1 + K_3x$	1
${}^{11}Z(K_2) = c_{13} + c_{123}K_2x$	$K_1K_3x^2$
${}^{01}Z(K_2) = 1 + c_{23}K_2x$	K_3x
${}^{10}Z(K_2) = 1 + c_{12}K_2x$	K_1x
${}^{00}Z(K_2) = 1 + K_2x$	1
${}^{11}Z(K_1) = c_{23} + c_{123}K_1x$	$K_2K_3x^2$
${}^{01}Z(K_1) = 1 + c_{13}K_1x$	K_3x
${}^{10}Z(K_1) = 1 + c_{12}K_1x$	K_2x
${}^{00}Z(K_1) = 1 + K_1x$	1
$\dim(\sigma) = 3$, $\dim(\tau) = 0$	
${}^{111}Z = c_{123}$	$K_1K_2K_3x^3$
${}^{011}Z = c_{23}$	$K_2K_3x^2$
${}^{101}Z = c_{13}$	$K_1K_3x^2$
${}^{110}Z = c_{12}$	$K_1K_3x^2$
${}^{100}Z = 1$	K_1x
${}^{010}Z = 1$	K_2x
${}^{001}Z = 1$	K_3x
${}^{000}Z = 1$	1

tween global and local effects, as will be demonstrated in the following sections.

6. The site-specific Hill plot

A simple measure of homotropic cooperativity in the description of global linkage effects is represented by the slope of the Hill plot [42], where

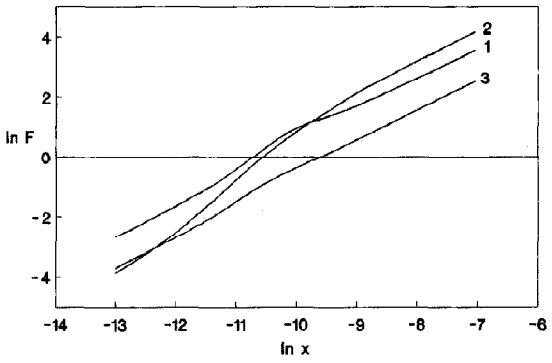


Fig. 1. Site-specific Hill plots for repressor binding to the three sites of λ-phage operator, O_L^1, O_L^2, and O_L^3, as indicated ($F = X_j/(1 - X_j)$). Continuous lines were drawn according to eq. 47 for $t = 3$, using parameter values reported elsewhere [26]. From these plots it results clear that sites O_L^1 and O_L^2 bind the repressor with higher cooperativity than site O_L^3. The spacing between the two asymptotes for each plot is proportional to the site-specific free energy of interaction (see eq. 50) and has a maximum for site O_L^2.

$\ln[X/(t - X)]$ is plotted vs $\ln x$, and is called the Hill coefficient, n_H. Wyman has outlined the peculiar properties of the Hill plot [5]. In particular, the Hill coefficient

$$n_H = \frac{d \ln[X/(t-X)]}{d \ln x} = \frac{t}{X(t-X)} \frac{dX}{d \ln x} \tag{44}$$

is equal to 1 for $x \to 0$ and $x \to \infty$, and cannot exceed t, the total number of binding sites. Also, n_H must always be positive since the binding capacity, $dX/d \ln x$, is necessarily a positive quantity * as we have seen in sections 2 and 3. Ligand binding cooperativity is positive when $n_H > 1$ and negative when $n_H < 1$. The free energy per site associated with stabilizing (positive cooperativity) or destabilizing (negative cooperativity)

* The binding capacity $dX/d \ln x$ has a sound statistical significance when one recognizes it as the mean square fluctuation of the ligation intermediates around X, or else the variance of the distribution of ligated species. This fact was first pointed out by Linderstrøm-Lang in the description of acid-base titration curves.

interactions among the sites is given by the integral equation [5]

$$\Delta G^{\mathrm{I}} = RT\int_{-\infty}^{+\infty}(n_{\mathrm{H}}-1)\,\mathrm{d}\ln x \qquad (45)$$

In the case of a monomeric macromolecule ($t=1$) without site heterogeneity the Hill plot yields a straight line of slope 1, and ΔG^{I} is zero. In the case of positive cooperativity $n_{\mathrm{H}}>1$ within the asymptotic regions of the Hill plot and $\Delta G^{\mathrm{I}}>0$, while in the case of negative cooperativity one has $n_{\mathrm{H}}<1$ and $\Delta G^{\mathrm{I}}<0$.

Analogous thermodynamic quantities can be defined and calculated in the case of local linkage effects. Consider the fractional saturation of site j written in terms of CPFs as follows

$$X_j = \frac{{}^1Z(\{K_{i\neq j}\})K_j x}{{}^1Z(\{K_{i\neq j}\})K_j x + {}^0Z(\{K_{i\neq j}\})} \qquad (46)$$

Consideration of a site-specific Hill plot * leads to the equation

$$\ln\frac{X_j}{1-X_j} = \ln K_j + \ln x + \ln\frac{{}^1Z(\{K_{i\neq j}\})}{{}^0Z(\{K_{i\neq j}\})} \qquad (47)$$

