J Math Chem (2013) 51:672–695
DOI 10.1007/s10910-012-0107-6

ORIGINAL PAPER

On the interaction of two different types of ligands binding to the same molecule part I: basics and the transfer of the decoupled sites representation to systems with n and one binding sites

Johannes W. R. Martini · Martin Schlather · G. Matthias Ullmann

Received: 7 October 2012 / Accepted: 18 October 2012 / Published online: 8 November 2012

Abstract The decoupled sites representation (DSR) for one type of ligand allows to regard complex overall titration curves as sum of classical Henderson-Hasselbalch (HH) titration curves. In this work we transfer this theoretical approach to molecules with different types of interacting ligands (e.g. protons and electrons), prove the existence of decoupled systems for n_1 and one binding sites for two different ligands, and point out some difficulties and limits of this transfer. A major difference to the DSR for one type of ligand is the loss of uniqueness of the decoupled system. However, all decoupled systems share a unique set of microstate probabilities and each decoupled system corresponds to a certain permutation of these microstate probabilities. Moreover, we show that the titration curve of a certain binding site in the original system can be regarded as linear combination of the titration curves of the individual sites of the decoupled system if the weights of the linear combination are substituted by functions in the activity of the second ligand. In the underlying model with only pairwise interaction, an important observation of our theoretical investigation is the following: Even though the binding sites of ligand L_1 may not interact directly, they can show secondary interaction due to the interaction with the second type of ligand. This means, if the activity of the second ligand is fixed and we regard the 1-dimensional titration

J. W. R. Martini (✉)
Institut für Mathematische Stochastik, Georg-August Universität Göttingen, Göttingen, Germany
e-mail: jmartin2@gwdg.de

M. Schlather
Institut für Mathematik, Universität Mannheim, Mannheim, Germany
e-mail: schlather@math.uni-mannheim.de

G. M. Ullmann
Bioinformatik/Strukturbiologie, Universität Bayreuth, Bayreuth, Germany
e-mail: Matthias.Ullmann@uni-bayreuth.de

curve of an individual binding site for ligand L_1 depending on its activity, we may observe a strong deviation from the classical HH shape in spite of non-interacting sites for ligand L_1.

Keywords Decoupled sites representation · Protonation · Electron binding · Different ligands · Binding polynomial · Interaction energy · Binding energy · Transport · Transfer · Photosynthesis · Receptor

1 Introduction

Let us regard a chemical substance M dissolved in a solution containing another solute L (the ligand). If L is able to bind reversibly to one special binding site of substance M, the average binding of ligand L to the binding site in equilibrium will be described by the rational function

$$\langle X \rangle = \frac{a\Lambda}{a\Lambda + 1}, \tag{1}$$

where Λ denotes the ligand activity and a is a constant, depending on the temperature (which is assumed constant) and the binding energy of this binding site. Equation (1) represents the classical sigmoid Henderson-Hasselbalch (HH) titration curve [10,11]. Mathematically more challenging is the description of overall titration curves as well as titration curves of individual sites of molecules with several interacting binding sites. Interaction can lead to strong deviations from classical HH curves [1,2,4,5,17]. One step towards understanding the nature of these altered titration curves was the decoupled sites representation which showed that for any molecule with interaction between its binding sites, there exists a hypothetical molecule without interaction exhibiting the same overall titration behaviour [14,15,12]. In this work, we transfer the decoupled sites representation to molecules with two different types of ligands e.g. protons and electrons. This problem is of biophysical interest as proteins involved in electron transfer are often located in membranes with pH-gradient. Moreover, it can be applied to any other transporter molecule with proton binding sites or receptors with different types of ligands which are frequently a subject of investigation [3,8,9,13,18,19]. Also from a mathematical point of view this transfer is not trivial as one has to deal with polynomials of the polynomial ring in two variables $\mathbb{C}[\Lambda, \kappa]$. In the following we will summarize the mathematical basics of ligand binding in equilibrium where we use a model in which every microstate energy is the sum of binding and pairwise interaction energies. This model can be generalized by allowing any state energy without using this additive structure, which corresponds to an extended model incorporating additional interaction terms of three and more binding sites. Even though we did not write down this extended model explicitly, our main results are true for the extended model as well, as in the decoupled systems we can set all additional interaction terms to zero which leads to the same systems of equations as in the presented model.

2 One type of ligand

2.1 The model

Let M be a molecule with n ligand binding sites. Then the ligand binding properties of M in equilibrium can be described by an $\frac{n(n+1)}{2}$-tuple

$$M = (g_1^M, \ldots, g_n^M, w_{1,2}^M, \ldots, w_{n-1,n}^M) \in \mathbb{C}^{*m}, \tag{2}$$

where $\mathbb{C}^* = \mathbb{C} \setminus \{0\}$ and $m := \frac{n(n+1)}{2}$. We call its entries "binding constants" and "interaction constants", which are given by

$$g_i^M := e^{-\beta G_i^M}$$

and

$$w_{i,j}^M := e^{-\beta W_{i,j}^M}$$

where G_i^M denotes the binding energy of site i, $W_{i,j}^M$ the interaction energy of sites i and j, and β a constant depending on the temperature (which is assumed constant). For the sake of uniqueness of the corresponding tuple we use an equivalence relation "$\sim$" on the set of energies defined by Definition 1 (compare [12]).

Definition 1 Let

$$A = (g_1^A, g_2^A, \ldots g_n^A, \ldots w_{n-1,n}^A)$$

and

$$B = (g_1^B, g_2^B, \ldots g_n^B, \ldots w_{n-1,n}^B) \in \mathbb{C}^{*m}.$$

Then A is equivalent to B (Notation: $A \sim B$) if and only if a permutation σ of $(1, \ldots, n)$ exists such that

$$A = (g_{\sigma(1)}^B, g_{\sigma(2)}^B, \ldots g_{\sigma(n)}^B, w_{\sigma(1),\sigma(2)}^B, w_{\sigma(1),\sigma(3)}^B, \ldots, w_{\sigma(n-1),\sigma(n)}^B). \tag{3}$$

Thus, Eq. (2) can be changed to

$$M \in {}^{\mathbb{C}^{*m}}\!/\!_{\sim} =: \mathbb{H}. \tag{4}$$

We call an element $M \in \mathbb{H}$ a molecule and use a tuple as representative for the notation. For the definition of a map which maps M to a rational function in the ligand activity, describing the average ligand binding of a certain site in equilibrium, the use

of microstates facilitates notation. A microstate k is an n-tuple illustrating the binding state of an individual molecule

$$k = (x_1^k, \ldots, x_n^k) \qquad x_i^k \in \{0, 1\} \ \forall i \in \{1, \ldots, n\}$$

and

$$x_i^k = 1 \iff \text{in microstate } k, \text{ a ligand is bound to site } i.$$

We use the notation K for the set of all microstates k. Obviously, the number of all possible microstates is $\#K = 2^n$, if a binding site can only exist in two possible states: occupied and unoccupied. In the underlying model with only pairwise interaction, we call

$$g(k) := \left(\prod_{i=1}^{n} \left(g_i^{x_i^k} \prod_{j=i+1}^{n} w_{i,j}^{x_i^k x_j^k} \right) \right) \tag{5}$$

the microstate constant of microstate k which equals $e^{(-\beta G(k))}$ with $G(k)$ the energy of microstate k. Thus, the binding polynomial (bp) in the ligand activity Λ of molecule M (which determines the overall titration) writes

$$P_M(\Lambda) = \sum_{k \in K} g(k) \Lambda^{l(k)} \tag{6}$$

with

$$l(k) := \sum_{i=1}^{n} x_i^k$$

[5,16,20]. Note, that the map $M \mapsto P_M(\Lambda)$ is well-defined as

$$M \sim N \Rightarrow P_M(\Lambda) = P_N(\Lambda).$$

In the following, we will have a closer look on the rational functions describing the titration of a certain site and the overall titration curve. The average ligand binding at site r, dependent on the ligand activity, is given by

$$\langle x_r \rangle = \frac{\sum_{k \in K, x_r^k = 1} g(k) \Lambda^{l(k)}}{\sum_{k \in K} g(k) \Lambda^{l(k)}} =: \frac{E_M^r(\Lambda)}{P_M(\Lambda)}. \tag{7}$$

Equation (7) means that the polynomial in the numerator only consists of summands given by microstates in which site r is occupied. Thus, the overall titration curve, which only depends on the bp, is given by

$$\langle X \rangle = \frac{\sum_{r=1}^{n} E_M^r(\Lambda)}{P_M(\Lambda)} \tag{8}$$

[5,16,20]. To distinguish between overall titration curves and titration curves of individual sites we use a capital letter for the overall curve.

