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The Meaning of the Decoupled Sites Representation in Terms of Statistical Mechanics and Stochastics

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Abstract

The investigation of thermodynamic properties of ligand binding is a classical field of (bio)chemistry and (bio)physics. Commonly, an algebraic description using polynomials (e.g. the binding polynomial) and rational functions (e.g. titration curves) is used to characterize systems of molecules and their ligand(s). However, the algebraic model is a result of the probabilistic setup of statistical mechanics and its concept of the Grand Canonical Partition Function. In this work, we reconsider the decoupled sites representation (DSR), a theoretical tool to regard an overall titration curve as sum of classical Henderson-Hasselbalch ligand binding curves from a stochastic point of view. Our work closes the circle from the initial stochastic model, to an algebraic description in which the DSR was developed and analyzed, back to its meaning in statistical mechanics and stochastics. In this regard, we translate results in the periphery of the DSR which were derived within the algebraic model into stochastics. The shifted point of view facilitates some proofs and physical interpretations and provides the basis for future work which might investigate how certain phenomenona of the algebraic concept can be interpreted stochastically.

1 Introduction

A classical field of chemistry is the investigation of pH-dependent binding of protons to different binding sites of a molecule in solution. In basic experiments a certain amount of protons (e.g. HCl) is added and the change of free protons ($\sim$pH) is measured. The number of protons which are absorbed by the solute is given by the difference of added protons and increase of free protons. Thus, the overall binding curve can be measured, which represents the average number of protons bound to a single solute molecule. From a stochastic point of view, a single molecule M with n binding sites can be interpreted as an n-tuple of Bernoulli variables $M = (X_1, ..., X_n)$ with $X_i \in \{0, 1\}$ indicating whether binding site i is occupied ($X_i = 1$) or not ($X_i = 0$). Its distribution on the set $\{0, 1\}^n$ depends on the proton activity $\Lambda = 10^{-pH}$. This pH-dependent distribution of M defines a pH-dependent distribution of the sum of occupied binding sites S ("overall binding")

$$S(X_1, ..., X_n) := \sum_{i=1}^{n} X_i \tag{1}$$

which takes values in $\{0, ..., n\}$. The overall proton binding of the whole population of a certain type of molecule is what can be measured by the previously described experiments. Assuming that the individual molecules bind protons stochastically independently, these rescaled overall binding curves of the whole population can be interpreted as the expected value of the pH-dependent distribution of S, according to the Law of Large Numbers. Thus, the overall titration curve $\Psi(\Lambda)$ is given by

$$\Psi(\Lambda) := \mathbb{E}_\Lambda S, \tag{2}$$

with $\mathbb{E}_\Lambda S$ denoting the expected value of S, depending on Λ ($\mathbb{E}_\Lambda$ is an operator acting on the adjacent variable). Analogously, the pH-dependent binding curve of an individual site i is given by

$$\Psi_i(\Lambda) := \mathbb{E}_\Lambda X_i. \tag{3}$$

For a molecule with only one binding site, $\Psi(\Lambda) = \Psi_1(\Lambda)$ is of shape

$$\Psi(\Lambda) = \frac{10^{pK_a}\Lambda}{10^{pK_a}\Lambda + 1} \tag{4}$$

where pK_a is the negative common logarithm of the activity of the ligand at which the expected value of the site being occupied is $\frac{1}{2}$. Binding curves of the structure described by Eq. (4) are called Henderson-Hasselbalch titration curves [1, 2]. For molecules with several binding sites, the overall titration curve is in general not of the shape presented in Eq. (4), since it is the sum of the binding curves of the individual sites (Eqs. (1),(2)). Additionally, the titration curves of the individual sites can also show huge deviations

from curves described by Eq. (4) which is a result of interaction between (or dependency of) the binding sites [3, 4, 5, 6]. Onufriev et al. [7] showed that for any given overall titration curve a hypothetical molecule exists, which is defined by the binding energies of its sites, such that each titration curve of an individual site is of shape given by Eq. (4) and all sum up to the given overall titration curve. This statement is called the decoupled sites representation (DSR).

In this work, we translate the DSR into stochastics. As natural first step, we show that decoupled sites in terms of the algebraic model (which means every binding site exhibits a standard Henderson-Hasselbalch binding curve) correspond to the stochastic independence of the Bernoulli variables describing the binding state of the individual sites, for every activity Λ.

Secondly, we reconsider the DSR for molecules with two different types of interacting binding sites [8, 9] –each type capable to bind only a certain type of ligand (L_1 or L_2, e.g. protons or electrons)– from a stochastic point of view. A result for molecules with two different types of ligands is that, if both overall titration curves (for both ligands) shall be preserved, only the binding sites for the same type of ligand can be decoupled [8, 9]. However, due to secondary interaction of the binding sites, the one-dimensional titration curves for fixed activity of the other ligand can deviate from the classical Henderson-Hasselbalch shape. We show that the decoupling with two types of ligands, presented by Martini et al. [8] corresponds to conditional stochastic independence of the Bernoulli variables describing the binding state of the individual sites for the same type of ligand. More precisely, the Bernoulli variables describing the binding sites for ligand L_1 are stochastically independent, if a microstate k_2 for ligand L_2 is fixed. This observation also shows that the two-dimensional titration curves of individual sites can be regarded as superposition of one-dimensional Henderson-Hasselbalch curves in a decoupled system.

2 The decoupled sites representation for one type of ligand

2.1 The biophysical setup

We summarize the theoretical algebraic setup of ligand binding and the decoupled sites representation (for more details on the DSR see [7] and [10]).

2.1.1 Molecules with several ligand binding sites

We consider a certain molecule M with n ligand binding sites and investigate the ligand-activity dependent average binding to the binding sites. To characterize the ligand binding properties of the molecule we use a model which incorporates binding constants and pair-

wise interaction constants. Thus, molecule M can be characterized by n binding constants $g_1^M, ..., g_n^M$ and $\frac{n(n-1)}{2}$ pairwise interaction constants $w_{1,2}^M, ..., w_{1,n}^M, ..., w_{n-1,n}^M$, where $w_{i,j}^M$ is the interaction constant of the i-th and j-th ligand binding sites. This model is not the most general one, since here it is assumed that the overall interaction of e.g. three binding sites is composed of three pairwise interactions which it not necessarily true. A more general model would incorporate interaction constants of higher order (e.g. $w_{1,2,3}^M$). Even though we are aware of this possible generalization, we use the simplified model since the results on which our work is based, such as the DSR, can be transfered directly to the more general model. For instance, for a molecule being decoupled means, in the simplified model, that all pairwise interaction constants are equal to one, which translates to a decoupled molecule in the generalized model by setting all additional interaction constants of higher order to one, too. Thus, the simpler model facilitates notation and is absolutely sufficient for our purposes. Moreover, this model –with only pairwise interaction– has already frequently been used in scientific literature (e.g. [4, 7, 10, 11]).
It is useful to allow $g_i, w_{i,j} \in \mathbb{C} \setminus \{0\} =: \mathbb{C}^* \ \forall i, j \in \{1, ..., n\}$. Thus, every molecule can be identified with at least one element

$$M \in {\mathbb{C}^*}^{\frac{n(n+1)}{2}}. \tag{5}$$

As the binding sites do not have any natural order, we use an equivalence relation $\sim$ to get a well defined mapping of a molecule to an equivalence class of tuples [10]. We use the notation

$$\mathbb{H} := \frac{{\mathbb{C}^*}^{\frac{n(n+1)}{2}}}{\sim}$$

for the set of molecules (equivalence classes of tuples).