As in the case of the global description, the site-specific Hill plot has two asymptotes

$$\lim_{x\to 0}\ln\left[X_j/(1-X_j)\right] = \ln K_j + \ln x \qquad (48a)$$

$$\lim_{x\to\infty}\ln\left[X_j/(1-X_j)\right] = \ln K_j + \ln\left(c_{\{i\}}/c_{\{i\neq j\}}\right) + \ln x \qquad (48b)$$

where $c_{\{i\}}$ and $c_{\{i\neq j\}}$ are the interaction constants of the highest power of x in the two CPFs of eq. 47, i.e., $c_{\{i\}} = c_{12\ldots t}$ and $c_{\{i\neq j\}} = c_{12\ldots j-1j+1\ldots t}$. The site-specific Hill plot thus provides independent information on the apparent equilibrium constant K_j, which can be resolved with high precision from the asymptote for $x\to 0$. Also, it provides an independent measure of the ratio of two interaction constants entering the definition of the canonical partition function. In this respect one should note that the two asymptotes of a site-specific Hill plot will not coincide, unless $c_{\{i\}} = c_{\{i\neq j\}}$.

Site-specific isotherms map the thermodynamic behavior of an individual site, but this behavior can be quite different from that of a single-site macromolecule. Indeed, site-specific effects can be 'cooperative' due to interaction of that site with the remaining ones [11,41]. Therefore, it seems appropriate to define a site-specific Hill coefficient by differentiating eq. 47 with respect to $\ln x$, so that

$$n_j = 1 + {}^1X(\{K_{i\neq j}\}) - {}^0X(\{K_{i\neq j}\}) \qquad (49)$$

where ${}^1X(\{K_{i\neq j}\}) = \partial\ln{}^1Z(\{K_{i\neq j}\})/\partial\ln x$ is the number of ligands bound to the remaining $t-1$ sites other than j when site j is ligated, and ${}^0X(\{K_{i\neq j}\}) = \partial\ln{}^0Z(\{K_{i\neq j}\})/\partial\ln x$ is the number of ligands bound to the remaining $t-1$ sites other than j when site j is unligated. Site-specific cooperativity is thus uniquely defined by the difference ${}^1X(\{K_{i\neq j}\}) - {}^0X(\{K_{i\neq j}\})$. Cooperativity is positive when loading site j increases the number of ligands bound to the remaining $t-1$ sites, and negative otherwise. Absence of cooperativity corresponds to the fact that loading site j has no influence on the remaining sites. However, the site-specific Hill coefficient n_j becomes equal to 1 when $x\to 0$ and $x\to\infty$, regardless of interactions among the sites, just as in the case of n_{H} in eq. 44. This is because both ${}^1X(\{K_{i\neq j}\})$ and ${}^0X(\{K_{i\neq j}\})$ tend to 0 for $x\to 0$, and to $t-1$ for $x\to\infty$.

The site-specific free energy of interaction can be defined according to eq. 45 as

$$\Delta G_j^{\mathrm{I}} = RT\int_{-\infty}^{+\infty}(n_j-1)\,\mathrm{d}\ln x \qquad (50)$$

* The site-specific Hill plot has a bearing on the individual site 'binding constant'

$${}^*K_i = X_i/[(1-X_i)x]$$

as defined by Ackers [11]. The logarithm of this quantity corresponds exactly to a site-specific Hill plot rotated 45° clockwise. It should be pointed out, however, that the important features of the site-specific Hill plot introduced here stem from the connection between the properties of this plot and the binding properties of the contracted macromolecule. Therefore, it is the approach based on CPFs that makes the site-specific Hill plot particularly relevant in the analysis of local linkage effects.

and will be positive, negative or zero depending upon the sign of $c_{\{i\}} - c_{\{i \neq j\}}$. In the case of the macromolecule as a whole, ΔG^{I} equals the free energy of binding the first ligand minus the free energy of binding the last ligand to the macromolecule [5]. In the case of an individual site ΔG_j^{I} can also be written as

$$\Delta G_j^{\mathrm{I}} = RT \int_{-\infty}^{+\infty} \left[{}^{1}X\left(\{K_{i \neq j}\}\right) - {}^{0}X\left(\{K_{i \neq j}\}\right) \right] \mathrm{d} \ln x \qquad (51)$$

But

$$(t-1) \ln \langle {}^{1}x\left(\{K_{i \neq j}\}\right) \rangle = \int_{0}^{t-1} \ln x \, \mathrm{d}\, {}^{1}X\left(\{K_{i \neq j}\}\right) \qquad (52a)$$

$$(t-1) \ln \langle {}^{0}x\left(\{K_{i \neq j}\}\right) \rangle = \int_{0}^{t-1} \ln x \, \mathrm{d}\, {}^{0}X\left(\{K_{i \neq j}\}\right) \qquad (52b)$$

where $\langle {}^{1}x(\{K_{i \neq j}\}) \rangle$ and $\langle {}^{0}x(\{K_{i \neq j}\}) \rangle$ are the mean * ligand activities for the remaining $t-1$ sites when site j is ligated or unligated, respectively. Hence, integration by parts of eq. 51 gives

$$\Delta G_j^{\mathrm{I}} = RT(t-1) \left[\ln \langle {}^{0}x\left(\{K_{i \neq j}\}\right) \rangle - \ln \langle {}^{1}x\left(\{K_{i \neq j}\}\right) \rangle \right] \qquad (53)$$

which shows that the free energy of interaction of site j is an important thermodynamic quantity. It equals the difference in the work done to load the remaining $t-1$ sites when site j is unligated with respect to when site j is ligated. Construction of a site-specific Hill plot thus provides useful information on a given site and the rest of the macromolecule. An example of this plot is given in fig. 1 for the case of repressor binding to λ-phage operator [26].