2.2 The decoupled sites representation

In this context, the decoupled sites representation can be expressed as proposition.

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

$$P_M(\Lambda) = P_L(\Lambda).$$

Moreover, the energies $g_i^L,\ i \in \{1, \ldots, n\}$ *of the decoupled systems are given by*

$$g_i = -\frac{1}{\Lambda_i}$$

with $(\Lambda_1, \ldots, \Lambda_n)$ *denoting any permutation of the roots of the bp.*

Proof For the proof of Proposition 1 see [12]. □

The aim of this work is to transfer the presented theory including the DSR to a situation with two different types of ligands. For this purpose the following corollary of the DSR will turn out to be a very useful tool.

Corollary 1 *Let V be the affine algebraic variety defined by the polynomials*

$$a_n = \prod_{j=1}^{n} y_j$$

$$a_{n-1} = \sum_{i=1}^{n} \left(\prod_{j=1, j\neq i}^{n} y_j \right)$$

$$a_{n-2} = \sum_{\{(i,k)|i<k\}} \left(\prod_{j=1, i\neq j\neq k}^{n} y_j \right)$$

$$\vdots$$

$$a_1 = \sum_{j=1}^{n} y_j$$

in $\mathbb{C}[y_1, \ldots, y_n]$. *Then* $dim(V) = 0$ *and V is the set of all permutations of* $(-\frac{1}{\Lambda_1}, \ldots, -\frac{1}{\Lambda_n})$ *with* Λ_i *denoting the roots of*

$$a_n \Lambda^n + a_{n-1} \Lambda^{n-1} + \cdots + a_1 \Lambda + 1.$$

Proof The energies g_i of a decoupled system are the elements of V. Thus, the statement of Corollary 1 is a consequence of the DSR (or of Vieta's formulas). □

3 The model for two different types of ligands

At first, we transfer the setup described in Sect. 2 to the situation of two different types of ligands. We assume that the ligands do not share binding sites, which means that there are two disjunct sets of binding sites which can only be occupied by one type of ligand. Analogously to Eqs. (2)–(8) we receive the following framework: The equilibrium binding properties of a molecule M with n_1 binding sites for ligand L_1 (sites $1, 2, \ldots, n_1$) and n_2 binding sites for ligand L_2 (sites $A_1, A_2, \ldots, A_{n_2}$) are described by an $m := \frac{(n_1+n_2)(n_1+n_2+1)}{2}$-tuple

$$\begin{aligned}&\left(g_1^M, \ldots, g_{n_1}^M, g_{A_1}^M, \ldots, g_{A_{n_2}}^M, w_{1,2}^M, \ldots, w_{1,A_{n_2}}^M, \ldots, w_{A_1,A_2}^M, \ldots, w_{A_{n_2-1},A_{n_2}}^M\right)\\ &= M \in {}^{\mathbb{C}^{*m}}\!/\!_{\sim}.\end{aligned} \tag{9}$$

However, the equivalence relation of Definition 1 has to be adapted.

Definition 2 Let $\mathbb{C}^{*m}$ with $m = \frac{(n_1+n_2)(n_1+n_2+1)}{2}$ be the set of all tuples describing molecules with n_1 binding sites for ligand L_1 and n_2 binding sites for ligand L_2. Moreover, let

$$M = \left(g_1^M, \ldots, g_{n_1}^M, g_{A_1}^M, \ldots, g_{A_{n_2}}^M, w_{1,2}^M, \ldots, w_{1,A_{n_2}}^M, \ldots, w_{A_{n_2-1},A_{n_2}}^M\right)$$

and

$$N = \left(g_1^N, \ldots, g_{n_1}^N, g_{A_1}^N, \ldots, g_{A_{n_2}}^N, w_{1,2}^N, \ldots, w_{1,A_{n_2}}^N, \ldots, w_{A_{n_2-1},A_{n_2}}^N\right).$$

Then M is equivalent to N (Notation: $M \sim N$) if and only if two permutations σ_1 of $(1, \ldots, n_1)$ and σ_2 of $(1, \ldots, n_2)$ exist such that

$$M = \left(g_{\sigma_1(1)}^N, \ldots, g_{A_{\sigma_2(n_2)}}^N, w_{\sigma_1(1),\sigma_1(2)}^N, \ldots, w_{\sigma_1(1),A_{\sigma_2(n_2)}}^N, \ldots, w_{A_{\sigma_2(n_2-1)},A_{\sigma_2(n_2)}}^N\right). \tag{10}$$

To simplify notation we will henceforth write g_i for g_i^M if it is clear to which molecule the binding constant belongs to. Moreover, we use the microstate notation and

$${}^{\mathbb{C}^{*m}}\!/\!_{\sim} =: \mathbb{G}_{n_1,n_2}$$

with "$\sim$" according to Definition 2. Analogously to Eq. (6) we define the binding polynomial in the ligand activities Λ and κ of a molecule M with n_1 binding sites for ligand L_1 and n_2 binding sites for ligand L_2 by

$$P_M(\Lambda,\kappa)=\sum_{k\in K} g(k)\Lambda^{l_1(k)}\kappa^{l_2(k)} \tag{11}$$

with $l_1(k) := \sum_{i=1}^{n_1} x_i^k$ and $l_2(k) := \sum_{i=n_1+1}^{n_1+n_2} x_i^k$ denoting the number of bound ligands of both types and $g(k)$ again the microstate constant of state k. The average amount of bound ligand to site r in equilibrium is given by

$$\langle x_r\rangle = \frac{\sum_{\{k\in K|x_r^k=1\}} g(k)\Lambda^{l_1(k)}\kappa^{l_2(k)}}{\sum_{k\in K} g(k)\Lambda^{l_1(k)}\kappa^{l_2(k)}} =: \frac{E_M^r(\Lambda,\kappa)}{P_M(\Lambda,\kappa)}. \tag{12}$$

Equation (12) leads to the following overall titration curves for ligands L_1 and L_2.

$$\langle X_1\rangle = \frac{\sum_{r=1}^{n_1} E_M^r(\Lambda,\kappa)}{P_M(\Lambda,\kappa)} \tag{13}$$

$$\langle X_2\rangle = \frac{\sum_{r=A_1}^{A_{n_2}} E_M^r(\Lambda,\kappa)}{P_M(\Lambda,\kappa)} \tag{14}$$

4 On decoupling molecules with two types of ligands

To get an idea, and to point out some problems with the transfer of the DSR, we give two simple examples of hypothetical molecules whose bp can be calculated easily. We will use the notation P_M for $P_M(\Lambda,\kappa)$ and E_M^r for $E_M^r(\Lambda,\kappa)$.

Example 1 Let $M=(g_1,g_A,w_{1,A})=(\frac{1}{2},2,\frac{1}{3})$ be a molecule with one binding site for each type of ligand. Then:

$$\begin{aligned} P_M &= \frac{1}{3}\Lambda\kappa+\frac{1}{2}\Lambda+2\kappa+1\\ E_M^1 &= \frac{1}{3}\Lambda\kappa+\frac{1}{2}\Lambda\\ E_M^A &= \frac{1}{3}\Lambda\kappa+2\kappa. \end{aligned}$$

Moreover, we see here that it is not possible to decouple this system as the map

$$P:(g_1,g_A,w_{1,A})\mapsto(g_1g_Aw_{1,A},g_1,g_A)$$

which gives the coefficients of the polynomial, is injective. Thus, it is impossible to find a molecule $(g_1',g_A',1)$ with the same binding polynomial.