2.1.2 The binding polynomial

To facilitate notation we use the following symbols:

- $k := (x_1^k, ..., x_n^k)$ denotes a realization of the random Bernoulli tuple $(X_1, ..., X_n)$. We use the term "microstate".
- $K := \{0, 1\}^n$ denotes the image set of $(X_1, ..., X_n)$, the set of all microstates.
- $S(k)$ denotes the realization of random variable S in Eq. (1) for microstate k.
- $g(k)$ denotes the probability constant of microstate k which is given by

$$g(k) := \prod_{i \in \{1,...,n\}} \left({g_i}^{x_i^k} \prod_{i<j} {w_{i,j}}^{x_i^k x_j^k} \right). \tag{6}$$

Eq. (6) is a result of the assumption that the energy of a state is the sum of the binding energies and pairwise interaction energies.

Using this notation, the binding polynomial (bp) in the variable Λ is defined by

$$\Phi(M) := \sum_{k \in K} g(k) \Lambda^{S(k)} \tag{7}$$

[3, 12, 13]. The bp of a molecule M characterizes the family of distributions of the random variable S (Eq. (1)) as function of the ligand activity Λ by

$$P_\Lambda(S = m) = \frac{\sum\limits_{\{k|S(k)=m\}} g(k) \Lambda^m}{\Phi(M)}$$

with $P_\Lambda(S = m)$ denoting the probability of S being equal to m at a certain ligand activity Λ (we will explain this in more detail in the next subsection). Note that the bp defines the overall titration curve (Eq. (2)) and that, since all microstates with the same number of bound ligand molecules are summarized, the titration curves of individual sites can not be regained from the bp of a molecule.

2.1.3 Titration curves of a certain site and of overall ligand binding

Let $M = (g_1, ..., g_n, w_{1,2}, ..., w_{n-1,n})$ be a molecule. Then the expected occupation of site i is given by

$$\Psi_i(\Lambda) = \frac{E_i(M)}{\Phi(M)} = \mathbb{E}_\Lambda X_i \tag{8}$$

with

$$E_i(M) = \sum_{\{k \in K | x_i^k = 1\}} g(k) \Lambda^{S(k)} \tag{9}$$

and $\Phi(M)$ denoting the bp of the molecule. Eq. (9) means that only those microstates in which site i is occupied, define the polynomial $E_i(M)$ [14, 7, 12, 13]. Consequently, the overall titration curve has the shape

$$\Psi(\Lambda) = \sum_{i=1}^{n} \Psi_i(\Lambda) = \frac{\sum_{i=1}^{n} E_i(M)}{\Phi(M)} = \sum_{i=1}^{n} \mathbb{E}_\Lambda X_i = \mathbb{E}_\Lambda S. \tag{10}$$

Proposition 1 shows how Eq. (10) can be rewritten.

Proposition 1 *Let M be a molecule with*

$$\Phi(M) = a_n \Lambda^n + a_{n-1} \Lambda^{n-1} + a_{n-2} \Lambda^{n-2} + ... + a_1 \Lambda + 1.$$

Then its overall titration curve is given by

$$\Psi(\Lambda) = \frac{na_n\Lambda^n + (n-1)a_{n-1}\Lambda^{n-1} + (n-2)a_{n-2}\Lambda^{n-2} + ... + a_1\Lambda}{\Phi(M)}. \tag{11}$$

A proof can be found in [10], an alternative proof within the stochastic setup is given below:

Proof. Use the stochastic setup, described in the next subsection to calculate the distribution of S and Eqs.(2), (10):

$$P_\Lambda(S=m) = \frac{\sum\limits_{\{k|S(k)=m\}} g(k)\Lambda^m}{\Phi(M)} = \frac{a_m\Lambda^m}{\Phi(M)}.$$

The titration curve is given by the expected value of S. □

With this framework we can express the DSR as a proposition (see [7, 10]):

Proposition 2 ***[The decoupled sites representation]***
Let $M = (g_1^M, ..., g_n^M, ..., w_{n-1,n}^M) \in \mathbb{H}$ be a molecule. Then a unique molecule $N = (g_1, ..., g_n, 1, ..., 1) \in \mathbb{H}$ exists, such that

$$\Phi(M) = \Phi(N).$$

Moreover, the entries of $(-\frac{1}{g_1}, ..., -\frac{1}{g_n})$ are the roots of $\Phi(M)$ (with multiplicity). (12)

2.2 A stochastic interpretation of this model

In the following, we investigate the stochastic features of the model presented in the previous subsection.

Remark 1 In the previously described setup, which our former work was based on [10, 8, 9], we allowed the binding and interaction constants to be complex valued. This makes the use of the fundamental theorem of algebra possible and facilitates theory. However, a complex valued binding constant of a certain binding site translates into a "complex probability measure". As a discussion of this phenomenon and of its possible physical interpretations is a separate topic, which we do not focus on in this work, we assume that all molecules which we use in this work have real binding and interaction constants. Moreover, instead of regarding equivalence classes we will focus on a map of tuples on measures. This is, in the following section, of advantage as otherwise the equivalence relation has also to be transfered to the image space of measures on K, which leads to a more complicated notation than necessary. However, all results can be directly transfered to the case with equivalence classes of tuples and of measures.

We define the map

$$P : \mathbb{R}^{+m} \longrightarrow \mathcal{L}(K)(\Lambda) \tag{13}$$

$$P(M)(\{k\}, \Lambda) = \frac{g(k)\Lambda^{S(k)}}{\Phi(M)} \tag{14}$$

which maps a tuple to a family of distributions on the set of microstates K which is parameterized by $\Lambda \in [0, \infty)$. $\mathcal{L}(K)(\Lambda)$ denotes the set of functions $f : [0, \infty) \longrightarrow \mathcal{L}(K)$ mapping the activity Λ onto a distribution on K.