One should consider the operationally useful aspects of constructing site-specific Hill plots. In fact, in the case of t binding sites one can resolve t apparent association constants K_j, along with t ratios of the form $c_{\{j\}}/c_{\{i \neq j\}}$. Since $c_{\{j\}}$ is the coefficient of the highest power of x in the partition function, eq. 31, it can be obtained independently from the mean ligand activity of the macromolecule as a whole by numerical integration [5]. Therefore, from the mean ligand activity of the macromolecule as a whole and the two asymptotes of t site-specific Hill plots, one can resolve a total of $2t+1$ parameters. Since eq. 31 contains a total of $2^t - 1$ parameters, one sees that for $t = 3$ all parameters can in principle be resolved without data fitting, as shown in table 2, and for $t = 4$ only six parameters strictly demand nonlinear least-squares analysis.

The most interesting point arising in connection with the definition of a site-specific Hill coefficient has a bearing on thermodynamic stability. The site-specific Hill coefficient can be expressed

Table 2

Parameter resolution for a trimeric macromolecule ($t = 3$)

Seven independent parameters entering the definition of the partition function can uniquely be determined from seven independent equations.

Partition function:	
$Z(K_1, K_2, K_3) = 1 + (K_1 + K_2 + K_3)x + (c_{12}K_1K_2 + c_{13}K_1K_3 + c_{23}K_2K_3)x^2 + c_{123}K_1K_2K_3x^3$	
Mean ligand activity:	
(whole macromolecule)	
$\langle x(K_1, K_2, K_3) \rangle = [c_{123}K_1K_2K_3]^{-1/3}$	
Site-specific Hill plots:	
Site 1 ($x \to 0$)	$\ln K_1$
($x \to \infty$)	$\ln K_1 + \ln c_{123} - \ln c_{23}$
Site 2 ($x \to 0$)	$\ln K_2$
($x \to \infty$)	$\ln K_2 + \ln c_{123} - \ln c_{13}$
Site 3 ($x \to 0$)	$\ln K_3$
($x \to \infty$)	$\ln K_3 + \ln c_{123} - \ln c_{12}$

* It should be pointed out that the *median* ligand activity, x_{m}, as defined by Wyman [5]

$$t \ln x_{\mathrm{m}} = \int_{0}^{t} \ln x \, \mathrm{d}X$$

is nothing but the *mean* value of $\ln x$, i.e.,

$$\ln x_{\mathrm{m}} = \int_{0}^{t} \ln x \, \mathrm{d}X \Big/ \int_{0}^{t} \mathrm{d}X$$

The quantity above is also the mean value of the ligand chemical potential expressed in RT units. For this reason, the term 'median' is replaced by 'mean' throughout this paper and the notation $\langle x \rangle$ introduced in eq. 52 follows quite simply from the above definition.

as a function of the site-specific binding capacity, $dX_j/d\ln x$, much like n_H in eq. 44, so that [41]

$$n_j = \frac{d\ln\left[X_j/(1-X_j)\right]}{d\ln x} = \frac{1}{X_j(1-X_j)}\frac{dX_j}{d\ln x} \tag{54}$$

and although the quantity $X_j(1-X_j)$ is always positive, nevertheless the binding capacity of site j can be positive or also negative due to interaction of site j with the remaining sites [41]. A negative value of $dX_j/d\ln x$ is perfectly consistent with the second law that is 'violated' only locally. In fact, the binding capacity of the macromolecule as a whole, given by the sum of t individual, site-specific binding capacities, remains always positive [41]. Local 'violation' of the second law necessarily demands ${}^1X(\{K_{i\neq j}\}) - {}^0X(\{K_{i\neq j}\}) < -1$ in eq. 49, so that binding to site j must be accompanied by a drastic decrease of the number of ligands bound to the remaining sites. The site-specific Hill plot in this case shows a *negative* slope. This feature can only be observed when dealing with local linkage effects and is depicted in fig. 2. Local violations of the second law have been documented in the case of redox equilibria of cytochrome a_3 [23], but their implication in connection with thermodynamic stability has not been recognized. From eq. 49 one also concludes that the maximum value of n_j is t, as in the case of n_H [5]. However, the minimum value of n_j is $2-t$ and not 0, as in the case of n_H [5], which shows quite simply that a local violation of the second law can only occur for $t > 2$ [41].

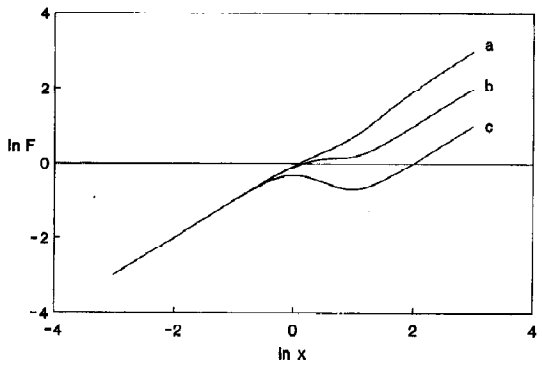


Fig. 2. Site-specific Hill plots for site 1 of a hypothetical trimeric macromolecule ($F = X_1/(1-X_1)$). Continuous lines were drawn according to eq. 47 for $t = 3$, using the following parameter values: $K_1 = 1$, $K_2 = K_3 = 0.1$, $c_{12} = c_{13} = 0$, $c_{123} = 1$. The value of c_{23} is equal to: 1 (curve a), 10 (curve b), 100 (curve c). Note how the slope of the plot becomes negative when $c_{23} > 10$, while both asymptotes retain a slope of unity regardless of the value of c_{23}.