Example 2 Let $M=(g_1,g_2,g_A,w_{1,2},w_{1,A},w_{2,A})=(\frac{1}{2},2,3,\frac{1}{2},2,\frac{1}{3})$ be a molecule with two binding sites for ligand L_1 and one for ligand of type L_2. Then,

$$P_M=\Lambda^2\kappa+\frac{1}{2}\Lambda^2+5\Lambda\kappa+\frac{5}{2}\Lambda+3\kappa+1.$$

In this situation of two and one bindings site(s) P is a map

$$P : \mathbb{G}_{2,1} \longrightarrow \mathbb{C}^{*5}.$$

Here, the image space $\mathbb{C}^{*5}$ represents the polynomials in two variables with 6 coefficients including the constant term which equals 1. Thus, it should not be injective and decoupling might be possible. However, intuitively, it is clear, that not all interaction energies can be trivial, as this would reduce the domain to $\mathbb{C}^{*3}/\sim$. Looking for another molecule with the same binding polynomial means searching for a solution $(g'_1, g'_2, g'_A, w'_{1,2}, w'_{1,A}, w'_{2,A})$ to the system

$$\begin{aligned} 1 &= g'_1 g'_2 g'_A w'_{1,2} w'_{1,A} w'_{2,A} \\ \frac{1}{2} &= g'_1 g'_2 w'_{1,2} \\ 5 &= g'_1 g'_A w'_{1,A} + g'_2 g'_A w'_{2,A} \\ \frac{5}{2} &= g'_1 + g'_2 \\ 3 &= g'_A. \end{aligned} \tag{15}$$

As g'_A is fixed, the solutions to system (15), that is all molecules with bp P_M, form an algebraic variety $V \subset \mathbb{C}^{*5}$ defined by four polynomials. This means, under certain conditions on the polynomials, $\dim(V) = 1$. The systems without interaction between the binding sites for the same type of ligand are given by

$$V \cap \{(g_1, g_2, w_{1,2}, w_{1,A}, w_{2,A}) \in \mathbb{C}^{*5} | w_{1,2} = 1\}.$$

We used the computer algebra program Maxima to calculate the solutions. In this special situation we receive the following tuples sharing the same binding polynomial but with non-interacting sites for ligand L_1:

$$(2.2807762, 0.2192236, 3, 1, 0.6288467, 1.0601419),$$
$$(0.2192236, 2.2807762, 3, 1, 1.0601419, 0.6288467),$$
$$(2.2807762, 0.2192236, 3, 1, 0.1018987, 6.5424460),$$
$$(0.2192236, 2.2807762, 3, 1, 6.5424460, 0.1018987).$$

Note that, as we are dealing with equivalence classes, the first and the second pair of the solutions coincide. Calculating the same with $w_{1,A} = 1$ or $w_{2,A} = 1$ does not give any solution. Remarkably, fixing $g_1 = 1$ or $g_2 = 1$ is solvable, however we will not investigate this phenomenon further, as we are interested in decoupling the system, which means setting interaction constants to 1.

Example 2 leads to the conjecture that it is possible to decouple the binding sites for the same type of ligand. However, it is not generally possible to decouple different

binding sites for different types of ligands which was illustrated by Example 1. For this reason we call a system decoupled if its binding sites for the same type of ligand do not interact directly. A very important point which is illustrated in Example 2 is the loss of uniqueness of the decoupled system which is given in the case of one type of ligand (Proposition 1). We formulate the DSR for two types of ligands the following way:

Conjecture 1 *Let*

$$M = \left(g_1^M, \ldots, g_{n_1}^M, g_{A_1}^M, \ldots, g_{A_{n_2}}^M, w_{1,2}^M, \ldots, w_{A_{n_2-1},A_{n_2}}^M\right)$$

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

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

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

and

$$P_M = P_N.$$

As we did not find a general proof for Conjecture 1 we will investigate the case $n_2 = 1$. The problem with proving this conjecture generally, is the following: One could use Hilbert's weak Nullstellensatz and show that the ideal generated by the polynomials [analogously to (15)] does not contain unity. Then the existence of a solution would be guaranteed. However, to use this approach, one has to calculate the ideal generated by the polynomials (e.g. the corresponding Gröbner basis) without writing down the polynomials explicitly as n_1 and n_2 are not fixed. Another similar argumentation—with the same problem— would be to calculate a Gröbner basis to find partial solutions in an elimination ideal and to extend theses solutions to full solutions of the system under the use of the Extension Theorem. We will illustrate this argumentation afterwards in detail. Another approach would be the use of a higher-dimensional analog of the Bezout-Theorem. Yet, this would only give a statement for varieties in projective space. The most promising idea might be to use the special structure of the polynomials to give a proof constructively by reducing the problem to the proof of the DSR for one type of ligand (Proposition 1). We will compare the second and the fourth approach to prove Conjecture 1 for $n_2 = 1$ in the next section and investigate which unique properties all decoupled molecules share. The fact that a decoupled system is not unique was illustrated by Example 2. However, Proposition 2 shows that at least the binding constants are unique (except for permutations):

Proposition 2 *Let*

$$P_M = a_{n_1,n_2}\Lambda^{n_1}\kappa^{n_2} + a_{n_1-1,n_2}\Lambda^{n_1-1}\kappa^{n_2} + \cdots a_{0,n_2}\kappa^{n_2} + a_{n_1,n_2-1}\Lambda^{n_1}\kappa^{n_2-1}\cdots + 1$$

be a bp of a molecule with n_1 binding sites for ligand L_1 and n_2 binding sites for ligand L_2. Let

$$N = (g_1^N, \ldots, g_{n_1}^N, g_{A_1}^N, \ldots, g_{A_{n_2}}^N, 1, \ldots, w_{n_1,A_{n_2}}^N, 1 \ldots, 1)$$

and

$$K = (g_1^K, \ldots, g_{n_1}^K, g_{A_1}^K, \ldots, g_{A_{n_2}}^K, 1, \ldots, w_{n_1,A_{n_2}}^K, 1 \ldots, 1)$$

be two different corresponding decoupled systems. Then there exist permutations σ_1 of $\{0, \ldots, n_1\}$ and σ_2 of $\{0, \ldots, n_2\}$ such that

$$(g_i^N)_{i=1}^{n_1} = (g_{\sigma_1(i)}^K)_{i=1}^{n_1}$$

and

$$(g_{A_i}^N)_{i=1}^{n_2} = (g_{A_{\sigma_2(i)}}^K)_{i=1}^{n_2}.$$

Proof A decoupled system is in the preimage of P_M with respect to the map $M \mapsto P_M$. In particular, it has to solve the subsystem of equations given by its coefficients $\{a_{i,0}\}_{i=1}^{n_1}$. As this subsystem is free of the binding constant variables $\{g_{A_i}\}_{i=1}^{n_2}$ of ligand L_2 it represents the case of the DSR for one type of ligand. Consequently, according to Corollary 1, the set $\{g_i\}_{i=1}^{n_1}$ can be calculated from the roots of

$$a_{n_1,0}\Lambda^{n_1} + a_{n_1-1,0}\Lambda^{n_1-1} + \cdots + 1,$$

which shows $(g_i^N)_{i=1}^{n_1} = (g_{\sigma_1(i)}^K)_{i=1}^{n_1}$. The same is true for the subsystem of equations given by $\{a_{0,i}\}_{i=1}^{n_2}$ which gives the second result. □

5 Molecules with n_1 to one binding sites

5.1 Decoupling

At first we will prove Conjecture 1 for the case $(n_1, n_2) = (2, 1)$ to compare the approach of calculating the Gröbner basis and using the Elimination and Extension Theorems to the use of the special structure of the varieties we are dealing with. Note that Proposition 4 includes the statement of Proposition 3. However, we will use Proposition 3 to illustrate the different approaches for proving Conjecture 1.

Proposition 3 *Let*

$$M = (g_1^M, g_2^M, g_A^M, w_{1,2}^M, w_{1,A}^M, w_{2,A}^M)$$

be a molecule with two binding sites for ligand L_1 and one binding site for ligand L_2. Then a molecule

$$N = (g_1, g_2, g_A, 1, w_{1,A}, w_{2,A})$$

exists such that

$$g_A = g_A^M \quad \textit{and} \quad P_M = P_N.$$

Proof Let

$$P_M = a_{2,1}\Lambda^2\kappa + a_{2,0}\Lambda^2 + a_{1,1}\Lambda\kappa + a_{1,0}\Lambda + a_{0,1}\kappa + 1$$

be the binding polynomial of molecule M. A molecule $N = (g_1, g_2, g_A, 1, w_{1,A}, w_{2,A})$ has to be a solution to the algebraic system

$$\begin{aligned} g_1^M g_2^M g_A^M w_{1,2}^M w_{1,A}^M w_{2,A}^M &= a_{2,1} = g_1 g_2 g_A w_{1,A} w_{2,A} \\ g_1^M g_2^M w_{1,2}^M &= a_{2,0} = g_1 g_2 \\ g_1^M g_A^M w_{1,A}^M + g_2^M g_A^M w_{2,A}^M &= a_{1,1} = g_1 g_A w_{1,A} + g_2 g_A w_{2,A} \\ g_1^M + g_2^M &= a_{1,0} = g_1 + g_2 \\ g_A^M &= a_{0,1} = g_A. \end{aligned} \tag{16}$$