Lemma 1 *The map P given by Eq. (14) mapping a tuple onto a family of measures is injective.*

Proof. Let M and N be two tuples and $P(M) = P(N)$. Then $P(M)(\{0\}^n, \Lambda) = P(N)(\{0\}^n, \Lambda)$ for every Λ. This implies that $\Phi(M) = \Phi(N)$. Since the measures of a state k with only one site occupied shall be identical for every value of Λ we receive $g_i^M = g_i^N$. Consequently, with the same argument for states with two sites occupied, this gives identical interaction constants of M and N. □

Since the map P is injective, we can use the letter M also for its image $P(M)$ (and M_Λ for $P(M)(\circ, \Lambda)$ with fixed Λ). Moreover, accordingly, we use the notation $M(\{k\})$ for $P(M)(\{k\})$ describing the probability of microstate k depending on Λ. Note that the definition given by Eq. (14) is the common interpretation of the probability of a certain state defined by the binding and interaction constants (for example see [15]). We will investigate which properties, the family of distributions of a tuple has. The random variables X_i will always denote the Bernoulli variables indicating whether site i is occupied $(X_i = 1)$ or not $(X_i = 0)$.

Lemma 2 *Let $M = (g_1, ..., g_n, 1, ..., 1)$ be a decoupled system. Then $\forall\, \Lambda \in [0, \infty)$*

$$M_\Lambda(X_i = 1) = \frac{g_i\Lambda}{g_i\Lambda + 1} \tag{15}$$

Proof. Let

$$M_{-i} := (g_1, ..., g_{i-1}, g_{i+1}, ..., g_n, 1, ..., 1)$$

denote the tuple with $n-1$ binding sites describing M as if site i was missing. Then,

$$M_\Lambda(X_i = 1) = \mathbb{E}_\Lambda X_i \overset{(8)}{=} \frac{E_i(M)}{\Phi(M)} \overset{(6),(9)}{=} \frac{g_i\Lambda\Phi(M_{-i})}{\Phi(M)} \overset{(12)}{=}$$

$$= \frac{g_i\Lambda \prod\limits_{k\neq i}(\Lambda + \frac{1}{g_k}) \prod\limits_{k\neq i} g_k}{\prod\limits_k (\Lambda + \frac{1}{g_k}) \prod\limits_k g_k} = \frac{\Lambda}{\Lambda + \frac{1}{g_i}} = \frac{g_i\Lambda}{g_i\Lambda + 1}.$$

□

Remark 2 Note that Eq. (15) also describes the Henderson-Hasselbalch titration curve, since for a Bernoulli variable the expected value equals the probability of being 1.

Proposition 3 *Let $M = (g_1, ..., g_n, 1, ..., 1)$ be a decoupled system, and let M_Λ be the corresponding family of measures on the power set of K. Let $\{X_i\}_{1 \le i \le n}$ be the Bernoulli variables which describe the occupation state of a certain site. Then $\forall \Lambda \in [0, \infty)$, $\{X_i\}_{1 \le i \le n}$ are stochastically independent.*

Proof. Let $m_1 + m_2 = n$ and let k_0 denote the corresponding microstate of the following event. Then

$$M_\Lambda(X_{i_1} = ... = X_{i_{m_1}} = 0, X_{j_1} = ... = X_{j_{m_2}} = 1) = M_\Lambda(\{k_0\}) =$$

$$\overset{(14)}{=} \frac{\left(\prod_{l=1}^{m_2} g_{j_l}\right) \Lambda^{m_2}}{\Phi(M)} = \frac{\prod_{l=1}^{m_2} (g_{j_l}\Lambda) \prod_{l=1}^{m_1} 1}{\Phi(M)} \overset{(12)}{=} \frac{\prod_{l=1}^{m_2} (g_{j_l}\Lambda) \prod_{l=1}^{m_1} 1}{\prod_{i=1}^{n} (g_i \Lambda + 1)} =$$

$$\overset{(15)}{=} \prod_{l=1}^{m_1} M_\Lambda(X_{i_l} = 0) \prod_{l=1}^{m_2} M_\Lambda(X_{j_l} = 1).$$

The Law of Total Probability shows that the probability can be factored also in the case of $m_1 + m_2 < n$. □

Proposition 3 shows that the natural intuition, saying that decoupled sites in the algebraic system correspond to stochastic independence of the Bernoulli variables in the stochastic setup, is correct. This gives the following view on the decoupled sites representation for one type of ligand: A molecule M with n binding sites for the ligand is given. It corresponds to a family of measures on $\{0, 1\}^n$ which is parametrized by the ligand activity Λ. We look for a family of product measures N on $\{0, 1\}^n$ such that $S(M) = S(N)$ for any choice of Λ. $S(M)$ denotes the distribution of the function S with respect to the measure M on the domain.

Thus, the question arises how a decoupled molecule with two different types of ligands can be interpreted stochastically, since we showed in a former work that not all interaction constants can be set to 1 and that the one-dimensional titration curves do not have to be of classical HH shape [8]. We will investigate this phenomenon in the next section.

3 The decoupled sites representation for two types of ligands

3.1 The biophysical setup

Analogously to the structure of the previous section we shortly summarize the theoretical setup of ligand binding for different types of ligands. For more details on the adapted equivalence relation and the DSR for two types of ligands see [8].

3.1.1 Molecules with several ligand binding sites for two different types of ligands

Analogously to subsection 2.1.1, we identify a molecule M with n_1 binding sites for ligand L_1 and n_2 ligand binding sites for ligand L_2 with an equivalence class of tuples. The entries of the tuple represent binding constants of the sites and pairwise interaction constants. To distinguish between sites for the different ligands we use the indices $1, ..., n_1$ for the binding sites for ligand L_1 and $A_1, ..., A_{n_2}$ for those for ligand L_2.

3.1.2 The binding polynomial

Again, $K = \{0,1\}^n$ denotes the set of all microstates, with $n := n_1 + n_2$, and $g(k)$ the probability constant of microstate k. However, we split S and k and extend the model to

- $S_1(k) := \sum_{i=1}^{n_1} x_i^k$, denoting the number of bound ligands L_1 in microstate k
- $S_2(k) := \sum_{i=A_1}^{A_{n_2}} x_i^k$, denoting the number of bound ligands L_2 in microstate k
- $k = (k_1, k_2) = (\underbrace{x_1^k, ..., x_{n_1}^k}_{=:k_1}, \underbrace{x_{A_1}^k, ..., x_{A_{n_2}}^k}_{=:k_2})$, denoting a microstate.

Thus, as generalization of the single ligand case, the binding polynomial can be written

$$\Phi(M) := \sum_{k \in K} g(k) \Lambda^{S_1(k)} \kappa^{S_2(k)} \tag{16}$$

with κ the activity of ligand L_2.