7. Site-specific partition coefficients

In the global description of linkage effects there is an important case arising when two different ligands compete for the same site. A prototypic example of this situation is provided by the competitive binding of oxygen and carbon monoxide to human hemoglobin. The thermodynamic quantity of interest in this case is the partition coefficient M

$$M = \frac{X}{Y}\frac{y}{x} \tag{55}$$

where X and Y are the number of ligands bound of each type and x and y the respective ligand activities. The partition coefficient gives a simple measure of the affinity of one ligand relative to the other. When M is independent of x and y, then X and Y have the same form and hence the two ligands have the same binding capacity and cooperativity. This situation is embodied by the famous Haldane law for the partitioning of oxygen and carbon monoxide to human hemoglobin [43]. In general, M is a function of both x and y, as also illustrated by human hemoglobin [44].

The isomorphism ligands $\leftrightarrow$ sites discussed in section 4 brings out another parallel of interest in the description of local linkage effects. Here one can imagine two sites competing for the same ligand, which is just the opposite of what we see in the case of global linkage effects. Site-specific partition coefficients can be defined accordingly as the affinity of one site relative to another, so that

$$M_{ij} = \frac{X_i}{X_j}\frac{K_j}{K_i} \tag{56}$$

which in terms of CPFs becomes

$$M_{ij} = \frac{{}^1Z(\{K_{h\neq i}\})}{{}^1Z(\{K_{h\neq j}\})} \tag{57}$$

Interestingly, M_{ij} tends to 1 for $x \to 0$ and to K_j/K_i for $x \to \infty$, and therefore a necessary (although not sufficient) condition for M_{ij} to be constant is that $K_i = K_j$. For practical purposes the use of site-specific partition coefficients can be exploited in a plot of $\ln(X_i/X_j)$ versus $\ln x$. This plot has two asymptotes equal to $\ln(K_i/K_j)$ for $x \to 0$ and to 0 for $x \to \infty$. The slope of the plot gives the interesting linkage relationship

$$\frac{\partial \ln(X_i/X_j)}{\partial \ln x} = \frac{\partial \ln M_{ij}}{\partial \ln x} = {}^1X(\{K_{h\neq i}\}) - {}^1X(\{K_{h\neq j}\}) \tag{58}$$

where ${}^1X(\{K_{h\neq i}\})$ is the number of ligands bound to the remaining $t-1$ sites other than i when site i is ligated, and ${}^1X(\{K_{h\neq j}\})$ is the analogous quantity for site j. Finally, integration of eq. 58 yields

$$\int_{-\infty}^{+\infty} \frac{\partial \ln(X_i/X_j)}{\partial \ln x} \mathrm{d} \ln x = \int_{-\infty}^{+\infty} \left[{}^1X(\{K_{h\neq i}\}) - {}^1X(\{K_{h\neq j}\})\right] \mathrm{d} \ln x = (t-1)\left[\ln\langle {}^1x(\{K_{h\neq j}\})\rangle - \ln\langle {}^1x(\{K_{h\neq i}\})\rangle\right] \tag{59}$$

that can be used in connection with eq. 53 to calculate the mean ligand activities of contracted macromolecules. An illustration of site-specific partition coefficients is given in fig. 3.

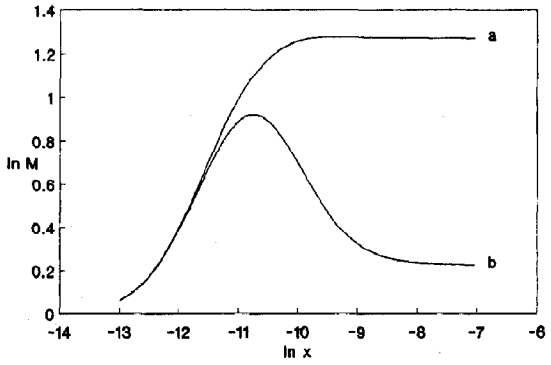


Fig. 3. Logarithm of the site-specific partition coefficients M_{21} (a) and M_{23} (b) for repressor binding to the three sites of λ-phage operator, O_L^1, O_L^2, and O_L^3. Continuous lines were drawn according to eq. 57 for $t = 3$, using parameter values reported elsewhere [26].