We regard these equations as polynomials in

$$\mathbb{C}[g_1, g_2, g_A, w_{1,A}, w_{2,A}, a_{2,1}, a_{2,0}, a_{1,1}, a_{1,0}, a_{0,1}]$$

and use the computational algebra system Magma to calculate the Gröbner basis GB of the corresponding ideal (w.r.t. the lexicographic order $g_1 > g_2 > g_A > w_{1,A} > \cdots > a_{0,1}$, see [7]). Note that in this situation it is not enough to see that $GB \neq \{1\}$ as this only implies that there exists a solution to the system, however this does not show, that there exists a solution for any choice of $(a_{2,1}, a_{2,0}, a_{1,1}, a_{1,0}, a_{0,1})$. The last of 17 polynomials of the Gröbner basis is

$$\begin{aligned} P_{17} = {} & w_{2,A}^4 a_{2,0}^2 a_{0,1}^2 - w_{2,A}^3 a_{2,0} a_{1,1} a_{1,0} a_{0,1} - 2w_{2,A}^2 a_{2,1} a_{2,0} a_{0,1} \\ & + w_{2,A}^2 a_{2,1} a_{1,0}^2 a_{0,1} + w_{2,A}^2 a_{2,0} a_{1,1}^2 - w_{2,A} a_{2,1} a_{1,1} a_{1,0} + a_{2,1}^2 \end{aligned}$$

and it defines the fourth elimination ideal (Elimination Theorem). Since the product $a_{2,0}a_{0,1} \neq 0$ and $a_{2,1} \neq 0$ we will find four solutions for $w_{2,A}$. As the leading coefficient of the 16th polynomial (regarded as polynomial in $w_{1,A}$)

$$\begin{aligned} P_{16} = {} & w_{1,A} a_{2,1} a_{2,0} a_{0,1} + w_{2,A}^A a_{2,0}^2 a_{0,1}^2 - w_{2,A}^2 a_{2,0} a_{1,1} a_{1,0} a_{0,1} \\ & - 2w_{2,A} a_{2,1} a_{2,0} a_{0,1} + w_{2,A} a_{2,1} a_{1,0}^2 a_{0,1} + w_{2,A} a_{2,0} a_{1,1}^2 - a_{2,1} a_{1,1} a_{1,0} \end{aligned}$$

will not vanish in the solutions of $P_{17} = 0$, all solutions of $w_{2,A}$ can be extended to solutions of the third elimination ideal (Extension theorem). Continuing this procedure leads to four solutions of the full system (only two of them are different w.r.t. the equivalence relation of Definition 2). For more information on the Elimination and Extension Theorems see [6,7]. A list of the corresponding Gröbner basis can be found in the supporting information. □

Note that the proof of Proposition 3 also showed that

$$P(\mathbb{G}_{2,1}) \supset \left\{ a_{2,1}\Lambda^2\kappa + a_{2,0}\Lambda^2 + a_{1,1}\Lambda\kappa + a_{1,0}\Lambda + a_{0,1}\kappa + 1 \middle| a_{i,j} \in \mathbb{C}^* \right\},$$

which means all polynomials of this shape have a preimage w.r.t. P. To calculate the energies of molecule N in Proposition 3 one can use a computational algebra system (Magma, Maxima) to solve (16) or use some special properties of (16): In general, not only for this choice of n_i, the coefficients $(a_{i,0})_{i=1,\ldots,n_1}$ define a system of algebraic equations which allows to calculate $(g_1, \ldots, g_{n_1})$ (proof of Proposition 2). Analogously, $(a_{0,i})_{i=1,\ldots,n_2}$ give $(g_A, \ldots, g_{A_{n_2}})$. In system (16), with the same argument, $w_{1,A}$ and $w_{2,A}$ are given by

$$w_{1,A} = -\frac{1}{g_1 \Lambda_{z_i}}$$
$$w_{2,A} = -\frac{1}{g_2 \Lambda_{z_j}}$$

where Λ_{z_i} are the roots of

$$\frac{a_{2,1}}{g_A}\Lambda^2 + \frac{a_{1,1}}{g_A}\Lambda + 1.$$

This calculation can also be used to prove Proposition 3 and will be used in the following to prove the more general case of $(n_1, 1)$ binding sites.

Proposition 4 *Let*

$$M = \left(g_1^M, \ldots, g_{n_1}^M, g_A^M, w_{1,2}^M, \ldots, w_{n_1,A}^M\right)$$

be a molecule with n_1 binding sites for Ligand L_1 and one binding site for ligand L_2 (which is denoted as site A). Then a molecule

$$N = (g_1, \ldots, g_{n_1}, g_A, 1, \ldots, w_{n_1,A})$$

exists with $w_{i,j} = 1\ \forall i, j \in \{1, \ldots, n_1\}$ and

$$P_M = P_N.$$

Proof Let

$$P_M = a_{n_1,1}\Lambda^{n_1}\kappa + a_{n_1,0}\Lambda^{n_1} + \cdots + a_{1,1}\Lambda\kappa + a_{1,0}\Lambda + a_{0,1}\kappa + 1$$

be the bp of M. Again, a decoupled molecule N is a point of the algebraic variety V in the variables $g_1, \ldots, g_A, w_{1,A}, \ldots, w_{n_1,A}$ defined by the coefficients $a_{i,j}$ and the corresponding equations. The equations of coefficients $a_{n_1,0}, \ldots, a_{1,0}$ are free from the variables $w_{i,j}$ since we are looking for a decoupled system. Thus, Corollary 1 gives

$$(g_1, \ldots, g_{n_1}) = \left(-\frac{1}{\Lambda_{z_1}}, \ldots, -\frac{1}{\Lambda_{z_{n_1}}}\right)$$

with Λ_{z_i} the roots of

$$a_{n_1,0}\Lambda^{n_1} + a_{n_1-1,0}\Lambda^{n_1-1} + \cdots + a_{1,0}\Lambda + 1. \tag{17}$$

Moreover, $a_{0,1}$ gives g_A. The remaining equations can be rewritten

$$\begin{aligned}
\frac{a_{n_1,1}}{g_A} &= \prod_{i=1}^{n_1} g_i w_{i,A} \\
\frac{a_{n_1-1,1}}{g_A} &= \sum_{j=1}^{n_1} \prod_{i=1, i\neq j}^{n_1} g_i w_{i,A} \\
&\vdots \\
\frac{a_{1,1}}{g_A} &= \sum_{i=1}^{n_1} g_i w_{i,A}.
\end{aligned}$$

Thus, the products $g_i w_{i,A}$ are determined by the roots of

$$\frac{a_{n_1,1}}{g_A}\Lambda^{n_1} + \frac{a_{n_1-1,1}}{g_A}\Lambda^{n_1-1} + \cdots + \frac{a_{1,1}}{g_A}\Lambda + 1. \tag{18}$$

Consequently, the interaction energies $w_{i,A}$ can be calculated as the binding energies g_i are already known. □

5.2 The maximal number of decoupled molecules and properties they share

The proof of Proposition 4 also shows how many different decoupled molecules exist at most.

Corollary 2 *Let*

$$M = (g_1^M, \ldots, g_{n_1}^M, g_A^M, w_{1,2}^M, \ldots, w_{n_1,A}^M)$$

be a molecule with n_1 binding sites for Ligand L_1 and one binding site for ligand L_2. Then there exist at most $n_1!$ different decoupled molecules.

Proof The proof of Proposition 4 shows that at most $(n_1!)^2$ tuples exist which correspond to the different permutations of the roots of polynomial (17) and polynomial (18) and which solve the system. However, $n_1!$ tuples represent the same molecule. Thus, the maximal number of different decoupled molecules is $n_1!$.

Example 3 We illustrate the binding curves of individual sites of a system with two binding sites for ligand L_1 and one binding site for ligand L_2 and its corresponding decoupled systems. We used other hypothetical binding and interaction constants than in Example 2 to observe titration curves which can be distinguished by eye. To this end, let

$$M = (g_1, g_2, g_A, w_{1,2}, w_{1,A}, w_{2,A}) = (900, 900, 300, 10^{-4}, 1000, 2000)$$

be a molecule. Its decoupled molecules are given by

$$\begin{aligned} N &= (1799.955, 0.04500113, 300, 1, 1500.004, 1333.33) \\ K &= (1799.955, 0.04500113, 300, 1, 0.03333491, 59997167). \end{aligned}$$

The titration curves of the individual sites are illustrated in Fig. 1. Ligand L_1 is regarded as electron and ligand L_2 as proton.