3.1.3 Titration curves of a certain site and of overall ligand binding

Let $M = (g_1, ..., g_{A_{n_2}}, w_{1,2}, ..., w_{A_{n_2-1}, A_{n_2}})$ be a molecule. Then the expected occupation of site i is given by

$$\Psi_i(\Lambda, \kappa) = \frac{E_i(M)}{\Phi(M)} = \mathbb{E}_{\Lambda,\kappa} X_i \tag{17}$$

with

$$E_i(M) = \sum_{\{k \in K | x_i^k = 1\}} g(k) \Lambda^{S_1(k)} \kappa^{S_2(k)} \tag{18}$$

and $\Phi(M)$ the bp of the molecule. The splitting of the number of all binding sites into two different groups leads to two overall titration curves of shape

$$\Psi_{L_1}(\Lambda, \kappa) = \sum_{i=1}^{n_1} \Psi_i(\Lambda, \kappa) = \frac{\sum_{i=1}^{n_1} E_i(M)}{\Phi(M)} = \mathbb{E}_{\Lambda,\kappa} S_1 \tag{19}$$

$$\Psi_{L_2}(\Lambda, \kappa) = \sum_{i=1}^{n_2} \Psi_{A_i}(\Lambda, \kappa) = \frac{\sum_{i=1}^{n_2} E_{A_i}(M)}{\Phi(M)} = \mathbb{E}_{\Lambda,\kappa} S_2 \tag{20}$$

The overall titration curves can be rewritten, analogously to Proposition 1.

Proposition 4 *Let M be a molecule with*

$$\Phi(M) = a_{n_1,n_2} \Lambda^{n_1} \kappa^{n_2} + a_{n_1,n_2-1} \Lambda^{n_1} \kappa^{n_2-1} + ...$$

$$... + a_{n_1,0} \Lambda^n + a_{n_1-1,n_2} \Lambda^{n_1-1} \kappa^{n_2} + ... + a_{0,1} \kappa + 1.$$

Then

$$\sum_{i=1}^{n_1} E_i(M) = n_1 \left(\sum_{i=0}^{n_2} a_{n_1,i} \kappa^i \right) \Lambda^{n_1} +$$

$$+ (n_1 - 1) \left(\sum_{i=0}^{n_2} a_{n_1-1,i} \kappa^i \right) \Lambda^{n_1-1} + ... + \left(\sum_{i=0}^{n_2} a_{1,i} \kappa^i \right) \Lambda \tag{21}$$

and

$$\sum_{i=1}^{n_2} E_{A_i}(M) = n_2 \left(\sum_{i=0}^{n_1} a_{i,n_2} \Lambda^i \right) \kappa^{n_2} +$$

$$+ (n_2 - 1) \left(\sum_{i=0}^{n_1} a_{i,n_2-1} \Lambda^i \right) \kappa^{n_2-1} + ... + \left(\sum_{i=0}^{n_1} a_{i,1} \Lambda^i \right) \kappa. \tag{22}$$

Eqs. (21) and (22) imply that the bp determines both overall titration curves.

Proof. Use the stochastic setup (Eq. (24)) to calculate the distribution of S_1 depending on Λ and κ and use Eq. (19). □

3.1.4 The decoupled sites representation for two types of ligands

Martini et al. [8] showed that in the case of two different types of ligands and the constraint that both overall titration curves of a molecule M shall be preserved, there is not in general a molecule N, in which all interaction constants are trivial and which possesses the same overall titration curves. Thus, we call a molecule with different types of ligands decoupled if all interaction constants of binding sites for the same type of ligand

are equal to one. We have already previously formulated the following conjecture which was proven for the cases of $(n_1, 1)$ and $(2, 2)$ binding sites [9, 8].

Conjecture 1 *Let*

$$M = (g_1^M, ..., g_{n_1}^M, g_{A_1}^M, ..., g_{A_{n_2}}^M, w_{1,2}^M, ..., w_{A_{n_2-1},A_{n_2}}^M)$$

be a molecule with n_1 binding sites for ligand L_1 and n_2 binding sites for ligand L_2. Then at least one molecule

$$N = (g_1, ..., g_{n_1}, g_{A_1}, ..., g_{A_{n_2}}, w_{1,2}, ..., w_{A_{n_2-1},A_{n_2}})$$

exists, with $\quad w_{i,j} = 1 \qquad \forall \{i,j\} \subset \{1, 2, ..., n_1\},$ *or* $\{i,j\} \subset \{A_1, A_2, ..., A_{n_2}\}$

and

$$\Phi(M) = \Phi(N).$$

The molecule N is called "decoupled".

Martini et al. [8] showed that even though in a decoupled molecule the binding sites for the same type of ligands do not interact, the one-dimensional titration curves, given when the activity of the second ligand is fixed, are not in general of HH shape. In the following we will investigate how this non-HH shape can be interpreted stochastically and where HH titration curves are hidden.

3.2 A stochastic interpretation of this model

All tuples in this section are assumed to have real valued, positive interaction and binding constants. Analogously to subsection 2.2, we consider the map

$$P : \mathbb{R}^{+m} \longrightarrow \mathcal{L}(K)(\Lambda, \kappa) \tag{23}$$

$$P(M)(\{k\}, \Lambda, \kappa) = \frac{g(k)\Lambda^{S_1(k)}\kappa^{S_2(k)}}{\Phi(M)} \tag{24}$$

We will again use the letter M as well for its image $P(M)$ to facilitate notation. Moreover, note that we defined the map P again on the set of tuples, without using the equivalence relation, to avoid transferring it to the set of measures. Using the equivalence relation would only lead to a more complicated notation. Before we prove some general statements, we will give an illustrating example.

Example 1 We choose a hypothetical decoupled molecule

$$M = (g_1, g_2, g_A, w_{1,2}, w_{1,A}, w_{2,A}) = (2, \frac{3}{2}, 2, 1, \frac{3}{2}, \frac{8}{3})$$

with two binding sites for ligand L_1 and one binding site for ligand L_2. These binding and interaction energies were chosen as example in which the polynomial and the probabilities of the states are easy to calculate. For a temperature of 300° Kelvin these constants translate to the binding and interaction energies of $-(1.7, 1.0, 1.7, 0, 1, 2.5)$ in kJ/mol, since $g_i = \exp(-\beta G_i)$ with $\beta = \frac{1}{RT}$ and T the absolute temperature in Kelvin and R the Boltzmann constant. We will not present the translation of constants into energies anymore, in the following examples. Thus, let X_1, X_2 be the Bernoulli variables describing the binding state of the sites 1 and 2. The binding polynomial of M is given by

$$\Phi(M) = 24\Lambda^2\kappa + 3\Lambda^2 + 14\Lambda\kappa + 3.5\Lambda + 2\kappa + 1.$$

Moreover,

$$M_{\Lambda,\kappa}(X_1 = 1) = \frac{24\Lambda^2\kappa + 3\Lambda^2 + 6\Lambda\kappa + 2\Lambda}{\Phi(M)} \tag{25}$$

$$M_{\Lambda,\kappa}(X_2 = 1) = \frac{24\Lambda^2\kappa + 3\Lambda^2 + 8\Lambda\kappa + 1.5\Lambda}{\Phi(M)} \tag{26}$$

$$M_{\Lambda,\kappa}(X_1 = 1, X_2 = 1) = \frac{24\Lambda^2\kappa + 3\Lambda^2}{\Phi(M)} \tag{27}$$