8. The free energy of linkage

From a mathematical point of view linkage is readily understood in terms of reciprocity between two effects. Consistent with this mathematical feature, it may be appropriate to decouple the energetics of macromolecular interactions in terms of pairwise contributions, although interactions can of course involve all sites of the macromolecule. In doing so, we focus our attention on linkage between two particular sites of the macromolecule relative to a given configuration of the remaining $t-2$ sites in the contraction domain σ. The linkage unraveled by this contraction draws attention to some peculiar features of the interaction of the two sites between each other and with the rest of the macromolecule. The partition function for the two sites, i and j, is equal to the following CPF

$${}^\sigma Z(K_i, K_j) = c_\sigma + (c_{\sigma i}K_i + c_{\sigma j}K_j)x + c_{\sigma ij}K_iK_jx^2 \tag{60}$$

where the domain τ contains only the fields K_i and K_j, and σ stands for one of the 2^{t-2} possible configurations of the contraction domain. By virtue of eq. 41, the CPF above can also be written as

$$\begin{aligned} {}^\sigma Z(K_i, K_j) &= {}^{\sigma 1}Z(K_j)K_ix + {}^{\sigma 0}Z(K_j) \\ &= {}^{\sigma 1}Z(K_i)K_jx + {}^{\sigma 0}Z(K_i) \end{aligned} \tag{61}$$

The fractional saturation of site i, ${}^\sigma X_i$, is derived from the logarithm of eq. 61 by differentiation with respect to $\ln K_i$, and likewise differentiation with respect to $\ln K_j$ yields ${}^\sigma X_j$. Linkage between

the two sites is expressed by the reciprocity relation

$$\partial {}^{\sigma}X_i/\partial \ln K_j = \partial {}^{\sigma}X_j/\partial \ln K_i = {}^{\sigma}g_{ij} = {}^{\sigma}g_{ji} \qquad (62)$$

which is a consequence of the symmetry of the matrix $\boldsymbol{G}$ contracted over σ, as shown in section 3. Differentiation of the CPFs in eq. 61 gives the expression for the linkage between sites i and j relative to the contraction domain σ

$${}^{\sigma}g_{ij} = \frac{K_i K_j x^2}{{}^{\sigma}Z(K_i,K_j)^2}\left[{}^{\sigma 11}Z\,{}^{\sigma}Z(K_i,K_j) - {}^{\sigma 1}Z(K_i)\,{}^{\sigma 1}Z(K_j)\right] \qquad (63)$$

The nature of this linkage, i.e., whether it is positive or negative, is uniquely determined by the sign of the expression in brackets at the numerator. Straightforward calculations lead to *

$$\begin{aligned}\operatorname{sgn}({}^{\sigma}g_{ij}) &= \operatorname{sgn}(c_\sigma c_{\sigma ij} - c_{\sigma i}c_{\sigma j}) \\ &= \operatorname{sgn}(c_\sigma c_{\sigma ij}/c_{\sigma i}c_{\sigma j} - 1) \\ &= -\operatorname{sgn}(\Delta G^{c}_{\sigma} + \Delta G^{c}_{\sigma ij} - \Delta G^{c}_{\sigma i} - \Delta G^{c}_{\sigma j}) \\ &= -\operatorname{sgn}({}^{\sigma}\Delta G^{L}_{ij}) \end{aligned} \qquad (64)$$

The interesting result embodied by eq. 64 is that the linkage between sites i and j, for a given configuration of the contraction domain σ, depends solely on the cooperative free energy levels of the four configurations entering the definition of the CPF ${}^{\sigma}Z(K_i,K_j)$. The particular relationship among these cooperative free energy levels yields a measure of the interaction of sites i and j relative to the configuration σ. This measure is the free energy of linkage, ${}^{\sigma}\Delta G^{L}_{ij}$, between the two sites, i and j, relative to the configuration σ of the contraction domain.

The free energy of linkage is a measure of *net* interaction, or coupling, between the two sites and should not be confused with the free energy of interaction defined in eq. 45. In fact, calculation of ΔG^{I} for the contracted macromolecule described by the CPF in eq. 61 yields

$${}^{\sigma}\Delta G^{I} = RT \ln\left[4c_\sigma c_{\sigma ij}K_iK_j/(c_{\sigma i}K_i + c_{\sigma j}K_j)^2\right] \qquad (65)$$

while for the free energy of linkage one has

$${}^{\sigma}\Delta G^{L}_{ij} = -RT \ln\left[c_\sigma c_{\sigma ij}/c_{\sigma i}c_{\sigma j}\right] \qquad (66)$$

The basic difference to be outlined here is that the free energy of linkage is independent of the apparent association constant of the two sites, while the free energy of interaction is not. A simple example can be revealing of the consequence of such a difference. When all cs are identical the free energy of linkage vanishes, while the free energy of interaction does not, unless $K_i = K_j$. This explains why the Hill plot can show negative cooperativity even in the absence of interactions (all cs = 1 or identical) when $K_i \neq K_j$.

The concept of free energy of linkage can be very useful in the analysis of macromolecular interactions. Its relevance can best be appreciated by consideration of particular mechanistic models of ligand binding and linkage. Free energies of linkage can be calculated for both the concerted (MWC) [45] and sequential (KNF) [46] allosteric models as follows. Consider the partition function for the MWC model [45]

$$Z_{\mathrm{MWC}} = \frac{L(1+k_{\mathrm{T}}x)^t + (1+k_{\mathrm{R}}x)^t}{L+1} \qquad (67)$$

where L is the allosteric constant, while k_{T} and k_{R} are the affinity constants of the two states ($k_{\mathrm{R}} > k_{\mathrm{T}}$). The free energy level associated with a particular configuration with w ligated sites is given by

$$\begin{aligned}\Delta G_w &= -RT \ln\frac{Lk_{\mathrm{T}}^w + k_{\mathrm{R}}^w}{L+1} \\ &= -wRT \ln k_{\mathrm{R}} - RT \ln\frac{La^w + 1}{L+1}\end{aligned} \qquad (68)$$

where $a = k_{\mathrm{T}}/k_{\mathrm{R}} < 1$. Since ΔG_w is given by the sum of the binding free energies of the individual sites plus the corresponding cooperative free energy level, and due to the fact that all sites are