An interesting observation is the fact, that in the titration curves of individual sites of decoupled molecules the area of transition between 0.1 and 0.9 probability of occupation is comparatively small. However, it is difficult to quantify this feature. Moreover, regarding the titration curves of the decoupled molecules it seems that the titration curve of site 1 of molecule K is a composition of the "left part" of site 1 and the "right part" of site 2 of molecule N. Analogously, the curve of site 2 of molecule K seems to be composed of the remaining parts of the curves of sites 1, 2 of molecule N. This observation leads to the conjecture, that there is a unique set of "bricks" all decoupled molecules are built of. We will investigate this in the following. As the titration curves of individual sites are sums of the probabilities of the microstates in which the individual site is occupied, the constants of the microstates, which are listed in Table 1 can give information about this observation. We see here, that in the decoupled systems of Example 3 the probabilities of the two events in which one electron and the proton is bound are permuted. These probabilities are the unique "bricks" all decoupled molecules are built of. Before we formulate this as Proposition, we define the term macrostate.

Definition 3 Let M be a molecule with (n_1, n_2) binding sites. It is said to be in macrostate (i, j), $i \leq n_1$, $j \leq n_2$ if —in its current microstate— exactly i ligands of type L_1 and exactly j ligands of type L_2 are bound.

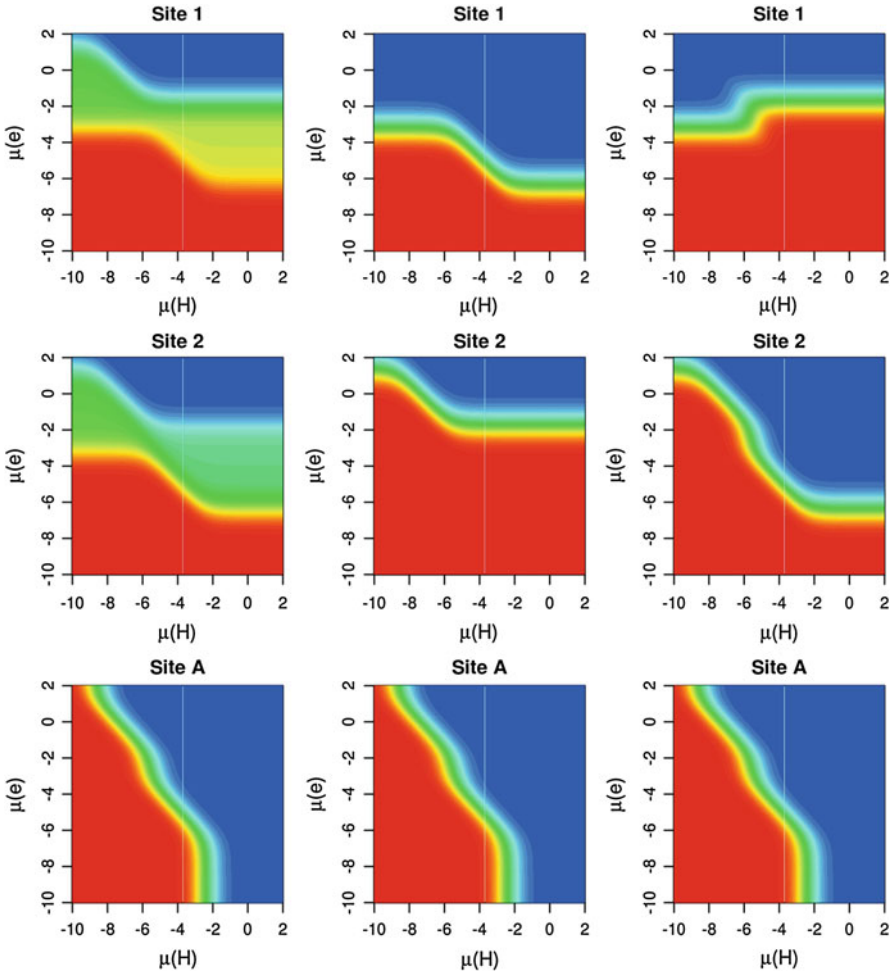

Fig. 1 Activity dependent ligand binding to each site of the original molecule M (*left column*) and of the decoupled molecules N (*middle column*) and K (*right column*) of Example 3. A logarithmic scale of the ligand activity is used: The chemical potentials $\mu(\mathrm{e}) := \log(\Lambda)$ and $\mu(\mathrm{H}) := \log(\kappa)$. In the case of protons as second ligand $\log(\kappa)$ equals the negative pH. *Dark blue area*: probability of occupation is equal to or higher than 0.9. *Red area*: probability of occupation is less than or equal to 0.1. *Green area*: probability of occupation between 0.25 and 0.75. (Color figure online)

Proposition 5 *Let*

$$M = (g_1^M, \ldots, g_{n_1}^M, g_A^M, w_{1,2}^M, \ldots, w_{n_1,A}^M)$$

be a molecule with n_1 binding sites for ligand L_1 and one binding site for ligand L_2. Moreover, let the order of the sites in the decoupled molecules be fixed to the same permutation. Then the following statements hold:

Table 1 Constants of all microstates of the different molecules of Example 3: $M = (900, 900, 300, 10^{-4}, 1,000, 2,000)$ and N, K the corresponding decoupled molecules

Microstate	M	N	K
(0, 0, 0)	1	1	1
(0, 0, 1)	300	300	300
(0, 1, 0)	900	0.04500113	0.04500113
(1, 0, 0)	900	1799.955	1799.955
(1, 1, 0)	81	81	81
(0, 1, 1)	5.4×10^8	18000.4	8.09982×10^8
(1, 0, 1)	2.7×10^8	8.09982×10^8	18000.4
(1, 1, 1)	4.86×10^{10}	4.86×10^{10}	4.86×10^{10}

The binding sites 1 and 2 for the first type of ligand are described by the first and the second entry of the microstate. The third entry of the microstate corresponds to site A, the binding site for the second ligand

(*a*) *For every microstate k with unoccupied site A, all decoupled molecules share the same microstate constant $g(k)$.*

(*b*) *For every macrostate $(i, 1)$ with occupied site A and i occupied sites for ligand L_1, there exist $\binom{n_1}{i}$ numbers such that for any decoupled molecule the tuple of its constants of microstates belonging to this macrostate is a permutation of these numbers.*

(*c*) *The permutation of microstate constants of macrostate $(1, 1)$ fixes the permutations of the microstate constants of all other macrostates $(i, 1)$.*

(*d*) *Every decoupled molecule can be identified one to one with a permutation of the microstate constants of macrostate $(1, 1)$.*

Proof (a) Let k be a microstate in which site A is unoccupied. Then its constant is the product of the binding constants of the sites which are occupied. According to Proposition 2 all decoupled molecules share the same binding constants which gives the first statement, since the permutation of $\{g_1, \cdots, g_{n_1}\}$ was assumed fixed, previously.

(b) Let k be a microstate in which site A is occupied. Eq. (5) states that its constant is the product of the binding constants of all occupied sites and their interaction constants. As the interaction constants of any pair of binding sites for the same type of ligand are 1, this reduces to

$$g(k) = g_A \prod_{i=1}^{n_1} g_i^{x_i^k} w_{i,A}^{x_i^k}. \tag{19}$$

Since the decoupled systems share the binding constant g_A and since the products $(g_i w_{i,A})_{i=1,\ldots,n_1}$ correspond to the permutations of the roots of polynomial (18), the microstate constants of different decoupled systems belonging to the same macrostate are permutations.

(c) Let a permutation of the microstate constants belonging to macrostate $(1, 1)$ be chosen. Then all interaction constants are determined as the microstate constants

are given by a product of $g_i g_A w_{i,A}$ and g_i, g_A are known. Thus, the molecule is known and all other constants are determined.

(d) Let N, K be two different molecules. Then their permutation of the microstate constants of macrostate (1, 1) differs, as otherwise $N = K$ due to identical interaction constants (injectivity). Conversely, every permutation of the microstate constants solves the system described by polynomial (18) (surjectivity). □

Remark 1 In Proposition 5, we used the term permutation for a permutation of numbers. This means that different permutations of the symmetric group $\mathcal{S}_n$ can be regarded as equal if some numbers are equal.