For the choice $(\Lambda, \kappa) = (1, 1)$ we receive

$$M_{1,1}(X_1 = 1) \cdot M_{1,1}(X_2 = 1) = \frac{35}{47.5} \cdot \frac{36.5}{47.5} \neq \frac{27}{47.5} = M_{1,1}(X_1 = 1, X_2 = 1) \tag{28}$$

which shows that the random variables X_1 and X_2 are not stochastically independent for all choices of (Λ, κ). The situation changes if we consider the conditional distribution on microstates $\{k \in K | k_2 = 0\}$ (distribution on the microstates in which site A is unoccupied) or on $\{k \in K | k_2 = 1\}$ (distribution on the microstates in which site A is occupied):
Let $M_{\Lambda,\kappa}(\cdot | k_2 = i)$ denote the conditional distribution on $\{k \in K | k_2 = i\}$. A conditional binding polynomial of M is given by

$$\Phi(M)_{|k_2=0} = 3\Lambda^2 + 3.5\Lambda + 1.$$

and thus

$$M_{\Lambda,\kappa}(X_1 = 1 | k_2 = 0) = \frac{3\Lambda^2 + 2\Lambda}{\Phi(M)_{|k_2=0}} \tag{29}$$

$$M_{\Lambda,\kappa}(X_2 = 1 | k_2 = 0) = \frac{3\Lambda^2 + 1.5\Lambda}{\Phi(M)_{|k_2=0}} \tag{30}$$

$$M_{\Lambda,\kappa}(X_1 = 1, X_2 = 1 | k_2 = 0) = \frac{3\Lambda^2}{\Phi(M)_{|k_2=0}} \tag{31}$$

which demonstrates independence of X_1 and X_2 with respect to the family of conditional

distributions for any choice of (Λ, κ):

$$M_{\Lambda,\kappa}(X_1 = 1|k_2 = 0) \cdot M_{\Lambda,\kappa}(X_2 = 1|k_2 = 0) = \frac{(3\Lambda^2 + 2\Lambda)(3\Lambda^2 + 1.5)}{\Phi(M)^2_{|k_2=0}} =$$

$$= \frac{3\Lambda^2\Phi(M)_{|k_2=0}}{\Phi(M)^2_{|k_2=0}} = \frac{3\Lambda^2}{\Phi(M)_{|k_2=0}} = M_{\Lambda,\kappa}(X_1 = 1, X_2 = 1|k_2 = 0). \tag{32}$$

This result might be obvious as in a decoupled system the one-dimensional titration curves of an individual site is of HH shape if the activity κ of the second ligand equals zero. However, conditional stochastic independence is also given if the condition is changed to $k_2 = 1$:

$$\Phi(M)_{|k_2=1} = 24\Lambda^2\kappa + 14\Lambda\kappa + 2\kappa.$$

$$M_{\Lambda,\kappa}(X_1 = 1|k_2 = 1) = \frac{24\Lambda^2\kappa + 6\Lambda\kappa}{\Phi(M)_{|k_2=1}} \tag{33}$$

$$M_{\Lambda,\kappa}(X_2 = 1|k_2 = 1) = \frac{24\Lambda^2\kappa + 8\Lambda\kappa}{\Phi(M)_{|k_2=1}} \tag{34}$$

$$M_{\Lambda,\kappa}(X_1 = 1, X_2 = 1|k_2 = 1) = \frac{24\Lambda^2\kappa}{\Phi(M)_{|k_2=1}} \tag{35}$$

which gives

$$M_{\Lambda,\kappa}(X_1 = 1|k_2 = 1) \cdot M_{\Lambda,\kappa}(X_2 = 1|k_2 = 1) = M_{\Lambda,\kappa}(X_1 = 1, X_2 = 1|k_2 = 1) \ \ \forall(\Lambda, \kappa) \in [0, \infty)^2.$$

We will formulate the observation of Example 1 generally in Proposition 5.

Proposition 5 *Let M be a decoupled molecule with n_1 binding sites for ligand L_1 and n_2 binding sites for ligand L_2. Then the random variables $\{X_i\}_{i=1}^{n_1}$ are conditionally stochastically independent for every condition $k_2 = c$ with $c \in \{0, 1\}^{n_2}$. Moreover, $\forall\,(\Lambda, \kappa) \in [0, \infty)^2$ and $1 \leq i \leq n_1$ a $g'_{c,i} \in \mathbb{R}^+$ exists such that*

$$M_{\Lambda,\kappa}(X_i = 1|k_2 = c) = \frac{g'_{c,i}\Lambda}{g'_{c,i}\Lambda + 1}. \tag{36}$$

Proof. Let $k_2 = c \in \{0, 1\}^{n_2}$ describe the state

$$X_{A_{\sigma(1)}} = ... = X_{A_{\sigma(l)}} = 1 \text{ and } X_{A_{\sigma(l+1)}} = ... = X_{A_{\sigma(n_2)}} = 0$$

with σ a permutation of $\{1, ..., n_2\}$ and $l \leq n_2$. Let $K_{|k_2=c} := \{k \in K|k_2 = c\}$ and let $k \in K_{|k_2=c}$. Then

$$g(k) = \prod_{i=1}^{l} g_{A_{\sigma(i)}} \cdot \prod_{j=1}^{n_1} \left(g_j \prod_{i=1}^{l} w_{j,A_{\sigma(i)}} \right)^{x_j^k} \tag{37}$$

and, due to the definition of conditional probability:

$$M_{\Lambda,\kappa}(X_i = 1|k_2 = c) = \frac{\sum\limits_{k\in K_{|k_2=c}, x_i^k=1} g(k)\Lambda^{S_1(k)}\kappa^l}{\sum\limits_{k\in K_{|k_2=c}} g(k)\Lambda^{S_1(k)}\kappa^l} =$$

$$\overset{(37)}{=} \frac{\sum\limits_{k\in K_{|k_2=c}, x_i^k=1} \left(\prod\limits_{j=1}^{n_1}\left(g_j \prod\limits_{i=1}^{l} w_{j,A_{\sigma(i)}}\right)^{x_j^k} \Lambda^{S_1(k)}\right)}{\sum\limits_{k\in K_{|k_2=c}} \left(\prod\limits_{j=1}^{n_1}\left(g_j \prod\limits_{i=1}^{l} w_{j,A_{\sigma(i)}}\right)^{x_j^k} \Lambda^{S_1(k)}\right)}$$

The last term equals the description of the titration curve of site i in a decoupled molecule with only n_1 binding sites for one type of ligand and binding constants

$$g'_{c,j} := g_j \prod_{i=1}^{l} w_{j,A_{\sigma(i)}}. \tag{38}$$

Eq. (38) proves the statements (and allows to calculate $g'_{c,i}$). □

Remark 3 In other words, Proposition 5 means that if we use the condition that the second ligand occupied its binding sites according to a fixed microstate, the complex of the molecule and the bound molecules of the second ligand can be regarded as a new molecule with different binding constants, but with independent sites. Note here that the condition necessarily has to be that strict (microstate of the second ligand). A relaxation is not possible, if independence of the sites for ligand L_1 shall be guaranteed.