* One in fact has

${}^{\sigma 11}Z = c_{\sigma ij}$

${}^{\sigma 1}Z(K_i) = c_{\sigma j} + c_{\sigma ij}K_i x$

${}^{\sigma 1}Z(K_j) = c_{\sigma i} + c_{\sigma ij}K_j x$

and hence eq. 64 in the text.

alike, one can take $-RT \ln k_R$ as the apparent free energy of binding to an individual site, so that

$$\Delta G_w^c = -RT \ln \frac{La^w + 1}{L + 1} \tag{69}$$

gives the cooperative free energy level of the w-ligated macromolecule, which is identical for all possible configurations [22]. The free energy of linkage between any two sites, i and j, is given by

$$\begin{aligned} {}^{\sigma}\Delta G_{ij}^L &= \Delta G_w^c + \Delta G_{w+2}^c - 2\Delta G_{w+1}^c \\ &= -RT \ln \frac{La^w + 1}{L + 1} - RT \ln \frac{La^{w+2} + 1}{L + 1} \\ &\quad + 2RT \ln \frac{La^{w+1} + 1}{L + 1} \\ &= -RT \ln \frac{(La^w + 1)(La^{w+2} + 1)}{(La^{w+1} + 1)^2} < 0 \end{aligned} \tag{70}$$

and is always negative, independent of the contraction domain σ, and for any value of the allosteric constant L and the ratio of affinity constants a *. Its (negative) value changes with the degree of ligation w and approaches 0 at low and high saturations. In fact, at high saturation one has $1 \gg La^w$ and both the numerator and denominator of eq. 70 tend to 1, while at low saturation one has $La^{w+2} \gg 1$ and both the numerator and denominator of eq. 70 tend to $L^2a^{2(w+1)}$. Interestingly, the same conclusion is reached even in the presence of site heterogeneity within either state, as a consequence of eq. 66. In this case the cooperative free energy level associated with a particular configuration is given by

$$\Delta G_w^c = -RT \ln[(La_1a_2 \ldots a_w + 1)/(L + 1)] \tag{71}$$

where a_i gives the ratio k_{iT}/k_{iR} for site i ($a_i < 1$ for any i). Calculation of the free energy of linkage between any two sites i and j ($w < i, j$) gives

$$\begin{aligned} {}^{\sigma}\Delta G_{ij}^L &= \Delta G_w^c + \Delta G_{wij}^c - \Delta G_{wi}^c - \Delta G_{wj}^c \\ &= -RT \ln\{(La_1 \ldots a_w + 1) \\ &\quad \times (La_1 \ldots a_w a_i a_j + 1)\} \\ &\quad \times \{(La_1 \ldots a_w a_i + 1) \\ &\quad \times (La_1 \ldots a_w a_j + 1)\}^{-1} < 0 \end{aligned} \tag{72}$$

which again is always negative as the reader can easily verify. In the case of the KNF model [46] calculation of the free energies of linkage is straightforward. The free energy level of each configuration in this model can be expressed as the sum of the apparent free energies of binding of the ligated sites plus a cooperative free energy level which takes into account all possible pairwise interactions among the ligated sites [46,47]. The relevant relations for the free energy of linkage between sites i and j are

$$\Delta G_\sigma^c = -RT \ln c_\sigma \tag{73a}$$

where c_σ is the product of all pairwise interaction constants within the contraction domain σ, and accordingly

$$\Delta G_{\sigma i}^c = -RT \ln c_\sigma - RT \ln c_{\sigma i} \tag{73b}$$

$$\Delta G_{\sigma j}^c = -RT \ln c_\sigma - RT \ln c_{\sigma j} \tag{73c}$$

$$\Delta G_{\sigma ij}^c = -RT \ln c_\sigma - RT \ln c_{\sigma i} - RT \ln c_{\sigma j} - RT \ln c_{ij} \tag{73d}$$

where $c_{\sigma i}$ and $c_{\sigma j}$ are the products of all pairwise interaction constants between site i or j and the sites within the contraction domain, while c_{ij} is the pairwise interaction constant between sites i and j. Combination of the equations above according to eq. 64 yields the result

$${}^{\sigma}\Delta G_{ij}^L = \Delta G_\sigma^c + \Delta G_{\sigma ij}^c - \Delta G_{\sigma i}^c - \Delta G_{\sigma j}^c = -RT \ln c_{ij} \tag{74}$$

Hence, the free energy of linkage in the KNF model can be positive, negative or zero, unlike the case of the MWC model, and is always independent of the particular configuration of the con-

* Expansion of eq. 70 in fact yields

$L^2c^{2w+2} + Lc^{w+2} + Lc^w + 1 > L^2c^{2w+2} + 2Lc^{w+1} + 1$

which reduces to $c^2 + 1 > 2c$ or equivalently $(1 - c)^2 > 0$ for any $c \neq 1$, as is the case by hypothesis.

traction domain and hence saturation. It depends solely on the pairwise interaction constant between the two sites.