5.3 Titration curves of individual sites

Onufriev et al. [14] and Martini and Ullmann [12] showed, that it is possible to regain the titration curve of each individual binding site as linear combination of the titration curves of the individual sites of the decoupled system in the case of only one type of ligand. We will show that this can be generalized to the situation of two types of ligands in a certain way. We use the notation $\langle x_i \rangle$ for the average amount of bound ligand L_1 at site i and $\langle y_j \rangle$ for the average amount of bound ligand L_2 at site j (as a function of ligand activities). In the following we investigate the case $(n_1, n_2) = (2, 1)$. With $M = (g_1^M, g_2^M, g_A, w_{1,2}^M, w_{1,A}^M, w_{2,A}^M)$ a molecule, $N = (g_1, g_2, g_A, 1, w_{1,A}, w_{2,A})$ a corresponding decoupled system, and bp

$$P_M = P_N = a_{2,1}\Lambda^2\kappa + a_{2,0}\Lambda^2 + a_{1,1}\Lambda\kappa + a_{1,0}\Lambda + g_A\kappa + 1,$$

the titration curves of individual sites are given by:

$$\begin{aligned} P_M\langle x_1\rangle_M &= a_{2,1}\Lambda^2\kappa + a_{2,0}\Lambda^2 + g_1^M g_A w_{1,A}^M \Lambda\kappa + g_1^M\Lambda \\ P_N\langle x_1\rangle_N &= P_M\langle x_1\rangle_N = a_{2,1}\Lambda^2\kappa + a_{2,0}\Lambda^2 + g_1 g_A w_{1,A}\Lambda\kappa + g_1\Lambda \\ P_N\langle x_2\rangle_N &= P_M\langle x_2\rangle_N = a_{2,1}\Lambda^2\kappa + a_{2,0}\Lambda^2 + g_2 g_A w_{2,A}\Lambda\kappa + g_2\Lambda. \end{aligned}$$

Thus, due to equality of the coefficients, the solutions (b_1, b_2) to

$$\langle x_1\rangle_M = b_1\langle x_1\rangle_N + b_2\langle x_2\rangle_N$$

coincide with the solutions to the linear system

$$\begin{pmatrix} 1 & 1 \\ g_1 g_A w_{1,A} & g_2 g_A w_{2,A} \\ g_1 & g_2 \end{pmatrix} \begin{pmatrix} b_1 \\ b_2 \end{pmatrix} = \begin{pmatrix} 1 \\ g_1^M g_A w_{1,A}^M \\ g_1^M \end{pmatrix} \tag{20}$$

Obviously, the problem in this situation is the following: there are three (possibly linearly independent) equations and only two variables. However, a linear combination can always be found the following way:

Proposition 6 *Let* $M = (g_1^M, g_2^M, g_A, w_{1,2}^M, w_{1,A}^M, w_{2,A}^M)$ *be a molecule with two binding sites for ligand* L_1 *(sites 1 and 2), one binding site for ligand* L_2 *(site A) and with bp* P_M*. Moreover, let* $N = (g_1, g_2, g_A, 1, w_{1,A}, w_{2,A})$ *be a corresponding decoupled molecule. Then a continuous function* $b = b(\kappa)$ *on* $\mathbb{R} \setminus \{\frac{g_2 - g_1}{(g_1 w_{1,A} - g_2 w_{2,A}) g_A}\}$ *exists, such that*

$$\langle x_1 \rangle_M = b \langle x_1 \rangle_N + (1 - b) \langle x_2 \rangle_N \tag{21}$$

and

$$\langle x_2 \rangle_M = b \langle x_2 \rangle_N + (1 - b) \langle x_1 \rangle_N. \tag{22}$$

The function $b(\kappa)$ *is given by*

$$\frac{g_A(g_1^M w_{1,A}^M - g_2 w_{2,A})\kappa + g_1^M - g_2}{g_A(g_1 w_{1,A} - g_2 w_{2,A})\kappa + g_1 - g_2}. \tag{23}$$

Note that $g_i g_A w_{i,A}$ *are microstate constants.*

Proof

$$P_M \langle x_1 \rangle_M = (g_1^M g_2^M g_A w_{1,2}^M w_{1,A}^M w_{2,A}^M \kappa + g_1^M g_2^M w_{1,2}^M)\Lambda^2 + (g_1^M g_A w_{1,A}^M \kappa + g_1^M)\Lambda \tag{24}$$

$$P_N \langle x_1 \rangle_N = P_M \langle x_1 \rangle_N = (g_1 g_2 g_A w_{1,A} w_{2,A} \kappa + g_1 g_2)\Lambda^2 + (g_1 g_A w_{1,A} \kappa + g_1)\Lambda \tag{25}$$

$$P_M \langle x_2 \rangle_N = (g_1 g_2 g_A w_{1,A} w_{2,A} \kappa + g_1 g_2)\Lambda^2 + (g_2 g_A w_{2,A} \kappa + g_2)\Lambda \tag{26}$$

As N and M share the same bp:

$$g_1^M g_2^M g_A w_{1,2}^M w_{1,A}^M w_{2,A}^M \kappa + g_1^M g_2^M w_{1,2}^M = g_1 g_2 g_A w_{1,A} w_{2,A} \kappa + g_1 g_2 \qquad \forall \kappa.$$

This means that $(b_1, b_2) = (b_1(\kappa), b_2(\kappa))$ is a solution to Eq. (21) iff

$$A \begin{pmatrix} b_1 \\ b_2 \end{pmatrix} = \begin{pmatrix} 1 \\ g_1^M g_A w_{1,A}^M \kappa + g_1^M \end{pmatrix},$$

where $A := \begin{pmatrix} 1 & 1 \\ g_1 g_A w_{1,A} \kappa + g_1 & g_2 g_A w_{2,A} \kappa + g_2 \end{pmatrix}$.
If $\kappa \neq \frac{g_2 - g_1}{(g_1 w_{1,A} - g_2 w_{2,A}) g_A}$ then $\det(A) \neq 0$ and there exists a unique solution for (b_1, b_2) with $b := b_1 = 1 - b_2$ which gives Eq. (23). Moreover, since

$$\langle x_1 \rangle_N + \langle x_2 \rangle_N = \langle x_1 \rangle_M + \langle x_2 \rangle_M = b \langle x_1 \rangle_N + (1 - b) \langle x_2 \rangle_N + \langle x_2 \rangle_M$$

this proves Eq. (22). □

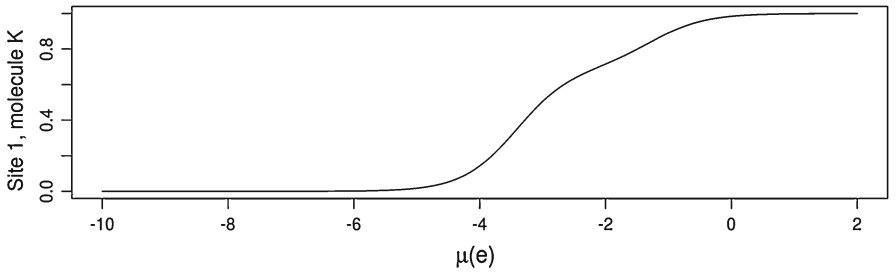

Fig. 2 Electron-activity ($\mu(e) := log(\Lambda)$) dependent electron binding to site 1 of molecule K of Example 3 for fixed pH $= 6 = -\mu(H)$.

5.4 The existence of pointwise decoupled systems for every activity κ

Regarding the 1-dimensional titration curve of site 1 of molecule K of Example 3 for fixed ligand activity pH $= 6$, we see that it is not of classical Henderson-Hasselbalch form (Fig. 2). This means that even though the electron binding sites have a trivial interaction constant in the decoupled molecule, the sites do not bind the ligand independently. This result may be counterintuitive as the electron binding sites do not interact directly. However, a secondary interaction of the electron binding sites results from the interaction with the proton. Let $M = (g_1, g_2, g_A, w_{1,2}, w_{1,A}, w_{2,A})$ be a molecule with two binding sites for ligand L_1 (sites 1, 2) and one binding site for ligand L_2 (site A). We investigate the following question: Which conditions on the binding and interaction constants are necessary to let the titration curves of the individual sites $\langle x_1 \rangle$ and $\langle x_2 \rangle$ be decoupled for all κ?