Corollary 1 *The two-dimensional titration curve of a certain site of a decoupled molecule is a parameterized convex combination of one-dimensional HH curves.*

Proof.

$$M_{\Lambda,\kappa}(X_i = 1) = \sum_{c\in\{0,1\}^{n_2}} M_{\Lambda,\kappa}(X_i = 1|k_2 = c) M_{\Lambda,\kappa}(k_2 = c),$$

where $M_{\Lambda,\kappa}(X_i = 1|k_2 = c)$ is a HH curve, according to Proposition 5, and

$$\sum_{c\in\{0,1\}^{n_2}} M_{\Lambda,\kappa}(k_2 = c) = 1.$$

□

We will illustrate the statement of Corollary 1 with an example.

Example 2 *Let* $M = (g_1, g_2, g_A, w_{1,2}, w_{1,A}, w_{2,A}) = (2, \frac{3}{2}, 2, 1, \frac{3}{2}, \frac{8}{3})$ *be the decoupled molecule of Example 1. Then*

$$M_{\Lambda,\kappa}(X_1 = 1) =$$

$$= M_{\Lambda,\kappa}(X_1 = 1|k_2 = 0)M_{\Lambda,\kappa}(k_2 = 0) + M_{\Lambda,\kappa}(X_1 = 1|k_2 = 1)M_{\Lambda,\kappa}(k_2 = 1). \qquad (39)$$

Note that, since in this example, there is only one binding site for ligand L_2, $k_2 \in \{0,1\}$ and thus $M_{\Lambda,\kappa}(k_2 = 1) = M_{\Lambda,\kappa}(X_A = 1)$ is the titration curve of site A and $M_{\Lambda,\kappa}(k_2 = 0) = 1 - M_{\Lambda,\kappa}(k_2 = 1)$. Thus, the titration curve of site 1 is a convex combination of two HH curves weighted by the curve of site A. To calculate (39) we need to know the distribution of X_A:

$$M_{\Lambda,\kappa}(X_A = 0) = \frac{\overbrace{3\Lambda^2 + 3.5\Lambda + 1}^{=\Phi(M)_{|k_2=0}}}{\Phi(M)} \qquad M_{\Lambda,\kappa}(X_A = 1) = \frac{\overbrace{24\Lambda^2\kappa + 14\Lambda\kappa + 2\kappa}^{=\Phi(M)_{|k_2=1}}}{\Phi(M)} \qquad (40)$$

and consequently

$$M_{\Lambda,\kappa}(X_1 = 1) = \frac{3\Lambda^2 + 2\Lambda}{\Phi(M)_{|k_2=0}}\frac{\Phi(M)_{|k_2=0}}{\Phi(M)} + \frac{24\Lambda^2\kappa + 6\Lambda\kappa}{\Phi(M)_{|k_2=1}}\frac{\Phi(M)_{|k_2=1}}{\Phi(M)} =$$

$$= \frac{24\Lambda^2\kappa + 3\Lambda^2 + 6\Lambda\kappa + 2\Lambda}{\Phi(M)} = (25).$$

The curves $M_{\Lambda,\kappa}(X_1 = 1|k_2 = 0)$, $M_{\Lambda,\kappa}(X_1 = 1|k_2 = 1)$, $M_{\Lambda,\kappa}(k_2 = 1)$ as well as the convex combination $M_{\Lambda,\kappa}(X_1 = 1)$ are illustrated in Fig 1.

The previous results draw the following picture: The algebraic decoupling of molecules with two different types of ligands described by Martini et al. [8] corresponds to finding a conditionally stochastically independent system with the same overall titration curves. In detail, this means that, for a given family of measures M on $\{0,1\}^{n_1+n_2}$, we look for a family of measures N such that all conditional measures $N_{|k_2}$ are product measures on $\{0,1\}^{n_1}$ for every $k_2 \in K_2$, $N_{|k_1}$ are product measures for every $k_1 \in K_1$ and $S_1(M) = S_1(N), S_2(M) = S_2(N)$. Compared to the setup with only one type of ligand we have an additional constraint since the function S was split into two parts. This constraint makes it impossible to find a family of product measures on $\{0,1\}^{n_1+n_2}$ for any given M. Consequently, the constraint of being a product measure is relaxed. However, the weakening of the constraints leads to the existence of several different solutions. A naturally arising question is which features the different distributions of different decoupled molecules share. We will compare the decoupled molecule of Examples 1, 2 with the other decoupled molecule sharing the same overall titration curves.

Example 3 Let $M = (g_1, g_2, g_A, w_{1,2}, w_{1,A}, w_{2,A}) = (2, \frac{3}{2}, 2, 1, \frac{3}{2}, \frac{8}{3})$ be the molecule of Example 1 and let $N = (2, \frac{3}{2}, 2, 1, 2, 2)$ be the second decoupled molecule with the same bp (the maximal number of decoupled systems is 2, except for permutations, compare