The peculiar properties of the two major allosteric models [45,46] of macromolecular interactions appear evident when linkage effects are analysed at the local level. Both models may satisfactorily describe the global cooperative properties of a macromolecular system displaying negative or positive cooperativity. However, the interpretation of macromolecular energetics at the local level given by these models is insufficient to account for all possibilities. As a matter of fact, there is no a priori reason for the free energy of linkage between two sites of a macromolecule to be always negative, as predicted by the MWC model. Likewise, there is no a priori reason for the free energy of linkage to be independent of the particular configuration of the contraction domain and hence saturation, as implied by the KNF picture.

The detailed cooperative free energy levels of all ligation intermediates have recently been solved for human hemoglobin [20,48]. From these levels the free energies of linkage can readily be calculated for any subunit pair. The results are summarized in table 3 for the CN-Met and Mn(III) model systems *. The free energy levels for the CN-Met system are either negative or zero, and do not seem to be affected by the configuration of the contraction domain. This is in sharp disagreement with the prediction of the MWC model, but perfectly consistent with a KNF picture with subunit interactions limited to $\alpha^1\beta^1$ and $\alpha^2\beta^2$ pairs. The free energy levels for the Mn(III) system cannot be calculated for all configurations because the cooperative free energy level of the $\alpha^1\beta^2$ ligated intermediate is not known [21]. However, it is clear from the other free energies of linkage listed in table 3 that significant interactions do exist for all subunit pairs, unlike the CN-Met system. These interactions can be positive ($\alpha^1\beta^2$ and $\beta^1\beta^2$ pairs), or even negative ($\alpha^1\alpha^2$ and $\beta^1\beta^2$ pairs), and also change with saturation ($\alpha^1\alpha^2$ and $\beta^1\beta^2$ pairs), which is in sharp disagreement with the predictions of both the MWC and KNF models. There seems to be no unique 'code' for subunit interactions arising from these values of free energy of linkage. In the CN-Met system interactions are independent of saturation and only occur within $\alpha^1\beta^1$ and $\alpha^2\beta^2$ pairs, while in the Mn(III) system significant interactions also involve the homologous $\alpha^1\alpha^2$ and $\beta^1\beta^2$ pairs and depend on saturation. Only the interactions within $\alpha^1\beta^1$ and $\alpha^2\beta^2$ pairs seem to be independent of saturation and conform to a simple KNF model in both systems. Therefore, the code is likely to be set by interactions within the remaining $\alpha^1\beta^2$, $\alpha^2\beta^1$, $\alpha^1\alpha^2$ and $\beta^1\beta^2$ pairs, i.e., by the linkage between subunits across the interface generated by association of the two $\alpha^1\beta^1$ and $\alpha^2\beta^2$ dimers into the tetrameric

Table 3

Free energies of linkage for human hemoglobin

The configurations of the contraction domain 00, 01, 10 and 11 are given in lexicographic order, with α preceding β and 1 preceding 2. For example, in the case of the $\alpha^1\beta^1$ pair, 00 represents both α^2 and β^2 unligated, 01 stands for β^2 ligated alone and so forth. Due to symmetry, the free energy levels of the $\alpha^2\beta^2$ and $\alpha^2\beta^1$ pairs are not shown as they are identical to those of the $\alpha^1\beta^1$ and $\alpha^1\beta^2$ pairs, respectively. The values are given in kcal/mol. Errors were propagated from the cooperative free energy levels calculated elsewhere assuming noncooperative dimers [20,22,48].

	00	01	10	11
CN-Met system				
$\alpha^1\beta^1$	-3.3 ± 0.5	-3.1 ± 0.6	-2.8 ± 0.6	-3.4 ± 0.5
$\alpha^1\beta^2$	-0.1 ± 0.5	0.1 ± 0.6	0.4 ± 0.6	-0.2 ± 0.5
$\alpha^1\alpha^2$	0.0 ± 0.5	-0.5 ± 0.6	-0.5 ± 0.6	0.2 ± 0.5
$\beta^1\beta^2$	-0.5 ± 0.5	-0.3 ± 0.6	-0.3 ± 0.6	-0.9 ± 0.5
Mn(III) system [a]				
$\alpha^1\beta^1$	-3.2 ± 0.5	–	–	-2.7 ± 0.5
$\alpha^1\beta^2$	–	0.6 ± 0.6	-0.8 ± 0.6	–
$\alpha^1\alpha^2$	1.0 ± 0.5	–	–	0.1 ± 0.5
$\beta^1\beta^2$	-0.8 ± 0.5	–	–	0.7 ± 0.5

[a] For the Mn(III) system, the cooperative free energy level relative to the $\alpha^1\beta^2$ or $\alpha^2\beta^1$ ligated intermediates is not available [22].

* It should be pointed out that the free energies of linkage listed in table 3 have been derived from cooperative free energy levels calculated from the association of noncooperative dimers into tetramers [20,48]. These cooperative levels, as well as the resulting free energies of linkage, would be different if dimers are assumed to be cooperative. For example, in the case of the CN-Met system the data could be interpreted equally well in terms of cooperative dimers associating into noncooperative tetramers.

form. In general, this code does not conform to either the MWC or the KNF models and is very sensitive to the particular ligand under consideration. It should be stressed that such a 'minimal phenomenological scheme' can be arrived at by consideration of basic thermodynamic quantities such as the cooperative free energy levels and the free energies of linkage, independent of any mechanistic assumption. Consequently, any mechanistic interpretation of hemoglobin functional properties should be able to account for the properties of this minimal phenomenological scheme.