Proposition 7 *Let $M = (g_1, g_2, g_A, w_{1,2}, w_{1,A}, w_{2,A})$ be a molecule.*

Then the 1-dimensional titration curves of sites 1 *and* 2 *are decoupled for all κ if and only if $w_{1,2} = 1$ and ($w_{1,A} = 1$ or $w_{2,A} = 1$).*

Proof "$\Longrightarrow$" Let M be a molecule as described. Its bp is given by

$$\begin{aligned} P_M(\Lambda, \kappa) = {} & g_1 g_2 g_A w_{1,2} w_{1,A} w_{2,A} \Lambda^2 \kappa + g_1 g_2 w_{1,2} \Lambda^2 \\ & + (g_1 g_A w_{1,A} + g_2 g_A w_{2,A}) \Lambda \kappa + (g_1 + g_2) \Lambda + g_A \kappa + 1. \end{aligned}$$

As the sites 1 and 2 are decoupled for all κ they are in particular decoupled for $\kappa = 0$. This implies $w_{1,2} = 1$. Thus, the titration curve of site 1 has the shape

$$\frac{(g_1 g_2 g_A w_{1,A} w_{2,A} \kappa + g_1 g_2) \Lambda^2 + (g_1 g_A w_{1,A} \kappa + g_1) \Lambda}{P_M(\Lambda, \kappa)}. \tag{27}$$

We know that for all fixed κ the molecule is decoupled, which is equivalent to all titration curves being of HH shape, which implies in particular that (27) can be rewritten (for fixed κ) as

$$\frac{g_1'(\kappa)\Lambda}{g_1'(\kappa)\Lambda+1}. \tag{28}$$

where $g_1'(\kappa)$ depends on κ but not on Λ (see [12,14]). This means that there exists a factor $a(\kappa) \in \mathbb{C}[\Lambda]$ ($a(\kappa)$ has to be of degree one) such that

$$a(\kappa)g_1'(\kappa)\Lambda = (g_1g_2g_Aw_{1,A}w_{2,A}\kappa + g_1g_2)\Lambda^2 + (g_1g_Aw_{1,A}\kappa + g_1)\Lambda \tag{29}$$

and

$$a(\kappa)(g_1'(\kappa)\Lambda + 1) = P_M(\Lambda,\kappa). \tag{30}$$

Regarding these polynomials as elements of $\mathbb{C}[\Lambda]$ we see that the constant term of $P_M(\Lambda,\kappa)$ is given by $g_A\kappa + 1$. Moreover, the constant term of $(g_1'(\kappa)\Lambda + 1)$ is 1. This implies that $a(\kappa)$ has constant term $g_A\kappa + 1$. As $g_1'(\kappa)$ is independent of Λ Eq. (29) implies

$$g_1'(\kappa) = \frac{(g_1g_Aw_{1,A}\kappa + g_1)}{g_A\kappa + 1} \tag{31}$$

$$a(\kappa) = \frac{(g_A\kappa+1)g_2(g_Aw_{1,A}w_{2,A}\kappa+1)}{(g_Aw_{1,A}\kappa+1)}\Lambda + (g_A\kappa+1). \tag{32}$$

The same arguments for site 2 show that its titration curve is of shape (28) with

$$g_2'(\kappa) = \frac{(g_2g_Aw_{2,A}\kappa + g_2)}{g_A\kappa + 1}. \tag{33}$$

As the overall titration curve is the sum of the individual curves we have necessarily

$$\begin{aligned}&\frac{g_1'(\kappa)\Lambda}{g_1'(\kappa)\Lambda+1} + \frac{g_2'(\kappa)\Lambda}{g_2'(\kappa)\Lambda+1}\\ &= \frac{2(g_1g_2g_Aw_{1,A}w_{2,A}\kappa + g_1g_2)\Lambda^2 + (g_1g_Aw_{1,A} + g_2g_Aw_{2,A}\kappa + g_1 + g_2)\Lambda}{P_M(\Lambda,\kappa)}.\end{aligned} \tag{34}$$

Hence, a $b(\kappa)$ must exist which is independent of Λ such that

$$(g_1'(\kappa)\Lambda + 1)(g_2'(\kappa)\Lambda + 1)b(\kappa) = P_M(\Lambda,\kappa). \tag{35}$$

Again, a comparison of the constant term of the polynomials gives $b(\kappa) = (g_A\kappa + 1)$. Thus, comparing the leading coefficients of the polynomials of Eq. (35) yields

$$\frac{(g_1g_Aw_{1,A}\kappa + g_1)(g_2g_Aw_{2,A}\kappa + g_2)}{g_A\kappa + 1} = g_1g_2g_Aw_{1,A}w_{2,A}\kappa + g_1g_2 \tag{36}$$

which gives

$$(w_{1,A} + w_{2,A})\kappa = (1 + w_{1,A}w_{2,A})\kappa \qquad \forall\kappa$$

and thus

$$w_{1,A}(1 - w_{2,A}) = 1 - w_{2,A}. \tag{37}$$

Eq. (37) shows that $w_{2,A} \neq 1$ implies $w_{1,A} = 1$.

"$\Longleftarrow$" Without loss of generality, let $w_{1,A} = 1 = w_{1,2}$. Then, using notation of Eqs. (31, 32)

$$a(\kappa)(g_1'(\kappa)\Lambda + 1) = P(\Lambda, \kappa)$$

and

$$a(\kappa)g_1'(\kappa)\Lambda = (g_1g_2g_Aw_{2,A}\kappa + g_1g_2)\Lambda^2 + (g_1g_A\kappa + g_1)\Lambda.$$

Thus, the 1-dimensional titration curve for fixed κ (Eq. 27) reduces to

$$\frac{(g_1g_2g_Aw_{2,A}\kappa + g_1g_2)\Lambda^2 + (g_1g_A\kappa + g_1)\Lambda}{P(\Lambda, \kappa)} = \frac{g_1'(\kappa)\Lambda}{g_1'(\kappa)\Lambda + 1}. \tag{38}$$

Moreover, the titration curve of site 2 for fixed κ is given by

$$\begin{aligned}&\frac{(g_1g_2w_{2,A}\kappa + g_1g_2)\Lambda^2 + (g_2g_Aw_{2,A}\kappa + g_2)\Lambda}{P(\Lambda, \kappa)}\\ &= \frac{(g_2g_Aw_{2,A}\kappa + g_2)\Lambda(g_1\Lambda + 1)}{(g_1\Lambda + 1)a(\kappa)} = \frac{g_2'(\kappa)\Lambda}{g_2'(\kappa)\Lambda + 1}.\end{aligned}$$

This means that sites 1 and 2 are decoupled. □

Proposition 7 can be interpreted the following way: The different binding sites of a molecule are decoupled for all activities of the second ligand if and only if they are not "connected". To avoid different interpretations, we give an exact definition of this term:

Definition 4 Let M be a fixed tuple with binding sites $1, \ldots, n$ for ligand L_1 and binding sites $A_1, \ldots, A_m$ for Ligand L_2. Moreover, let $i, j \in \{1, \ldots, n, A_1, \ldots, A_m\}$. The sites i and j are called connected if a path $I = \{(i, k_1), (k_1, k_2), \ldots, (k_p, j)\}$ exists with $w_i \neq 1\ \forall i \in I$.

Using this definition we conjecture that Proposition 7 can be generalized to molecules with more binding sites.

Conjecture 2 *Let M be a molecule with binding sites $1, \ldots, n$ for ligand L_1 and binding sites $A_1, .., A_m$ for ligand L_2. Then the binding sites for ligand L_1 are 1-dimensionally decoupled for all κ if and only if all binding sites for ligand L_1 are pairwise not connected.*

6 Summary and outlook

We formulated the DSR for molecules with binding sites for two different types of ligands and showed that if the overall binding curves of both ligands shall be preserved not all interactions can be trivial. However, the binding sites for the same type of ligand can be decoupled under the loss of uniqueness of the decoupled system which is given when only one type of ligand is present. We proved this statement for the case of $(n_1, 1)$ binding sites and presented different approaches for proofs. From a theoretical point of view a general proof for any choice of n_1 and n_2 would be favorable. Moreover, we showed, that even though there are several different decoupled systems all of them share the same binding constants and seem to be built of a unique set of "bricks" which are combined differently. For the case of $(n_1, 1)$ binding sites we identified these "bricks" as microstate constants (or mircrostate probability functions) and showed that the maximal number of different decoupled systems is given by $n_1!$. Furthermore, we showed that regaining original individual titration curves as linear combination of decoupled molecules is possible if the weights of the linear combinations are functions of the ligand activity of the second ligand. Regarding the titration curves of decoupled systems revealed that even though the binding sites for the same type of ligand in the decoupled molecule do not interact the 1-dimensional titration curve, when the activity of the second ligand is fixed, are not in general of classical HH shape. This is counterintuitive but a result of secondary interaction, which is not present if the different sites are not connected.