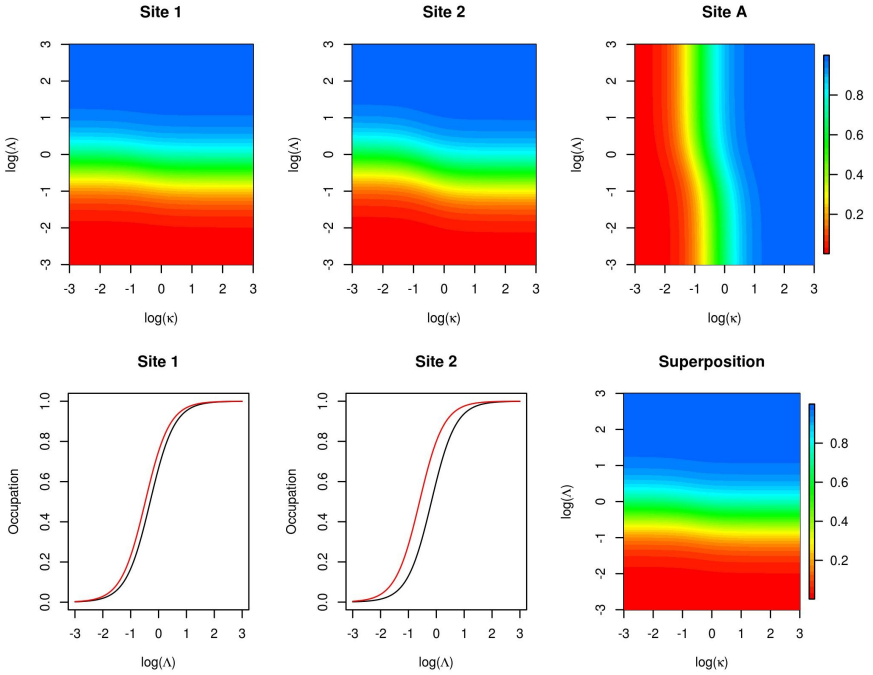

Figure 1: First row: Activity dependent ligand binding to each site of the tuple M of Example 2. Logarithmic scales of the activities of the ligands are used. The probability of a site being occupied is encoded by colors, according to the color bar on the right side of the figure. Second row: Conditional binding curves of site 1 and 2 for the conditions "site A is unoccupied" (black line) and "site A is occupied" (red line). The superposition represents the titration curve of site 1, however was calculated using the conditional HH curves and the titration curve of site A: $M(X_1 = 1) = M(X_1 = 1|k_2 = 0)M(k_2 = 0) + M(X_1 = 1|k_2 = 1)M(k_2 = 1)$.

[8]). Then

$$N_{\Lambda,\kappa}(X_i = 1|k_2 = 0) = M_{\Lambda,\kappa}(X_i = 1|k_2 = 0),\ i = 1, 2$$

$$N_{\Lambda,\kappa}(X_1 = 1|k_2 = 1) = M_{\Lambda,\kappa}(X_2 = 1|k_2 = 1)$$

and

$$N_{\Lambda,\kappa}(X_2 = 1|k_2 = 1) = M_{\Lambda,\kappa}(X_1 = 1|k_2 = 1)$$

These equations mean that the decoupled molecules M and N share the same conditional HH titration curves. However they are permuted in the case of $k_2 = 1$.

We can formulate this observation as proposition.

Proposition 6 *Let M and N be two different decoupled molecules with n_1 binding sites for ligand L_1 and n_2 binding sites for ligand L_2 sharing the same binding polynomial. Moreover, let the order of the binding sites be equal, that is $g_i^M = g_i^N\ \forall i \in \{1, .., ., A_{n_2}\}$. Then the following statements hold:*

a) $N_{\Lambda,\kappa}(X_i = 1|k_2 = \{0\}^{n_2}) = M_{\Lambda,\kappa}(X_i = 1|k_2 = \{0\}^{n_2}) \quad \forall \Lambda, \kappa \in [0, \infty)$

b) A permutation σ of $\{1, ..., n_1\}$ exists such that

$$N^N_{\Lambda,\kappa}(X_i = 1|k_2 = \{1\}^{n_2}) = M_{\Lambda,\kappa}(X_{\sigma(i)} = 1|k_2 = \{1\}^{n_2})$$

Proof.

a) According to Eq. (38) $g'_{i,c} = g_i$, and $g_i^M = g_i^N$ since the order of the sites is assumed to be fixed.

b) Again, Eq. (38) proves the statement, since the products

$$g_j \prod_{i=1}^{n_2} w_{j,A_{\sigma(i)}}$$

have to solve a subsystem of equations given by the coefficients a_{i,n_2} of the bp and consequently are permutations of each other, since they correspond to the roots of a polynomial (compare [9], Proposition 3).

□

Remark 4 Proposition 6 b) states that every family of conditional stochastic independent measures, that is every decoupled molecule, corresponds to a permutation of the same set of conditioned HH curves (conditioned on the sites for ligand L_2 being fully occupied). If every permutation was realizable this would give at least $n_1!$ different molecules. As the same is true for the sites of ligand L_1 being fully occupied this adds the factor $n_2!$.

Since a molecule is not fully described only by the conditional curves of these "extreme" conditions, the number of decoupled molecules belonging to the same bp should be higher than $n_1!n_2!$. This observation is contrary to a former conjecture by Martini et al. [9] in which we supposed that the maximal number of molecules is equal to $n_1!n_2!$. We checked this conjecture intensively and saw that an additional factor is required.

To complete our illustrations, we give a final example of a system with two binding sites for each ligand. This example shows, how to deal with the weights of the superposition in the case of more than one binding site for both ligands.

Example 4 Let the decoupled tuple $M = (g_1, g_2, g_A, g_B, w_{1,2}, w_{1,A}, w_{1,B}, w_{2,A}, w_{2,B}, w_{A,B})$ $= (2, 16, 4, 8, 1, 8, 4, \frac{1}{16}, \frac{1}{32}, 1)$ be given. Its binding polynomial is

$$\Phi(M) = 64\Lambda^2\kappa^2 + 96\Lambda^2\kappa + 32\Lambda^2 + 2049\Lambda\kappa^2 + 136\Lambda\kappa + 18\Lambda + 32\kappa^2 + 12\kappa + 1$$

We calculate the conditional HH curves of site 1, exemplarily.

$$M(X_1 = 1|k_2 = (0,0)) = \frac{32\Lambda^2 + 2\Lambda}{32\Lambda^2 + 18\Lambda + 1} = \frac{2\Lambda}{2\Lambda + 1} \tag{41}$$

$$M(X_1 = 1|k_2 = (0,1)) = \frac{32\Lambda^2\kappa + 64\Lambda\kappa}{32\Lambda^2\kappa + 68\Lambda\kappa +} = \frac{4\Lambda^2 + 8\Lambda}{4\Lambda^2 + 8.5\Lambda + 1} = \frac{8\Lambda}{8\Lambda + 1} \tag{42}$$

$$M(X_1 = 1|k_2 = (1,0)) = \frac{64\Lambda^2\kappa + 64\Lambda\kappa}{64\Lambda^2\kappa + 68\Lambda\kappa + 4\kappa} = \frac{16\Lambda^2 + 16\Lambda}{16\Lambda^2 + 17\Lambda + 1} = \frac{16\Lambda}{16\Lambda + 1} \tag{43}$$

$$M(X_1 = 1|k_2 = (1,1)) = \frac{64\Lambda^2\kappa^2 + 2048\Lambda\kappa^2}{64\Lambda^2\kappa^2 + 2049\Lambda\kappa^2 + 32\kappa^2} = \frac{64\Lambda}{64\Lambda + 1} \tag{44}$$

The corresponding weights for the superposition are given by:

$$M(k_2 = (0,0)) = \frac{32\Lambda^2 + 18\Lambda + 1}{\Phi(M)} \tag{45}$$

$$M(k_2 = (0,1)) = \frac{32\Lambda^2\kappa + 68\Lambda\kappa + 8\kappa}{\Phi(M)} \tag{46}$$

$$M(k_2 = (1,0)) = \frac{64\Lambda^2\kappa + 68\Lambda\kappa + 4\kappa}{\Phi(M)} \tag{47}$$

$$M(k_2 = (1,1)) = \frac{64\Lambda^2\kappa^2 + 2049\Lambda\kappa^2 + 32\kappa^2}{\Phi(M)} \tag{48}$$

Thus, we receive the following representation of $M(X_1 = 1)$ with Eqs. $(41 - 48)$:

$$M(X_1 = 1) = (41) \cdot (45) + (42) \cdot (46) + (43) \cdot (47) + (44) \cdot (48) \tag{49}$$

The binding curves of the individual sites as well as the HH curves and the corresponding weights are illustrated in Fig. 2.