9. Discussion

Linkage phenomena in biological macromolecules can be approached at different levels. A global dimension involves effects arising from the mutual interference of different ligands, the macromolecule working as a transducer of the effects. Reciprocity of the effects is not limited to chemical fields, but also arises when dealing with physical fields such as temperature, pressure, surface tension, redox potentials and so forth. Each field represents a coordinate in F^n. The metric in F^n is positive definite and fully characterized in terms of the matrix G. Contraction in the field manifold corresponds to closure of the system to a particular set of fields and parallels the fundamental contraction of the secular equation of a quantum mechanical Hamiltonian, by which the interaction field-particle is cast in a mathematical form independent of the field [49]. The contraction changes the particular form of the matrix G in the various subspaces, thereby affecting linkage among the independent fields.

Through the basic isomorphism ligands $\leftrightarrow$ sites the field manifold F^n can be mapped 1 : 1 into F^t where linkage effects among individual sites of the macromolecule dominate the picture. The relevant fields are no longer chemical potentials but apparent, site-specific association constants. Contraction in F^t in this case yields 'contracted macromolecules', generated from the original macromolecule containing all t binding sites. The contraction domain contains the sites that are kept in a particular ligation state, while the contracted field manifold contains the remaining sites. Metal-substituted hemoglobins can be considered suitable models of such contracted macromolecules. The unligated Fe(II) atom in the heme pocket can functionally be mimicked by metals such as Mn(II) [22] which, unlike Fe(II), cannot combine with oxygen or carbon monoxide. A contraction domain wherein all sites are kept unligated can be constructed by specifically substituting the Fe(II) atom with Mn(II) at some of the four hemes. On the other hand, the ligated Fe(II) atom can functionally be mimicked by CN-Fe(III) [20], or Mn(III) [21], which again do not combine with oxygen or carbon monoxide. These metals provide useful models for ligated sites in the contraction domain. Interestingly, some of these contracted hemoglobins occur naturally as M hemoglobins [50] where the β chains contain a Fe(III) atom. In principle, all configurations in the contraction domain can be explored by suitable combinations of metal substitutions. If these substitutions do not alter the interactions within the macromolecule in a significant way, then the detailed energetics of global and local linkage effects can completely be mapped through this strategy. In the case of protein-DNA interactions contracted macromolecules can be obtained naturally or by site-directed mutagenesis as mutant forms that lack some of the binding sites, as illustrated by the phage λ repressor operator [26,51]. This idea can be pushed even further and extended to any system where 'perturbations' of a given binding site can be seen as a 'contraction' in the field manifold F^n leading to a particular configuration of σ.

The description of linkage effects in the local dimension, where the energetics of ligand binding is probed at the level of individual binding sites of the macromolecule, can be cast in terms of contracted partition functions. Each CPF depicts a particular contracted macromolecule in all its possible configurations, relative to a given configuration of the contraction domain. The use of CPFs leads to straightforward calculation of thermodynamic quantities such as site-specific Hill plots, partition coefficients and free energies of linkage, that share operationally useful aspects. The properties of these thermodynamic quantities in some

ways parallel those of analogous quantities defined in the global picture. However, site-specific quantities generally behave quite differently from their global counterparts, so that the parallel often breaks down. Of particular importance is the possibility of locally violating thermodynamic stability, which is a unique feature of site-specific binding phenomena. Negative binding capacities and site-specific Hill coefficients can only arise in the local picture as a result of the mutual interference of binding sites. Similar violations need not be restricted to chemical events. In fact, they can be predicted to occur also in the description of temperature and pressure effects as negative compressibilities and heat capacities [52], provided one could monitor these quantities at the level of single domains of multidomain macromolecules. Giant biochemical systems such as DNA or biological membranes are prototypic examples of multidomain structures. Local violations of thermodynamic stability in these systems could be used as important mechanisms of regulation. For example, a negative value of the binding capacity represents a local fluctuation, or instability, that can lead to a dynamical regime of bistability in biochemical systems working at steady state [53]. It is well known that thermodynamic instabilities are necessary prerequisites of biological complexity [54].

As we have seen, the local picture contains a metric isomorphic with the metric of the global picture, but it also contains thermodynamic effects of 'instability' that are totally absent in the global description. The second law tells us that at equilibrium thermodynamic stability must always hold for the system as a whole, but not necessarily for parts of the system taken separately. Indeed, an individual site can be seen as an open subsystem wherein thermodynamic stability may no longer apply due to a flow of energy from the outside generated by site-specific interactions. This brings out a direct connection between the theory developed here and the first two laws, from which it becomes evident that the thermodynamics of local linkage effects is by no means a special case of the global description. Rather, the mathematical structure of the global treatment is contained in that of the local description as a very special case. Linkage effects occurring locally, at specific sites of a biological macromolecule, are by far more intriguing than those involving the macromolecule as a whole. The theoretical treatment of these effects at equilibrium can be cast within a rigorous thermodynamic framework. The general implications in systems far from equilibrium hinge upon the possibility of modulating the dynamics of biochemical systems through the fluctuations of macroscopic quantities [53].

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