Prospective work might investigate which unique properties all decoupled molecules characterize for (n_1, n_2) systems and how decoupled molecules can facilitate understanding the binding behavior of ligands to the original molecule. Proteins of special biophysical interest such as proteins with electron and proton binding sites which play an important role in electron and proton transfer chains such as photosynthesis should be decoupled exemplarily. Moreover, the DSR can also be applied to other interacting systems consisting of a molecule and different types of ligands. Examples are transporter systems in membranes with pH-gradient, co-transporter, receptors with different types of signaling molecules, and toxin and target interaction. This may also be of pharmacological interest if the average amount of receptor bound drugs, dependent on their activity in blood, shall be calculated.

Acknowledgments We would like to thank Tobias Dorsch for helpful discussions.

Appendix: Supporting information

We used the following short Magma code for the proof of Proposition 3:

```
## Proposition 3

R<g1,g2,g3,w13,w23,a21,a20,a11,a10,a01>:=PolynomialRing(Rationals(),10);
eqns:=[
```

```
g1*g2*g3*w13*w23-a21,
g1*g2-a20,
g1*g3*w13 + g2*g3*w23-a11,
g1+g2-a10,
g3-a01
];
I:=ideal<R|eqns>;
GB:=GroebnerBasis(I);
GB;

## The corresponding Groebner basis

[
    g1 + g2 - a10,
    g2^2 - g2*a10 + a20,
    g2*w13*a21 - g2*w23*a21 + w13*w23*a20*a11 - w13*a21*a10,
    g2*w13*a11 - g2*w23*a11 - w13^2*a20*a01 - w13*w23*a10^2*a01 - w23^2*a20*a01
        + w23*a11*a10 + 2*a21,
    g2*w13*a01 - g2*w23*a01 - w13*a10*a01 + a11,
    g2*w23^2*a20*a01 - g2*a21 - w23*a20*a11 + a21*a10,
    g2*w23*a20*a11 - g2*a21*a10 + w23^2*a20^2*a01 - w23*a20*a11*a10 - a21*a20 +
        a21*a10^2,
    g2*w23*a10*a01 - g2*a11 + w13*a20*a01 - w23*a20*a01,
    g2*a21*a10^2*a01 - g2*a20*a11^2 + w13*a20^2*a11*a01 - w23^2*a20^2*a10*a01^2
        - w23*a20^2*a11*a01 + w23*a20*a11*a10^2*a01 + a21*a20*a10*a01 -
        a21*a10^3*a01,
    g3 - a01,
    w13^2*a20*a01^2 + w13*w23*a10^2*a01^2 - w13*a11*a10*a01 + w23^2*a20*a01^2 -
        w23*a11*a10*a01 - 2*a21*a01 + a11^2,
    w13*w23^2*a21*a10^2*a01 + w13*w23^2*a20*a11^2 - w13*w23*a21*a11*a10 +
        w13*a21^2 + w23^3*a21*a20*a01 - w23^2*a21*a11*a10 - 2*w23*a21^2,
    w13*w23^2*a20^2*a11^2 - w13*w23*a21*a20*a11*a10 + w13*a21^2*a20 +
        w23^3*a21*a20^2*a01 - w23^2*a21*a20*a11*a10 - 2*w23*a21^2*a20 +
        w23*a21^2*a10^2,
    w13*w23^2*a10^2*a01^2 - w13*w23*a11*a10*a01 + w13*a21*a01 + w23^3*a20*a01^2
        - w23^2*a11*a10*a01 - 2*w23*a21*a01 + w23*a11^2,
    w13*w23*a20*a01 - a21,
    w13*a21*a20*a01 + w23^3*a20^2*a01^2 - w23^2*a20*a11*a10*a01 -
        2*w23*a21*a20*a01 + w23*a21*a10^2*a01 + w23*a20*a11^2 - a21*a11*a10,
    w23^4*a20^2*a01^2 - w23^3*a20*a11*a10*a01 - 2*w23^2*a21*a20*a01 +
        w23^2*a21*a10^2*a01 + w23^2*a20*a11^2 - w23*a21*a11*a10 + a21^2
]
```

References

1. G.K. Ackers, M.A. Shea, F.R. Smith, Free energy coupling within macromolecules: the chemical work of ligand binding at the individual sites in co-operative systems. J. Mol. Biol. **170**, 223–242 (1983)
2. D. Bashford, M. Karplus, Multiple-site titration curves of proteins: an analysis of exact and approximate methods for their calculation. J. Phys. Chem. **95**(23), 9556–9561 (1991)

3. T. Becker, R.T. Ullmann, G.M. Ullmann, Simulation of the electron transfer between the tetraheme subunit and the special pair of the photosynthetic reaction center using a microstate description. J. Phys. Chem. B **111**(11), 2957–2968 (2007)
4. E. Bombarda, G.M. Ullmann, pH-dependent pKa values in proteins- A theoretical analysis of protonation energies with practical consequences for enzymatic reactions. J. Phys. Chem. B **114**(5), 1994–2003. PMID: 20088566 (2010)
5. C.R. Cantor, P.R. Schimmel, *Biophysical Chemistry. Part III. The Behavior of Biological Macromolecules*, 1st edn. (W. H. Freeman, 1980)
6. D. Cox, J. Little, D. O'Shea, *Using Algebraic Geometry* (2nd ed.). (Springer, New York, 2005
7. D.A. Cox, J. Little, D. O'Shea, *Ideals, Varieties, and Algorithms: An Introduction to Computational Algebraic Geometry and Commutative Algebra*, 3rd ed. (Springer, Secaucus, NJ, USA, 2008)
8. B.E. Garcia-Moreno, Probing structural and physical basis of protein energetics linked to protons and salt. Methods Enzymol. **259**, 512–538 (1995)
9. G. Gnacadja, A method to calculate binding equilibrium concentrations in the allosteric ternary complex model that supports ligand depletion. Math. Biosci. **232**(2), 135–141 (2011)
10. K. Hasselbalch, *Die Berechnung der Wasserstoffzahl des Blutes aus der freien und gebundenen Kohlensäure desselben, und die Sauerstoffbindung des Blutes als Funktion der Wasserstoffzahl*. (Julius Springer, Berlin, 1916)
11. L.J. Henderson, The Fitness of the Environment. (Macmillan Company, New York, 1913)
12. J. Martini, G. Ullmann (2012). A mathematical view on the decoupled sites representation. J. Math. Biol. 1–27 (2012). doi:10.1007/s00285-012-0517-x
13. E. Medvedev, A. Stuchebrukhov, Kinetics of proton diffusion in the regimes of fast and slow exchange between the membrane surface and the bulk solution. J. Math. Biol **52**, 209–234 (2006). doi:10.1007/s00285-005-0354-2
14. A. Onufriev, D.A. Case, G.M. Ullmann, A novel view of pH titration in biomolecules. Biochemistry **40**(12), 3413–3419 (2001)
15. A. Onufriev, G.M. Ullmann, Decomposing complex cooperative ligand binding into simple components: Connections between microscopic and macroscopic models. J. Phys. Chem. B **108**(30), 11157–11169 (2004)
16. J.A. Schellman, Macromolecular binding. Biopolymers **14**(5), 999–1018 (1975)
17. C. Tanford, J.G. Kirkwood, Theory of protein tiration curves. I. general equations for impenetrable spheres. J. Am. Chem. Soc. **79**(20), 5333–5339 (1957)
18. M.S. Till, T. Essigke, T. Becker, G.M. Ullmann, Simulating the proton transfer in gramicidin a by a sequential dynamical monte carlo method. J. Phys. Chem. B **112**(42), 13401–13410 (2008). PMID: 18826179
19. R.T. Ullmann, G.M. Ullmann, Coupling of protonation, reduction and conformational change in azurin from Pseudomonas aeruginosa investigated with free energy measures of cooperativity. J. Phys. Chem. B **115**, 10346–10359 (2011)
20. J. Wyman, S.J. Gill, Binding and Linkage: Functional Chemistry of Biological Macromolecules. (University Science Books, Mill Valley, California 1990)