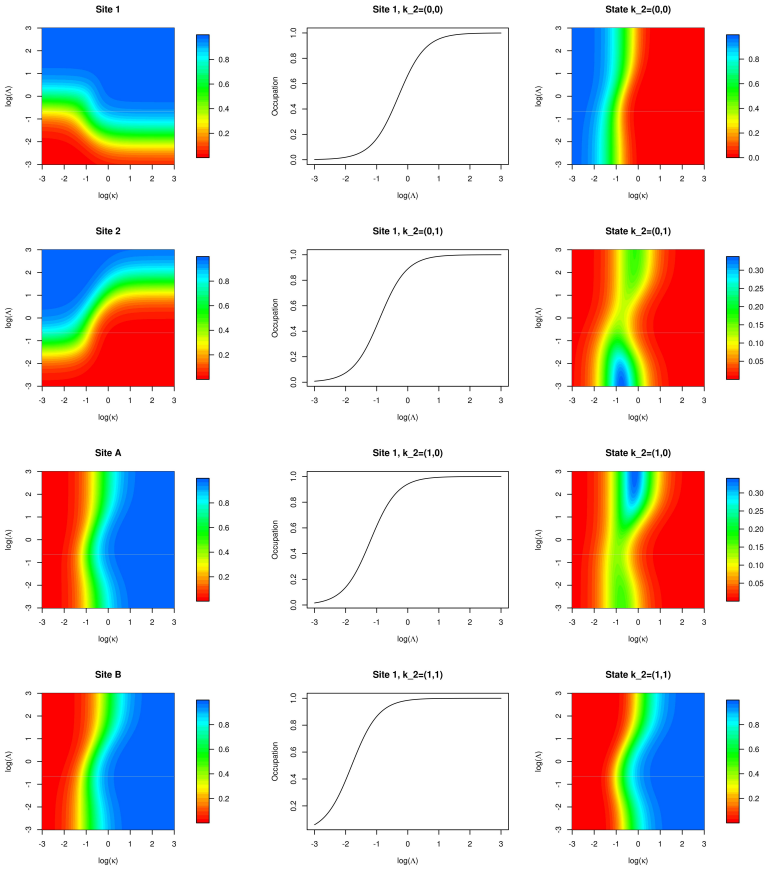

Figure 2: First column: Activity dependent ligand binding to each site of the tuple M of Example 4 . Logarithmic scales of the activities of the ligands are used. The probability of a site being occupied is encoded by colors, according to the color bar on the right side of each image. Second column: Conditional binding curves of site 1 for the different microstates of ligand L_2 ($k_2 \in \{(0,0),(0,1),(1,0),(1,1)\}$). Third column: probabilities of the different microstates of the binding sites for the second ligand.

Remark 5 In a decoupled molecule M with only one type of ligand, the following calculation rule for the binding constants of a microstate $k = (x_1^k, ..., x_n^k)$ is a direct result of stochastic independence of the binding sites:

$$M_\Lambda(k) = M_\Lambda(x_1^k, ..., x_n^k) = \prod_{i=1}^{n} \left(M_\Lambda(X_i = 1)^{x_i^k} \cdot M_\Lambda(X_i = 0)^{1-x_i^k} \right). \tag{50}$$

The sum of all probabilities of microstates belonging to the same macrostate gives the probability of the macrostate

$$M_\Lambda(S(k) = i) = \sum_{\{k|S(k)=i\}} M_\Lambda(k) = \frac{a_i \Lambda^i}{\Phi(M)} \tag{51}$$

which the decoupled molecule obviously shares with every molecule with the same binding polynomial, since the coefficient a_i of the polynomial as well as the polynomial $\Phi(M)$ are identical (the coefficient a_i is given by the sum of all constants of the microstates with macrostate i). These equations can be transfered to the case of a decoupled molecule N binding two ligands. For a microstate $k = (k_1, k_2) = (x_1^k, ..., x_{n_1}^k, ..., x_{A_{n_2}}^k)$, Eqs. (50- 51) translate to:

$$N_{\Lambda,\kappa}(k) = N_{\Lambda,\kappa}(x_1^k, ..., x_{A_{n_2}}^k) = N_{\Lambda,\kappa}(k_2) \prod_{i=1}^{n_1} \left(N_{\Lambda,\kappa}(X_i = 1|k_2)^{x_i^k} \cdot N_{\Lambda,\kappa}(X_i = 0|k_2)^{1-x_i^k} \right) \tag{52}$$

and

$$N_{\Lambda,\kappa}(S_1(k) = i, S_2(k) = j) = \sum_{\{k|S(k)=(i,j)\}} N_{\Lambda,\kappa}(k) = \frac{a_{i,j} \Lambda^i \kappa^j}{\Phi(M)} \tag{53}$$

Due to the symmetric role of the two ligands Eq. (52) can also be rewritten with k_1 instead of k_2 (and $i \in \{A_1, ..., A_{n_2}\}$). The coefficient $a_{i,j}$ is given by the sum of all constants of microstates belonging to macrostate (i, j).

4 Summary

In this work, we pulled back the DSR from the algebraic description of ligand binding – in which it was developed – to stochastics and statistical mechanics which are the basis of the algebraic setup. In this regard, we translated the DSR for one type of ligand from algebraic equations into stochastics and showed that for a molecule being decoupled means that the binding to the individual sites is stochastically independent for every activity of the ligand. Furthermore, we showed that the DSR for two types of ligands corresponds to conditional stochastic independence of the binding sites for one type of ligand if a microstate of the

second ligand is fixed. This view implies that the two-dimensional titration curves can be regarded as superposition of Henderson-Hasselbalch curves in a decoupled molecule. The weights are given by the marginal distribution of the microstates of the second ligand binding sites. The stochastic point of view helps to understand what the DSR for two types of ligands means and which restrictions lead to the loss of uniqueness, compared to the DSR for one type of ligand. Moreover, the secondary interaction in decoupled molecules illustrates that –in any molecule– the absence of direct interaction of a pair of binding sites (for the same ligand or for different ligands) –e.g. due to a great distance between them– is not sufficient for stochastic independence of their occupation. This observation also implies that the interaction of two sites for the same type of ligand can be different if other ligands are available in the environment. Regulatory networks of certain processes in biological systems might also take advantage of this mechanisms, in which a second ligand does not have to play an active role, but might only work as a transmitter of interaction. Future work should investigate how decoupled molecules can also facilitate the understanding of the binding behavior of ligands to molecules with interacting binding sites.

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